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A review on application of external carbon sources for denitrification for wastewater treatment

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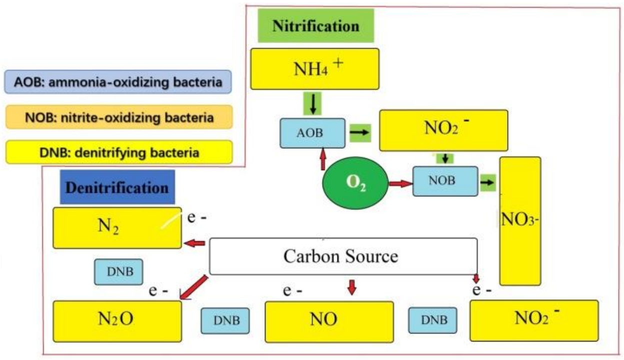
Graphical abstract



Abstract

With the increase in industrial and agricultural activities, huge amount of wastewater containing nitrogen is produced. Biological denitrification is currently the most widely used, sustainable and cost-effective process to remove nitrogen from wastewater. In biological denitrification, application of external carbon sources is important because it is the way to boost the denitrification process and it is necessary for wastewater treatment plants that have to meet very stringent effluent nitrogen limits. This review study focused with treatment of wastewater using biological denitrification as advantages. This also deals with advantages of different external carbon sources with emphasis on biological denitrification as the cost of biological denitrification is controlled by the carbon source because the cost of biological denitrification is controlled by the carbon source. Hence, use of alternative carbon sources such as agricultural wastes, and food waste is reviewed in this paper. This study recommends other waste material such as textile waste can be a part of denitrification because carbon source such as methanol, ethanol and acetic acid/acetate can be recovered from textile waste.

Keywords: Wastewater treatment, biological denitrification, nitrate, nitrite, external carbon source, microbial community.

1. Introduction

In the past 20 years, several pollutants have been identified which bring water pollution and nitrogen is one of them. The water quality is continuously diminished due to the waste discharge from municipal and industrial wastewater release without standard process to treat this water and drain into pure surface water and the using fertilizers for agriculture purpose. Nitrogen removal from water is also one of the challenges (Kamal *et al.*, 2019). Huge research has been carried out on nitrogen removal from wastewater treatment because the release of reactive nitrogen "N_r" into surface water has added to coastal hypoxia (oxygen depletion) and eutrophication (algae growth/excessive plant) in more than 400 estuaries globally, with few signs of development. Many thresholds for human health and ecosystem have been exceeded owing to N_r pollution, including those for drinking water (nitrates), air quality (smog, particulate matter, ground-level ozone), limate change, biodiversity loss, freshwater eutrophication, stratospheric ozone depletion, c and coastal ecosystems (dead zones). Each of these environmental effects can be magnified by the 'nitrogen cascade': a single atom of N_r can trigger a cascade of negative environmental impacts in sequence (Erisman *et al.*, 2013). Much of the "N_r" that arrives these systems is originated as agricultural and urban runoff. The main point-source of "Nr" is effluent from wastewater treatment plants (WWTPs) to these ecosystems (Du *et al.*, 2020; Hang *et al.*, 2016; He *et al.*, 2018; Nair and D'Souza, 2012). Anthropogenic sources of "N_r" (all forms of nitrogen except N_2) are now three times as great as the nitrogen inputs from natural sources. There are three main sources including uses of synthetic fertilizers to agricultural lands, combustion processes, and the purposeful planting of legumes. There is world-wide increase in the regulatory control of nitrogen since the last several decades (He *et al.*, 2018; Ji *et al.*, 2015; Li *et al.*, 2019). There are several methods have been introduced to deal with nitrogen pollution such as Ion Exchange (Huang *et al.*, 2020; Leaković *et al.*, 2000), Catalytic Reduction (Fan *et al.*, 2020; Guan *et al.*, 2011; Kim *et al.*, 2020; Zhu *et al.*, 2020), Electrodialysis (Schoeman, 2009; Vineyard *et al.*, 2020; Zhang *et al.*, 2020) and others (Dhamole, 2015) but research scope involves in several other suitable treatment methods, especially biological and physicochemical methods because nitrogen removal system provides a process for removing a large portion of the nitrogen concentration from wastewater effluent before it is discharged into environment.

In new methods, the most widely used technology is biological method (An *et al.*, 2020; Sun *et al.*, 2020). Studies related to denitrification, nitrogen removal or carbon source for denitrification (Burghate, 2013; Dhamole *et al.*, 2015; Hang *et al.*, 2016; Ji *et al.*, 2015; Kuypers *et al.*, 2018; Rajta *et al.*, 2020; Yang *et al.*, 2020; You *et al.*, 2020) have been conducted. Generally, the biological method for nitrogen includes two stages, nitrification and denitrification (Chen *et al.*, 2021; Du *et al.*, 2020; Li *et al.*, 2017). The nitrification reaction is the method of converting ammonia nitrogen into nitrate, which includes two basic reaction steps: the reaction of nitrite bacteria to convert ammonia nitrogen into nitrite; the reaction of nitrite bacteria to convert nitrite into nitrate (He *et al.*, 2018; Ji *et al.*, 2015; Li *et al.*, 2019). Role of microorganisms and enzymes also have been identified (Moura and Moura, 2001). Later, process of electron donating and accepting, electron donors in the denitrification process are various organic substrates (carbon sources) (Grebliunas and Perry, 2016; Her and Huang, 1995; Ling *et al.*, 2021; Yang *et al.*, 2020) and the nitrogen oxides act as terminal electron acceptors in the absence of oxygen, and the gaseous nitrogen species are the major end product of these reductive processes (Matějů, 1992). In this method, alternative carbon sources are required (Dhamole and D'Souza, 2015; Peng *et al.*, 2007; Rajta *et al.*, 2020), it has been widely studied. External carbon sources boost denitrification process. Different carbon sources were applied in different studies such as methanol (Louzeiro *et al.*, 2002), ethanol (Hardman *et al.*, 1996), acetic acid/acetate (Elefsiniotis and Li, 2006) with a good rate of denitrification, high amounts of denitrification are gained with these carbon sources. It's difficult and costly to get external carbon sources. Main carbon sources for denitrification are methanol, ethanol and acetic acid/acetate (Dhamole *et al.*, 2015). These days, several different external carbon sources are being studied such as external carbon source from agricultural product/waste, food waste and other (animal/plants). Research should be focused on different types of wastes which can provide these carbon sources so that waste should be managed, and carbon sources should also be gained. This study reviews about biological method for denitrification. It also explores all possible carbon sources for denitrification and different enzymes dozes for obtaining better result for pollutant removal from wastewater.

2. Sources of nitrogen

Nitrogen is an important element for life. Even though nitrogen and other ions have been recognized as major cause for water eutrophication, its effects on aquatic ecosystems have now become a major worry to society (Zhao *et al.*, 2020). Before the advent of the industrialization and the green movement, the rate of supply of nitrogen on Earth was limited to the rate of bacterial nitrogen fixation, but recent growth of urbanization and industrialization now has roughly doubled the rate. Variations have been noticed region viz, some regions have got little change and some regions have got great change. In addition, Nitrogen fluxes via the atmosphere and rivers have grown by 10–15-fold at most in certain parts of the Earth (Howarth, 2008). The main sources of nitrogen including geological sources (Howarth, 2008; Paredes *et al.*, 2019; Yang *et al.*, 2018), atmospheric precipitation (Gibrilla *et al.*, 2020; Howarth, 2008), urban waste, agricultural land (Ding, 2014), livestock and poultry operations (Barrios-González, 2018; Granéli and Granéli, 2008; Zhao *et al.*, 2020). Nitrate is the key form of nitrogen pollution that takes place in freshwater bodies. In current years, the extreme usage of agricultural fertilizers, the discharge of huge volumes of domestic sewage. Nitrate content in freshwater bodies is caused by animal manure, which postures a probable risk to the health of human-beings (Zhao *et al.*, 2020). Each of these environmental effects can be magnified by the 'nitrogen cascade': a single atom of N_r can trigger a cascade of negative environmental impacts in sequence (Erisman, 2013). The two main anthropogenic sources by which "Nr" creates energy production and fiber/food/biofuel production, by this way they come into the biosphere (Srinuanpan *et al.*, 2020). The discharge of one molecule of "Nr" in the form of NO_2 to the atmosphere will originates a series of air-related issues and then can be set down in the aquatic or terrestrial zones. Later conversion process may start, such as NH_3 or N_2O, and it will bring a chain of new issue. Transfers of "Nr" from the atmospheric system take place by deposition to aquatic or terrestrial zones of the biosphere (Galloway *et al.*, 2008; Kruk *et al.*, 2020; You *et al.*, 2020), see Figure 1. Identification of the contamination load that come into the water bodies is important for managing the nitrogen source pollution in water bodies, it will support to prevent and address the pollution in water bodies (Paredes *et al.*, 2019).

The major sources of nitrogen for municipal wastewater are, fecal matter, Urea, and food processing wastes (Zhao *et al.*, 2019). Domestic wastewater usually has a total nitrogen content that is approximately one-fifth of the biochemical oxygen demand (BOD) and concentrations of nitrogen ranging from 20 to 70 mg/L. Moreover approximately 30% to 40% is organic nitrogen, and 60% to 70% is ammonia-nitrogen, with less than 1% nitrite and nitrate nitrogen (Galloway *et al.*, 2008). There are several methods for nitrogen removal, but biological process is recommended. In biological treatment systems, the nitrogen removal process consists of four basic steps (Galloway *et al.*, 2008). The first step is the alteration of organic nitrogen to ammonia in a method called ammonification. Ammonia is then changed to nitrate in a two-step process called nitrification. The change of ammonia to nitrite is followed by the change of nitrite to nitrate. Finally, nitrate is changed to nitrogen gas by the process of denitrification. It is very easy and cost-effective methods. These methods should be deeply studied and make it more easily available and maintainable system.

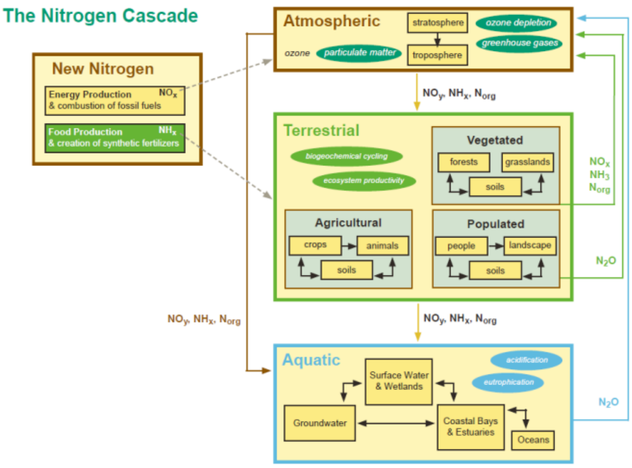


Figure 1. The nitrogen cascade (Source: U.S. EPA).

3. Biological process for wastewater treatment

Biological removal of nitrogen can be conducted using several treatment configurations (McCarty, 2018). It can be implemented using a single-unit process with many treatment zones or in separate phases such as combined systems, fixed growth, or suspended growth can be used (Rahimi *et al.*, 2020). Most of treatment systems used for this, they all need an aerobic zone for exchanging ammonia to nitrate and an anoxic zone for exchanging the nitrate to nitrogen gas (Hagemann *et al.*, 2016; Zhu *et al.*, 2008). One of the more known methods to retrofitting existing services is to prolong the aeration period to allow for nitrification, followed by a filtration system for denitrification (Du *et al.*, 2020). As organic carbon is taken by denitrifying bacteria, mostly in the extended aeration process, so in this process, especially when the discharge limits for total nitrogen are low, it is often essential to add a carbon source (Jia and Yuan, 2016). Nitrate is reduced to nitrogen gas under anaerobic/anoxic conditions and in presence of suitable electron donor (Megonigal *et al.*, 2003).

In the past 10–15 years, China has adopted rapid urbanization and industrialization. If there is industrialization then the need for standards for discharge and more effective wastewater treatment options are considered (Li *et al.*, 2021). As before, 35.5% of rivers in China were not suitable for drinking purpose because of its contamination and it brought water shortage issues in several regions (Han *et al.*, 2016). In the last few years, several existing WWTPs have been advanced to biological system for nitrogen removal using denitrification filters, with some external carbon source such as methanol (Zhang *et al.*, 2010). In Jiashan City, China, during year 2008, a WWTP (5.3-MGD) was designed and constructed by Severn Trent Services. The plant has three separate biofiltration systems. Nitrified wastewater is recycled to the head end of the plant for mixing with influent (Tan *et al.*, 2021). In the first phase, for denitrification, there is an up-flow anoxic filter in which raw sewage is used as the carbon source. The second phase is an up-flow aerated filter for BOD removal and nitrification. The third phase is a down-flow filter for polishing denitrification and suspended solids removal. Methanol is added to the final stage as needed to meet the discharge standards. Pilot-plant studies showed a reduction in total nitrogen (TN) from 88 to 11 mg/L and ammonia from 82 to 3 mg/L, meeting China's Class 1A effluent standards (Tan *et al.*, 2021).

In Europe, biological system was also adopted. The European Water Framework Directive (EU WFD) marked a shift in focus, from point-source control to an integrated prevention and control approach at the water-body level. Tertiary wastewater treatment systems were increased in 1990 around the globe, varies by region (Stephan *et al.*, 2021). The European Water Framework Directive (EU WFD) initiated the decrease from 10 mg/L to 2.2 mg/L for discharge standard for nitrogen in water. The objectives of this action were to "safeguard the water environment, stimulate sustainable water use, develop the status of aquatic ecosystems, lessen the effects of floods and droughts, and decrease pollution." The main strategy was to adopt of new wastewater treatment technologies and that is biological denitrification (Gutierrez *et al.*, 2019; Poikane *et al.*, 2019).

Biological denitrification is a good technology, and many natural things are involved. As it is conducted under anoxic conditions by a broad range of heterotrophic bacteria through the method of nitrate dissimilation, in which nitrite and/or nitrate play role as the electron acceptor rather than oxygen (Du *et al.*, 2020). These bacteria are referred to as facultative heterotrophs because these organisms can utilize either oxidized nitrogen or oxygen while oxidizing organic material (Martínez-Espinosa *et al.*, 2021; Zhang *et al.*, 2021). Generaly denitrification is often conducted after most of the organic matter has been consumed aerobically, then adding an organic carbon source play important role (Scheer *et al.*, 2020). Unlike nitrification, denitrification can increase the pH. Several studies have concluded that the optimal denitrification rate of pH is in of 7.0 to 7.5 (Shi *et al.*, 2020). There are several different tools and techniques to conduct biological denitrification. Denitrification technologies, such as Heterotrophic denitrification (HD) and Autotrophic denitrification (AD), they are commonly used in constructed wetlands (Ahmed Sanjrani *et al.*, 2020; Park *et al.*, 2015). Combined autotrophic and heterotrophic denitrification technology enhances the efficiency of nitrate removal in constructed wetlands. It is also good for treating hydroponic wastewater containing low organic carbon and high nitrate concentrations (Liu *et al.*, 2009). The use of combined HD and AD techniques improves nitrate removal in CWs (Zhao *et al.*, 2019). Autotrophic and heterotrophic denitrification process in HF–HF CWs together has been recommended than the heterotrophic denitrification alone (Park *et al.*, 2015). Nitrification, denitrification and anammox process coupled to iron redox were experienced in wetlands for domestic wastewater treatment (Ma *et al.*, 2021). Some Improvement in denitrification in surface flow constructed wetland (SFCW) planted with *calamus* was demonstrated (Fan *et al.*, 2021). Developments and challenges of sulfur-driven autotrophic denitrification (SDAD) for removal of nitrogen (Wang *et al.*, 2020) and partial denitrification-anaerobic ammonium oxidation process for municipal wastewater treatment for mainstream (Chen *et al.*, 2021) have been studied. Fig 2 shows all these full process by chemical equations (Ding *et al.*, 2019).

Chemical reaction of nitrogen fixation

$$N_2 + 8\,H^+ + 8\,e^- \longrightarrow 2\,NH_3 + H_2$$

Chemical reactions of ammonia oxidation carried out by bacteria

$$NH_3 + O_2 + 2\,e^- \longrightarrow NH_2OH + H_2O$$
$$NH_2OH + H_2O \longrightarrow NO_2^- + 5\,H^+ + 4\,e^-$$

Chemical reaction of nitrite oxidation

$$NO_2^- + \tfrac{1}{2}O_2 \longrightarrow NO_3^-$$

Chemical reaction of anaerobic ammonia oxidation (anammox)

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2\,H_2O$$

Reactions involved in denitrification

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO + N_2O \longrightarrow N_2$$
$$2\,NO_3^- + 10\,e^- + 12\,H^+ \longrightarrow N_2 + 6\,H_2O$$

Figure 2. Chemical reactions invloved for nitrogen (Nitrification and denitrification)- Source: Modified from Anne *et al.* (Bernhard, 2010).

Other methods by several environmentalist have been initiated for concentration removal such as an anoxic–aerobic sequencing batch reactor for nitrogen removal, biological denitrification in an anoxic sequencing batch biofilm reactor (Ding *et al.*, 2019), nitrification and aerobic denitrification (Alzate Marin *et al.*, 2016), simultaneous nitrification and denitrification in flat-panel air-cathode microbial fuel cells handling domestic wastewater for complete removal of nitrogen (Park *et al.*, 2017), denitrification in an integrated bioelectro-photocatalytic system (Lin *et al.*, 2017), an aerobic granular sludge-sequencing batch-reactor with high dissolved oxygen for simultaneous nitrification, denitrification and removal of phosphorus (Wang *et al.*, 2018), simulation of denitrification in groundwater from Chaohu Lake Catchment, China (Qian *et al.*, 2018), potential of sulfide-based denitrification for treatment of municipal wastewater (Van den Hove *et al.*, 2020) and biological nitrogen and phosphorus removal by a phosphorus-accumulating bacteria *Acinetobacter* sp. strain C-13 with the ability of heterotrophic nitrification–aerobic denitrification (Chen *et al.*, 2021). All above methods have demonstrated good results for concentration removal. It is also recommended that different new methods should be studied for improving removal efficiency while adding different external carbon sources.

4. Effects of external carbon source on nutrients removal

Several studies have proved that external carbon source for denitrification stimulated denitrification. Li et at (2018) investigated efficiency of external carbon sources. The average removal efficiencies of NH_4+-N were respectively 67.2%, 74.8%, and 75.2%, indicating that the external carbon source enhanced the NH_4+-N removal, and that corn straw was more efficient than sodium acetate (Li *et al.*, 2018), whereas rice straw was proven to be most effective in improving TN removal (Yang *et al.*, 2015). Municipal sewage was treated with addition of external carbon source with nitrogen removal efficiency of 92.8% (Du *et al.*, 2019). It was also reported that adding an external source under low C/N can significantly improve nitrogen removal efficiency (Luo *et al.*, 2018) and it also increases nitrate removal (Yang *et al.*, 2019). In addition, using lactic acid fermentation products from food waste as external carbon sources for nitrogen removal (Tang *et al.*, 2018), acidogenic liquid from food waste (Zhang *et al.*, 2016) and citric acid during simultaneous heterotrophic-autotrophic denitrification (HAD) and electrocoagulation (Kłodowska *et al.*, 2016), this acid type material proved to be a good external carbon source.

It is generally known as the phosphorus removal in CWs includes abiotic processes (e.g., sorption, settling on substrates, co-precipitation with minerals, precipitation and adsorption) and biotic processes (e.g., the uptake and growth of plants and microorganisms) (Li *et al.*, 2018; Luo *et al.*, 2017; Pietro and Ivanoff, 2015). Phosphorus removal is mostly done through adsorption/ precipitation in the media. In CWs, the phosphorus removal is stimulated with the addition of the carbon source, and it also cleans and unsaturated gravel media (Li *et al.*, 2018). Due to the low cost and good effect, it is feasible to use an external carbon source in CWs (Li *et al.*, 2018) or other external carbon source gained from agricultural or its waste or food/textile waste.

5. Carbon Sources from different materials for denitrification

It is often necessary to add an organic carbon source for denitrification because denitrification is often carried out after most of the organic matter has been consumed aerobically. In wastewater treatment, the respiratory denitrification depends on a carbon source as an electron donor. Concentration of available carbon in wastewater may not always be as enough as required for proper process, so that an external carbon source is needed (Kłodowska *et al.*, 2018), see Figure 3. Various electron donors for are studied (Pang and Wang, 2021), evaluation of sustainable electron donors for this process need to analyze (Fowdar *et al.*, 2015), some of the main requirements for a suitable external carbon source apart from low costs are a non-toxic/nondangerous nature, availability, low sludge yield and ability to stimulate a complete denitrification (Dhamole *et al.*, 2015).

Several studies have been conducted to find out a best solution (Wang *et al.*, 2021). Regarding finding about it, a new activated primary tank was developed for recovering carbon source and its application were revealed (Jin *et al.*, 2016). Most widely used carbon sources for denitrification are methanol, ethanol and acetic acid/acetate (Dhamole *et al.*, 2015). These carbon sources bring high rates of denitrification. However, they are expensive and 70 % of the operating cost is incurred (Dhamole *et al.*, 2015). These carbon sources were applied in different studies such as methanol (Louzeiro *et al.*, 2002), ethanol (Hardman *et al.*, 1996) acetic acid/acetate (Elefsiniotis and Li, 1996) with good rate of denitrification. Safety should also be considered for selection of carbon source. The use of external carbon sources such as acetic acid, methanol, and ethanol for enhancing pollutant removal has extended the scope of safety concerns in wastewater treatment services. The most significant concerns are that of flammability and explosion hazards because of methanol and ethanol (EPA, 2013).

Table 1. External carbon sources used for biological denitrification

Carbon source material	System/Wastewater	Key findings	Reference
External carbon source from Acid/Chmicals			
Citric acid	Post-denitrification biofilm reactors	Nutrients removal	Mielcarek *et al.*, 2020
Glycerine	Wastewater	Nitrite removal	Bernat *et al.*, 2015
Methanol and acetate	Anaerobic-Anoxic-Oxic-Membrane Bioreactor	Nutrient removals	Xu *et al.*, 2016
Methanol and ethanol	Post-denitrifying moving bed biofilm reactors	Removal of micropollutants	Torresi *et al.*, 2014
Acetate	Bioelectrochemical systems	Nitrogen Removal	Nguyen *et al.*, 2015
Citric acid	Heterotrophic-autotrophic denitrification (HAD) and electrocoagulation	Removal of nitrogen and phosphorus compounds	Kłodowska *et al.*, 2016
Sodium acetate	Water of Chaohu Lake Catchment in reactor (continuous up-flow)	Nitrogen Removal	Qian *et al.*, 2018
Citric acid	Biofilm reactor	Nitrogen Removal	Mielcarek *et al.*, 2017
Glycerol	Drinking water denitrification	Nitrate removal	Schroeder *et al.*, 2020
Polyvinyl alcohol sodium alginate (PVA-SA)	Low carbon nitrogen wastewater	Nitrogen removal	Xiong *et al.*, 2020
External carbon source from Agricultural products			
Rice husk	Long-term flooded constructed wetlands	Nitrogen removal	Luo *et al.*, 2018
Wheat straw (WS), rice straw (RS), corn stalk (CS), soybean hull (SH), corncob (CC) and soybean stalk (SS)	Wastewater	Nitrate removal	Xie *et al.*, 2019
Corncob	Artificial sewage treatment plant tail water	COD and NO3−-N removal	Cao *et al.*, 2015
Polycaprolactone-peanut shell	Drainage of municipal WWTP	Nitrogen removal	Xiong *et al.*, 2019
A. donax	Reverse osmosis concentrate treatment	Total nitrogen removal	Li *et al.*, 2019
Peanut shell (PS)	Low carbon nitrogen wastewater	Nitrogen removal	Xiong *et al.*, 2020
Corn flour	Sequencing batch reactors (SBR)	Nitrogen Removal	Zhu *et al.*, 2015
Starch/PCL blends	Constructed wetland	Nitrate removal	Shen *et al.*, 2015
corncob (CC)	Low carbon nitrogen wastewater	Nitrogen removal	Xiong *et al.*, 2020
Cotton	Groundwater	Nitrogen removal	Volokita *et al.*, 1996
Giant reed, liquiorice and G. verrucosa	Low C/N ratio wastewaters	Nitrogen removal	Ovez *et al.*, 2006
Wheat straw	Water denitrification	Nitrogen Removal	Soares *et al.*, 1998
corncob, peanut shell, retinervus luffae fructus, wheat straw, cotton stalk, rice straw, rice husk and reed	Membrane Bioreactor (MBR)	Total nitrogen removal.	Yang *et al.*, 2015
External carbon source from other different products			
Newspaper	Drinking water	Nitrogen removal	Volokita *et al.*, 1996
Sludge alkaline fermentation	Water denitrification	Nitrogen Removal	Hu *et al.*, 2020
Fumarate	Groundwater	Nitrate removal	Park *et al.*, 2019
Polycaprolactone	Slow sand filter (SSF)	Nitrogen removal	Yang *et al.*, 2021
Pig manure hydrolysate	Piggery wastewater	Nitrate removal	Yang *et al.*, 2019
Food waste fermentation	Sequencing batch reactors (SBR)	Total nitrogen removal	Qi *et al.*, 2020
Poly butylene succinate (PBS)	Low carbon nitrogen wastewater	Nitrogen removal	Xiong *et al.*, 2020
Catechol	The cyclic rotating bed reactor (CRBR)	Nitrate and toxic organic compounds	Moussavi *et al.*, 2015

Acidogenic liquid from food waste	synthetic wastewater treatment	Total nitrogen removal	Zhang *et al.*, 2016
Kitchen wastewater-derived carbon source	Municipal wastewater	Biological nutrient removal	Zheng *et al.*, 2018
Poly butylene succinate (PBS), polyvinyl alcohol sodium alginate (PVA-SA)	Low carbon nitrogen wastewater	Nitrogen removal	Xiong *et al.*, 2020
anaerobic fermentation slurry from food waste	Wastewater treatment	Nutrients removal	Tang *et al.*, 2019
Food waste-recycling wastewater (FRW)	Sewage wastewater treatment plant	Nitrate removal	Kim *et al.*, 2017
Fermented soybean liquids (FSL)	Deep denitrification of tail water	Nitrogen Removal	Xue *et al.*, 2018
Immobilized nitrifier pellets	Constructed wetlands	Nitrogen Removal	Wang *et al.*, 2016
Fermentation liquid from food waste (FLFW)	Wastewater treatment	Nitrogen removal	Tang *et al.*, 2018
Obsolescent rice (OR) and poly - caprolactone (PCL),	Low carbon nitrogen wastewater	Nitrogen removal	Xiong *et al.*, 2020
Microalgal biomass	Horizontal subsurface flow constructed wetlands	Nitrate removal	Zhong *et al.*, 2019
Multi-walled carbon nanotubes (MWCNT)	Denitrification performance of Alcaligenes sp. TB	Nitrate removal	Wang *et al.*, 2019
Solid-liquid separation on food waste fermentation products	Denitrification	Nutrient removals	Shasha *et al.*, 2021
Natural algal powder-derived	System with aerobic conditions	Biodegradation of tetracycline (TEC) and nitrogen conversion	Shao *et al.*, 2019
Recycling of food-waste leachate	Microbubble	Nutrient removals	Lim *et al.*, 2016

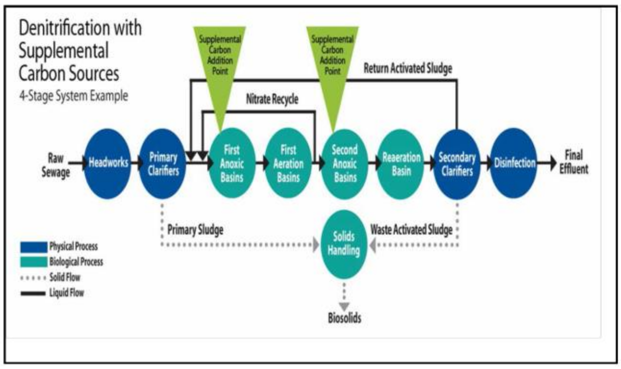


Figure 3. Design of process of wastewater treatment and additional carbon feed points (modified from US. EPA, 2013)

Rates of denitrification with many external carbon sources are not appreciably different from one another after acclimation (population selection rather than adaptation). Several studies have been conducted about efficiency of external carbon source such as the influence of carbon sources on nutrient removal and availability assessment of internal carbon source (Xu, 2016) and effect of carbon source on nitrogen removal in anaerobic ammonium oxidation (anammox) process (Zhu *et al.*, 2016). Regarding electron donors for denitrification, study (Di Capua *et al.*, 2019) was conducted about twelve electron donors for autotrophic denitrification, they were critically reviewed. Denitrification with non-organic electron donor for treating low C/N ratio wastewaters was also discussed (Tan and Yu, 2020). Acetate was applied as electron donor in BESs with biotic anode, whereas with abiotic anode, a direct current power was supplied as energy source in BESs. The efficiency of nitrogen removal increased from 18.1% to 43.0%. The highest efficiency autotrophic nitrogen removal was achieved as 78.0%, when electron transfer from the biotic anode chamber of BESs was used (Nguyen *et al.*, 2015). In addition, various alternative carbon sources have been investigated by many researchers see Table 1. Such solid substrates for denitrification are: giant reed (Ovez, 2006), liquiorice (Ovez, 2006), G. verrucosa (Ovez, 2006), wheat straw (Soares and Abeliovich, 1998), cotton (Volokita and Abehovich, 1996), newspaper (Volokita, *et al.*, 1996) with denitrifcation rate, 3.33 mg/l NO_3-N/g/d, 6.2 mg/l NO_3-N/g/d, 13.13 mg/l NO_3-N/g/d, 53 mg NO_3-N/l/d, 0.081 mg NO_3-N/l/d and 0.160 mg NO_3-N/l/d respectively. Moreover, these carbon sources can broadly be categorized into simple compounds and complex carbon sources. Complex carbon sources can additional be classified into solid substrates, based on the source, which are primarily the agricultural wastes and industrial byproducts or wastes (Dhamole *et al.*, 2015).

5.1. External carbon source from agricultural products

Agricultural wastes can be beneficial as carbon sources and biological carriers (Yang *et al.*, 2015). Ling et al (2021) studied on denitrification performance, release mechanism, and secondary pollutants while comparison of six types of agricultural wastes as solid carbon sources for nitrate removal. The main constituents of agricultural wastes are biodegradable cellulose, hemicellulose, and refractory lignin (Li *et al.*, 2019). Study recommended that it is good option for carbon source. Results show that C, N, and P in agricultural wastes demonstrate not only the release capacity of carbon sources but also the potential risk of secondary pollution (Xie *et al.*, 2019). Regarding C, N and P content, wheat straw (WS) has the highest C content, followed by rice straw (RS), corn stalk (CS), soybean hull (SH), corncob (CC) and soybean stalk (SS). The content of N in rice straw (RS) has been found the highest, which may have released more N and P pollutants and caused adverse effects on the effluent quality. By contrast, corncob (CC) has the lowest potential risk of secondary pollution (Liang *et al.*, 2021). Comparison of agricultural wastes and synthetic macromolecules as solid carbon source in treating low carbon nitrogen wastewater, agricultural waste is cost effective (Xiong *et al.*, 2020) because several studies have demonstrated that application of various plant biomass in CWs such as Cattail litter , E. Canadensis, Common reed, Cattail litter, Wheat straw, Corncob, Bulrush, Rice straw have shown better results for pollutant removal (Aerts and de Caluwe, 1997; ÆsØy *et al.*, 1998; Akunna *et al.*, 1993; Aslan and Türkman, 2004; Bachand and Horne, 1999; Ballantine *et al.*, 2014; Bastviken *et al.*, 2005; Beutel *et al.*, 2009; Bezbaruah and Zhang, 2003; Białowiec and Janczukowicz, 2011; Białowiec *et al.*, 2012).

The popular carbon sources used to enhance CW and bioreactor for denitrification are woodchip (eucalyptus woodchip, maize woodchip, pine bark, etc.), crop plants (liquorice, rice straw*, wheat straw, corncob*, etc.), chopped macrophytes (giant reed, P. austrails, E.canadensis, T. latifolia, etc.), macrophytes litters (Commelina communis, P. austrails, Ipomoea, and Pistia stratiotes, etc.), and other plants as sawdust, G. verrucosa, P. acerifolia leaf, cotton wools, etc (Hang *et al.*, 2016). Study on effects of external carbon source (corn straw) on COD removal was conducted, where the five constructed wetlands (CWs) reached a steady state after being cultivated with synthetic wastewater for four weeks, an external carbon source (corn straw) was added to system, after the constructed wetlands (CWs) were stable. Results show that the average COD removal efficiency was 74.5%. This specified that the constructed wetlands (CWs) had a low effluent COD concentration after adding corn straw (Li *et al.*, 2018). Corn straw and corncob as carbon source give good result in removal process. Corncob carbon source and bamboo charcoal filter was used in treatment of artificial secondary effluent for effective nitrogen removal (Cao *et al.*, 2016). Another approach for biological denitrification also gave better removal result, where polycaprolactone-peanut shell was used as slow-release carbon source for treating drainage of municipal WWTP (Xiong *et al.*, 2019). Several studies proved that agricultural waste is cost effective and easily available material. Substantial reduction in operating cost can be achieved by the application of wastes or by-products as an external carbon source for denitrification. Further, disposal of these wastes poses great economic and ecological problems Dhamole *et al.*, 2015. It is better to use this waste for good reason.

5.2. External carbon source from Acid/Chemicals

According to new legislation, water removal systems have been upgraded. In the last several years, most of existing WWTPs have been upgraded to biological system for removal nitrogen using e.g denitrification filters, with methanol as the additional carbon source. China represents an emerging market for tertiary wastewater treatment for nitrogen removal. Impact of additional external carbon dose of methanol and ethanol for the removal of micropollutants in post-denitrifying Moving Bed Biofilm Reactors was investigated. Addition of external carbon sources to post-denitrification systems is frequently used in wastewater treatment plants to enhance nitrate removal. Effects of methanol and ethanol as carbon sources on the removal of micro-pollutants in biofilm systems were assessed for better result (Torresi *et al.*, 2017).

Application of Citric acid for denitrification process support in biofilm reactor (Mielcarek *et al.*, 2017) and application of glycerol as carbon source for continuous drinking water denitrification using microorganism from natural biomass (Schroeder *et al.*, 2020) were investigate. Citric acid aiding the denitrification in AnSBBR was proved (Mielcarek *et al.*, 2017). Glycerine can be successfully used as sole carbon source for denitritation in nitrite removal and sludge production (Bernat *et al.*, 2015). Another approach, polycaprolactone as filtration medium (Li *et al.*, 2016) and polycaprolactone as solid carbon source (Yang *et al.*, 2021) proved to be better external carbon source for nitrogen removal for municipal wastewater and denitrification. Even though the way is different, (i) a solid-phase denitrifying biofilter with polycaprolactone as the carbon source and filtration medium (Li *et al.*, 2016) (ii) biofilm formation and microbial diversity during startup of slow sand filter using powdery polycaprolactone as solid carbon source (Yang *et al.*, 2021). Regarding microbial, denitrification performance and microbial diversity using starch-polycaprolactone blends as external solid carbon source and biofilm carriers for advanced treatment also demonstrated better result (Lim *et al.*, 2016).

5.3. External carbon source from food wastes

Although supplement of an external carbon source (e.g., acetic acid and methanol) resolved the deficiency of carbon source issue, but it also raises the operating cost of wastewater treatment plants (WWTPs). Alternatively, huge amounts of sludge are produced during biological sewage treatment, which comprise high concentrations of organic matter (Qi *et al.*, 2020; Wang *et al.*, 2021). Use of food waste-recycling wastewater as an alternative carbon source for denitrification process is best because a full-scale WWTP supplemented with food waste-recycling wastewater as carbon source achieved desired nitrate removal (Kim *et al.*, 2017). Sludge properties and performance of nutrients removal while using anaerobic fermentation slurry from food waste as an external carbon source for wastewater treatment was also investigated (Tang *et al.*, 2019).

In addition, food waste was used for anaerobic fermentation to prepare carbon sources. Effect of fermentation liquid from food waste as a carbon source for enhancing denitrification in wastewater treatment has demonstrated good results (Xue *et al.*, 2018). Generally, fermentation liquid showed similar denitrification result as sodium acetate (Zhang *et al.*, 2016). Food waste fermentation for carbon source production and denitrification in sequencing batch reactors played a vital role (Qi *et al.*, 2020). Moreover, advancement was adopted with solid-liquid separation on food waste fermentation products (Shasha *et al.*, 2021), recycling of food-waste leachate using Microbubble (Lim *et al.*, 2016) as external carbon source for denitrification. Another approach was made with microbial community and denitrification in MBBR using *A. donax* as carbon source and biofilm carriers for reverse osmosis concentrate treatment (Li *et al.*, 2019). Several studies related to food-waste as carbon source documented that it is also as better as sodium acetate. Using organic wastes as an alternative to commercial carbon sources could be beneficial by reducing costs and environmental impacts (Kim *et al.*, 2017).

5.4. External carbon source from animal/ microorganisms/alagal

A different approach by environmentalist, animal can be utilized for getting carbon source for denitrification. Addition of pig manure hydrolysate as a carbon source to improve nitrate removal efficiency from piggery wastewater under different HRTs (Yang *et al.*, 2021). The pig slurry was found to be C-sources that stimulated potential denitrification while assessing the potential of different carbon sources to stimulate denitrification (Dlamini *et al.*, 2020). In addition, the use of microalgal biomass as a carbon source for nitrate removal in horizontal subsurface flow constructed wetlands (HSFCW) (Zhong *et al.*, 2019). Effects of natural algal powder-derived carbon source, nitrogen source and carbon source on biodegradation of tetracycline (TEC) were investigated. It shows better result (Shao *et al.*, 2019).

6. Denitrification microbiology

Several types of bacteria are capable of growing anaerobically by reducing ionic nitrogenous oxides to gaseous products (Wahman and Pressman, 2014). This respiratory method leads to nitrates or nitrites serve as terminal electron acceptors instead of oxygen. It results in generation of ATP and is termed dissimilatory nitrate reduction or denitrification (Samocha and Prangnell, 2019). Specially facultative bacteria can perform a role for denitrification (Torres *et al.*, 2016). Aerobic heterotrophs-denitrifying bacteria which belong to taxonomically diverse genera like *Pseudomonas, Alcaligenes* and *Azospirillum* (Dhamole *et al.*, 2015) also important. Besides, some autotrophic organisms such as *Paracoccus denitrificans, Thiobacillus denitrificans, Rhodopseudomon as sphaeroides* are also capable for denitrification (Dhamole *et al.*, 2015). Those organisms which are known as denitrifying bacteria, their genera are *Acinetobacter, Aeromonas, Achromobacter, Gallionella, Halomona, Halobacterium, Hyphomicrobium, Janthinobacterium, Neisseria, Paracoccus (formerly Micrococcus), Propionibacterium, Pseudomonas, Rhizobium, Rhodobacter (formerly Rhodopseudomonas), Thiosphaera, Thiobacillus, Vibrio, Xanthomonas* (Matějů *et al.*, 1992). In addition, the nitrification and denitrification reactions are done by the action of nitrifying bacteria and denitrifying bacteria respectively (Sun *et al.*, 2019; Wang and Chu, 2016). Few denitrifying bacterial strains keep the complete pathway for denitrification (Zimmer *et al.*, 1984). However, these organisms still play role to the overall process of bacterial denitrification. Not only biological nitrogen but also phosphorus removal by a phosphorus-accumulating bacteria *Acinetobacter* sp. strain C-13 with the ability of heterotrophic nitrification–aerobic denitrification is done (Chen *et al.*, 2021). Denitrifying bacteria *Bacillus* and *Thauera* were the most abundant genera in a study (Shen *et al.*, 2015), the results showed that nitrate was removed mainly in the layer filled with SPCL (Shen *et al.*, 2015).

Several studies such as “microbiota involved in process of nitrogen removal and their roles in wastewater treatment” (Mai *et al.*, 2021), “performance evaluation, nitrous oxide emission and microbial community” (Ding *et al.*, 2019), “effect of microbial community structure and denitrification genes” (Xu *et al.*, 2018) and “microbial community and electron transport in an integrated nitrification and denitrification system for ammonium-rich wastewater treatment” (Zeng *et al.*, 2018) have demonstrated actual functions of these microorganisms during denitrification. The microorganisms play a vital role in removal processes nitrogen pollutant, but information about them is limited (Mai *et al.*, 2021). During this process of electrons transfer from the donor to the acceptor, these organisms get energy which can be utilized for the synthesis of a new cell mass and the repairs of the existing cell mass (Hendriks *et al.*, 2000). *Dechloromonas*, *Ignavibacterium, Nitrospira* and *Thauera* are the most abundant microbial genera in the A^2/O sludge (Kim *et al.*, 2013; Xiang *et al.*, 2021). The extracellular enzymes excreted by the microorganisms can hydrolyze cellulose and hemicellulose into soluble and small molecule substrates, which can be used by microbes during the denitrificationprocess (Her and Huang, 1995; Sun *et al.*, 2019). Further, *Nitrospira*, *Nitrosomonas*, and *Nitrobacter* have been identified as the key taxa for nitrite oxidation (Feng *et al.*, 2021; Wang *et al.*, 2019; Zhou *et al.*, 2021) and *Denitratisoma, Paracoccus* and *Truepera* were found to primarily conducting denitrification (Deng *et al.*, 2020; Wang *et al.*, 2020).

Fungi also play a significant role in the nitrogen cycle. Diverse fungi are recognized as microorganisms who to reduce nitrate or nitrite to gaseous nitrogen oxides such as nitrous oxide (N_2O), nitric oxide and dinitrogen via denitrification or co-denitrification (microbially mediated nitrosation), and to ammonium via ammonia fermentation (fungal dissimilatory nitrate reduction to ammonium) (Aldossari and Ishii, 2021). Boosted simultaneous nitrification and denitrification via addition of biodegradable carrier *Phragmites communis* in biofilm pretreatment reactor treating polluted source water (Feng *et al.*, 2015). Due to the diverse necessities for environmental conditions, these two processes cannot occur simultaneously, but can only sequentially, that is, the nitrification reaction occurs under aerobic conditions, and the denitrification reaction occurs under anoxic or anaerobic conditions (Mrkonjic *et al.*, 2007). Generally, the biological denitrification processes developed from this separate the anoxic zone from the aerobic zone, forming a hierarchical nitrification and denitrification process, so that nitrification and denitrification can be carried out independently (Xiujie *et al.*, 2019). There has been lot of work on this research of microorganisms involved in these processes but still there is gap as biotechnology is involved in environment field. Biotechnology and environment together can play a good role for advancement of these methods.

7. Role of Enzymes in nitrogen removal

Several enzymes which catalyze these reactions contain nitrite reductase, nitrate reductase, nitrous oxide reductase and nitric oxide reductase. These enzymes are synthesized when conditions are favorable for denitrification (Xiujie, *et al.*, 2019). The process of enzyme synthesis is typically a highly regulated process and the enzymes are inducible. Enzyme synthesis takes place under anaerobic conditions. However, it can take place even in the presence of oxygen (Hardman *et al.*, 1996). In some cases, enzyme induction may even require low concentrations of oxygen (Dhamole *et al.*, 2015)]. The enzymes associated with denitrification are synthesized when conditions become advantageous for denitrification (Liu *et al.*, 2020). Synthesis of denitrifying enzymes is typically a highly regulated process. It is generally held that the denitrifying enzymes are inducible (Xiang *et al.*, 2020). Significant levels of enzyme may be present as a consequence of anaerobiosis even in the absence of nitrate or other nitrogenous oxides (Matějů, *et al.*, 1992). In addition, nitrogen fixation is carried out by the enzyme nitrogenases which are found in microbes. The enzymes involved in the reduction of nitrogenous oxides are thought to be intermediates in denitrification processes (Hochstein and Tomlinson, 1988). Several studies have proved that denitrification process consists of four steps and each step is carried out by an array of enzymes. Nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase are the four main enzymes involved in denitrification. Ratja et al (2020) has studies about role of enzymes in denitrification and demonstrated that the first enzyme of the denitrification pathway is nitrate reductase which converts nitrate to nitrite. Nitrite reductase is the second most important enzyme involved in denitrification. Nitric oxide reductase is a membrane-bound enzyme. Nitrous oxide reductase is the last but one of the most important enzymes involved in denitrification as it converts N2O (greenhouse gas) to N2. This enzyme is required to carry out the complete denitrification process (Rajta *et al.*, 2020). There are several things are unknown which should be deeply analyzed.

8. Conclusions

Despite some limitations, biological denitrification is the most suitable method for nutrients removal. Significant development has been made in research on biological denitrification with adding of different factors such as different methods or mode of denitrification and addition of different external carbon sources. But still there is much scope for the further study in optimization of the denitrification process and by-products while using different factors and materials. Most important factor is external carbon source and significant fraction of operating cost of a denitrification unit is sustained by carbon source. Before, most of the published research regarding denitrification has the use of ethanol, methanol and acetic acid. It is reported that at high concentrations, ethanol and methanol becomes toxic to the microorganisms and they are highly inflammable, which leads to costly safety concern. Hence considering these factors, several different materials were explored for this purpose. Recently used carbon sources are mainly of agricultural wastes, industrial effluents or by products. Since most of these carbon sources are complex carbon compounds so the rates of denitrification are low. This study concluded that external carbon source from agricultural waste; food waste and textile waste are good choice because waste generated from these materials can be managed and there is also no secondary pollution. More research is needed on thermal and acid/base hydrolysis as enzymatic hydrolysis is slow. Further, there are very few studies which report the successful implementation of these carbon sources at a larger scale. Scales up studies are needed to check the sustainability of the process with alternative carbon sources. Hence, the policy of using alternative carbon source is recommended.

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