Application of Natural Biodegradable Fiber as Biofilm Medium and Carbon Source in DEnitrifying AMmonium OXidation (DEAMOX) Process for Nitrogen Removal from Wastewater

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Abstract

Nitrite (NO_2^-) accumulation and retaining of anammox bacteria are two decisive factors for stable operation of partial denitrification and anammox coupling process. Denitrifying ammonium oxidation (DEAMOX) process has been regarded as a promising way for nitrogen removal from wastewater containing both ammonium (NH_4^+) and nitrate (NO_3^-) pollutants simultaneously. However, performance and efficiency of DEAMOX process in biofilm reactors are still not fully understood. This study successfully implements biodegradable *Luffa Cylindrica* fiber as both carbon source and biofilm carrier in the DEAMOX system. 87% nitrate-to-nitrite transformation was achieved through the partial denitrification process. An average total nitrogen (TN) removal efficiency of about 98% was obtained with influent NH_4^+-N and NO_3^--N concentration of 100 mg L^{-1} in an up-flow packed bed biofilm reactor. The Field Emission-Scanning Electron Microscopy (FE-SEM) photographs showed that present conditions in the DEAMOX packed bed reactor favor the granulation of biofilm developed on *L. cylindrica*. The results imply that solid phase partial

denitrification and granulation of anammox bacteria was achieved using *L. cylindrica* as a carbon source and biofilm carrier respectively. Complete NO_3^- removal observed in this study supports the hypothesis that solid carbon source can support denitrification of NO_3^- produced through the anammox process.

Keywords: Partial-denitrification; Packed bed reactor; Anammox; Biofilm; Wastewater treatment; *Luffa cylindrica*

1. Introduction

High concentrations of nitrogen (N) compounds can deteriorate the aquatic environmental quality and threaten human health. Therefore, to protect water resources and public health from the detrimental effects of N compounds, stringent nutrient level for N compounds concentration of treated wastewater have been set by many countries [1]. Conventional NH_4^+ removal process is accomplished by nitrification/denitrification, associated with high costs and gives rise to an adverse effect on the environment due to the high biomass production and greenhouse gas emission, contributing to the global warming [2]. Although the biological process is economical for N removal, it is not highly effective for wastewater with low COD/N ratio due to the shortage of carbon source needed for denitrification [3]. One way to improve efficiency of N removal is adding external carbon sources, including methanol, ethanol, acetic acid and glucose, which is a costly process with the potential risk of insufficient or over dosing of carbon sources [3]. Insufficient carbon source leads to NO_2^- accumulation, while overdosing results in remains of organic carbon in the effluent [3]. In recent years, innovative and cost-efficient autotrophic nitrogen removal systems including SHARON, ANAMMOX, combined Partial SHARON/ANAMMOX process in two bioreactors, and combined Partial SHARON/ANAMMOX process in a single bioreactor (i.e. OLAND, CANON, and DEMON

process) have been put forward as promising alternatives for treatment of NH_4^+ rich wastewaters [4]. Interestingly, in all of these processes NO_2^- plays a key role in promotion of biochemical reactions involved in NH_4^+ removal. These innovations have rapidly finding favours due to their higher efficiency and lower cost than the conventional N-removal methods [5]. The anammox process was found cost-effective N removal technology compared to traditional nitrification-denitrification, given that anammox requires less oxygen and the addition of organic carbon is not essential [6, 7]. Nonetheless, slow start-up and strict operational conditions are main barriers for widespread use of autotrophic N removal systems [5]. In addition, during anammox reaction, 11% of N is converted to NO_3^-, resulting in a maximum efficiency of 87% for N removal [8]. High NO_3^- concentrations in effluent of anammox process have been reported [9], indicating that denitrification process is necessary to remove excess NO_3^-, while external carbon source must be added for the denitrification. Although NO_2^- accumulation is believed not to occur during denitrification as NO_2^- reduction rate is higher than that of NO_3^-, accumulation of NO_2^- has been reported in denitrification process [10, 11]. NO_2^- accumulation is attributed to microbial communities with two specific patterns of denitrification pathway including NO_3^- reducing only to NO_2^- and transient NO_2^- accumulation during the reduction of both NO_3^- and NO_2^- [1]. On the other hand, since oxidation of NH_4^+ to NO_3^- is more probable than NH_4^+ oxidation to NO_2^- in aerobic wastewater treatment [12], an innovative biological N removal process named DEnitrifying AMmonium OXidation (DEAMOX) has been regarded as a promising way to nitrogen removal from wastewater containing both NH_4^+ and NO_3^- simultaneously [13]. During the DEAMOX process, NO_3^- is partially denitrified to NO_2^- using organic carbon as an electron donor providing NO_2^- for anammox bacteria [14]. Therefore, NO_2^- reduction to N_2 should be

suppressed to achieve a desirable performance. Step-feeding organic carbon have been considered as a promising method for preventing NO_2^- reduction to N_2 by denitrifiers [14].

A number of studies [15-17] demonstrated the adverse effects of organic carbon on anammox systems. Complete inactivation or reduction of anammox activity is mainly due to the fact that at high organic carbon concentration, denitrifiers out-compete the anammox bacteria, resulting in abatement of anammox process [18]. Selective carbon source, as an electron donor is an important factor influencing the end products of microbial NO_3^- respiration so that particular carbon source may favor different respiratory pathways. Consequently, selective carbon source is a determining factor as to how it influences the microbial community [19]. Apart from liquid carbon sources, solid substrates have also been applied for denitrification process, i.e., solid-phase denitrification [20], offering a number of advantages including providing a constant carbon source and serving as solid matrices for development of biofilm [20]. Generally, there are two main kinds of solid carbon source including the natural plant materials and synthetic biodegradable polymers [21]. Since the yield coefficient of heterotrophic denitrifying bacteria (Y=0.27-0.3) [22] is much more than that of anammox bacteria (Y=0.066±0.01) [22], co-existence of the two kinds of bacteria cannot be achieved easily. However, the availability of NO_2^- has a pivotal role in effective co-existence of denitrifiers and anammox bacteria [13]. Due to long doubling time of 5.5-7.5 days [16] and slow growth rate (maximum specific growth rate of 0.0027 h^{-1}) [23] of the anammox bacteria, the biofilm reactors such as fixed bed and fluidized bed reactor have been used to ensure that enough biomass is available inside the reactors [24]. In comparison with conventionally used sequencing batch reactors, biofilm and granular systems improve anammox biomass retention [25] and enhance anammox start-up performance [26]. Moreover, biofilm reactors reduce the microbial washout, permitting the operation of the reactor at longer solid retention time (SRT) and shorter

hydraulic retention time (HRT), resulting in higher treatment efficiency and lower effluent solids [27]. This paper investigates the DEAMOX process in an up-flow packed bed biofilm reactor using *L. cylindrica* as an organic carbon source and biofilm carrier. Methodology for NO_2^- accumulation through the partial denitrification is successfully developed in order to facilitate anammox process promoting DEAMOX system. This study hypothesized that *L. cylindrica* can provide a constant and low-release carbon source favored to accumulate NO_2^- by denitrifiers. The proposed method is shown to improve the coupling of partial denitrification and anammox, enabling more stable DEAMOX process.

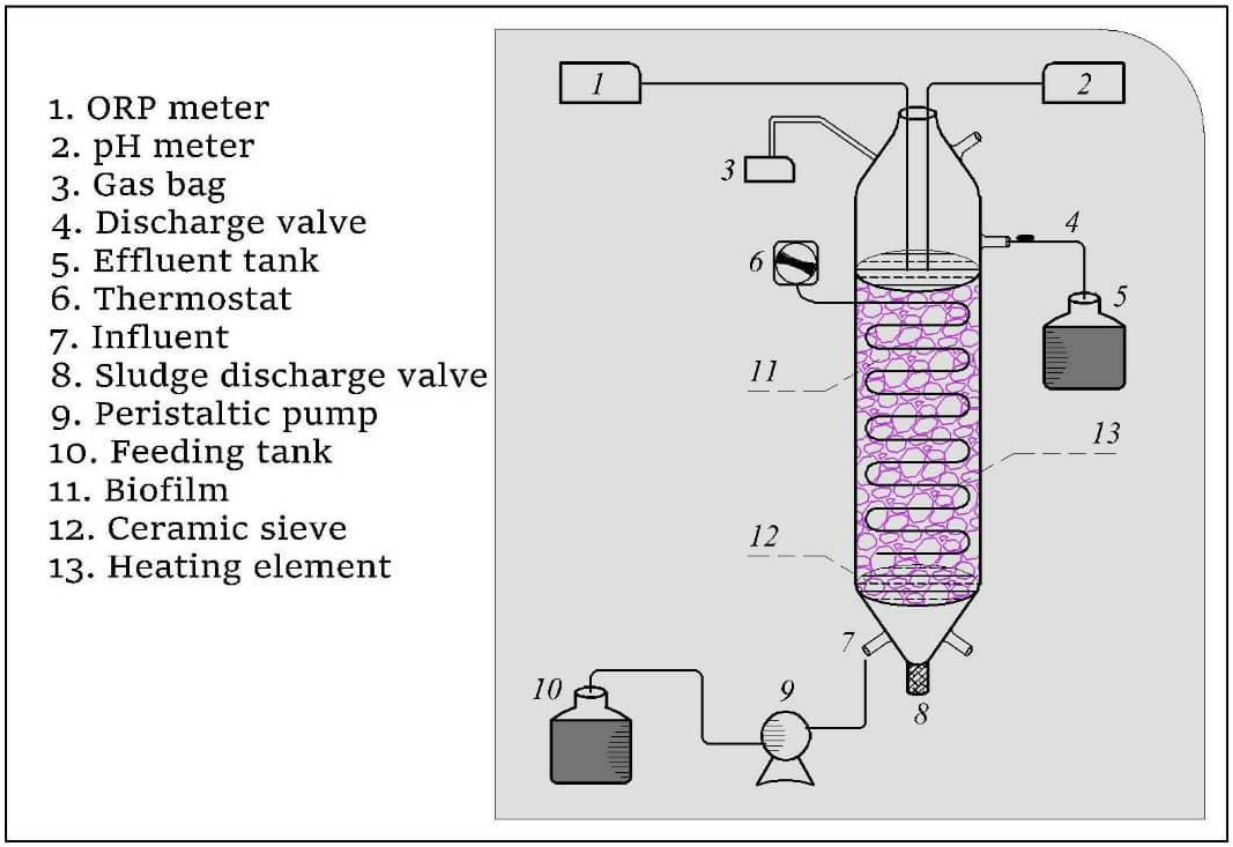


Fig. 1. Schematic diagram of up-flow packed bed biofilm reactor

2. Materials and Methods

2.1. Experimental setup

A lab-scale up-flow packed bed biofilm reactor (Fig. 1) with the working volume of 1.25 L and inner diameter of 80 mm was designed and operated continuously as DEAMOX system. The temperature of the bioreactor was controlled at 30°C using a thermostat and flexible silicone heating element coiled around the reactor. *L. cylindrica* was used as a biological carrier. In order to prevent penetration of light and maintain the constant temperature, the reactor was insulated by glass wool and aluminum foil. The experiment was undertaken for the duration of 205 days. The Redox and pH values were monitored continuously to ensure the stability of the biological processes in the reactor. The value of pH was maintained at 7.8 using 0.1 M HCl and 0.1 M NaOH. The ORP (Oxidation-Reduction Potential) was fluctuated between -76 and -117 mV. The reactor experiment was designed in five Phases (see table 1). During Phase I, the DEAMOX process was carried out at a same concentration of NH_4^+-N and NO_3^--N = 100 mg L^{-1} with HRT of 12 hr. In Phase II, HRT was decreased by 40% because of the biofilm developed on the medium. In Phase III, the HRT was adjusted again by decreasing the flow rate. During Phase IV, acetate as an external carbon source (COD/NO_3^- ratio of 3.0) was added to the feeding tank. Finally, in Phase V, the reactor was operated the same as Phase I. The percentage of anammox/denitrification contribution on TN removal was determined throughout the tests using Eq. 1 and 2 [28]:

$$\text{Anammox percentage} = \frac{[(\text{Inf. } NH_4^+\text{-N} - \text{Eff. } NH_4^+\text{-N}) + 1.32\ (\text{Inf. } NH_4^+\text{-N} - \text{Eff. } NH_4^+\text{-N})] \times 100\%}{(\text{Inf. TN} - \text{Eff. TN})} \quad (1)$$

$$\text{Denitrification percentage} = 100\% - \text{anammox percentage}\ (\%) \quad (2)$$

Table 1. Operation modes designed for up-flow packed bed biofilm reactor tests

Phase	Day	HRT (hr)	Influent Nitrogen Concentration (mg L^{-1})		
			NH_4^+-N	NO_3^--N	COD/NO_3^-
I	25-90	12	100	100	-
II	91-110	7.2	100	100	-
III	111-150	12	100	100	-
IV	151-180	12	100	100	3
V	181-205	12	100	100	-

2.2. Inoculation and biofilm formation

The initial seed for the enrichment of denitrifiers was obtained from an anoxic tank of municipal wastewater treatment plant based in Tehran, Iran. Then, the enrichment of denitrifiers biomass was performed in a batch system using synthetic wastewater containing NO_3^- and *L. cylindrica* as a N and carbon source, respectively. Following the enrichment of denitrifiers in the batch system, the biofilm developed on the medium were mechanically transferred to synthetic wastewater. Enriched biomass of anammox bacteria obtained from our previous study [29] was used as an inoculum. 1 L glass bottle congaing synthetic wastewater inoculated by 120 mL enriched anammox sludge and 40 mL denitrifier sludge were anaerobically introduced into the up-flow packed bed biofilm reactor using a peristaltic pump. The reactor was operated in a circulation loop for 25 days to immobilize the biofilm on the carrier.

2.3. Synthetic wastewater

The ratio of NO_3^--N/NH_4^+-N in the influent was kept at 1. The composition of synthetic wastewater was as follows (L^{-1}): 607.17 mg/L $NaNO_3$ (100 mg NO_3^--N), 0.397 g NH_4Cl (100 mg/L NH_4^+-N), 0.014 g KH_2PO4, 1.180 g $KHCO_3$, 0.110 g $MgCl_2.6H_2O$, 0.012 $FeSO_4.7H_2O$, 0.300 g $CaCl_2.2H_2O$, 1 mL trace element solution contained (L^{-1}): 15 g Na-EDTA, 0.43 g ZnSO4·7H2O, 0.24 g $CoCl_2·6H_2O$, 0.81 g $MnCl_2·2H_2O$, 0.25 g $CuSO_4·5H_2O$, 0.22 g $Na_2MoO4·2H_2O$, 0.19 g $NiCl_2·6H_2O$, 0.11 g $Na_2SeO_3·5H_2O$, 0.01 g $FeSO_4·7H2O$, and 0.07 g H_3BO_3 [29].

2.4. Biofilm carrier

Biodegradable *L. Cylindrica* fiber was used as both the carbon source and biofilm carrier. *L. cylindrica* was obtained from Luffa farm in Iran. It was washed with distilled water to remove impurity then dried in an oven at 80°C for 5 hr. Surface morphology of *L. cylindrica* and biofilm immobilized on the support were characterized by means of FE-SEM. The samples were prepared by fixation, dehydration, drying, mounting and coating [30]. Prepared samples were investigated using FE-SEM method.

2.5. Analytical methods

The effluent samples were taken every fifth day and analyzed immediately after sampling.

The samples were filtered through 0.45 µm membrane prior to determine the concentration of N compounds. NO_2^--N and NO_3^--N were measured using spectrophotometry method according to standard methods (4500-NO_2^-B and 4500 NO_3^- C respectively) [31]. NH_4^+-N was measured using volumetric method by titration after distillation of NH_4^+ (4500-NH_3 C) [31].

2.6. Batch experiments

In addition to continuous up-flow packed bed biofilm reactor, some batch tests were carried out to determine the NO_3^- to NO_2^- transformation rate (i.e., partial-denitrification activity). The batch test was conducted in a sealed 100 mL conical flask at 30°C. 1 g biofilm taken from up-flow packed bed biofilm reactor on day 75 was added to the flask containing 75 mL basal medium [32] with NO_3^--N concentration of 25 mg/L. Moreover, one flask with NO_3^- and lack of substrate was also run as a control test. The supernatant samples were collected and analyzed for NO_3^- and NO_2^- concentration. The N_2 was sparged into the flask for 2 min to develop anoxic condition and resazurin indicator was applied to monitor anoxic condition.

The NO_3^--N to NO_2^--N transformation ratio (NTR) was determined using Eq. 3 [33]:

$$\text{NTR (\%)} = (NO_2^- - N_t - NO_2^- - N_{initial})/(NO_3^- - N_{initial} - NO_3^- - N_t) \times 100 \quad (3)$$

3. Results and discussion

3.1. Partial-denitrification activity

The results obtained from batch experiments (Fig. 2) show 87% nitrate-to-nitrite transformation was achieved by denitrifiers immobilized on *L. cylindrica*, demonstrating that that *L. cylidrica* as a solid carbon source supported partial denitrification. During solid phase partial denitrification, NO_3^--N decreased gradually using *L. cylindrica* as organic carbon source, whereas NO_2^--N accumulated and reached the maximum value of 22 mg/L. Different NTR rates have been reported in different condition. For example, NO_2^--N accumulation of 71.7% under the acetate feast-famine condition has been observed [34]. While, with sludge fermentation liquid as a carbon source, the NTR of 80% was achieved [35]. However, use of other biopolymers (i.e., Polycaprolactone [PCL], Polylactic acid [PLA] and starch) as a solid substrate for denitrification, did not show significant

nitrite accumulation [36, 37]. It has been reported that microbial community and consequently end product of NO_3^- reduction can be altered by selective carbon sources [19]. For example, D-cellobiose induces *Klebsilla* to be enriched as a NO_2^- accumulator, while other carbon source enriched for an *Escherichia* nitrate ammonifier [19]. As the availability of NO_2^- is pivotal in the co-existence of denitrifiers and anammox bacteria [13], *L. cylidrica* was applied efficiently for solid-phase partial denitrification process.

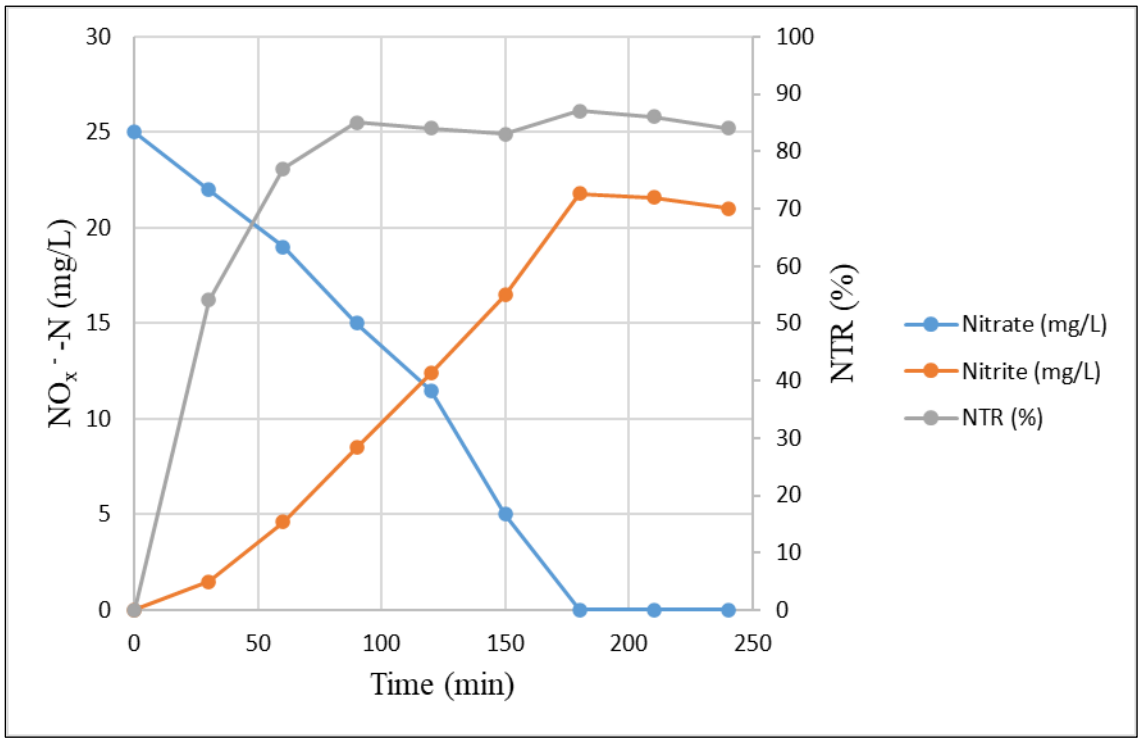


Fig. 2. NO_3^- to NO_2^- transformation rate (partial-denitrification activity)

3.2. N removal in DEAMOX reactor

Fig. 3 shows the variation of N compounds concentration and TN removal efficiency in the DEAMOX system. The figure indicates that NO_3^- and NO_2^- were completely removed during Phase I, with NH_4^+ effluent concentration of less than 5 mg L^{-1}. The average total nitrogen (TN) removal efficiency of about 98% was achieved during Phase I, demonstrating co-existence of

denitrification and anammox processes. Complete removal of NO_3^- supports the hypothesis that solid carbon source can support denitrification of NO_3^- produced through the anammox process. This result confirming the finding of studies reported the effects of adding polycaprolactone (PCL), as a solid carbon source, for the removal of the NO_3^--N produced during anammox process [38].

In Phase II, a reduction in TN removal efficiency was recorded. During phase 2. The TN removal efficiency was reduced considerably by about 30% and reached the minimum level of approximately 60% by day 110, indicating that HRT plays a key role on the efficiency of TN removal process. HRT reduction can be associated to biofilm development on the medium leading to a reduced working volume and subsequently HRT.

The measurements show that the HRT was increased during Phase III by decreasing of inflow rate in order to operate the reactor under previous HRT condition (12 hr). Data presented in Figure 3 for Phase III show that TN removal efficiency recovered after adjustment of HRT.

The effects of excess organic matter on N removal efficiency were investigated by adding sodium acetate (COD/NO_3^- ratio of 3.0) during Phase IV. During Phase IV, a significant 40% reduction in TN removal efficiency was recorded, revealing that denitrification was predominant process removing TN, while the average NH_4^+ removal efficiency was only 19%. The analysis of the test data show that NO_2^- was reduced to N_2 rather than being accumulated, resulting in inhibition of NH_4^+ oxidation by anammox bacteria. However, previous study found NO_2^- accumulation rate can increase when COD/NO_3^--N ratio was amplified [39]. Although acetate, as a carbon source, have high potential for NO_2^- generation, partial denitrification will not be affected by COD/NO_3^- -N ratio [40]. These variations in findings of previous studies can be associated with different characteristics of microbial communities for NO_3^- and NO_2^- reduction. Due to the different types

of metabolic pathways in various denitrifiers, different patterns of denitrification in wastewater treatment plants including partial denitrification, complete denitrification, and transient accumulation of NO_2^- has been reported [1]. Competition for electron donor between NO_2^- reductase and NO_3^- reductase found to be different among denitrifiers, even when the same carbon source was used for denitrification [33]. Moreover, existence of two carbon sources can change the pattern of NO_2^- accumulation [39]. In Phase V, an increase in TN removal efficiency was observed (Fig. 3), as addition of acetate to the feeding tank was stopped, and the reactor was operated the same as Phase I, indicating that the system was able to shift from denitrification to DEAMOX process.

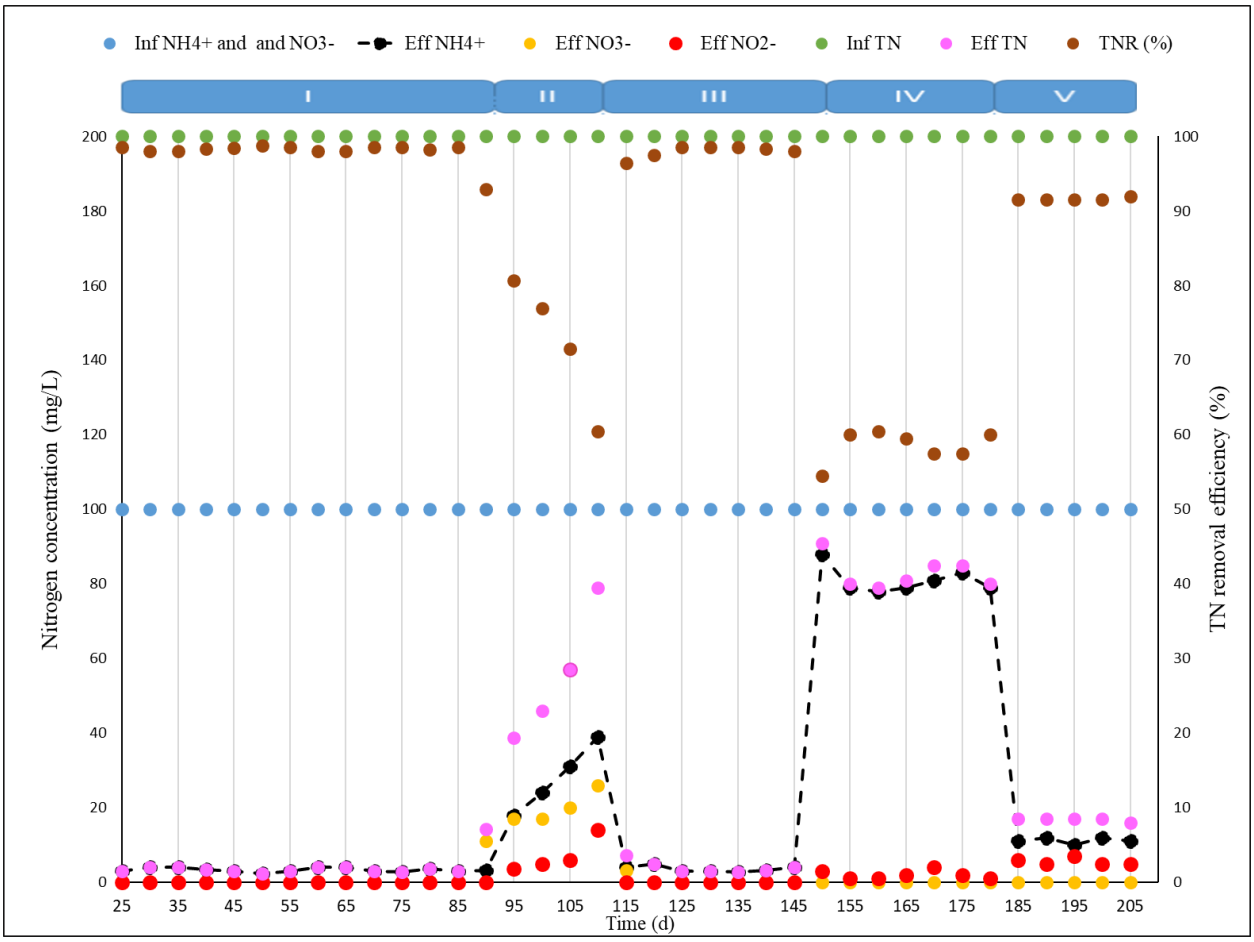


Fig. 3. Variation of nitrogen compounds concentration and TN removal efficiency in the DEAMOX system

Percentages of anammox and denitrification contribution on TN removal in DEAMOX process was determined in this study (Fig. 4). The results show that the N was removed predominantly by ANAMMOX process which accounted for over 90% in all Phases, except for phase IV (Fig. 4). During Phase IV, denitrification was the main pathway for N removal, demonstrating that high concentration of organic carbon (acetate) promotes complete denitrification instead of partial denitrification. Consequently, it was shown that relative lack of NO_2^- accumulation adversely affect ANAMMOX process due to the inhibition of NH_4^+ oxidation. This is consistent with a number of previous studies reporting adverse effect of organic carbon on ANAMMOX process, given that high concentration of organic carbon suppress the ANAMMOX bacteria [13, 41].

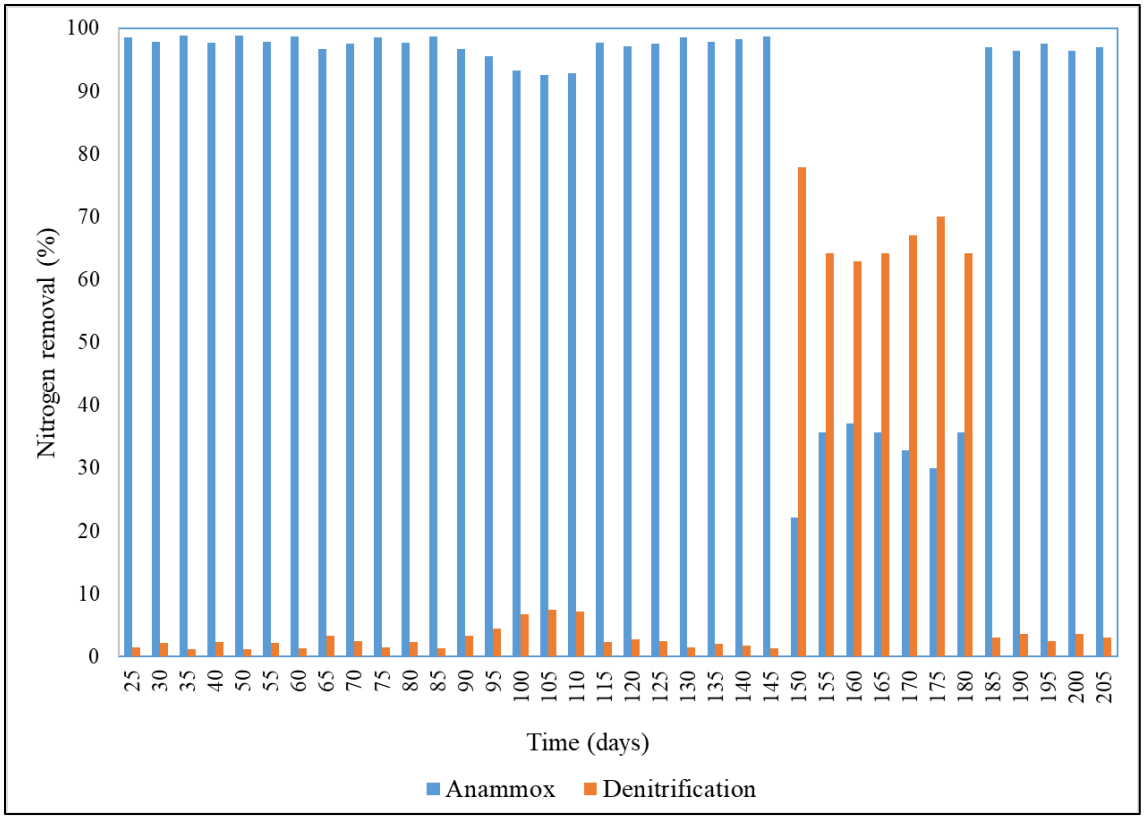


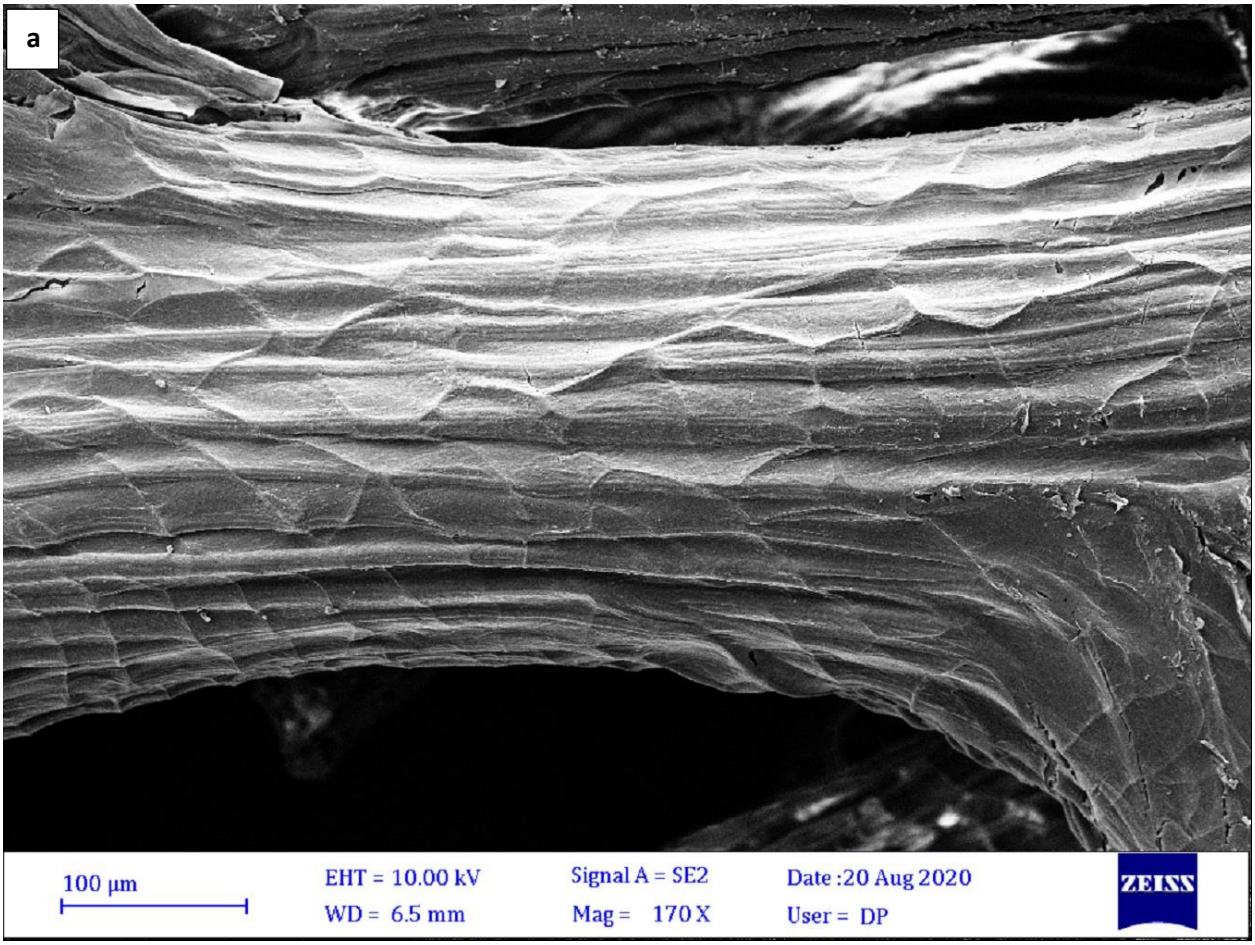
Fig. 4. Contribution of ANAMMOX/Denitrification on TN removal in the DEAMOX system

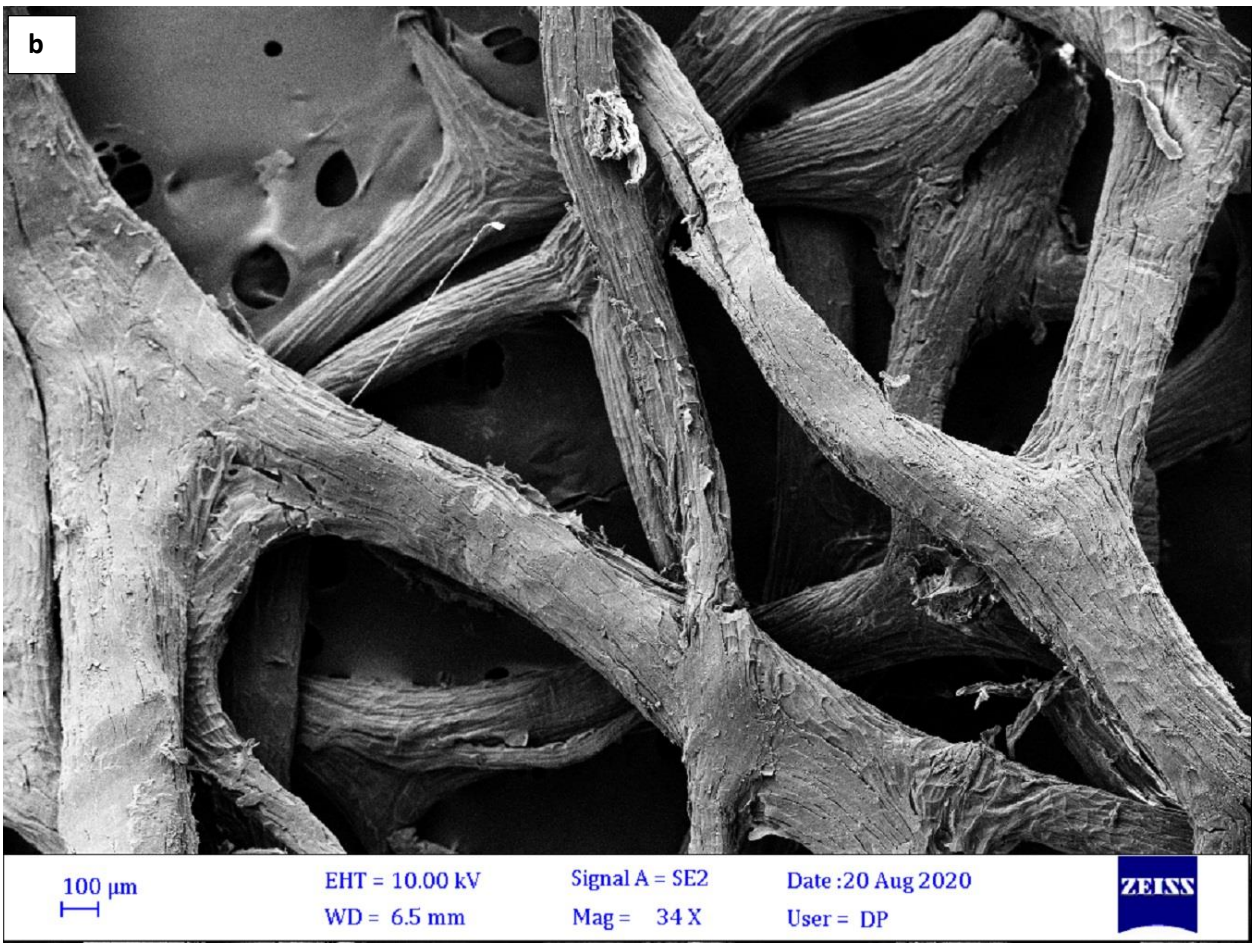
3.3. FE-SEM analysis of L. cylindrica as a carbon source and biofilm carrier

Natural *L. cylindrica* fiber has unique properties such as renewability, availability, low-cost, good hydrophilicity, biocompatibility, porous structure non-toxicity to microorganisms, and long-term sustainability to the natural environment [42, 43,] making this biopolymer efficient as bio-carrier for wastewater treatment. The average Brunauer-Emmett-Teller (BET) specific surface area of *L. cylindrica* fiber was determined to be 123 m^2/g [44] which is higher than other biodegradable polymer used for nitrogen removal. For example, BET surface area of 0.376 m^2/g and 55 m^2/g have been reported for polycaprolactone (PCL) and poly-3-hydroxybutyric acid (PHB) respectively [3, 45]. Overall, the results show that high surface area of *L. cylindrica* enhanced biofilm attachment, leading to rapid start-up performance. *L. cylindrica* have a netting-like fibrous

vascular structure serving like an open cell material [46]. Cellulose (62%), hemicellulose (20%), and lignin (11%) were found to be the main components of *L. cylindrica* fibers [42]. Moreover, *Luffa* fiber has solubility of 3.30% and 4.50% in hot water and cold water, respectively [47]. Initially, the biodegradable solid carbon source is hydrolyzed by extracellular enzyme excreted from microorganisms immobilized on medium and then decomposed into soluble and micro-molecular substrates [48]. Unlike liquid carbon source, *L. cylindrica* provides constant and slow-release carbon source favored to accumulate NO_2^- by denitrifiers indicating better condition for coupling of partial denitrification and anammox to achieve more stable DEAMOX process. Fig. 5 shows the FE-SEM of un-immobilized (Fig. 5a and b), immobilized (Fig. 5 c – f) and used (Fig. 5 g and h) *L. cylindrica*. Fig. 5d shows a specific region of Fig. 5c with higher magnification. The FE-SEM results show *L. cylindrica* has rough surface with a various fibrous cord and a large number of protrusions, making this matrix efficient as biofilm medium (Fig. 5a and b). Figs. 5 c – f illustrate thick and compact granular biofilm developed on the carrier. FE-SEM results show both netting-like fibrous structure and surface of fibrous threads were completely covered by bacteria. This study also shown that bacteria were aggregated along the threads and over the cords (Figs. 5c, e and f). Different models including inert nuclei model, synthetic and natural polymer-bonding model, spaghetti theory, and cell-to-cell communication model has been suggested for anaerobic granulation [49]. According to inert nuclei model, presence of nuclei or micro-size biocarrier is the first step for attachment of bacteria [49]. Protrusions disposed on the *L. cylindrica* favor the granulation and spherical biofilm formation (Fig. 5d). Development of granules with higher size and density due to polymer addition was reported [50]. Although, the biofilm is tightly integrated, some cracks observed (Fig. 5d), which can be attributed to microbial autolysis [51]. Figs. 5g and

h show the *used L. cylindrica* fiber. Microbial degradation of *L. cylindrica* fiber has resulted in concave cavities on the surface of *L. cylindrica* (Figs. 5 g and h).

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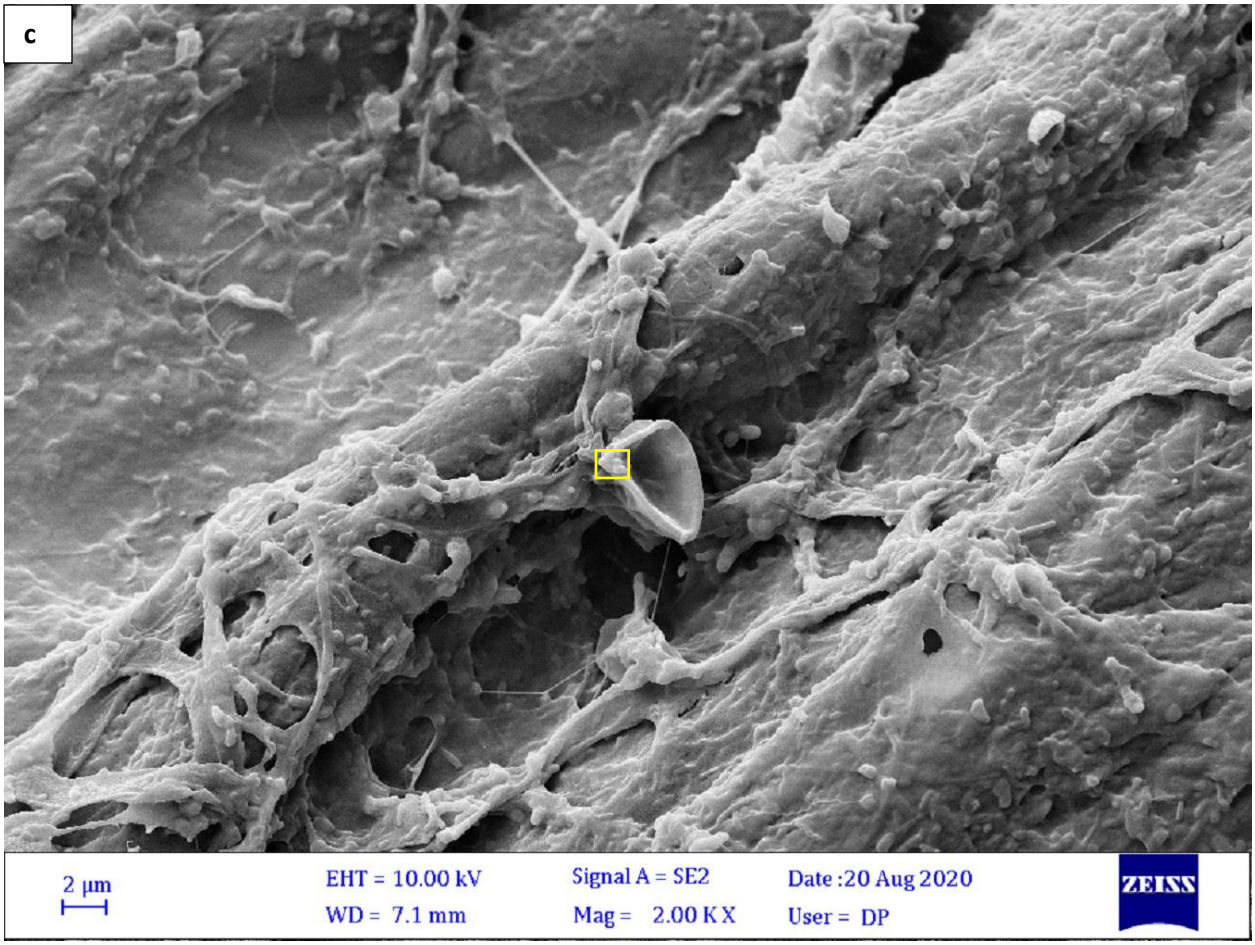
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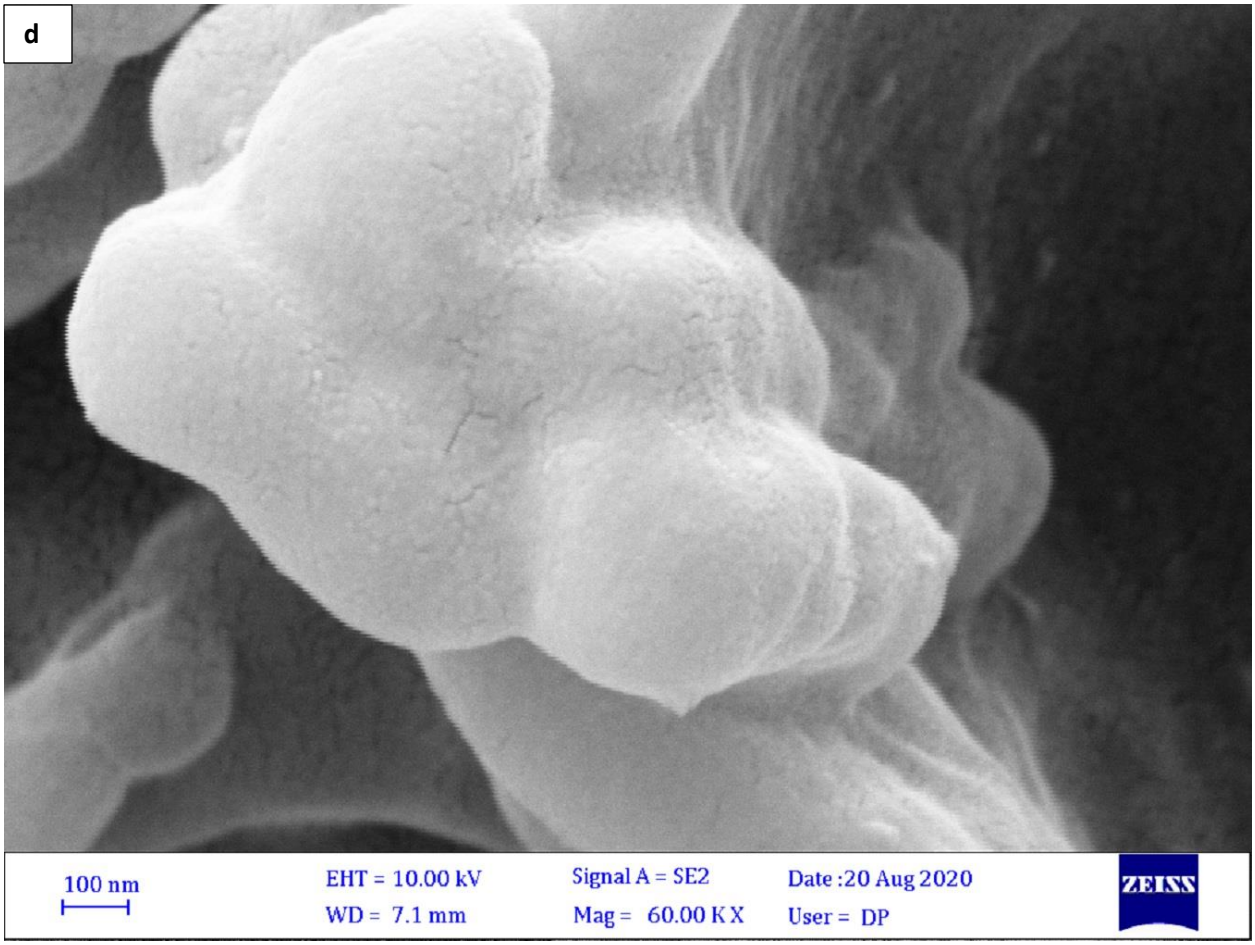
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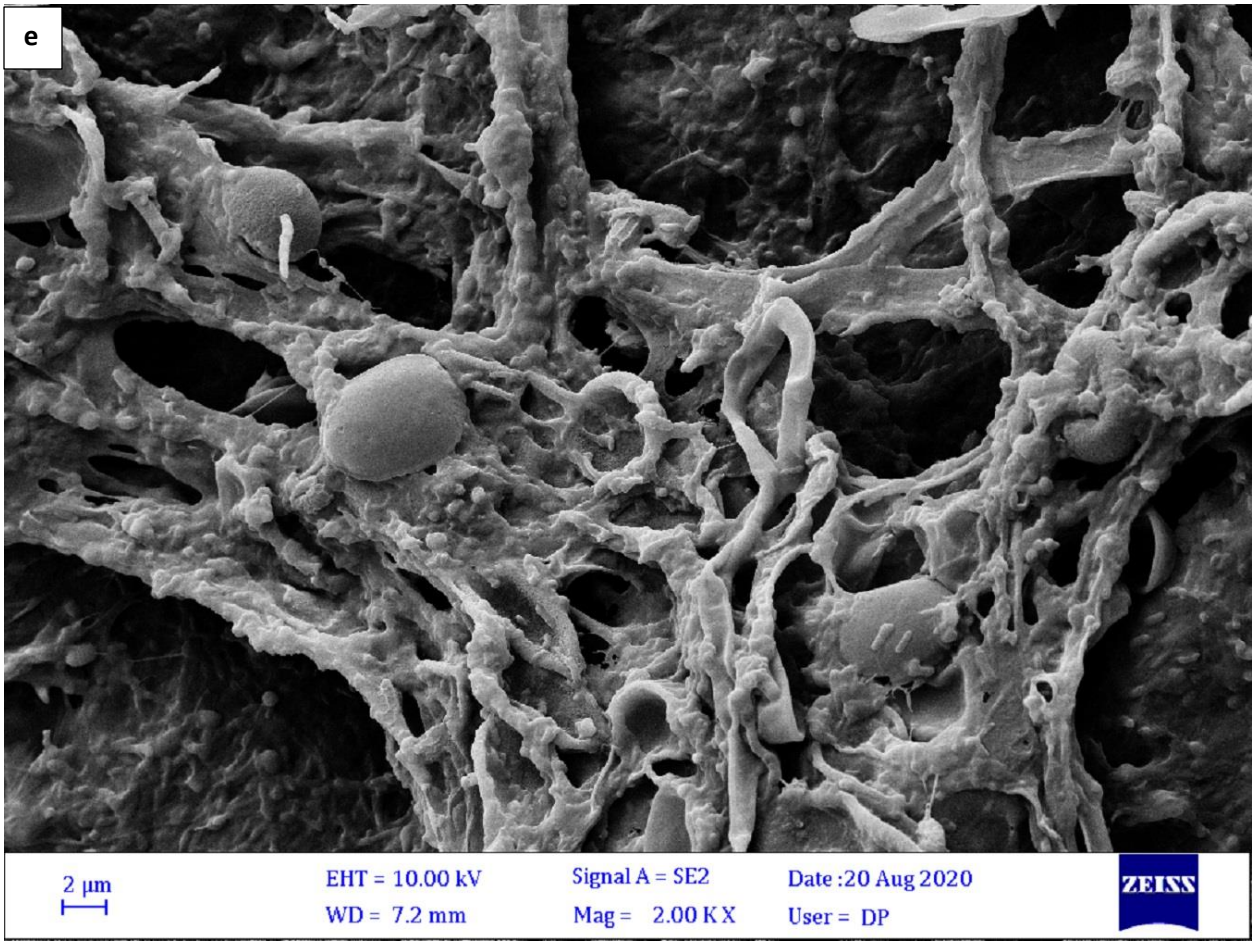
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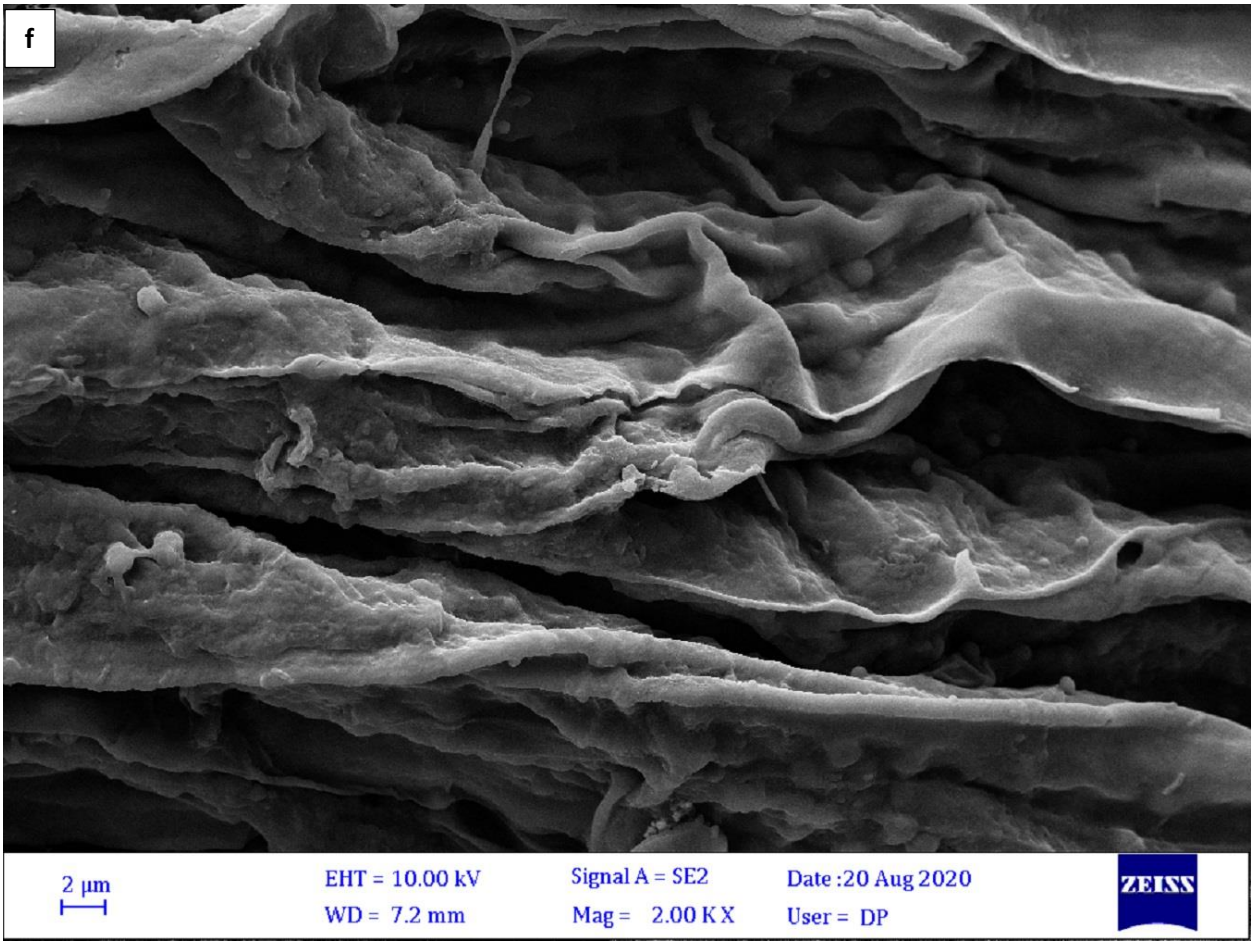
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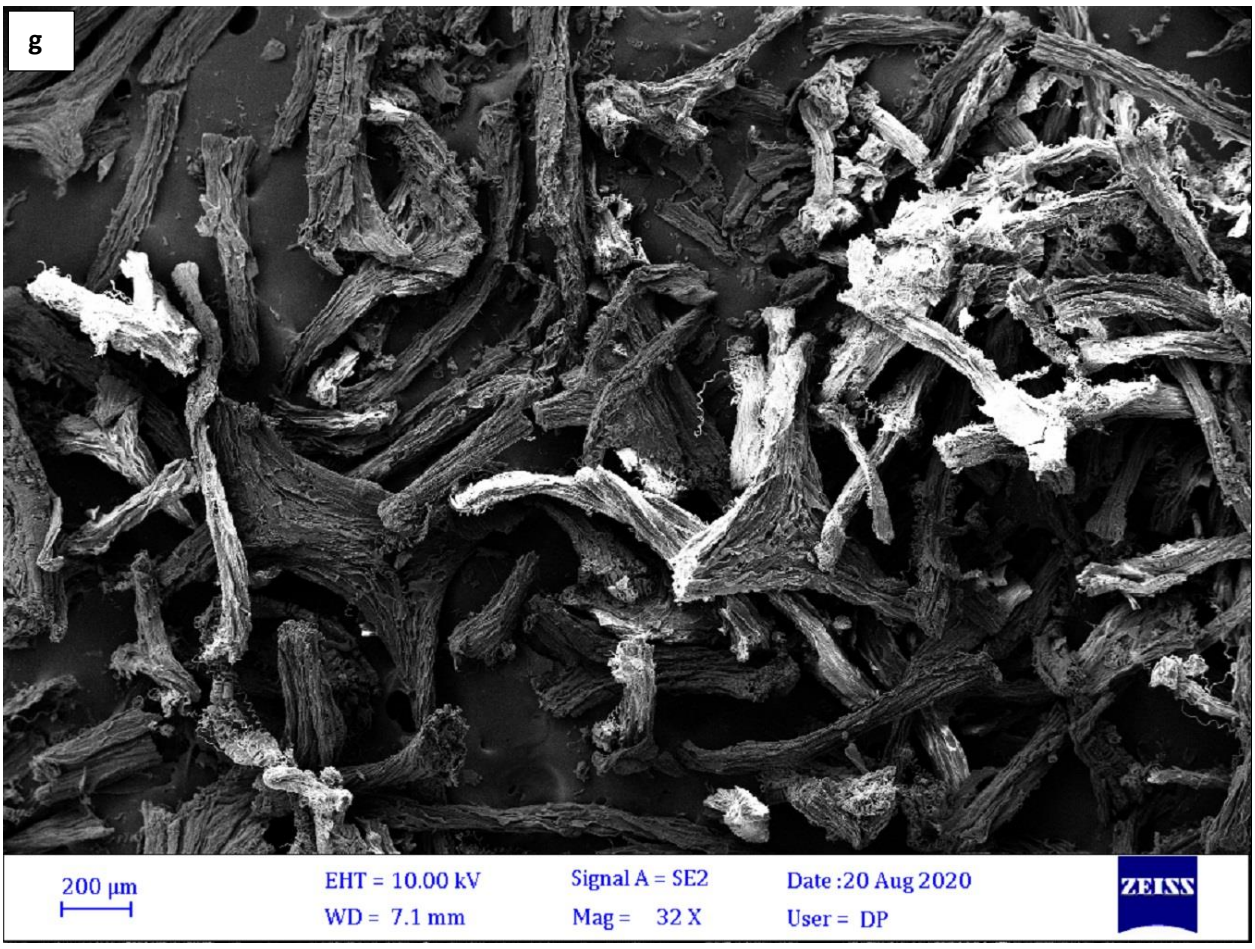
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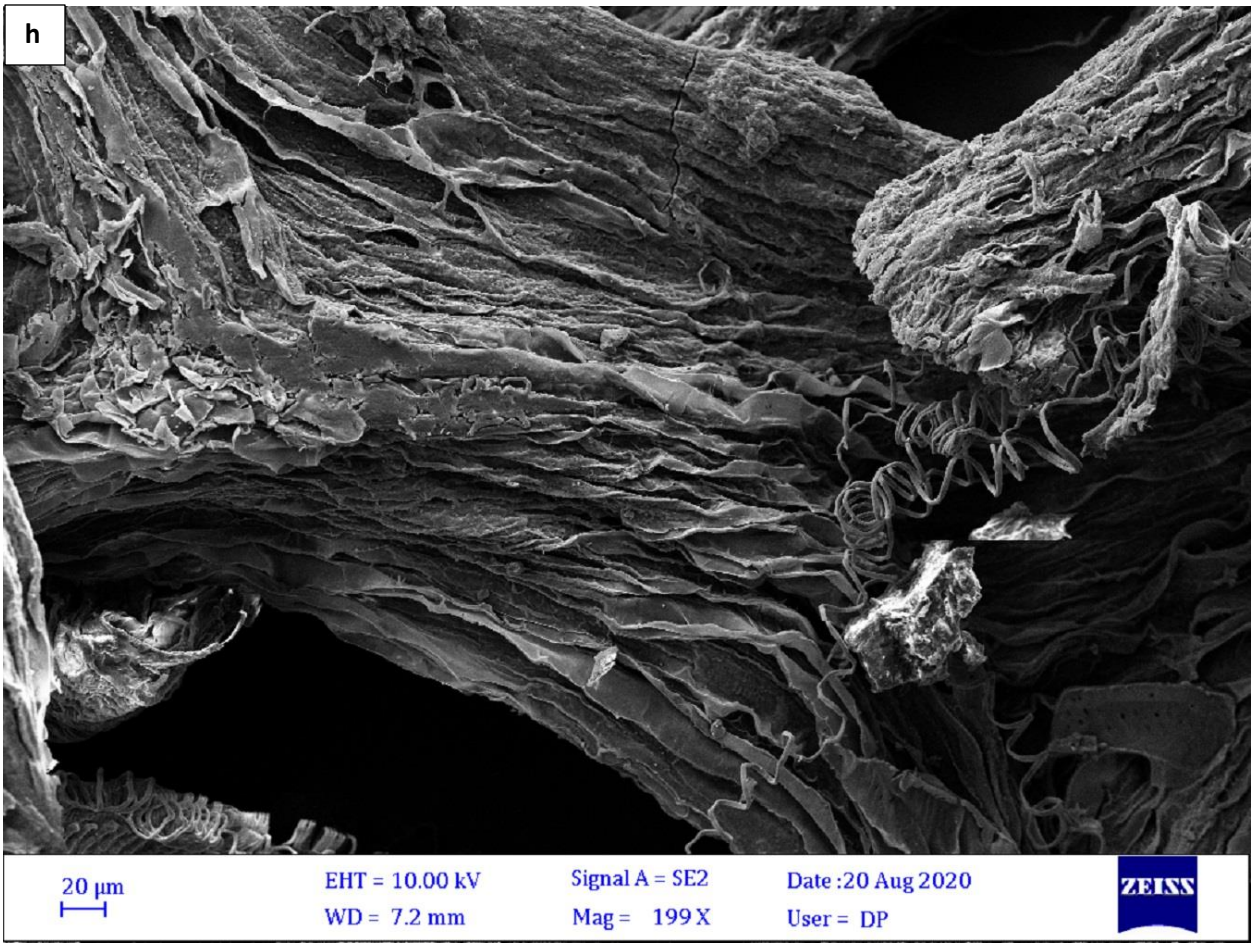

Fig. 5. FE-SEM photographs of un-immobilized, immobilized, and used *L. cylindrica*. (a & b) FE-SEM photographs of un-immobilized *L. cylindrica*. (c-f) immobilized *L. cylindrica*, and (g & h) used *L. cylindrica*.

4. Conclusion

Simultaneous partial denitrification and anammox process using *L. Cylindrica* as a solid carbon source and biofilm carrier in an up-flow packed bed biofilm reactor was successfully established. An average total nitrogen (TN) removal efficiency of 98% was achieved with influent NH_4^+-N and NO_3^--N concentration of 100 mg L^{-1} using the methodology outlined in this paper. *L. cylindrica* as a slow-release carbon source supported co-existence of partial denitrification and anammox

processes in the DEAMOX system and retention of anammox bacteria was successfully achieved due to biofilm granulation. The results imply that solid phase partial denitrification and granulation of anammox bacteria was achieved using *L. cylindrica* as a carbon source and biofilm carrier, respectively. Complete removal of NO_3^- supported the hypothesis that solid carbon source can support denitrification of NO_3^- produced through the anammox process.

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