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Valorization of Agro-Industrial By-products: Use of Rice Husk as a Source of Microorganisms to Denitrification of Water

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Abstract

Rice husk, which is an agricultural waste, provides a feasible alternative for the growth and propagation of denitrifying microorganisms. Nitrate and nitrite were removed using Immobilized Microorganisms (MO_{IM}) or Microorganisms in Solution (MO_{SO}). Microorganisms present in the rice husk biomass responsible for denitrification were identified as *Pseudomonas*, and other microorganisms have also been identified, as *Oerskovia spp. Enterococcus sp. Bacillus mycoides* and *Escherichia coli*. The influence of pH, temperature, C/N ratio and carbon source on biological denitrification were investigated. MO_{IM} and MO_{SO} consortium had optimal denitrifying performance at 25-30 °C and in pH 7-8. MO_{SO} has average denitrification efficiency larger than MO_{IM}. The MO_{IM} denitrification efficiency was more sensitive to pH changes than the MO_{SO}. Ethanol and sodium acetate were carbon sources for the denitrifying process. The efficiency of nitrate and nitrite removal using MO_{SO} and ethanol or acetate with 1:1, 1:2, 1:3 and 1:4 C/N ratios were equivalents and above 97.00%. The denitrifying process presented was robust and it presented nitrate removal close to 100% during 10 cycles.

Keywords: wastewater treatment, water, pollution, denitrification, rice husk

1. Introduction

Safe water is crucial for humans, plants, and animals (Abu Hasan et al., 2020). Nowadays, water quality preservation is an important environmental issue where agricultural activities, industrial and domestic effluents are the main contamination sources of water (Júnior et al., 2007; Shamsollahi & Partovinia, 2019).

In many parts of the world, groundwater and superficial waters are widely used as drinking water. Over the decades, human activity increased nitrate (NO_3^-) concentration in groundwater and superficial waters due to human activity (Hou et al., 2019; Wan et al., 2015). Several factors contribute to this problem, such as: industrial effluents, final disposal of domestic sewage and the indiscriminate use of fertilizers and pesticides in agriculture (Liu et al., 2020; Zhang et al., 2016).

At high concentrations, nitrate cause eutrophication and toxic algal blooms in receiving waters. It is identified as one of the hazardous contaminants in drinking water, it can cause blue baby syndrome and nitrate reduction to nitrite induce the formation of carcinogenic nitrosamines (Fan & Steinberg, 1996).

The US Environmental Protection Agency set the nitrate maximum admissible in drinking water as 10 mg L^{-1} as nitrate-nitrogen ($N\text{-}NO_3^-$) and the World Health Organization set a limit of 50 mg L^{-1} as nitrate (He et al., 2016).

Nitrite and nitrate have high solubility in water and, its removal via precipitation is impractical. Thus, nitrate removal using conventional water treatment technologies is a challenge (Chen et al., 2019; Hou et al., 2019). Nitrate removal was carried out using several treatment, such as: adsorption, (Chuah et al., 2005) membrane separation (Pan et al., 2020) and electrochemical processes (Garcia-Segura et al., 2018). The main drawbacks of these processes were generation of undesirable by-products and its high cost. Thus, biological nitrate removal process is a promising alternative (Di Capua et al., 2019).

Biological nitrogen compounds removal (NO_3^-, NO_2^- and NH_4^+) is a selective method, but it is more cost-effective than traditional physicochemical methods (Liu et al., 2012). The heterotrophic biological denitrification process demands an incessant supply of external organic matter – such as methanol, ethanol or sodium acetate - which provides source for bacterial growth, as well as, generate energy for the conversion of nitrate into gaseous nitrogen, according to equation 1 (He et al., 2018; Tian & Yu, 2020).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

Agro-industrial waste - such as sugarcane bagasse, wheat straw, corn stover and rice husk - are used in biological denitrification processes, where it can promote reproduction many microorganisms and it is also a carbon source for the denitrifying process (Maheshwari et al., 2014).

Some authors (Della et al., 2001; Shamsollahi & Partovinia, 2019) reviewed the pollutants removal by rice husk. They claimed, for example, that the rice husk is 20-25% w/w amount

of the whole paddy produced (Della et al., 2001). This is an agricultural waste that is widely available, and it has been used as an adsorbent to remove heavy metals, organic pollutants, and dyes.

The main constituents of the rice husks were cellulose (34.61% weight basis - wb), lignin (19.66% wb), ash (16.82% wb), hemicellulose (10.90% wb), and protein (3.16% wb) (João et al., 2020). Where, rice husk composition changes from one sample to another because soil, climate, and geographical location and cultivar differences (Shamsollahi & Partovinia, 2019).

Few studies used husk as a source of denitrifying microorganisms. Thus, the efficiency of the denitrification process under various conditions with the participation of rice husk microorganisms was evaluated.

2. Materials and Methods

All chemical reagents such as, sodium hydroxide, sulfuric acid, sodium nitrate, sodium nitrite, used were analytical grade. Deionized water was obtained using a Millipore Milli-Q system. Solutions were prepared using deionized water,

2.1 Rice Husk as Source of Microbiota

The study was carried out using rice husk collected at an agricultural rice processing cooperative (COPAGRO), in the south of Santa Catarina, Brazil. The microorganisms used in this study were immobilized in rice husk biomass (MO_{IM}) or suspended microorganisms in solution (MO_{SO}).

2.2 Denitrification Using Microorganisms Immobilized on the Rice Husk and Suspended in Solution

In a 500 mL Erlenmeyer, rice husk was weighed and homogenized using aqueous nitrate solution (35 mg L^{-1} $N\text{-}NO_3^-$) resulting in 15% (w/v). The solution was kept under Dubnoff orbital agitation at 30°C for 72 hours. This last cycle was repeated twice in a row for the growth and proliferation of denitrifying microorganisms. Subsequently, rice husks with immobilized microorganisms were separated from the crude bacterial extract both fractions (MO_{IM} and MO_{SO}) are used for denitrification experiments.

Initially, nitrate removal experiments were carried out using 100 mL crude extract (MO_{SO}) and sodium nitrate in a of 35 mg L^{-1} concentration. Nitrate removal experiments using microorganisms MO_{IM} were carried out using 100 mL of a 35 mgL^{-1} sodium nitrate solution. Both experiments were carried out using a Dubnoff water bath, in a 250 mL Erlenmeyer, at 30 °C and pH 7, under constant shaking at 30 rpm. The aliquots were collected at intervals of 6, 12, 18, and 24 hours.

Posteriorly, the reactions were carried out under different conditions of nitrate concentration (35, 70, 105,140 and 280 mg L^{-1} $N\text{-}NO_3^-$), pH (5, 6, 7, 8 and 9), temperature (15, 25, 30, 40 and 500 °C) and C/N Ratio (1:1, 2:1; 3:1, 4:1 and 5:1). Ethanol or sodium acetate were used an external carbon source.

The control treatment was carried out in the absence of an external carbon source. All

experiments were carried out in triplicate.

2.3 Identification of the Presence of Denitrifying Microorganisms

To generate the microorganisms, the rice husk biomass was washed with running water and mixed with aqueous nitrate solution (35 mg L^{-1} $N\text{-}NO_3^-$) resulting in 15% (w/v). After the complete removal of nitrate, the denitrified water was again contaminated with aqueous nitrate solution and a carbon source (C/N Ratio 1:1). After 72 h incubation, (shaking regime at water bath at 30 °C), samples were centrifuged (6000 rpm for 8 min) and bacterial pellet was collected. Thereafter, microorganisms were prior inoculated into 250 mL Erlenmeyer containing aqueous nitrate solution and carbon source for anaerobic conditions incubation. Last step was repeated three times in a row to produce microorganisms. Finally, microorganism samples were seed on Mueller-Hinton agar, where colonies growth, then, colonies were separated using successive sowing on Macconkey and Blood agar plates, to obtain pure cultures.

The colonies were morphologically characterized (color, shape, elevation), submitted to GRAM staining and biochemical tests (catalase and oxidase). The bacteria were identified at the UNISUL Biochemistry Laboratory (CENTEC), based on morphological and biochemical tests (João et al., 2020).

2.4 Assessment of Operational Robustness

Microorganism recycling experiments were carried out using MO_{SO} or MO_{IM}. After complete denitrification, different amounts of denitrified water were withdraw, and the resulting volume, called crude extract, was filled to 100 mL, with nitrate contaminated water (35 mg L^{-1} $N\text{-}NO_3^-$) and a source of carbon added. Volumes crude extract used in the different biological denitrification cycles were 10, 20, 30, 40, 50 and 100 mL.

2.5 Determination of Nitrogen Containing Species

Denitrification process was monitored by the analysis of nitrite and nitrate, by using methodologies 4500-NO_2^- B and 4500-NO_3^- B, respectively. These methods followed the description on 22th edition of the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). Analyses were carried out by using a UV/VIS spectrophotometer PHARO 300 (Merck). Obtained results were used to express nitrogen content, either in the bases of nitrate ($N\text{-}NO_3^-$) or nitrite ($N\text{-}NO_2^-$) anions. The water pH was measured by a portable pH meter (Hanna).

3. Results and Discussion

Pseudomonas (GRAM negative), *Oerskovia spp.* (GRAM positive Bacilli), *Enterococcus sp.* (GRAM positive Cocci), *Bacillus mycoide* (GRAM positive Bacilli), and *Escherichia coli* (GRAM negative) are isolated from rice husk biomass. These bacteria form a biofilm in the growth medium and these biofilms are responsible for the process of biological denitrification of the water samples tested. *Pseudomonas* are the principal microorganism responsible for the denitrification process. These microorganisms were used either Immobilized in Rice Biomass (MO_{IM}) or Suspended Microorganisms in Solution (MO_{SO}). There was no

denitrification using autoclaved rice husk, which means that selected MO were required for denitrification process.

The period of greatest removal of N was up to 6 hours of the experiment, in which N-NO_3^- was reduced from 35 to 22.6 mg L^{-1}, and N-NO_2^- from 35 to 19.6 mg L^{-1}, representing removals close to 40% (Figure 1).

At the beginning of the test, there were larger nutrients and N concentrations available to microorganisms than at any other time, and these larger concentrations induce the microbial activity (Bankston et al., 2020). Therefore, the rate of removal decreases progressively until reaching the equilibrium state (~99% of removal) after 24 hours.

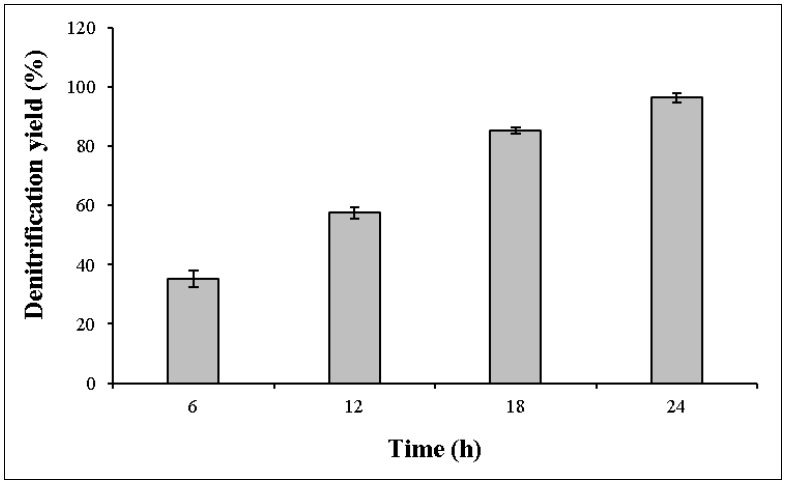

Figure 1. Denitrification efficiency versus experiment time. Initial N-NO_3^- concentration was 35 mg L^{-1}, 100 mL of MO_{SO}, T = 30 °C, pH = 7

3.1 Effect of Temperature on the Denitrification Process

The temperature represents one of the most important factors for the success of denitrification. Here, Using MO_{SO}, optimal denitrification occurred between 25 °C and 30 °C (Figure 2).

The rate of denitrification using MO_{SO} (Figure 2A) and MO_{IM} (Figure 2B) increases with increasing temperature until the optimum value of 30 °C. Above 30 °C, there was a decrease in efficiencies in the denitrification process.

At 40 °C and 50 °C and using MO_{SO}, denitrification processes were less efficient than at 25 °C and 30 °C. These data corroborate with most of the reported studies that indicate an ideal denitrification temperature between 20 °C and 30 °C (Figure 2A) (Bucco et al., 2014; Liu et al., 2012).

At 25 °C and 30 °C, MO_{IM} (48 h) and MO_{SO} (24 h) provided equivalent denitrification rates. However, in 24 h, MO_{SO} provided larger denitrification rates (double) than MO_{IM}, because MO_{IM} activity is limited and reduced when compared to free cells in suspension MO_{SO} (Figure 2) (Bankston et al., 2020; Gan et al., 2019; Willaert, 2009).

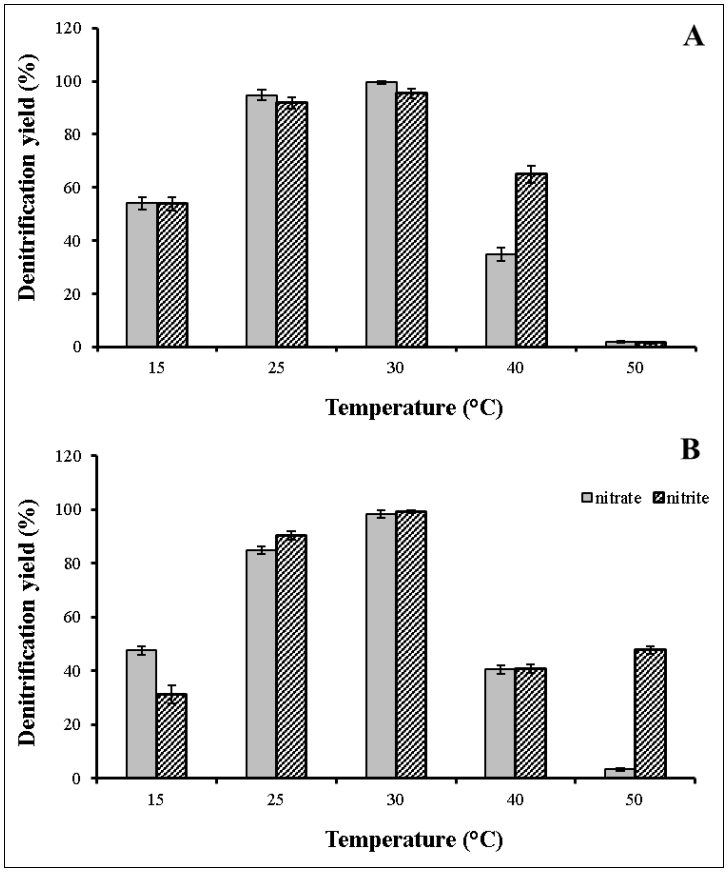

Figure 2. The efficiency of denitrification at different temperatures using MO_{IM} and MO_{SO} as a source of denitrifying microorganisms in water contaminated with 35 mg L^{-1} $N\text{-}NO_3^-$ (A) MO_{SO} within 24 h, (B) MO_{IM} within 48 h

3.2 Effect of pH on the Denitrification Process

Temperature and pH are crucial parameter in the biological denitrification process. Here, the best denitrification efficiencies were obtained at a pH 6-8 (Figure 3). Nancharaiah et al. (Nancharaiah et al., 2017) reported that denitrifying microorganisms tolerate pH between 6-8 and there was low denitrification out of this pH range.

The efficiency in nitrate removal using MO_{SO} increases with pH increase from 5 to 8 (Figure 3A). However, at pH 9, nitrate and nitrite removal decrease, and best results were found at pH 7-8. At pH 7-8 and using MO_{IM}, nitrate removal was optimal at pH 7-8. Nitrate removal efficiency increased from 59 % to 94% when the pH of the medium raised from 5 to 8 (Figure 3B). However, when the pH goes from 8 to 9, the nitrate removal efficiency drastically decreased from 94% to 40%. This behavior agrees with other literature reports, which indicates that pH 6-8 is the optimum range for denitrifying microorganisms grow (Liu et al., 2012).

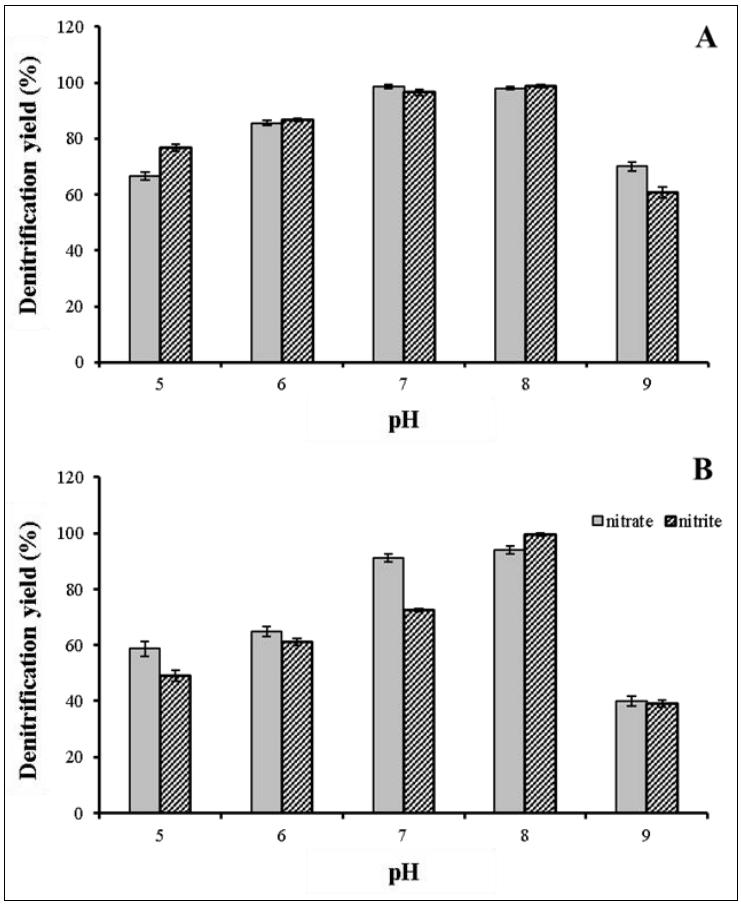

Figure 3. The efficiency of denitrification at different pH using MO_{IM} and MO_{SO} as a source of denitrifying microorganisms in water contaminated with 35 mg L^{-1} $N\text{-}NO_3^-$ (A) MO_{SO} within 24 h (B) MO_{IM} within 48 h

3.3 Nitrate Concentration

Figure 4 shows the efficiency of denitrification at different nitrate initial concentration using MO_{SO} (Figure 4A) and MO_{IM} (Figure 4B) as a source of denitrifying microorganisms in water contaminated with nitrate and nitrite.

At 35 and 70 mg L^{-1} $N\text{-}NO_3^-$ concentrations, the denitrification process using MO_{SO} is optimal, where nitrate and nitrite denitrification rates were equivalent, with an average close to 100% (Figure 4A). The denitrification process using MO_{IM} is optimal, where nitrate denitrification rates were larger that nitrite denitrification rates, where nitrate denitrification efficiency were close to 100% and nitrite denitrification efficiency were close to 70% (Figure 4B).

At 140 mg L^{-1} $N\text{-}NO_3^-$ concentration, nitrate removal using MO_{SO} and MO_{IM} was 52% and 59%, respectively. Nitrite removal using MO_{SO} and MO_{IM} were 87% and 60%, respectively. At 280 mg L^{-1} $N\text{-}NO_3^-$ concentrations, the nitrate removal efficiency using MO_{IM} and MO_{SO} were 42% and 39%, respectively, nitrite removal using MO_{SO} and MO_{IM} were 45 and 80%, respectively. It was possible to verify that nitrate concentration is a worth parameter for denitrifying bacteria growth rate and denitrifying efficiency was reduced in high nitrogen concentrations.

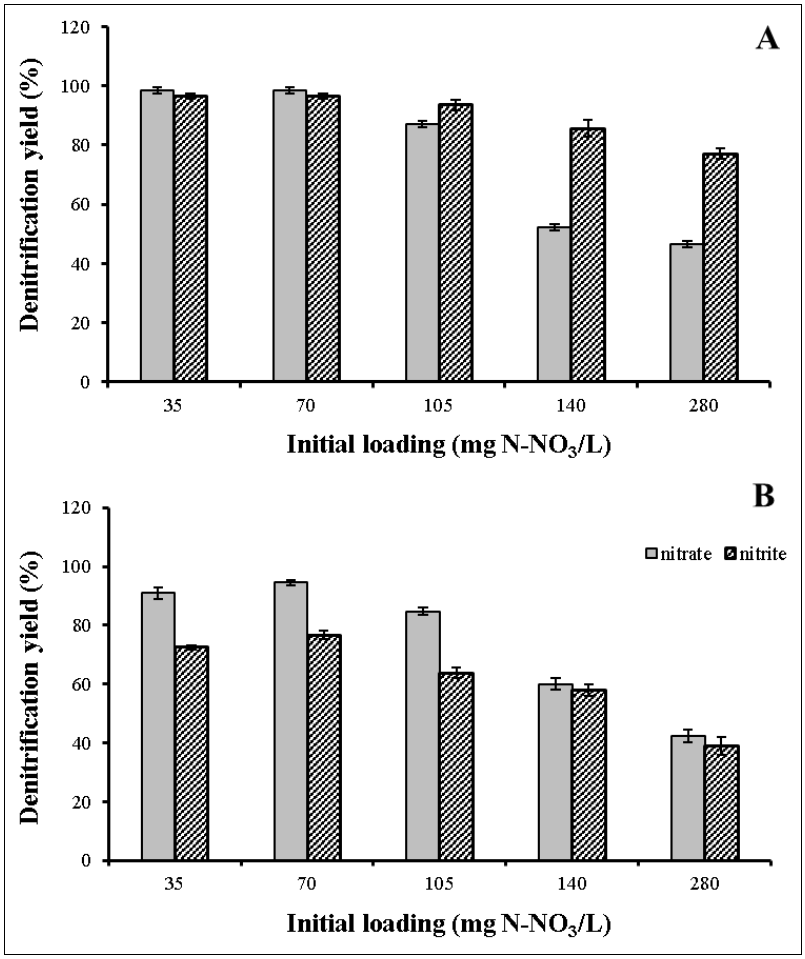

Figure 4. The efficiency of denitrification at different nitrate initial concentration using MO_{IM} and MO_{SO} as a source of denitrifying microorganisms in water contaminated with 35-280 mg L^{-1} $N\text{-}NO_3^-$ (A) MO_{SO} within 24 h (B) MO_{IM} within 48 h

3.4 Carbon Sources

In water denitrification process, organics compounds, which are electron donors, contribute to the process. As facultative heterotrophic bacteria, the denitrification process requires organic substrates as electron donors to effectively support the reduction of nitrate to N_2. Therefore, the concentration of bioavailable organic substrates as carbon sources is a mandatory parameter for the process (Wei et al., 2017). Methanol, ethanol, and sodium acetate are carbon sources. Methanol is toxic, ethanol and sodium acetate have been used as an organic carbon source since these compounds have low toxicity than methanol (Tian & Yu, 2020).

Denitrification efficiency using ethanol and sodium acetate in different stoichiometry ratios were shown in Figure 5 and Figure 6, respectively. Considerable denitrification was identified even without addition of an external carbon source. Thus, we identified that denitrifying microorganisms grow and denitrify using rice husk biomass as carbon source.

The efficiency of nitrate and nitrite removal using MO_{SO} and ethanol with 1:1, 1:2, 1:3 and 1:4 C/N ratios were equivalent, with removal above 97% (Figure 5A). The efficiency of

nitrate removal using MO_{IM} and ethanol with 1:1, 1:2, 1:3 and 1:4 C/N ratios were equivalent, showing an average of 80% of removal for the investigated C/N ratio, nitrite removal was close to 100% (Figure 5B).

Aqueous solutions containing C/N ratio of 3~5 support the metabolism of heterotrophic microorganisms (He et al., 2016). When the C/N ratio is too low to provide sufficient electron donors to the bacteria, the denitrification process requires biodegradable organic matters as external carbon sources (Wei et al., 2017).

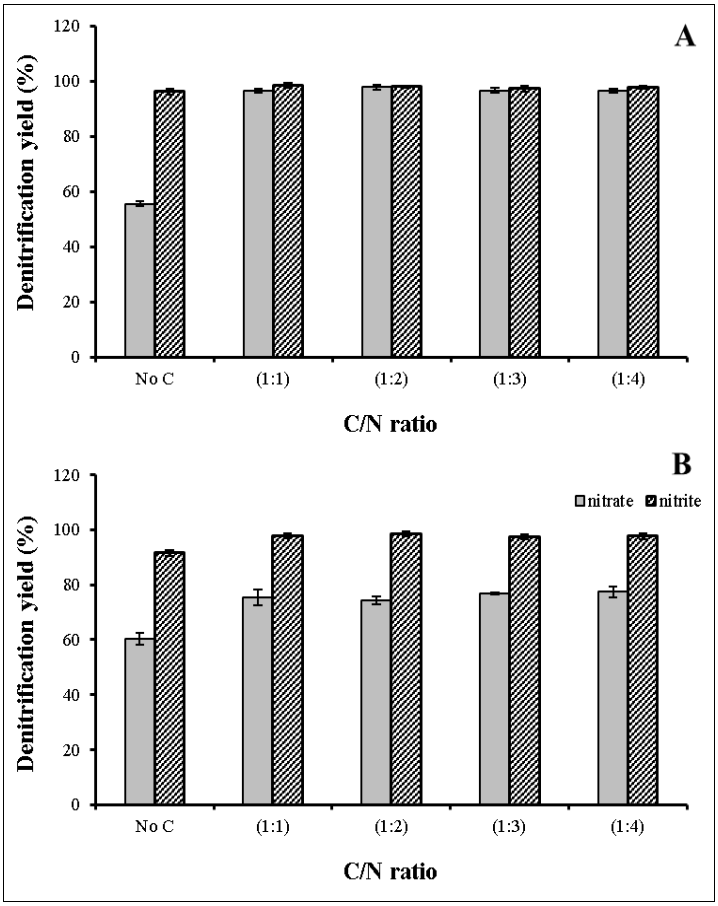

Figure 5. Efficiency of denitrification using ethanol in different stoichiometric ratios. (A) MO_{SO} within 24 h (B) MO_{IM} within 48 h

When denitrification process was carried out using sodium acetate as carbon source and MO_{SO} (Figure 6A), in all C/N ratios, nitrite and nitrate removal was close to 100%, without a carbon source, nitrate removal was just 55%. When denitrification process was carried out using sodium acetate as carbon source and MO_{IM} (Figure 6B), in all C/N ratios, nitrate removal was close to 77% and nitrite removal was close to 96%, without a carbon source, nitrate removal was just 55%.

Without a carbon source, MO_{SO} and MO_{IM} provided equivalent nitrate and nitrite removal. In addition, the efficiency of nitrate and nitrite removal using MO_{IM} was larger than those obtained using MO_{SO} (Figure 6).

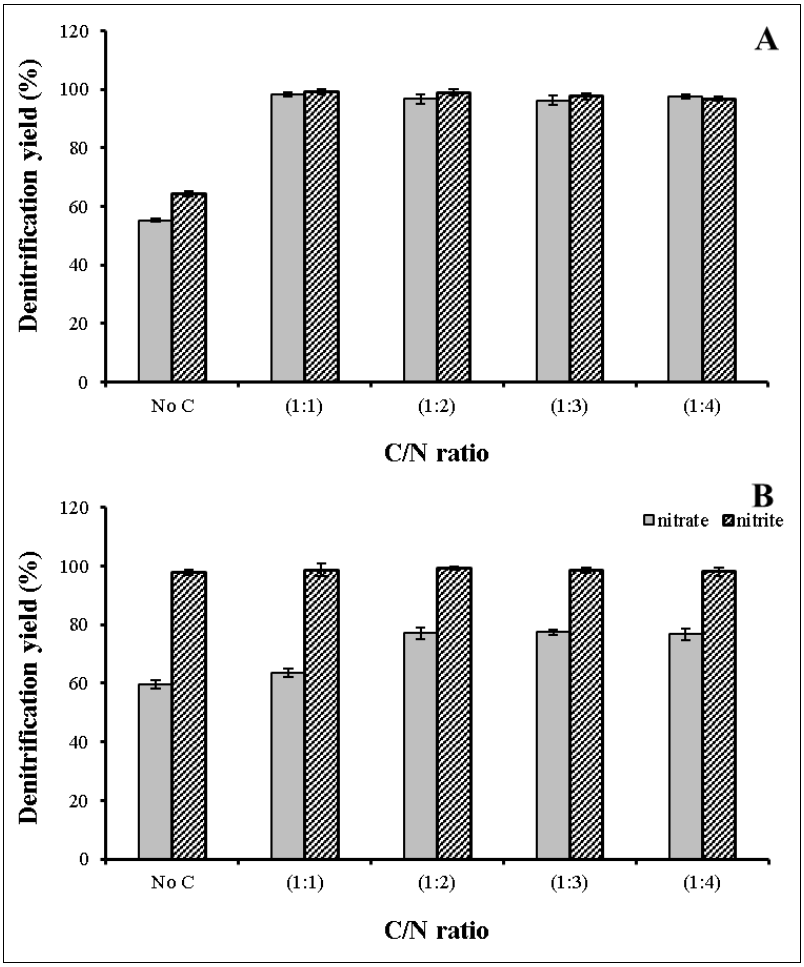

Figure 6. Efficiency of denitrification using sodium acetate in different stoichiometric ratios. (A) MO_{SO} within 24 h (B) MO_{IM} within 48 h

In scientific literature other external carbon sources are being studied for denitrification purposes. Hu et al. (Hu et al., 2017) studied denitrification efficiency in soil using sawdust. They claimed that the denitrification efficiency of sawdust was low because of its poor carbon availability. Thus, they treated sawdust with lime and peracetic acid to enhance the carbon availability. As a result, they increase denitrification efficiency and it was mainly attributed to the removal of lignin from the biomass.

3.5 Operational Robustness Assessment

The first cycle for the generation of microorganisms with the crude extract required 72 h for the complete removal of nitrate. On the other hand, subsequent cycles required only 24 h. During 10 cycles of experiment, $N\text{-}NO_3^-$ removal had a strong and robust performance when fed with the same amount of substrate (C/N), indicating the possibility of biomass reuse in several cycles of denitrification (Table 1).

Table 1. Denitrification for different subtract percentages, %. 35 mg de $N\text{-}NO_3^-L^{-1}$, each cycle 24 hours and T = 30 °C

	Concentrations of the crude extract					
cycles	100%	50%	40%	30%	20%	10%
1	100	100	100	100	100	79
2	100	100	100	100	100	78
3	100	99	100	99	100	80
4	99	99	99	99	98	80
5	99	99	99	100	98	82
6	98	99	99	99	99	82
7	100	98	99	99	98	78
8	99	98	98	98	98	79
9	97	98	99	98	97	81
10	98	98	98	99	98	79

After 10 cycles, concentrations of 20% a 100% of the crude extract provided nitrate removal close to 100%. In this crude extract range, according any-away ANOVA, nitrate removals were equivalent ($p \leq 0.05$). However, using 10% of the extract, nitrate removal efficiency decreased to 80%.

The microorganisms produced from the rice husks have great potential to be employed on nitrogen removal. The denitrifying process presented was robust and it presented nitrate removal close to 100% during 10 cycles. The only concern found, after 10 cycles, was the appearance of a yellowish color of the aqueous solution, indicating accumulation of chromophores along the biological treatment.

4. Conclusion

The principle of denitrification in this research was biological denitrification. The denitrification of microorganisms in MO_{SO} was always higher than in MO_{IM} under different conditions because the microorganisms are dispersed in the solution which improves the contact area between the microorganisms and nitrogen compounds.

Biological denitrification of contaminated water using wild type microorganisms from rice husk were an effective and promising approach. This immobilized and suspended MO had optima denitrifying efficiency at 25-30 °C and at pH 6-8.

The average denitrification efficiency using MO_{SO} was larger than it was obtained using MO_{IM}. Moreover, MO_{IM} approach was more pH sensitive than the MO_{SO} approach. Ethanol and sodium acetate were good carbon sources for MO denitrifying for both approaches. The efficiency of nitrate and nitrite removal using MO_{SO} and ethanol with 1:1, 1:2, 1:3 and 1:4 C/N ratios were equivalents, with removal above 97%. Using sodium acetate and MO_{SO}, the denitrification efficiencies were close to 100% for all C/N ratios, without any carbon source, nitrate removal was 55%. Similar results were obtained using MO_{SO}.

We conclude that the presented process was efficient and robust since nitrate and nitrate were removed with efficiencies close to 100% in 10 cycles.

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