Gel immobilization: a strategy to improve the performance of anaerobic ammonium oxidation (anammox) bacteria for nitrogen-rich wastewater treatment

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Abstract

Anaerobic ammonium oxidation (anammox) process appears a suitable substitute to nitrification-denitrifcation at a lower C/N ratios. Anammox is a chemolithoautotrophic process, belong to phylum *Planctomycetes,* and they are slow growing bacteria. Different strategies, e.g., biofilm formation, granulation and gel immobilization, have been applied to maintain a critical mass of bacterial cells in the system by avoiding washout from the bioreactor. Gel immobilization of anammox appears the best alternative to the natural process of biofilm formation and granulation. Polyvinyl alcohol-sodium alginate, polyethylene glycol, and waterborne polyurethane are the most reported materials used for the entrapment of anammox bacteria. However, dissolution of the gel beads refrains its application for long term bioprocess. Magnetic powder could coat on the surface of the beads which may increase the mechanical strength and durability of pellets. Application and problem of immobilization technology for the commercialization of this technology also addressed.

Keywords: Anammox; Gel immobilization; Polyethylene glycol; Polyvinyl alcohol-sodium alginate; Waterborne polyurethane.

Contents

1.   Introduction

Nitrogen compounds in wastewater discharged directly into rivers, canals, and lakes can cause severe threats to the water bodies and human health. Eutrophication of lakes, rivers and other water bodies is the primary outcome of nitrogenous wastewater (Quan et al., 2011). The use of nitrogen cycle players for nitrogen removal from wastewater has been reported in common, and function of different nitrogen cycle bacteria is shown in Fig.1. The different nitrogen cycle players have symbiotic or competitive functions in wastewater treatment plants. Customarily, nitrogenous species have been removed from wastewater through the conventional process of nitrification-denitrification (N/DN) (Choi et al., 2020; Yang et al., 2019). Higher carbon to nitrogen (C/N) ratio is an essential parameter for the successful application of the conventional N/DN process. However, some wastewater has characteristics of high ammonium concentration and lower organic matter which increases the cost of treatment due to the addition of artificial carbon source for the growth of heterotrophic denitrifying bacteria (Kimura et al., 2011a; Ribeiro et al., 2018; Quan et al., 2011).

Fig. 1

The advancement in the nitrogen removal process is desired under the conditions of lower C/N ratio. In the late twentieth century, a new mean of anaerobic ammonium oxidation (anammox) was reported (Mulder et al., 1995). Later studies proved that anammox is an active biological pathway to treat nitrogenous wastewater (Jetten et al., 2001). Generally, the anammox reaction produced di-nitrogen gas with an amount of nitrate by using nitrite and ammonium as an essential substrates (Mulder et al., 1995). Autotrophic nature of anammox growth, sluggish growth behavior, and lower cell yield could increase the start-up period of wastewater treatment process (Chen et al., 2015; Wang et al., 2020). So, for the slow-growing anammox, more diversifying techniques such as the design of the reactor, restriction of cell mass by immobilization and proper functioning conditions (Bae et al., 2015) reduce the anammox initiation time and maintain higher microbial biomass for stable nitrogen removal from wastewater. Suspended growth is advantageous because of a suitable distribution of substrate in the reactor for effective reaction (Bae et al., 2015). However, the major challenge in the suspended growth wastewater treatment is the retaining of anammox biomass in the reactor (Magrí et al., 2012). Plodding growth of anammox makes the cell vulnerable to washout from the reactor (Zhu et al., 2014; Quan et al., 2011). Thus, it is necessary

to lessen the loss of slow-growing anammox and to make anammox as dominate bacteria in the reactor's microbial community (Chen et al., 2016) for the sustainability of the removal process.

Cell immobilization is a new technique that attracted the scientific community as a competitive alternative to avoid cell washout from the reactor (Lu et al., 2018). The immobilization can protect anammox cells from inhibitory effects caused by substrates, organics, and inorganic compounds. Also, the immobilization of microbial cell is preferred because of the consistent and effective retention of anammox biomass (Bae et al., 2015). So, immobilization of anammox is looking to have a high potential to improve the performance of anammox bacteria for wastewater treatment. The techniques practiced for the immobilization of anammox cell includes development of granules by a natural process (Xue et al., 2020), attachment of anammox microbial cell on an exterior of the carrier (Biofilm) (Ni et al., 2010; Tsushima et al., 2007), and entrapment of anammox biomass inside of gel (Liu et al., 2020; Wang et al., 2020). Among these methods, entrapment of anammox microbial cell inside of gel carrier is more advantageous in terms of shorter start-up time (Ali et al., 2015; Bae et al., 2015), maintaining proper density of cell, and protection against the unfavorable environment (Hsia et al., 2008; Zhu et al., 2009). In the developed countries like USA and Japan, nitrification and denitrification with gel immobilization have already been practicing (Isaka et al., 2007). However, full-scale or pilot-scale study of anammox gel immobilization, except with polyethylene glycol gel (PEG), is missing in the literature. Previous studies depicted good removal performance and short start-up period of anammox with gel immobilization under laboratory conditions (Tuyen et al., 2020). As anammox bacteria are very sensitive to changing conditions in the surrounding environment, the immobilization techniques must be studied under real wastewater conditions. The growing number of publications in recent years on anammox immobilization increases the interest of wastewater engineers working on anammox. The broad review of this topic is the need of the time. The review article published by Manonmani and Joseph (2018) focused mainly on the characteristics of different materials and factors affecting anammox growth inside of gel beads (Manonmani and Joseph, 2018). Another review article partially discussed materials, techniques, and start-up of anammox with immobilization technology in one heading (Ali and Okabe, 2015). However, a critical review about the detail of anammox immobilization mechanisms, the behavior of different materials and associated loopholes, and full-scale applications needs to be demonstrated.

This review aims to, 1) highlight the opportunities and challenges in using cell immobilization for enhancing the efficacy of wastewater nitrogen removal by anammox bacteria, 2) disclose the existing technologies for the immobilization of anammox and examine the sustainability of different gel carrier, 3) discuss the application of anammox and partial nitrification (an essential pre-treatment step to achieve the required ratio of ammonium and nitrite for successive anammox process) with immobilization technology, 4) highlight the economic, safty and environment concerns of immobilization technology.

2. Anammox bacteria and their metabolic process

Anammox bacteria are belonging to phylum *Planctomycetes* and metabolized very slowly (Strous et al., 2006). The physiology of anammox bacterial cell showed that cytoplasm is divided by three membranes. The outermost layer is called cytoplasmic membrane which lined inside of the cell wall, the second membrane known as intracytoplasmic membrane separates the paryphoplasm from the riboplasm, and the third membrane separates the machinery of anammox known as anammoxosome from the riboplasm (Van Niftrik et al., 2008). Further, different anammox related genera have a diverse range of cell size. The anammox cells belonging to the genera *Candidatus Brocadia fulgida* and *Candidatus Kuenenia stuttgartiensis* have size about 880 nm in diameter with 61% of cell volume occupied by anammoxosome. The *Candidatus Scalindua spp.* has an average diameter of around 950 nm. The largest cell diameter is about 1100 nm which is reported to the genus *Candidatus Anammoxoglobus propionicus* (Van Niftrik et al., 2008).

Anammox species have versatility in their metabolism. Although it was assumed that anammox bacteria oxidize ammonia with nitrite as an essential electron acceptor, recent studies reported that anammox could use alternative electron acceptor rather than nitrite (Liu et al., 2008). Guven et al. (2005) described that organic acids (propionate) have competition with ammonium for nitrite as an electron acceptor. The later study proposed the name of that novel anammox as *Anammoxoglobus propionicus* (Kartal et al., 2007). With the freshly discovered anammox strain, it was concluded that the anammox process has a comprehensive application for the removal of nitrogen and organic acids from wastewater. It was also found that iron could be oxidized with nitrite as an electron acceptor (Strous et al., 2006).

$$Fe_2^+ + NO_2^- \rightarrow Fe_3^+ + N_2 \quad (1)$$
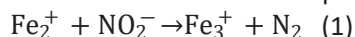
Another study postulated the anaerobic realization of elemental sulfur and nitrogen gas from ammonium and sulfate (Fdz-Polanco et al., 2001), but the exact mechanism of this conversion was not reported. A few years later, purified strain by per-coll density gradient centrifugation was applied for the removal of ammonium and sulfate. Completely autotrophic removal of ammonium and sulfate was observed with pure culture, and phylogenetic analysis confirmed that the new strain had 92% similarity with *Planctomycetes* (phylum to which anammox belongs), provisionally named as *Anammoxoglobus sulfate* (Liu et al., 2008). *Anammoxoglobus sulfate* used sulfate as an electron acceptor with ammonium as an electron donor to produce nitrite and elemental sulfur. After nitrite concentration becomes high, the traditional anammox process started and caused the removal of remaining ammonium and nitrite (Liu et al., 2008). Chemical mechanism of autotrophic sulfate-reducing ammonium oxidation reaction is given in Eq. 2, and further traditional anammox process removes the remaining ammonium with nitrite as an electron acceptor (Eq. 3) (Liu et al., 2008).

$$SO_4^{2-} + NH_4^+ \rightarrow NO_2^- + S + 2H_2O \quad (2)$$
$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (3)$$
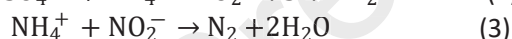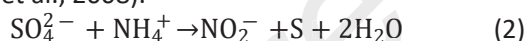
Recently, it has also been reported that anammox can perform dissimilatory nitrate reduction to ammonium DNRA (Yang et al., 2019). The metabolism of anammox bacteria are environment-dependent and are governed by the existing pollutants, either the contaminant is ammonium, propionic acid, urea, sulfate, etc., which shows the metabolic versatility of anammox species. The detailed mechanism of anammox metabolism was explored by Kartal et al., (2011) in anammox species *Kuenenia stuttgartiensis*. The nitric oxide instead of hydroxylamine is the precursor of hydrazine (Fig.2A). The cd_1 nitrite/nitric oxide oxidoreductase (NirS) has been reported as responsible for nitrite reduction to NO (Fig.2A). However, in "*Ca*. Brocadia sinica" hydroxylamine is the precursor of hydrazine instead of NO (Fig.2B) (Oshiki et al., 2016). Though the responsible genes for the production of hydroxylamine are yet unknown, it has been proposed that the Hao like protein may cause the reduction of nitrite into hydroxylamine (Fig.2B). The presence of Nrf enzyme in anammox bacteria has also been reported, but their role is still ambiguous (Kartal and Keltjens, 2016). Nrf enzyme in anammox metabolism plays a role to detoxify hydroxylamine and NO_2^- into ammonium (Fig.2B) (Yang et al., 2019) or organic compound oxidation by using nitrite as an electron acceptor to generate ammonium (Kartal et al., 2013). However, the mechanism and involved protein for the production of nitrate is an open question. The production of nitrate is proposed to keep the electron balance. But, this hypothesis is proposed theoretically, which is missing the experimental proof.

Fig. 2

3.   Cell immobilization: a strategy to improve microbial wastewater treatment

3.1 What is cell immobilization?

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The artificial technique for the immobilization of cells is defined as "The confinement of microorganisms within the specific material to enhance the time course of the process and make sure the presence of a microbial cell in the system" (Leenen et al., 1996). Through the immobilization, the retention time of the cell can be increased and eventually amplifies the process efficiency. After the discovery of anammox, main objectives of many researchers were to address the washout problems of anammox. Different strategies were practiced ranging from the natural process of granulation, biofilm, and artificial gel immobilization techniques.

3.2 Different approaches for immobilization of anammox

In general, the immobilization of microorganisms can be divide into three methods (1) granulation, (2) natural adsorption to a support matrix (Biofilm immobilization), and (3) entrapment into cross-linked polymers (Gel immobilization). That means the microorganism also has the property to adhere on the solid surface, and this happens as long as the conditions are appropriate.

3.2.1   Granulation

Self-aggregation is also known as self-immobilization of bacteria without any support materials (Gómez-Basurto F et al., 2020). The granules have a clear spherical structure with diameter of 0.14–5 mm and settling velocity of 18–100 m/h (Schmidt and Ahring, 1996). The granules settling ability closely related to particle size and density, while the surface hydrophobicity of granules was also higher than activated sludge (Li et al., 2013; Liang et al., 2017; Zheng et al., 2005). The granulation also leads to a variety of strains which means the coexistence of aerobic and anaerobic chamber, decreased specific surface area and concentrated carbohydrates and proteins in the extracellular polymeric substances, better tolerance to various types of high-strength and toxic wastewaters (Hamza et al., 2016; Liu et al., 2002, 2015; Lourenço et al., 2015; Zheng et al., 2005; Zhu et al., 2015). The formation of granules depends on various factors such as input power, nitrogen loading rate, and reactor configurations (Li et al., 2014; Tang et al., 2017) also biological interaction of different microorganisms (Manonmani and Joseph, 2018). Different reactor configurations have been used for the formation of granules. Usually, up-flow sludge blanket reactor (UASB) (Graaf et al., 1996; Tang et al., 2010), sequencing batch reactor (SBR) (´Lopez et al., 2008; Arrojo et al., 2006), gas lift reactor (Dapena-Mora et al., 2004), anaerobic baffled reactor (ABR) (Ismail et al., 2019a),and membrane bioreactor (Li et al., 2014) have been applied for granules formation of anammox bacteria.

The entrapment of gas inside big size granules reduces the granular density, which leads toward the floatation of anammox sludge and then the poor nitrogen removal efficiency (Chen et al., 2010;Xue et al., 2020). The control of granule size in the running system is also tedious (Li et al., 2018). Different strategies have been proposed for the control of granule floatation such as hydrodynamic control, gas automatic circulation, and high hydraulic loading (Li et al., 2018). Further, input power is also reported as a strategy to control the size of granules (Arrojo et al., 2006). Li et al. (2014) reported the dispersion of anammox particle at the input power of 0.8 kW/m³, which reduced the mean particle size below 144 um. However, the decrease of input power from 0.8 to 0.4 kW/m³ was proved to be effective to upgrade the particle size and achieved 80% of nitrogen removal efficiency. Very care is needed for hydrodynamic control, gas automatic circulation, and high hydraulic loading strategy because a little increment or decrement of the control factor can lead to the failure of the treatment system. Also, the proper selection of the input power to control the required size of granules needs technicality and much caution. Besides, taken out of floated anammox granules and breaking them into small granules is also laborious work.

3.2.2   Biofilm formation or adsorption

Biofilm formation is capable of reducing the washout of microbial biomass from the system with effective sludge bulking, and good tolerance to environmental pressure (Gilbert et al., 2015). The attachment of

anammox sludge on the wall of the reactor gets the attention of many researchers toward biofilm technology of anammox. One of the critical points in biofilm formation is the presence of a carrier or surface for the development of biofilm. So far, the use of different carrier materials has been practiced for the enrichment of anammox by developing biofilm on the carrier surface (Li et al., 2018). The materials reported as a support for the development of anammox biofilm include non-woven fabric membrane (Ni et al., 2010), zeolite particle (Fernández et al., 2008), sponge and volcanic rock (Lu et al., 2017), and Anoxic Kaldnes K3 (Kowalski et al., 2017). The size of the carrier is an essential parameter for the development of biofilm (Adams et al., 2020). Carrier size of 15 mm exhibited an excellent result compared to large and small carrier size (Ahmad et al., 2017). The biofilm techniques are also suitable for the provision of ammonium rich micro-environment due to ammonium adsorption by carrier (Lv et al., 2019). The nitrogen removal rate of 1047.5 mg N/l/d was achieved after 8 months of operation with nitrogen loading rate of 1263 mg N/l/d by using non-woven fabric membrane (Ni et al., 2010). The use of zeolite particle as biofilm support material could decrease the biomass washout up to 3 mg/L of effluent (Fernández et al., 2008). The application of sponge and volcanic rock could improve the nitrogen removal efficiency and reached 93.4% after 105 days of operation (Lu et al., 2017). Recently, anaerobic baffled biofilm reactor has been applied for the enrichment of anammox by using modified carriers (non-woven fabric wrapped around the honey-comb like material) and achieved reactor start-up within 47 days (Wang et al., 2019). The maximum nitrogen removal rate of 0.9 kg N m^{-3} d^{-1} was achieved with nitrogen loading rate of 1.0 kg N m^{-3} d^{-1} after 161 days. Ahmad et al. (2017) had studied the development of biofilm and reported that carbohydrate, protein amid and deoxyribonucleic acid/ ribonucleic acid (DNA/RNA) were the responsible factors for the growth of biofilm. There are two mechanisms for the anammox attachment on the carrier surface. First one is the production of EPS which act as glue (Ismail et al., 2019b) and the second is the generation of active site on the carrier (Li and Cheng, 2019).

As for the concern of biofilm technology, the biofilm initiation to maturity is a long time process. The presence of undesired conditions is sensitive to biofilm, which can cause the detachment of bacteria and reduce the process performance. Also the dissaggregation and pathogenic induce cell death can cuase the detachment of cell cluster from the biofilm surface.

3.2.3   Gel entrapment

Except bacterial biofilm and granulation, there is also an artificial approach to immobilize the biomass. The incorporation of bacterial biomass within gel materials is a novel and advantageous approach (Chen et al., 2015). The bacterial cells could be fixed inside the gel beads artificially. The net-like structure of the beads could provide a suitable environment for the growth of entraped anammox (Wang et al., 2020) by avoiding the leakage of cells from inside the beads.The microbial cell and enzyme incorporation by different gel materials without affecting their activity increase the application potential of immobilization techniques in the wastewater treatment. This incorporation technique effectively avoided biomass washout and enhanced microbial activity (Bae et al., 2015b; Chen et al., 2015). The artificial incorporation technique is a simple, practical, and effective approach. Different natural and artificial materials are ideal carriers for the immobilization of enzymes and bacteria (Wang et al., 2020). The natural materials are easy to biodegrade due to organic in nature, while synthetic materials are resistant to the biodegradation. So, in wastewater treatment, artificial materials are more attractive because of their stability and excellent durability against the shock loading. The details of the well-reported materials used for anammox immobilization are discussed in the following section.

4.   Why is gel immobilization advantageous?

The biofilm formation and granulation process are based on the exploration of the natural properties of microorganisms, which have some demerits. Small granules may cause the saturation of oxygen in the single step of partial nitrification-anammox process; also, large granules limit the transfer of substrate in

the core of pellets. The gas pocket developed inside the granules may cause the floating of granules and ultimately leads toward the lower efficiency of the system (Song et al., 2017). Similarly, the properties of carriers, especially surface properties, would affect the development of biofilm and the process efficiency. Moreover, large carrier size reduces the penetration of substrate deep inside the biofilm, and small carrier size causes the detachment of biofilm under the influence of large shear force (Ahmad et al., 2017). The biomass detachment from the specific surface under limited conditions reduces the process efficiency. The increase in the shear stress decreases the nitrogen removal efficiency due to the obliteration of the anaerobic zone and ultimately washout of anaerobic microbes due to the detachment. Therefore, in the wake of the above-described loopholes of granulation and biofilm technologies, gel immobilization seems a better alternative approach to shorten the start-up time of the anammox process and improve anammox bacterial activity. The retention of biomass was enhanced by immobilization of anammox bacteria within the gel material even at a short hydraulic retention time. The easy liquid and solid separation by gel immobilization can be achieved, and biomass washout from the treatment system can be reduced (Tuyen et al., 2020). Furthermore, the stirring can remove the formation of the gas pocket inside the gel beads. The anammox could perform the removal process within the gel beads efficiently (Adeel et al., 2019). The gel cubes are easy to handle for the further investigation of anammox characteristics such as anammox identification and visual observation of the anammox process (Isaka et al., 2007). Optimal temperature range for the immobilized biomass can be extended using gel beads. Moreover, the microbial cell can be easily separated from inhibitory substances in the surrounding environment.

The comparison of the start-up period of different methods is depicted in Table 1. The start-up time of anammox was 20 days when entrapped inside of the gel while the start-up of anammox with granulation technology was achieved within 65 days. Further, activated sludge was entrapped by gel materials for the enrichment of anammox, and the anammox process start-up was 42 days which is also shorter compared with enrichment of anammox from anaerobic granular sludge. Wang et al., (2019) used aerobic activated sludge for the enrichment of anammox in anaerobic baffled biofilm reactor and the start-up of anammox was observed after 47 days. The use of anaerobic granuler sludge for the enrichment of ananmmox failed to observe anammox activity even after 57 days, but the addition of external anammox sludge quickened the start-up process (Wang et al., 2018). The possible phenomenon of the shorter start-up period of anammox process using gel immobilization is the provision of a suitable anaerobic environment inside of gel beads and avoidance of oxygen inhibition.

5.   Gel materials used for the immobilization of anammox

Nitrogen removal activities by immobilized cell were reviewed. It was concluded that the anammox biomass immobilizing in the gel carrier were protected from colloidal solids present in the wastewater (Magrí et al., 2012). Various synthetic polymers including waterborne polyurethane (Chen et al., 2015), Polyethylene glycol (Isaka et al., 2007), polyvinyl alcohol (PVA) (Magrí et al., 2012), and mixed media of polyvinyl alcohol with sodium alginate (PVA/SA) (Hsia et al., 2008) have been applied for the entrapment of anammox bacteria to reduce the effects of some inhibitor such as nitrite and best substitute to avoid the washout problems (Isaka et al., 2006; Magrí et al., 2012).

5.1 PVA an PVA/SA

Polyvinyl alcohol/sodium alginate was used for entrapment of microbial cell over the past decades. Carboxyl group of SA and hydroxyl group of PVA would develop bonding which made the rugged structure of the carrier (Zhu et al., 2009). The concentration of pre-polymer and reinforcing procedure of the gel carrier affect the micro-pores of the beads (Quan et al., 2011) and the exchange of the substrate between solid and liquid phases. Cells can be entrapped within the PVA by two mechanisms. The dissimilarity between these two methods is in the second stage of immobilization, which involves the hardening of

beads accomplished by freezing or chemical means (Magrí et al., 2012). The chemical way of immobilization is more sustainable than the freezing method, especially when the process temperature is adjusted above 30 °C. However, the duration and concentration of cross-link solution affect the stiffness of the beads and ultimately process efficiency. It was reported that insertion of SA improved the substrate transfer and microspores structure of the PVA beads (Chen et al., 2015). Most reported solutions used for the crosslink included calcium chloride (Ali et al., 2014; Zhu et al., 2009), calcium chloride/sodium nitrate (Hsia et al., 2008; Quan et al., 2011), and calcium chloride/boric acid (Bae et al., 2015). It was reported that anammox activity was less affected when calcium chloride used as a crosslink solution (Zhu et al., 2009). Conversely, Crosslinking time and concentration of the solution are crucial factors if it consists of boric acid, which may increase the time of process start-up and stability of the beads.

The porous structure of PVA/SA which is confirmed by the SEM micrograph facilitated the transformation of the substrate into the gel beads (Quan et al., 2011; Zhu et al., 2009). Microscopic observation of PVA beads showed their smoothed surface with a diameter of about 3-4 mm (Hsia et al., 2008). Entrapment of anammox by PVA and SA have been practiced from a few years back (Hsia et al., 2008; Zhu et al., 2014). The concentration of PVA affects the properties of the beads and ultimately process performance. The PVA of 4-6% is considered the most preferred concentration to immobilize anammox bacteria.

5.2 Waterborne Polyurethane

There is another material known as waterborne polyurethane which has been applied for the immobilization of anammox bacteria. A comparative study was conducted to verify the mechanical strength, stability, and long term application of different materials on anammox immobilization including waterborne polyurethane (WPU), PV, PVA/SA, and SA (Chen et al., 2015). It was concluded that WPU has the best stability and mechanical strength compared to other synthetic and natural materials (Chen et al., 2015). WPU revealed higher mechanical stability compared to PVA, PVA/SA, and SA, which showed the clear superiority of WPU for the immobilization of microbes. Unlike other material such as carboxymethyl cellulose (CMC), PVA, and PEG, WPU also has superiority in term of mechanical stability (Chen et al., 2015). The apparent dissimilarity in the mechanical stability of different materials is due to the variance in the properties of immobilized materials and the way of cross-linking of immobilized granules (Chen et al., 2016). WPU, when compared with PEG, has a different chemical structure and relative molecular mass. The WPU has small space in their three-dimensional structure, which makes WPU less absorbance of water during swelling and ultimately increases the mechanical strength of immobilized granules (Chen et al., 2016). Another significant parameter is the absence of the spherical shell on the WPU granules, which increases the mass transfer property of WPU granules (Chen et al., 2015).

Moreover, WPU immobilized anammox have lower half-saturation constant (K_s), which increases the substrate affinity of immobilized granules and aids the anammox process in the competitive environment (Chen et al., 2016). During the continuous experiment, it was reported that WPU has an excellent sludge retaining ability and good mechanical strength (Chen et al., 2015). WPU provided a suitable environment for the proliferation of anammox inside the gel beads, which is the strong evidence for the increased of anammox activity inside the gel carrier (Chen et al., 2015). Despite the high performance of WPU as an immobilized material, there were fewer studies utilized this material for anammox immobilization. So, WPU is demanding more research for their application in the full-scale treatment arrangements.

5.3 Polyethylene glycol gel

Anammox bacteria entrapped in the PEG pre-polymer were also used for the removal of nitrogen from wastewater. The colorless PEG material provides an easy characterization of anammox consortia with the changing of the beads color. In the initial of the experiment, the small light brownish-red aggregates can be seen and later the whole cube turned into bright red (Isaka et al., 2007). The different aspects of anammox process were examined under the immobilization of anammox within the PEG. The

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performance of immobilized bacteria depends on the distribution of ammonium inside the gel material. The calculation of ammonium distribution coefficient was carried out according to the equation reported in the study of Sumino et al. (1992).

$$K_p = \frac{C_s'}{C_s} = \frac{V_o}{V_1 - V_o} \frac{C_{so} - C_{s1}}{C_{s1}} \quad (3)$$

Where Kp represents the ammonium distribution coefficient, V_o and V_1 portray the volume of the solution before and after the addition of gel cubes, C_{so} and C_{s1} are the ammonium concentrations before and after the addition of gel cubes. The ammonium distribution coefficient of 2.2 is ideal for the negatively charged PEG (Sumino et al., 1992). Different molecular weights PEGs are available in the market. The price is an important concern when dealing with PEG, which needs further research on this material for the provision in the lower-income country with an affordable price.

6. Application of cell immobilization in anammox and Partial nitrification

6.1 Application of immobilized anammox

The full-scale application of immobilized anammox was only reported with PEG (Isaka et al., 2017). However, other materials also had great potential to upgrade the immobilization to pilot scale and then full-scale application. The lab-scale study and the feasible option of anammox application in the future were disclosed in this section. The brief results and objectives from different studies have been compiled in Table 2. The immobilization can effectively achieve the rapid start of anammox by alleviating the washout of slow growth anammox bacteria (Ali et al., 2015). Anammox bacteria were sheltered from the surrounding, and the loss of anammox cell from the reactor was avoided by immobilizing in the PVA/SA (Hsia et al., 2008; Lu et al., 2018). The rapid start-up of anammox process was achieved by the conditions of immobilization, initial higher concentration of anammox sludge, and retaining of microbial biomass due to entrapment (Quan et al., 2011).

Recently, activated sludge (AS) immobilized within the PVA/SA gel beads was applied to initiate the anammox process, specifically in the countries with the absence of full-scale anammox treatment process (Cho et al., 2017). The anammox activity with pre-cultured anammox bacteria (PAB) started very shortly after the operation of the system, but the activity of anammox started after 93 days with the AS. The PAB reached an average nitrogen removal rate (NRR) of 0.35 Kg-N/m³/day in the last phase of the operation while the NRR of 0.36 Kg-N/m³/day with AS was achieved with immobilization techniques. The bacterial composition on the base of 16S rRNA of both reactor was entirely different during the start-up period, but at the end of the operation, the anammox bacterial population was comparable in both reactors (Cho et al., 2017). Another point of importance for the application of anammox in the place where there is no full-scale application is to transfer the anammox sludge to the long distance. It was reported that anammox immobilized and stored at the temperature of -8 °C for 17 h recovered their activity very quickly (Magrí et al., 2012). Transportation of PVA/SA immobilized anammox sludge, preserved at room temperature, is another feasible option to promote the application of anammox (Ali et al., 2014). Therefore, the immobilized anammox can be transferred to a long distance without the fatal damage of their activity. Immobilizing anammox within the PVA/SA beads strategically increased the nitrogen loading rate (NLR) from 0.32±0.04 to 1.26±0.04 within three phases (cell lysis phase, anammox start-up phase, and anammox stability phase), but failed to achieve the reported stoichiometry ratio probably due to AOB which can adopt lower dissolved oxygen or from the oxidant (superoxides or hydroxyl radical) resulted from the enrichment process of biomass (Bae et al., 2015).

After the lag phase, anammox performance was increased and an average nitrogen conversion rate of 3.4 kg N/m³/d was achieved from 0.2 kg N/m³/day after 60 days which indicated the growth of anammox in the PEG gel carrier (Isaka et al., 2007). The maximum nitrogen loading rate of reactor with immobilized anammox have been increased to 14.76 kg-N m⁻³ d⁻¹ while control reactor only achieved 5.77 kg-N m⁻³ d⁻¹.

The nitrogen removal rate about 13.9 kg m^{-3} d^{-1} was achieved with immobilized anammox as compared to control 5.3 kg m^{-3} d^{-1} (Wang et al., 2020). Furthermore, the effect of initial biomass concentration also was verified, and the start-up period was reduced by increasing the biomass concentration in the gel carrier. The biomass to gel carrier ratio of 1.4 % w/v dramatically reduced the start-up time, and 11 kg N/m^3-carrier nitrogen conversion rate was achieved within 25 days, which was very short compared to 62 and 39 days at the concentrations of 0.24 and 0.52 % w/v, respectively (Isaka et al., 2007).

The color of PEG beads changed from light brownish-red to bright red, confirming the growth of anammox in the gel carrier (Isaka et al., 2007). Similar to nitrifying bacteria, anammox bacteria can also grow in the core of bead (Isaka et al., 2007). The successful operation of anammox wastewater treatment depends on the activity of anammox biomass. T-RFLP analysis showed two peaks (40 and 285 bp) characterizing *HPT-WU-N01* clone and *HPT-WU-A01* clone, while clone *HPT-WU-A01* became the dominant anammox population in PEG prepolymer carrier (Date et al., 2008). It was observed that anammox population highly correlated with anammox activity (Date et al., 2008). As wastewater also contains a complex mixture of heavy metals and other chemicals. The laboratory experiment to study the effects of different heavy metals on anammox activity under gel immobilization condition was also disclosed. Up to 2 mg/L Zn, Ni, Co, and Cu did not affect the anammox activity, but as the concentration increased anammox activity showed a decreasing trend. The effects of Zn, Ni, Co and Cu metals on anammox activity were reversible, while the effects of Mo were irreversible (Kimura and Isaka, 2014). The addition of methanol irreversibly inhibited the immobilized anammox (Isaka et al., 2008b). The nitrogen gas bubbles formation is another point of consideration to avoid the washout of the sludge. Nitrogen gas bubbles were observed on the exterior of the carrier, but stirring can remove the gas from the carrier peripheral and settle down the carrier (Isaka et al., 2007). Moreover, the salinity level in the wastewater can also affect anammox performance. The immobilized anammox could adopt as high as 10g/L of salinity (Liu et al., 2020). The concentration of inorganic carbon (IC) was directly proportional to the anammox activity immobilized by PEG (Kimura et al., 2011b). As the concentration of IC increased, the activity of anammox also increased at the same time.

Zhu et al. (2014) reported that nitrogen removal efficiency of immobilized anammox was higher when compared with suspended growth. Nevertheless, the laboratory scale study performed well with immobilization, but the scenario of full-scale wastewater treatment is always different. The next state of the art strategy is required to promote immobilization technology to full-scale scenario.

6.2 Application of immobilized partial nitrifying bacteria

Conversion of ammonium into nitrite by suppressing the growth of nitrite reducing bacteria is known as partial nitrification (nitritation). The nitritation process was carried out by slow-growing autotrophic bacteria. It was reported that SA beads were proved as an excellent carrier material for partial nitrification (Yan and Hu, 2009). The growth of nitrite-oxidizing bacteria (NOB) is another point of concern which decreased the stability of the PN process and ultimately anammox process also. It is an accepted fact that anammox uses ammonia and nitrite as a particular substance, so the suppression of NOB is required to avoid the nitrite oxidation. Inhibition of nitrite oxidizer can be achieved by controlling the DO (Chou et al., 2012b). Immobilization of nitrifying bacteria within the PVA-SA carrier can suppress the growth of NOB due to the diffusion limitation of DO (Chou et al., 2012b). Because immobilized beads affect the transfer of oxygen inside the balls and support the growth of AOB. Stable partial nitrification was achieved using immobilized cells with a range of 0.5-2 mg/L DO concentration (Chou et al., 2012b). The 90% conversion of ammonium into nitrite was achieved with DO concentration below 0.5 mg/L in the biofilm system, while the increase of DO led to a higher nitrate concentration. Practically, control of oxygen below 0.5 mg/L is difficult, which may affect nitritation efficiency. So, the immobilization process is the best alternative to inhibit the growth of NOB. The nitritation with polyurethane carrier applied with heat shock appeared another option to inhibit the activity of NOB. Ammonium removal started after 12 days using waterborne

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polyurethane carrier treated at 60°C, and 90% of nitrite accumulation rate was achieved with no observed nitrate concentration (Chen et al., 2016c). Partial nitrification immobilized with PEG gel carrier was observed after 14 days with heat shock treatment, and stable nitritation was achieved from 24 to 74 days (Isaka et al., 2008a). While, nitritation process of the gel beads without heat shock appeared immediately but after some day's nitrate was observed in the effluent (Chen et al., 2016c; Isaka et al., 2008a). Duration and temperature for the treatment of sludge beads are essential parameters to be concerned. As temperature and duration increased, start-up time also increased (Chen et al., 2016c; Isaka et al., 2008a). Further, nitrification process with immobilization adopted to salinity up to 15 g /L (Gao et al., 2020).

Furthermore, the effects of temperature on the microbial community were carried out by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis. By comparing different bands, it was concluded that AOB was less affected compared to NOB after heat shock (Chen et al., 2016c). Higher effluent ammonium concentration may be due to the change in the microbial community caused by heat treatment. Chen et al. (2016c) reported the disappearance of bands related to *proteobacterium* (JF514815.1) and uncultured *Nitrosomonas sp.* (GU073372.1) in the heat shock sludge, which are more ammonium-loving. However, after 74 days, the growth of NOB appeared which caused by high DO concentration (over 4 mg/L). Free ammonia (FA) approach was also applied for the suppression of NOB in the suspended growth. However, the FA inhibition to NOB is limited due to the limitation of FA diffusion inside the beads, while at the same time DO diffusion restriction within the pallets suppressed the growth of NOB (Khan and Bae, 2014). 97% nitrite accumulation at the DO concentration of 1.7 mg/L was achieved using immobilization of activated sludge. While gradually increase in the DO concentration from 1.7 to 3.0 mg/L didn't change nitrite accumulation rate, which contradicts with the study of Chou et al. (2012b). The probable reason for this stable performance may be the constraint of DO diffusion (Khan and Bae, 2014). Above all, immobilization overlooks the suppression of NOB by FA stress. DO concentration and heat shock are two critical parameters which can be adapted to inhibit the nitrite oxidation for immobilized sludge.

7.   Economical, safety and environmental concerns of immobilizing technology

The immobilization technology for the removal of nitrogen practiced in modern nations like Japan and U.S. was reported (Isaka et al., 2007). However, the application of immobilization technology in lower-income countries is lacking. Further, the practical, industrial and full-scale application of immobilization technology in anammox process is limited due to the complex composition of real wastewater, operational complications, and special construction specifications. The laboratory scale study had no significance until it can be upgraded to a commercial scale. Therefore, it is essential to study the bottlenecks of anammox immobilization technology for their promotion to commercial scale. In terms of cost and life, care should be taken to select the most suitable immobilization carrier for the engineering application of anammox immobilization.

For instance, the capital cost of such a considerable quantity of beads, the construction and operation cost of treatment plant should be considered carefully. The commercial-scale application needs a large number of gel beads from 30 to 1500 m^3 depending on the volume of the treatment plant. The preparation, application, and management of large amounts of beads are laborious work. Moreover, PEG with different molecular weight is available in the market with different prices. Using higher molecular weights of PEG increases the capital cost and seems difficult to be adopted at full scale. So, study on the production of low cost and log time stable material is desired. Additionally, further studies are required about the substrate diffusion inside the bead, bead dissolution behavior, and lifespan of the beads.

Although immobilization shows good performance on the laboratory-scale, the large scale applications for an extended period have some other limitations. It was reported that the PVA was not equally distributed in the water under lower temperature (Tacx et al., 2000). Thus, higher temperatures are needed for the equal distribution of PVA into the water for stable beads formation, which increases the cost and the risk of human safety. The higher temperature needed for dissolution of PVA solution which

can burn the skin because of fallen drops. Self-condensing and water swelling properties of several hydroxyl groups in PVA (Tacx et al., 2000) may cause the adherence of PVA granules with each other and influence the mass transfer (Chen et al., 2015). Therefore, these factors limit the application of PVA-SA gel beads on a large scale. The dissolution of PVA is a matter of time, but possibly the time of decay can be extended to a long period.

Another critical point of concern to the environment is the disposal of the gel material. The start-up of the experiment depends on the packaging ratio as well as the sludge inside the beads (Isaka et al., 2007). Increasing the concentration of sludge inside the beads may decrease their stability due to the growth of bacteria which may cause the breakage of beads. The decrease of gel beads stability and ultimate breakage of the gel beads raise the question about the disposal of these beads. The study of cost estimation about the immobilization is needed, which for sure provides a useful contribution in this area of study. The study should include the cost of material, human labor, and gel material disposal and management.

8. Future directions

The most of the studies were carried out at laboratory scale under ideal conditions of anammox growth. The upgradation of immobilized anammox to pilot scale and full scale is awaited. So, more preliminary studies are encouraged to scale-up this technology at pilot scale first and then upgrad to full scale. Since natural biofilm formation is lengthy process, by using gel material, biofilm formation on specific surface can be achieved quickly and keep in using for long time.

Actual wastewater treatment plant is loaded with various pollutants and it has been proved that anammox is very sensitive to changing environment conditions. We proposed that gel material may provide the shield to anammox by adsorption of heavy metals and other emerging pollutants. However, there is a gap for the study of emerging pollutants on immobilized anammox. Incorporation of some effective adsorbent in the gel beads is also an option to avoid the influence of toxic substances on anammox. Iron-based adsorbent such as nZVI can be incorporated in the gel beads with multiple functions. Combined PN-Anammox in a single bead should also be study to evaluate the fast start-up of one-satge PN-anammox process. Although, there are some studies on immobilized PN and Anammox, they are mostly based on two reactor system or separately enriched PN bacteria and anammox then combining in one reactor, which is time consuming and laborious process. Over all, the studies on combind PN-Anammox with immobilization are not enough to draw conclusion. Gel immobilization can also be applied to the integration of anammox with dissimilatory nitrate reduction to ammonium (DNRA), partial denitrification, and denitrifying anaerobic methane oxidation (DAMO).

9. Conclusion

In conclusion, immobilization is a feasible and applicable option for slow growing anammox enrichment and long term process stability. The difference in the result of laboraotry scale study may be caused due to difference in materials, sludge concentration and filling ratio. Immobilization is also an effective approach for the pre-treatment of ammonium rich wastewater with partial nitrification to feed into subsequent anammox reactor. However, the full-scale application of immobilization technology still needs to solve some questions. The cost, human safty and environmental concern can be adress properly. More study on immobilization is awaited for their upgradation to full scale.

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Figure legends

Fig. 1. Nitrogen conversion processes: Comammox= Complete ammonium oxidation to nitrate,

Anammox= Anaerobic ammonium oxidation, DNRA= dissimilatory nitrate reduction to

ammonium, and DAMO = denitrifying anaerobic methane oxidation

Fig. 2. (a) The molecular mechanisms of anammox metabolism drawn from (Kartal et al., 2011)

included; NIR=nitrite reduction; HZS=Hydrazine synthesis; HDH=Hydrazine dehydrogenase. (b)

picture drawn from (Kartal and Keltjens, 2016; Oshiki et al., 2016; Yang et al., 2019) included

Nrf= ammonium generating cytochrome c nitrite reductase, and Hao= hydroxylamine oxido-
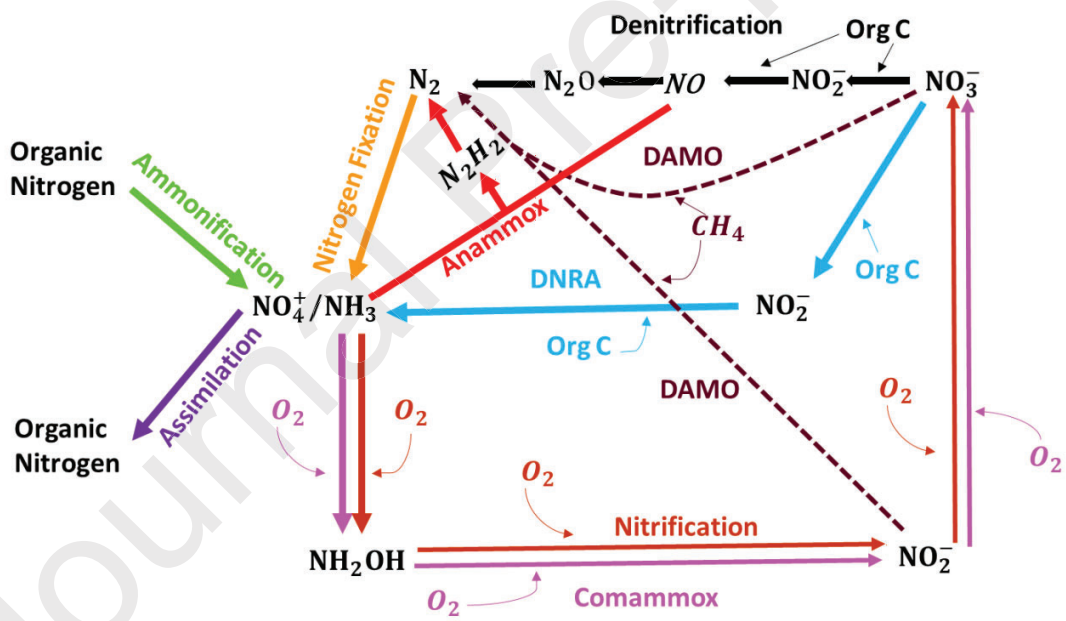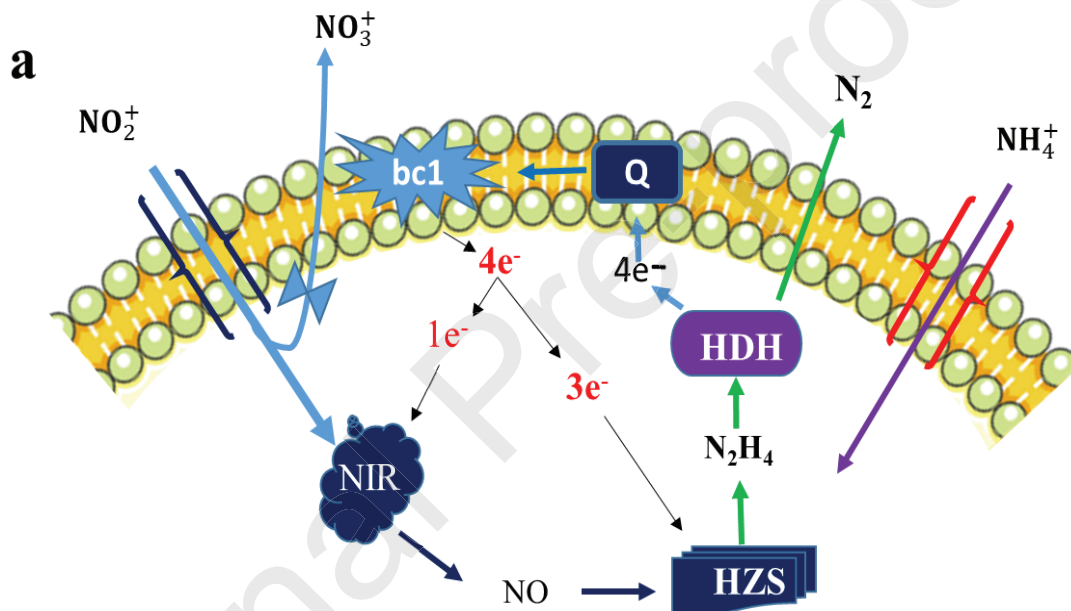
reductase

Fig. 1. Nitrogen conversion processes: Comammox= Complete ammonium oxidation to nitrate, Anammox= Anaerobic ammonium oxidation, DNRA= dissimilatory nitrate reduction to ammonium, and DAMO = denitrifying anaerobic methane oxidation
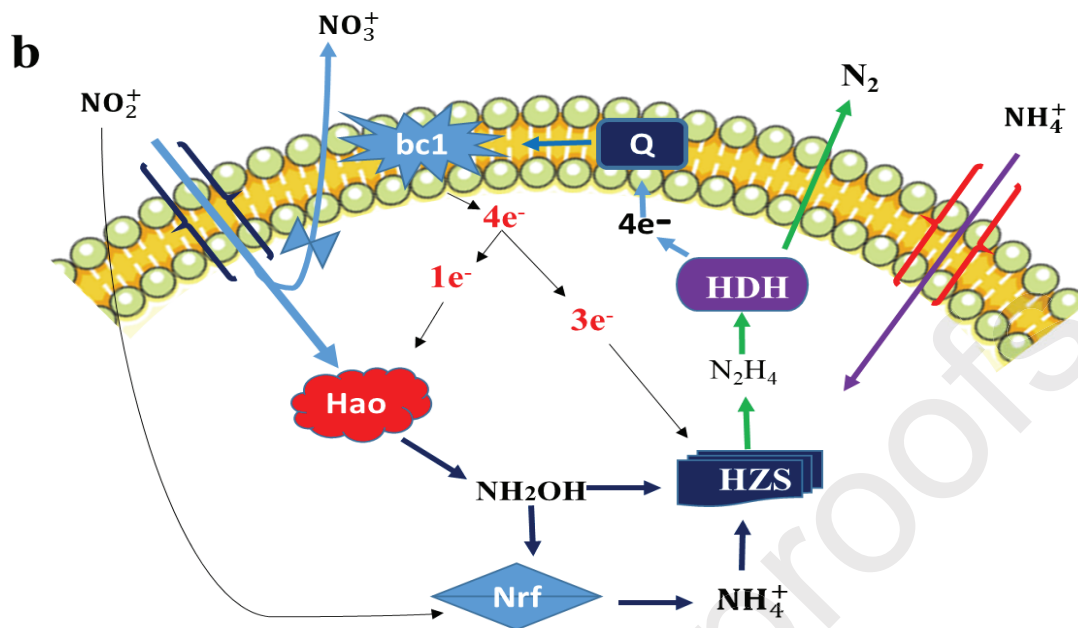
Fig. 2. The molecular mechanisms of anammox metabolism, (a) modified from Kartal et al. (2011) and includes NIR=nitrite reductase; HZS=Hydrazine synthesis; HDH=Hydrazine dehydrogenase, (b) reproduced from (Kartal and Keltjens, 2016; Oshiki et al., 2016; Yang et al., 2019) and includes Nrf= ammonium generating cytochrome c nitrite reductase, Hao= hydroxylamine oxidoreductase.

Tables Legends

Table 1. Comparison of the start-up periods between different immobilization techniques

Table 2. The studies of immobilized anammox for evaluation of the different factors affecting the anammox activity

Table 1. Comparison of the start-up time between different immobilization techniques

Immobilization techniques	Origin of the sludge	Sludge concentration	Start-up time (days)	Removal efficiency	Dominant Strain	Removal rate kg-N/m³/d	Reference
Granulation	Enriched anammox sludge	2124 mg VSS L⁻¹	62	88%	NA	NA	(Li et al., 2014)
Granulation	Anaerobic granular sludge	93900 mg L⁻¹ MLSS	63	NA	*Candidatus jettenia, Brocadia,* and *kuenenia*	1.16	(Wang et al., 2017)
Gel entrapment (PEG)	Enriched anammox sludge	0.72 g (dry SS)	20	NA	NA	3.7	(Isaka et al., 2007)
Granulation	Anaerobic granular sludge	2 g VSS L⁻¹	>55	29.8%	NA	NA	(Wang et al., 2018)
Gel entrapment (PVA/SA)	Enriched anammox sludge	0.33 g VSS L⁻¹	35	NA	NA	10.8	(Ali et al., 2015)
Biofilm	Aerobic activated sludge	MLVSS 1.68 g L⁻¹	47	70%	NA	7.64×10^{-2}	(Wang et al., 2019)
Gel entrapment (PVA/SA)	Activated sludge	25.3 g L⁻¹ (TSS)	42	89%	*Candidatus Brocadia sinica*	1.12	(Bae et al., 2015)
granulation	Enriched anammox biomass	1700 mg L⁻¹ (MLVSS)	24	81%	NA	NA	(Qiao et al., 2009)
Gel entrapment (PVA-SA)	Anammox sludge	MLVSS 1.5 g/L	20	80%	NA	0.2	(Tuyen et al., 2020)

| Biofilm (Sponge + volcanic rock) | N/A | 2000 mg L^{-1} (MLVSS) | 70 | 91% | NA | 0.19 | (Lu et al., 2017) |

Table 2. The studies of immobilized anammox for evaluation of the different factors affecting the anammox activity

Material	Packaging ratio	Reactor type	Reactor volume	Objectives	Remarks	Reported value	Level of study and duration	References
PEG	30%	CSTR	1L	To evaluate the entrapped anammox activity	Comparatively lower nitrogen removal achieved which can be increased by further optimization of the different conditions	NRR of 3.4 kg N m⁻³ d⁻¹	Continuous test for about 100 d	(Isaka et al., 2007)
PVA	20%	CSTR	1.4 L	Usefulness of anammox biomass immobilized by PVA Steadiness of anammox system using synthetic as well as partially nitrified swine wastewater	Anammox bacteria perform well inside the gel beads Successful recovery of nitrite to ammonium ratio with the application of swine wastewater	NCE of 73% with partially nitrified swine wastewater	Continuous test for about 160 d	(Magri et al., 2012)
PVA-SA	40%	ASBR		To check the effects of various pH, HRT and temperature on immobilized anammox To check the Nitrogen removal performance	Achieved stable performance of anammox, broadened pH range, HRT was 25 d, and temperature of 35 ºC to achieve a stable nitrogen removal	NCR of 0.58 kg N m⁻³ d⁻¹	Continuous test about 90 d	(Zhu et al., 2014)
PEG-PVA-SA	N.A	UASB	5L	To study the effects of salinity on immobilized anammox	Immobilized anammox can resist to 10 gL-1 salinity but high salinity than 10 inhibit anammox process	Nitrite and ammonium RR of 80%	Continuous study about 4 months	(Liu et al., 2020)
PEG	20%	CSTR	500 mL	To evaluate the inhibition and reversibility of anammox activity under different concentrations of heavy metals	Anammox activity was affected by higher levels of heavy metals, but the effects of Zn, Ni, Cu, and Co were reversible, and	NCR of 4.0 kg N m⁻³ d⁻¹ with Zn, Ni, Cu and Co concentration	Batch test	(Kimura and Isaka, 2014)

Material	Concentration	Reactor	Volume	Objective	Findings	Performance	Test	Reference
PEG	20%	CSTR	500 mL	To examine the impact of nitrite on anammox activity	the impact of Mo was irreversible / Impact of nitrite was reversible but the time to recover the anammox activity depended on the duration of nitrite inhibition	below 2 mg L^{-1} ACR 1.8 kg N m^{-3} d^{-1}	Continuous test	(Kimura et al., 2010)
WPU, PEG, PVA, and carboxy methyl cellulose	Not mentioned	Serum bottles as reactor	500 mL	To check the stability of anammox for long term operation immobilized by different gel carrier	Compared to all other material WPU was more stable in term of mechanical stability, shock loading, and anammox activity	ARR of 0.455 kg N m^{-3} d^{-1}	Continuous test with WPU, 65 days	(CHEN et al., 2015)
WPU-, SA, PVA, PVA–SA	=	Serum bottle as reactor	500 mL	To compare the mechanical stability, effects of shock loading on the performance of immobilized anammox by different material	In term of mechanical stability and shocked loading, WPU was the suitable material compared to PVA, SA, and PVA-SA	NCR of 80.98%	Continuous test with WPU, 100 days	(Chen et al., 2015)
PEG	20%	CSTR	500 mL	Evaluation of the different concentration of methanol as well as ethanol on the anammox activity	Anammox has more sensitivity for the methanol compared to ethanol. Methanol inhibited anammox activity irreversibly	NCR of 2.9 kg N m^{-3} d^{-1}. Before the application of methanol	Batch test	(Isaka et al., 2008b)
PVA	100 capsule per reactor	Capsule bioreactor	1 L	To evaluate the anammox performance by immobilizing within the novel PVA capsule and nitrite inhibition	Improvement in the anammox process achieved which showed the stability of a novel capsule bioreactor	Not reported	Continuous test for about 100 days	(Chou et al., 2012a)
PVA-CS-Fe	40%	Up-flow	1.3L	Elucidate the role of iron in the immobilize anammox process	Fe significantly improved the nitrogen removal rate and provide resistance to anammox against substrate shock	NRR of 13.9 kg m^{-3} d^{-1}	Continuous study	(Wang et al., 2020)

Material	Concentration	Reactor	Volume	Objective	Findings	NRR	Test	Reference
PVA-SA	70%	Up-flow column reactor	10 mL	To optimize the concentration of anammox in the gel beads to fasten the start-up of the anammox process	Achievement of the anammox process at the concentration of 0.33 gVSS L^{-1}	NRR 7 kg N m^{-3} d^{-1}	Batch test	(Ali et al., 2015)
PVA-SA	20%	Conical flask	1 L	To retain the anammox biomass and evaluation of anammox nitrogen removal as well as stoichiometric value	On the base of the stoichiometric value and nitrogen removal process, anammox retained in the gel beads successfully	Not reported	Continuous test	(Hsia et al., 2008)
Na-CMC, PVA, SA, and PVA-SA	20%	Conical flask	250 mL	To optimize the characteristics of different materials for the successful application of the anammox process	Only PVA and SA were unfit for the application of anammox. Na-CMC have good transferability but have inferior long-term stability. The mixture of PVA-SA was a suitable option for the successful application.	Not reported	Batch test	(Zhu et al., 2009)
PEG	30%	CSTR	1 L	To evaluate the change in the anammox population in the co-existing environment	On the base of real-time PCR result, anammox bacteria associated with clone HPT-WU-N03 were the dominant population	Not reported	Continuous test	(Date et al., 2008)
PVA-SA	18.5%	CSTR	14 L	To validate the activated sludge as a potential source of the anammox process and long-term constancy	Anammox bacteria enriched by using activated sludge. Anammox activity start after 93 days	0.36 kg N m^{-3} d^{-1}	Continuous test about 1.5 year	(Cho et al., 2017)
PEG	20%		500 mL	To verify the effects of inorganic carbon on the activity of immobilized anammox	The decrease in the anammox activity observed under low concentration of inorganic carbon. The higher nitrogen conversion rate observed at 60 mg/L inorganic carbon	NCR of 7.0 kg N m^{-3} d^{-1}	Continuous test	(Kimura et al., 2011b)

NCR=nitrogen conversion rate, NRR=Nitrogen removal rate, ARR=Ammonium removal rate, ACR=Ammonium conversion rate

NCE=nitrogen conversion efficiency, CSTR= Continuous stirred tank reactor,