

Assessing the efficacy of waste organic solids as carbon sources in denitrification bio-filters for abating nitrate toxicity from wastewater

Dissertation report to be submitted in partial fulfillment of the requirements for the degree

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in
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by

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Under the guidance of

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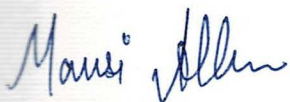


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JULY, 2022

DECLARATION

I hereby declare that the work that has been presented in the dissertation report **Assessing the efficacy of waste organic solids as carbon sources in denitrification bio-filters for abating nitrate toxicity from wastewater** submitted by Mansi Achhoda (302001008) in partial fulfilment of the requirement for the award of the Masters of Science in Biotechnology at Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab 147 004, India, is an original record of my own work done during the period from January 2022 to June 2022, carried out under the guidance of Dr. Prangya Ranjan Rout. This dissertation report has not been submitted in part or full to any other university or institute in India or abroad for the award of any degree.



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Place: Patiala

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CERTIFICATE

This is certified that the thesis entitled **Assessing the efficacy of waste organic solids as carbon sources in denitrification bio-filters for abating nitrate toxicity from wastewater** submitted by Mansi Achhoda (Registration No. 302001008) a postgraduate student of Department of Biotechnology in partial fulfillment for the award of degree of Masters of Science at Thapar Institute of Engineering and Technology, Patiala, Punjab 147 004, India, is a record of student's own work carried out under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this institute or any other university in India or abroad for the award of any degree.

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LIST OF SYMBOLS AND ABBREVIATIONS

SEM	Scanning Electron Microscope
FTIR	Fourier Transform Infrared spectroscopy
XRD	X-ray Diffraction
et al.	and others
mg/L	Milligrams per Liter
MCL	Maximum contaminant level
pH	Potential of Hydrogen
SCS	Solid carbon sources
CW	Constructed wetlands
C/N	Mass of carbon to Mass of nitrogen
N	Nitrogen
C	Carbon
N/A	Not Available
SPD	Solid phase denitrification
°C	Degree Celsius
STP	Sewage Treatment Plant
RPM	Rotation per Minutes
%	Percent
mL	Milliliter
g	gram
ppm	Parts per Million
Cm	Centimeter
mL/minute	Milliliter per Minute
min	Minute
nm	Nanometer
mg	Milligram
L	Liter
g/L	Grams per Litre
μL	Microliter
OD	Optical density
eV	Electron-volt
keV	Kiloelectron volt

ABSTRACT

Wastewater treatment is used to preserve the quality of scarce freshwater resources and improve their usability. Solid-phase denitrification is a method that has the potential to improve nitrate removal from wastewater. Being heterotrophic by nature, the source of organic carbon determines how well the denitrification process proceeds because both a lack of and an abundance of organic carbon cause disruptions in the heterotrophic denitrifiers' usual metabolism. So, choosing the right carbon source is crucial for the best denitrification process. Therefore, the purpose of this study is to evaluate a few locally available waste organic solids as potential carbon source for heterotrophic denitrifiers. The primary research variables included in this project were the influent flow rate, influent nitrate concentration, and amount of carbon source and its effect on denitrification rate. Batch and continuous mode of experiments were used to thoroughly study and assess the denitrification potential of agricultural wastes, namely rice straw (RS), wheat straw (WS), tender coconut shells (CS) and mix of the three. Maximum denitrification efficiency of 96.13% was achieved in continuous mode of operation, surpassing the efficiency of each individual carbon source of 72.6% (WS), 60.3% (CS), and 58.6%. (RS). When the physical features of the organic wastes were examined using SEM, FTIR, and EDS, the findings showed significant morphological changes in samples after denitrification as compared to original samples. The nitrate reduction ability of the microbial strains isolated from the denitrifying column as confirmed by biochemical characterization validated the experimental evidences of this study. These results provide a theoretical framework for effectively using agricultural waste as a solid carbon source and improving denitrification efficiency in wastewater treatments.

Keywords: Solid carbon sources, Heterotrophic denitrification, Wheat straw, Rice straw, Waste organic solids

Chapter 1

Introduction

1.1 Background of the study

The increasing population has led to a significant increase in water pollution, with ever-increasing water pollution nitrogen levels have spiked in the domestic wastewater. Agricultural runoffs and the discharge of poorly treated domestic wastewater are the main sources of nitrate pollution (How et al., 2021). The presence of nitrogen along with other elements like phosphorus leads to water bodies becoming enriched with anthropogenic nutrients and eutrophication of water bodies occurs. Methemoglobinemia, also known as blue baby syndrome, a condition where the body is oxygen-deprived and the skin turns blue-gray, can be brought on by excessive exposure to nitrates and nitrites. Mothers who are exposed to ambient nitrates and nitrites run a higher risk of developing preeclampsia, anemia, premature labor, miscarriage, and acute poisoning in some cases. While there are naturally occurring levels of nitrate in groundwater, quantities larger than 3 mg/L typically signify contamination (Madison and Brunett, 1984). The maximum contamination limit for nitrate has been set by the United States Environmental Protection Agency (USEPA) at 10 mg/L in order to prevent health hazards. But domestic discharge ranges from 20-70 mg/L NO_3^- - N, which is quite above the permissible limit. Even municipal wastewater treatment plants (WWTPs) contribute to nitrogen loading into the ground and surface waters with approximately 80,000 tonnes/year (Rahimi et al., 2020). Ion exchange, reverse osmosis (RO), ammonia volatilization, and electro-dialysis are the methods that can be used to eliminate nitrate from wastewater (Shrimali and Singh, 2001). Ion exchange and RO are relatively very expensive processes if employed on an industrial scale, electro dialysis and RO have high maintenance and energy costs and water requires pre and post-treatments like pH correction and removal of chemicals like chloride or bicarbonate. Low efficiency, production of large amounts of waste brine, and significant change in effluent pH are other disadvantages of such

techniques (Matei and Racoviteanu, 2021). Biological denitrification is the most economical and reproducible method for nitrate removal and is the only process that directly targets nitrate and does not change the concentration of any other ions. It's an anoxic process that requires organic carbon as an electron donor in which nitrates (nitrogen) are the terminal electron acceptors used by denitrifying bacteria in place of oxygen throughout their respiratory process. The sequential reduction of nitrates to harmless nitrogen gas by the action of enzymes of denitrifying bacteria (heterotrophs) as demonstrated in Figure 1.1 is termed as denitrification. The process is catalysed by enzymes like nitrate, nitrite, nitrous oxide, and nitric oxide reductase, and their synthesis occurs mostly in anaerobic environments. Nitrate Reductase plays the most significant role among these enzymes in denitrification (Ahmed et al., 2022).

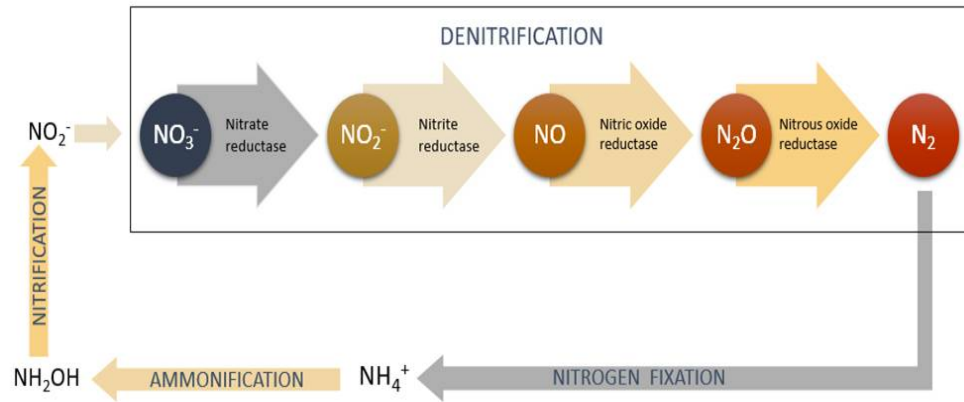


Figure 1.1: Nitrogen cycle

The majority of wastewater in use lacks considerable amounts of organic materials necessary for nitrate removal. Therefore, there is a need for an additional external carbon source to accomplish the required denitrification performance (Kim et al., 2017). The cost of the carbon source used, the rate of denitrification, the amount of sludge produced, availability, and the ease of storage are some of the variables that influence the choice of an external carbon source. The source of organic carbon for the heterotrophic denitrification process can be liquid or solid. Ethanol, succinate, acetate, and methanol, like liquid carbon sources possess the problem of insufficient doses, overdosing, storage, and transportation issue. These problems can easily be tackled by solid carbon sources which are comparatively stable, and economical and ensure controlled release of carbon via microbial enzyme mediated degradation. Biological denitrification where the carbon source employed is in a solid state, is termed as solid phase denitrification. Solid carbon sources can be used in their

natural forms like wood chips, fruit shells, and agricultural waste. Agricultural waste can be proved to be a good carbon source on large scale because of the enormous availability and, by eliminating nitrate from wastewater, we may achieve the motto of "treat waste by waste" (Peng et al., 2021). Artificially synthesized biopolymers like poly(ρ -caprolactone), polyhydroxyalkanoates, poly (l-lactic acid), polyvinyl alcohol, poly3-hydroxybutyrate-co-3-hydroxyvalerate, poly (butylene succinate) are also used (Xu et al., 2018). Primary sludge formed in any wastewater treatment plant can also be used as an external carbon source (Cao et al., 2020). However, there may be some drawbacks to solid phase denitrification, such as poor electron donor availability, accumulation of nitrite oxide, release of dissolved organic carbon (DOC) and production of nitrous oxide as side products. Operational factors and environmental conditionals also affect the heterotrophic denitrification (Zhong et al., 2020).

Carbon sources in solid-phase denitrification can also be used as a biofilm carrier for denitrifying microbes. The biofilm's microbial enrichment directly affects how well contaminants are removed. In continuous flow solid-phase denitrification bioreactors, Betaproteobacteria were demonstrated to play a major role in denitrification by using biodegradable polymers as a carbon source (Yang et al., 2021). Biofilm's nitrate removal effectiveness is decreased because of the greater shear stress and heavy volume load brought on by shorter hydraulic retention times (HRT). In comparison to conventional suspended biomass treatment methods, fixed film bioreactors have a number of advantages, such as increased biomass concentration, prolonged microbial retention time, high hydraulic loading rates, decreased bioreactor size, and consistency with regard to variable wastewater composition and flow. They, therefore, perform better than other types of treatment (Cheikh et al., 2013).

1.2 Motivation and Objective

With a predominant agrarian economy, Punjab has a huge reserve of agricultural waste biomass in the form of harvest build-ups, rice and wheat straw (stubble) in particular. Stubble burning (burning of crop residues) is a widespread practice that leads to environmental hazards like air pollution, loss of nutrient from the soil, human health risk, etc. In fact, stubble burning is one of the key factors of environment pollution in north India and therefore measures to curb stubble burning needs immediate attention. The utility of such large amount of agricultural waste biomass generated to minimize the stubble burning issues still appears to be an unanswered predicament. In order to find some additional utility of agricultural waste biomass, solid phase denitrification using stubbles as potential carbon sources

has been proposed in this study.

The objective of this research work is to evaluate the potential of wheat and RS (stubble) to be used in denitrifying bioreactors as solid carbon sources and biofilm carrier for heterotrophic microorganisms. The use of stubble for denitrification of course will have several added advantages including, reduced dependency on harmful chemical carbon sources, increase the use of locally and easily available natural waste organic substances, minimization of stubble burning mediated environmental pollution, and reduced nutrient load to the aquatic ecosystems. The possible utilization of degraded stubble as organic fertilizer after composting will ensure the realization of circular bio economy through the conversion of wastes (stubble) into wealth (organic fertilizer).

Chapter 2

Review of literature

2.1 Solid Carbon Sources (SCS) studied

Constructed wetlands (CW) are water treatment systems that use microbial assemblages, wetland vegetation, and soils to improve water quality through natural processes. Denitrification of a CW may be further accelerated by the addition of co-immobilizing solid carbon sources and denitrifying microorganisms. Denitrifying bacteria are able to maintain their biological activity and stability, grow swiftly, and do so without being leached out of CW. Additionally, by increasing the wastewater's C/N ratio by addition of carbon source, problem of lack of carbon sources during the denitrification process solves and hence improves the effluent water quality from CWs. In particular, at a C/N ratio of 3, the use of biochar in Constructed wetlands (CW) led to significantly improved removal efficiencies for COD (92%), $\text{NH}_4^+ \text{-N}$ (50%), and TN (50%) (Zhou et al., 2018). According to (Li and Chen, 2018), incomplete pyrolytic biochar could yield dissolved organic matter that could serve as bioavailable carbon sources for denitrification. WS, apricot pits, walnut shells, and cotton waste have all been employed as external carbon sources in CWs, which increased N removal performance (Si et al., 2018; Jia et al., 2020). According to research by (Yang et al., 2018), polyacetic acid and poly-3-hydroxybutyrate-co-3-hydroxyvalerate were both shown to be effective carbon sources for CWs used in denitrification, with a maximum efficiency of 97.3%. Using corn starch-polycaprolactone as a carbon source, nitrate was removed from the layer containing solid carbon sources, and the removal efficiency of $\text{NO}_3^- \text{-N}$ was up to 98.23% (Shen et al., 2015). Polycaprolactone is the other most commonly used synthetic carbon source for slow sand filters. The powdery polycaprolactone (PCL) with different particle sizes (40 and 200 mesh) PCL200 could rapidly release more organic compounds compared to PCL40. Ideal denitrification results were achieved in the PCL200 system due to higher denitrifying bacterial biomass (62.12%) and abundance compared to the PCL40 reactor.

Compared to PCL40 (121.2 mg/L), PCL200 could release organic carbon up to 273.9 mg/L. The PCL200 proved helpful for the quick start-up of the SSF and obtaining the desired removal efficiency of NO_3^- -N (>95%) and TN (>80%) at the early stage of operation because of the increased organic carbon release and greater surface area (Yang et al., 2021). Further the biofilm gets rigidly attached to the clear pores on the surface of PCL carriers and bacteria that could degrade PCL carriers to obtain electron donor for denitrification were found on the used surface of PCL (Zhang et al., 2019).

Other than synthetic carbon sources agricultural waste can be of huge importance as a carbon source for denitrifying bacteria. Based on their widespread availability and minimal or no cost, the four waste organic solids sugar cane bagasse (SB), aegle shell (AS), groundnut shell (GS), coconut shell (CS) were chosen as potential carbon sources and substrates for biofilm attachment to facilitate denitrification. SB, AS, GS, CS, and Mix showed the highest denitrification efficiency in batch cultures, which was 94.55, 90.53, 97.9, 96.38, and 100%, respectively. Beginning with a feed nitrate concentration of 100mg/L, the nitrate concentrations decreased to less than 10mg/L in each case. The duration of the denitrification process follows the order Mix>CS>GS>SB>AS, where the mixture of all the substrates had 97% denitrifying efficiency (Rout et al., 2017). The reason for such good efficiency can be because Mix substrate had the advantage of being both easily degradable and difficult to degrade, where the former contributed to a quicker onset of the denitrification process and the latter to a longer ability to do so. Mix substrate as a carbon source supports a wide range of microbial consortia and helps to achieve increased microbial activity (Gilbert et al., 2008). (Peng et al., 2021) demonstrated that when carrying out denitrification of secondary effluent which has a very low C/N ration, crosslinking between polybutylene succinate (PBS) and peanut shell (PS) with polyvinyl alcohol–sodium alginate (PVA-SA) as carrier operated more effectively and durably than individual sources because of the sustainable release of carbon during the entire process. Individual carbon sources, such as PS, somewhat improved denitrification but increased the DOC in the effluent. Only SCS that maintained DOC below 20 mg/L, was the cross-linked polymer which had a 68 percent efficacy rate in removing nitrate. Immobilization of bacterial species directly on carbon surface has also been proved helpful for denitrifying purposes one such example was illustrated by (Yu et al., 2019), where bacteria *Pseudomonas fluorescens* was immobilized on the surface of rice husk (P1). These P1 granules were studied against bacterial colonies (P2) and just rice husk (P3) immobilized alone. *P. fluorescens* is a mesophilic species, that explains best removal efficiencies using either P1 or P2 of 95.2% and 45.7%, respectively, at 30 °C and maintaining that efficiency at various pH values of 6.5, 7.0, 7.5, and 8.0. The main reason for such observation was that the

lack of a sufficient carbon supply inhibited denitrifying bacteria from growing and reproducing, which had a significant negative impact on the denitrification process (Yu et al., 2019). A list of potential denitrifiers, their sources, nutritional types and redox conditions required for denitrification are summarised in Table 1. A combination of natural and synthetic carbon sources can also be employed in a bioreactor. Two floating constructed wetlands (FCWs) were created by employing light ceramsite (FCW-LC) and RS (FCW-RS) as a substrate for microorganisms (Cao et al., 2016). When utilising RS, the TN concentration dropped from 1.32-2.97 to 0.05-0.66 mg/L; and when using LC, it declined to 0.05-1.32 mg/L. Densities of biofilms present on the RS and light ceramsite were 6.73 g/m² and 14.27 g/m². As a result, RS proved to have a higher TN removal effectiveness than LC. When compared to other agricultural waste, such as banana stalks, watermelon skins, loofah sponges, sorghum stalks, wheat husks, and sweet potato stalks, the six agricultural wastes were found to be capable of completely eliminating NO₃⁻ and NO₂⁻ over the course of the 181-hour denitrification process. The maximum TN removal efficiencies were achieved by Sorghum, sponges, and wheat husk, and loofah sponges (LS) exhibited the highest TP (total phosphorus) removal and the lowest cumulative COD. As a consequence, LS was preferable to the other five agricultural wastes as a solid carbon source for denitrification (Gao et al., 2022).

Table 2.1: Characteristics of denitrifying bacteria

S.No.	Denitrifying Microorganisms	Source	Types	Redox Conditions	Gram stain	References
1	<i>Paracoccus denitrificans</i>	N/A	Autotrophic	Aerobic	Gram-negative	(Baumann et al., 1996)
2	<i>Thiobacillus denitrificans</i>	ANANOX pilot plant	Autotrophic	Anoxic	Gram-negative	(Zou et al., 2016)
3	<i>Thiobacillus thioparus</i>	Gas work effluent	Autotrophic	Facultative anaerobic	Gram-negative	(Rezvani et al., 2019)
4	<i>Acidithiobacillus thiooxidans</i>	Coal waste	Autotrophic	Aerobic	Gram-negative	(Yang et al., 2019)
5	<i>Pseudomonas pseudoalcaligenes</i>	Soil, waste water	Heterotrophic	Aerobic	Gram-negative	(Rezvani et al., 2019)
6	<i>Bacillus niacini</i>	N/A	Heterotrophic	Aerobic	Gram-positive	(Martienssen and Schöps, 1999)
7	<i>Thiospaera pantotropha</i>	Wastewater treatment system	Heterotrophic	Aerobic	Gram-negative	(Robertson and Kuenen, 1990)
8	<i>Microvirgula aerodenitrificans</i>	Activated sludge	Heterotrophic	Anoxic	Gram-negative	(Patureau et al., 2000)
9	<i>Alcaligenes faecalis</i>	Wastewater treatment system	Heterotrophic	Aerobic	Gram-negative	(Joo et al., 2006)
10	<i>Comamonas denitrificans</i>	N/A	Heterotrophic	Anoxic	Gram-negative	(Khan et al., 2007)
11	<i>Thaurea mechernichensis</i>	Activated sludge	Heterotrophic	Aerobic	Gram-negative	(Etchebehere and Tiedje, 2005)
12	<i>Acinetobacter junii</i>	Waste water	Heterotrophic	Aerobic	Gram-negative	(Gu et al., 2022)
13	<i>Acinetobacter pittii</i>	N/A	N/A	Aerobic	Gram-negative	(Shi et al., 2022)

2.2 Effect of HRT on denitrification

The hydraulic retention time (HRT) is a measure of how long the water/liquid phase is typically retained inside the digester. The nitrate removal capacity of any bioreactor can be improved with increasing HRT however if HRT is too high, the effectiveness of the process would decline, and the economic viability of a bioprocess is directly influenced by the hydraulic retention time. The efficacy of COD removal was reported to have steadily dropped based on the drop in HRT during the denitrification process (Kim et al., 2008). Increasing HRT improved COD removal efficiency at high influent TN levels, but had the opposite effect on COD removal at low TN concentrations (Li et al., 2010). At high HRTs, the slow water flow rate permits heterotrophic denitrifying bacteria to flourish, allowing for better nitrate removal but the construction costs of bioreactors would rise (Xiao et al., 2014; He et al., 2016). Due to the sufficient interaction between denitrifying bacteria and wastewater, independent of the carbon source used, the removal efficiency of NO_3^- , NO_2^- , and TN improved with HRT increasing within a particular range (Wang et al., 2015). Notably, the denitrifying bacteria are affected by HRT, it also affects the structure of the total bacterial community present in the bioreactor. When HRT is set to 2 hours, maximum efficacy of nitrate removal of 85% can be attained in denitrifying bioreactor (Chen et al., 2021). The system will malfunction if the HRT is too short because the slowly growing bacteria required for biological denitrification will be washed out (Zaher et al., 2009). When (Wu et al., 2021) varied HRT from 12 h to 0.5 h with 5 mg/L DO, optimum aeration of low carbon SPD bioreactor was achieved at 6 hours, even though DO was reduced to 3 mg/L due to microbial activity.

2.3 Effect of C/N rations on denitrification

The primary factor affecting the efficiency of denitrification is the C/N ratio, which specifies how much of a carbon source is used relative to how much nitrogen molecules are reduced in a process (Sobieszuk and Szewczyk, 2006). Hou et al (Hou et al., 2017) compared C/N ratios of 2, 2:1, and 5:1, and it was found that the bioreactor with the highest C/N ratio had the best nitrate removal rate and that system's nitrate concentration fell by 71.8%. This shows that by including an external carbon source to the water-saturated denitrifying section of any bioreactor, the denitrifying capacity can be increased (Fan et al., 2013). When an external carbon source was added, the experiment's preliminary results revealed relatively minor nitrogen removal, which suggests that the microbes in the second stage needed time to adapt to the changing conditions. Bacteria also requires time to acclimate to the surface of a SCS.

Once there's biofilm formation and the experiment's duration is prolonged, denitrifying capability increased. This shows that the 2:1 C/N ratio increased the second stage denitrifying capacity to some extent by encouraging the growth of denitrifying bacteria, but it was unable to meet the rising carbon demand as the denitrifying bacteria multiplied, and the C/N ratio of 5 performed better than a ratio of 2. The best TN performance obtained from these three ratios resulted in 79.8% TN removal (Fang et al., 2018). The denitrification process is also affected by the $\text{NO}_2\text{-N}$ concentration. Accumulation of $\text{NO}_2^- \text{-N}$ mainly occurs due to carbon insufficiency when the C/N ratio are below 5, indicating that the denitrification process is incomplete (Ding et al., 2012).

2.4 Effect of temperature

Si et al (Si et al., 2018) conducted a study where four solid carbon sources (WS, cotton, poly(butylene succinate) (PBS), and newspaper) were utilized as supplementary electron donors to traditional liquid carbon sources in denitrification. The experiment was conducted in both high and low temperatures. Since temperature fluctuations affect the physiological activity of microorganisms participating in biodegradation and denitrification (Spieles and Mitsch, 1999), denitrification performances varied between low- and high-temperature stages, especially in newspaper, WS, and cotton which are cellulose-rich solid carbon sources (Volokita et al., 1996). WS and newspaper both had a significant total nitrogen removal efficiency in high-temperature settings, with respective values of 90.82 and 89.62 percent, which is much greater than cotton's (65.81%) and PBS's (23.08%). WS's effectiveness as a solid carbon source for denitrification in CWs dropped to 69.42% because it was more sensitive to temperature variations. Other carbon sources also performed better in comparatively high temperatures, but in both low- and high-temperature conditions, the newspaper can accelerate the biological heterotrophic denitrification process in artificial wetlands. Hence nitrate removal efficiency of SCS is better at higher temperatures (Si et al., 2018). But too high temperature will prevent denitrifiers from working properly, which will quickly reduce the efficiency of denitrification. According to studies, the temperature's upper threshold was between 35°C and 40°C (Zhong et al., 2020).

2.5 Effect of DO

Depending on the concentration, dissolved oxygen (DO) can affect the denitrification process. When oxygen is scarce, nitrate outperforms other electron acceptors. The majority of

denitrifying bacteria are facultative anaerobes, and denitrification can occur without anaerobic conditions (Gutierrez-Wing et al., 2012) . However, a concentration of DO that is too high, lowers denitrification rate (Healy et al., 2012) and results in incomplete denitrification (Elgood et al., 2010). The impact of DO inhibition on denitrification rates is also influenced by nitrate levels and can be reduced when the influent’s nitrate concentration is low (e.g., 2mg/L NO_3^- -N) (Lynn et al., 2015).

2.6 Redox conditions

Biodegradable plastic was utilised to create the aerated, anoxic, and low-oxygen experimental treatment groups in an experiment conducted by (Zhang and Zhang, 2018). The author concluded that the removal rates of nitrate under anoxic and low oxygen conditions were 30.6g NO_3^- -N $\text{m}^{-3} \text{d}^{-1}$ and 30.8 g NO_3^- -N $\text{m}^{-3} \text{d}^{-1}$ at 83 hours, respectively. Both of these conditions showed denitrification rates that were higher than those in the aerated group. In terms of nitrate elimination, there was no noticeable difference between both the anoxic and low-oxygen treatments. Additionally, the anoxic and aerated groups had lower nitrite concentrations than the low-oxygen group did. By the end of the experiments, no nitrite was found in any of the experiments.

2.7 Adverse effects of solid-phase denitrification

When the organic carbon source undergoes anaerobic oxidative breakdown, CO_2 and maybe CH_4 are produced as a byproduct, which is a potential detrimental effect of denitrification. Methanogens are likely to start growing inside the biofilm that is attached to the solid substrate if microbial nitrate consumption exceeds nitrate diffusion into the biofilm. CH_4 fluxes were moderate when nitrate levels in the bioreactor were high enough to keep methanogens in check, but they increased when nitrate levels declined and could now compete with denitrifiers for carbon supplies (Schipper et al., 2010). Another significant issue with SPD is the leaching of DOC as well as ammonium and nitrite buildup.

Chapter 3

Materials and methods

This project was conducted to establish the efficacy of rice straw (RS), wheat straw (WS), and tender coconut shells (CS) as the primary carbon sources for denitrifying bacteria. Agricultural waste was collected from around the campus of TIET, Patiala. All three samples were thoroughly washed and dried prior to use. Upon drying them properly, they were cut into smaller pieces and stored at room temperature.

3.1 Preparation of flasks

Five flasks were prepared with different quantities of RS, WS and CS and two with the mixture of agricultural waste as mentioned hereunder. Along with the carbon source, sludge samples obtained from the sewage treatment plant (STP) of TIET, Patiala, was added as a microbial source for biological denitrification to occur. To check the efficiency of mentioned carbon sources in the nitrate removal process, 75 ppm nitrate solution was added to the 250 mL Erlenmeyer flasks. Stock solution of 1000 ppm nitrate solution was prepared by adding 1.631 g of KNO_3 to 1 L of distilled water. Working volumes of 25 ppm, 50 ppm, and 75 ppm were made by further diluting the stock solution. These flasks were kept in an incubator shaker at 120 RPM and at 37°C. All the experiments were conducted in triplicates.

- **Flask 1** – 8 g WS + 25 mL sludge + 100 mL distilled water
- **Flask 2** – 8 g CS + 25 mL sludge + 100 mL distilled water
- **Flask 3** – 10 g WS + 25 mL sludge + 100 mL 75 ppm nitrate solution
- **Flask 4** – 10 g CS + 25 mL sludge + 100 mL 75 ppm nitrate solution
- **Flask 5** – 5 g WS + 5 g CS + 25 mL sludge + 100 mL 75 ppm nitrate solution

- **Flask 6** – 3.5 g WS + 3.5 g CS + 3.5 g RS + 25 mL sludge + 100 mL 75 ppm nitrate solution
- **Flask 7** – 8 g RS + 25 mL sludge + 100 mL 75 ppm nitrate solution

Flask 1 and 2 were initially prepared with distilled water as control to check nitrate present in sludge. Later on, these two flasks were incubated under similar conditions as other flasks. The purpose of these sets of experiments were to assess the feasibility of the heterotrophic denitrification process.

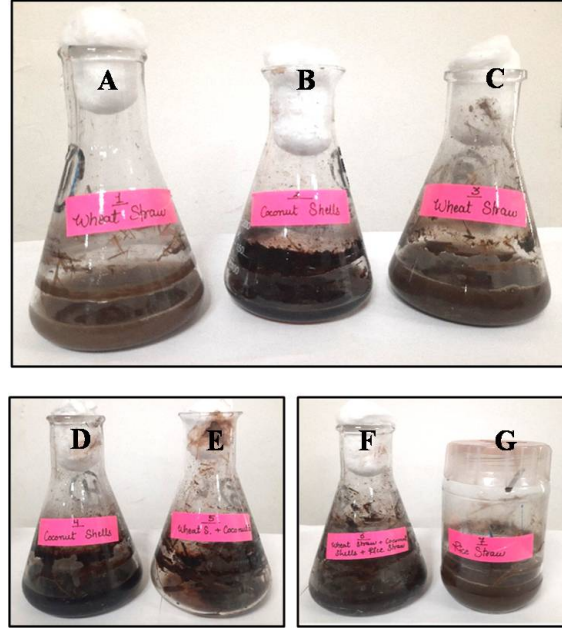


Figure 3.1: Batch experiments carried out in Flasks

3.2 Preparation of wash bottles

During the preliminary experiments discussed in the **section 3.1**, anoxic conditions were not maintained. As a pre-requisite of heterotrophic denitrification process, experiments should be conducted under anoxic conditions. Therefore, further set of experiments were conducted using wash bottles to ensure anoxic condition by sealing the small openings of the bottles through parafilm/clean film as shown in **Figure 3.2**. All the experimental conditions of the wash bottles are mentioned below:

- **Wash bottle 1** – liquid contents of flask one were emptied into the wash bottle and 6 g of WS and 100 mL nitrate solution were added.
- **Wash bottle 2** – liquid contents of flask two were emptied into the wash bottle and 6 g of CS and 100 mL nitrate solution were added.
- **Wash bottle 3** – liquid contents of flask seven were emptied into the wash bottle and 6 g of RS and 100 mL nitrate solution were added.

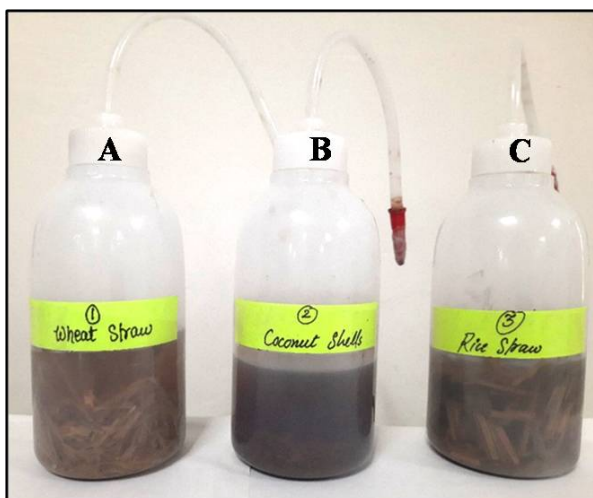


Figure 3.2: Denitrification experiments carried out in wash bottles

The purpose of transferring the contents from specific Erlenmeyer flasks to the wash bottles was to monitor or compare the denitrification efficiencies of the similar carbon sources and microbial inoculum under anoxic condition.

3.3 Continuous flow mode

Along with the batch mode of study, these three agricultural wastes were also packed in a polyacrylic column for continuous mode of denitrification. Packed bed column mode is believed to stimulate the field scale denitrification condition better than the batch mode (Rout et al., 2017). The fabricated column was having a length of 50 cm and diameter of 10 cm. 27 g CS, 13 g WS, 10 g RS, and 200 mL of sludge were added to the column up to 10 cm height and kept for 10 days for microbial growth and attachment to the solid carbon sources as shown in Figure 3.3 and 3.4. Nitrate solution of 75 ppm was pumped to the column through the inlet using a peristaltic pump and water level was maintained 5 cm above the packed substrates to ensure anoxic condition. The top of the column was

sealed with parafilm tape and aluminium foil to minimize evaporation mediated loss of the nitrate solution (Figure 3.4). The column was operated in up flow mode with a flow rate of 1 mL/min, resulting in a HRT of approximately 20 h.

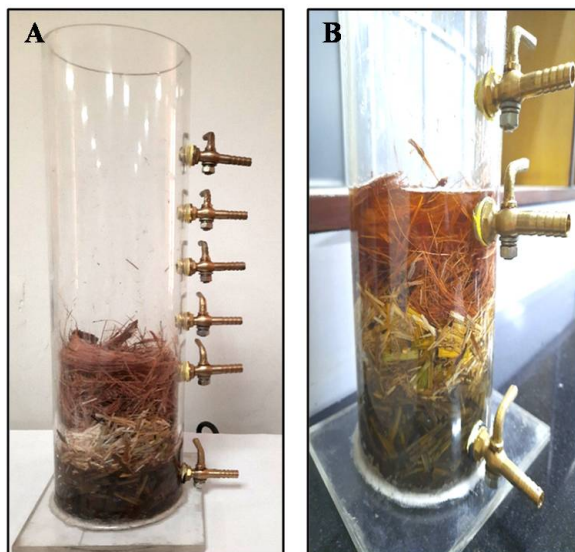


Figure 3.3: (a)Packed column bed with RS, WS and CS, (b)Column filled with 75ppm nitrate solution

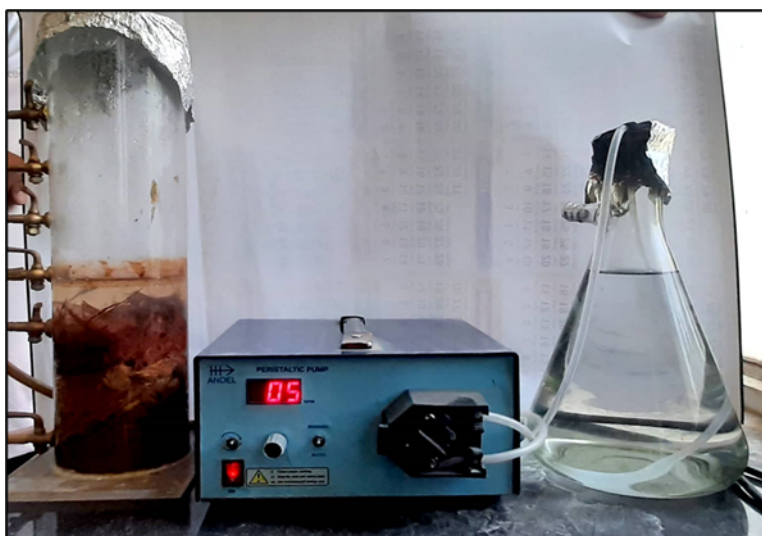


Figure 3.4: Working model of fixed bed column

3.4 Nitrate estimation by sodium salicylate method

Nitrate measurement was done by sodium salicylate method. 10 mL sample was taken in a falcon tube and centrifuged at 10,000 RPM for 10 min at room temperature, the supernatant was collected in a glass bottle. Fresh sodium salicylate solution (1%) was prepared by adding 0.1 g sodium salicylate in 10 mL distilled water and 1 mL of this solution was added to the sample. The bottle was kept at 100°C in the oven till complete evaporation of the samples. After cooling down to room temperature, 1 mL of concentrated H_2SO_4 was added to dissolve the residues, after 10 min 7 mL 10M NaOH was added, and the final volume was made up to 50 mL by adding distilled water (Figure 3.5). After 10 minutes of incubation time optical density (OD) of the samples were measured at 410 nm using a UV-Visible spectrometer (Bartosová et., 2012).

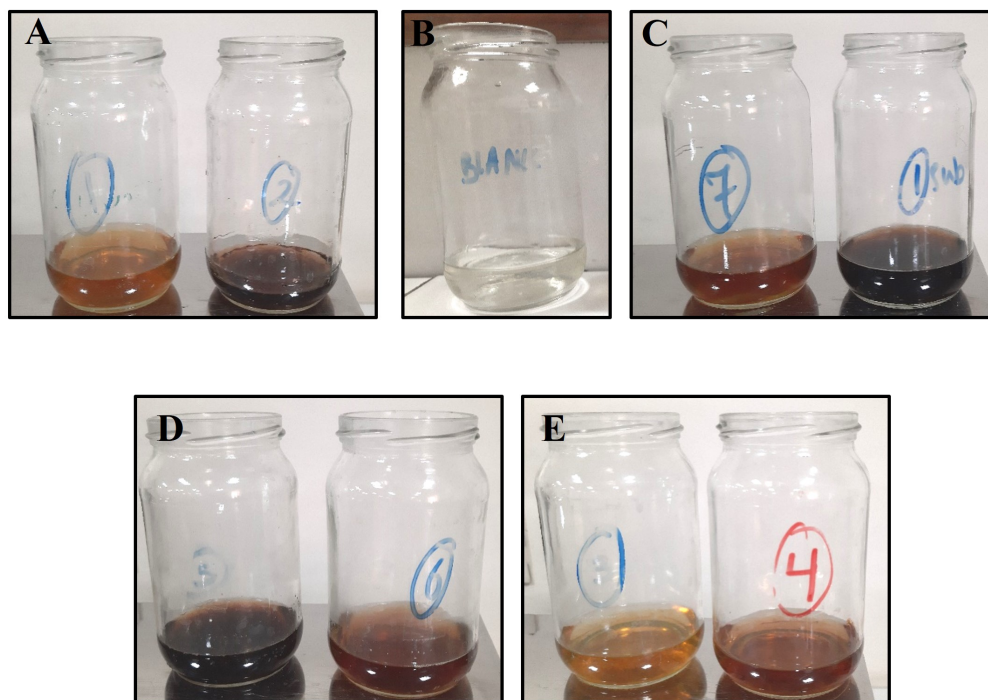


Figure 3.5: Samples after addition of Sodium Salicylate

3.5 Preparation of standard curve (Calibration curve)

A standard curve was plotted by considering the known concentrations of nitrate solutions. By diluting the stock solution, 2, 4, 6, 8, 10, 15, and 20 mg/L nitrate solutions were prepared. The samples and the blank were further prepared following the steps mentioned above in sodium salicylate method (section 3.4) and OD of the sample were measured at 420 nm. The standard curve was plotted by taking concentration in X-axis and OD in Y-axis (Figure 3.6). The generated straight-line equation from the standard curve was further used to estimate the nitrate concentration of the treated samples.

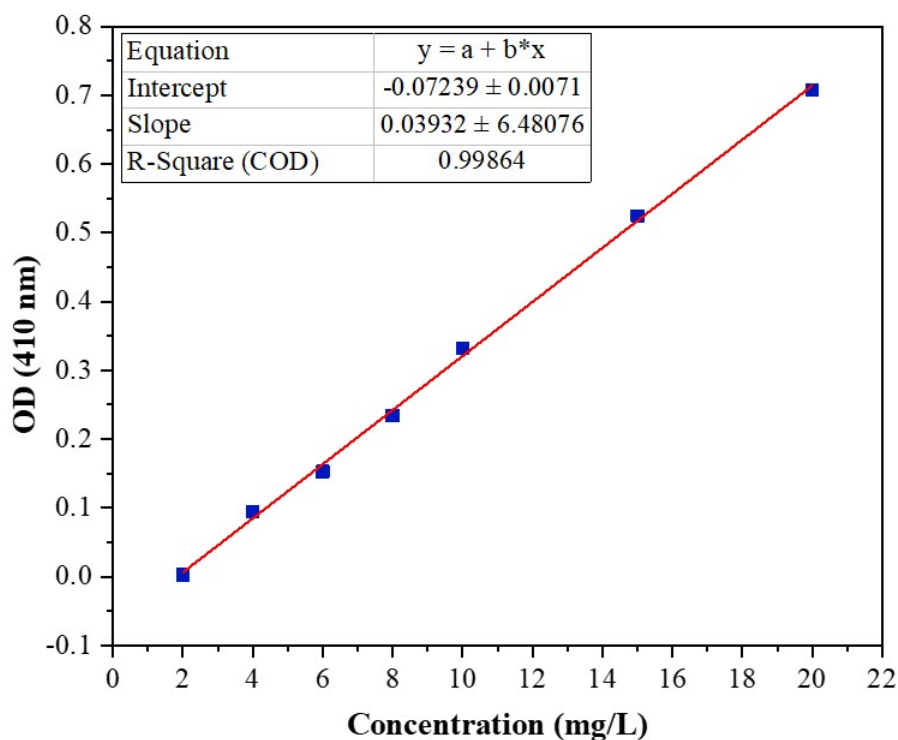


Figure 3.6: Standard Curve

3.6 Analytical methods

Modified procedure of American public health association (APHA) standard methods for the examination of water and wastewater were followed for all the analytical measurements of this work.

3.6.1 COD (Chemical Oxygen Demand) measurement

Open reflux COD measurement was carried out in a COD digester. To a 10 mL sample, 5 mL 0.041M $K_2Cr_2O_7$, and 15 mL digestion reagent ($Ag_2SO_4 + H_2SO_4$) and 1 mL $HgSO_4$ were added. The open end of the condenser was covered and reflux was set for 2 hours at 150°C. After cooling down the tubes to room temperature the samples were titrated with standard ferrous ammonium sulfate (FAS) using 2-3 drops of ferroin indicator. COD was calculated using the formula given in Equation 1:

$$COD \text{ as mg } O_2/L = (B-A) \times M \times 8000 / \text{Volume of sample} \quad (1)$$

B = mL FAS used for sample

A = mL FAS used for blank

M = molarity of FAS

*For diluted samples multiply df with the final value of COD for that particular sample.

3.6.2 TOC (Total Organic Carbon) measurement

TOC measurement was done in an open COD digester using the same reagents mentioned in section (3.6.1). The digester was set at 100°C for 30minutes and the sample were titrated against orthophosphoric acid. TOC was calculated using the formula given in Equation 2:

$$TOC = (B-A) \times M \times 12000 \times M / \text{Volume of sample} \times 4 \quad (2)$$

B = Volume used in blank

A = Volume used in Sample

M = molarity of FAS

3.6.3 TKN (Total Kjeldahl Nitrogen) measurement

To 100 mL sample in Kjeldahl digestion tube add 10 g K_2SO_4 and 1g $CuSO_4$, after proper mixing of salts, add 25 mL concentrated H_2SO_4 dropwise. Set block digester at 350° for 3

hours. Digestion of sample is followed by Steam distillation. After cooling down, 40% NaOH is added to the remaining volume of sample in the tube till the sample color turns blue (200 mL). The tube was placed in the Kjeldahl distillation unit where steam was passed into the sample for 15 min. The samples were collected in 25 mL 4% boric acid solution in a flask with the mixed indicator in it. The collected samples were titrated against 0.04 N H₂SO₄ till color changed from green to purple as shown in **Figure 3.7**. TKN was estimated as per the formula in Equation 3.

$$\% \text{ of N in sample} = 1.4 V \times N / W \quad (3)$$

V= Acid used in titration (mL)

N = Normality of standard acid

W = Weight of sample (g)



Figure 3.7: Change in colour of mixed indicator before and after titration

3.6.4 DO (Dissolved Oxygen) and BOD (Biochemical Oxygen Demand) measurement

For measuring the BOD, samples needed to be diluted with dilution water. 1L of dilution water was required per sample. Dilution water was prepared by aerating tap water overnight, followed by addition of FeCl₂, CaCl₂, MgSO₄, and Phosphate buffer in it (1 mL each for 1L of aerated water). Dilutions of each sample were made according to their respective COD values. The 1 L sample was distributed in three DO bottles and labelled properly. The DO bottles were separated for day zero, day three, and day five BOD calculations. The bottles were placed in BOD incubator at 20°C. For day zero bottles, 1 mL each of KI and MnSO₄

solution and 2 ml H_2SO_4 were added in every DO bottle and mixed well. After mixing the reagents 201 mL of sample was measured, transferred in Erlenmeyer flask, and titrated against 0.025 sodium thiophosphate using few drops of 2% starch indicator till the blue color of sample disappeared. The volume of sodium thiosulphate used gave the exact value of DO of a given sample. BOD was calculated using the formula presented in Equation 4.

$$\text{BOD} = (\text{Titrant used on 1st day for Blank} - \text{Titrant used on 5th day}) - (\text{Titrant used on 1st day for sample} - \text{Titrant used on 5th day}) \quad (4)$$

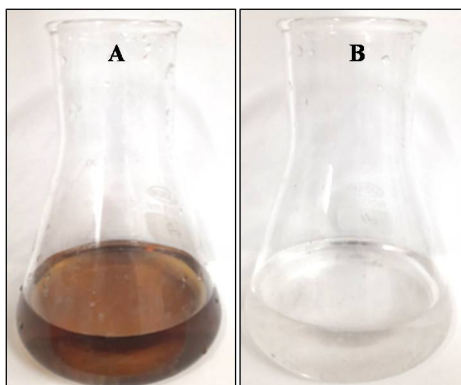


Figure 3.8: (a)After adding starch indicator, (b)After complete titration

3.6.5 MLSS (Mixed Liquor Suspended Solids) and MLVSS (Mixed Liquor Volatile Suspended Solids) measurement

MLSS (total solids weight) and MLVSS (volatile fraction of the total solids) were measured using weight of the sewage sludge in an empty crucible and ash less filter paper, respectively. 20 mL sludge was filtered through the filter paper and placed in an oven at 100°C overnight and further weighed after cooling down. The same filter paper was placed with filtered sludge contents in an empty crucible at 550°C in a muffle furnace for 30 min. After cooling down, the crucible was placed in a desiccator, and weighed again. From the difference between the initial and final weight of the filter paper and crucible, MLSS and MLVSS, respectively were measured.

3.7 Isolation of potential denitrifiers

In order to isolate denitrifying bacteria, effluent from the column was collected. Further serial dilutions were performed for diluting the collected sample. Serial diluted samples were plated in nitrate agar. The composition of nitrate agar is presented in Table 3.1. 0.9 mL of distilled water was taken in 7 autoclaved Eppendorf. The dilutions were prepared up to 10^{-7} by taking 0.1 mL of sample in 0.9 mL of distilled water and labelled the same as 10^{-1} and the process continued till reaching 10^{-7} dilution. 100 μ L of diluted samples were spreaded on nitrate agar plates. The bacterial growth was monitored after 24 hours of incubation. Further sub culturing of the isolated microbes were repeated through streak plate method to obtain pure single colonies of denitrifiers.

Table 3.1: Nitrate agar composition

Ingredients	g/L
Peptone	5
Peptone B	3
Potassium nitrate	1
Agar	12
Final pH	6.8 ± 0.2

Chapter 4

Results and discussion

4.1 Mechanism of working of the denitrifying column

Synthetic nitrate solution was pumped in up flow mode into the denitrifying column using a peristaltic pump. The denitrification column employed solid media (WS, RS, and CS) under anoxic as well as saturated condition. Under these conditions the solid organic carbon acted not only as an electron donor for the denitrification process but also as attachment surfaces for the growth, proliferation and development of biofilm of the denitrifying microorganisms. With the full-fledged establishment of the biofilm of the denitrifying microorganism, heterotrophic denitrification driven NO_3^- -N elimination was achieved in the denitrifying column. The heterotrophic microbes immobilized on the waste solid carbon derived their requisite electrons and carbons from the WS, RS, and CS for the smooth functioning of their heterotrophic metabolism. The schematic representation of the working mechanism of the column is depicted in Figure 4.1.

4.2 Characteristics of treated samples under different denitrification conditions

The synthetic nitrate solution denitrified under different experimental conditions like Erlenmeyer flasks, wash bottles, and denitrifying column are subjected to various analytical characterizations. The results are summarized in Table 4.1. The presence of bacteria in suspension cultures in wash bottles (WB1, WB2, and WB3), in biofilm mode inside the

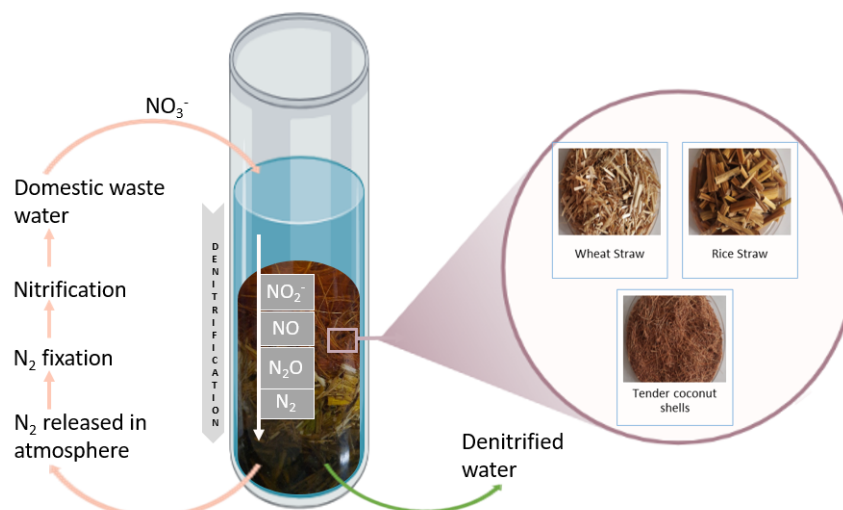


Figure 4.1: Working mechanism of the denitrifying column

denitrifying column and the addition of the external carbon sources are responsible for the rise in organic matter, TOC as compared to the original STP sample. BOD is the criterion for assessing the quality of water. Increase in BOD values under all the denitrification experimental conditions indicated enrichment of the denitrifying microbial consortia. An essential component of managing water quality is determining the amount of organic matter in a water body and how it affects the ecosystem. The primary sludge from the STP has a low C/N ratio, which correlates to a low level of organic compounds in the wastewater. Since denitrifying bacteria require carbon sources, the higher TOC values for the column and all of the wash bottles a desirable criteria for effective denitrification. The addition of WOSS raises the system's carbon content while simultaneously accelerating the bacterial growth, which accounts for the relatively high BOD and COD levels. The COD/N ratio is one of the key controlling elements for the proliferation of microorganisms and is crucial for both denitrification and nitrification. A higher COD/N ratio in bioreactor can be observed due to higher formation of carbohydrates, and proteins (Das and Das., 2020). In the treated effluent BOD or COD rose if the C/N ratio was higher than the ideal value. While the column setup functioned as a fix bed bioreactor with a rather stable biofilm, contributing to a better C/N ratio, the higher values of these parameters in wash bottles are mainly due to the presence of both SCS and microbial species in suspension medium. MLSS and MLVSS of the activated sludge sample collected from TIET, STP is measured to be 143.8 and 30.04 g/L respectively.

Table 4.1: **Value of different parameters of experimental Setup**

S.No.	Test	STP	Coloumn outlet	WB1	WB2	WB3
1.	pH	9	8.2	8.6	7.3	8.9
2.	COD (mg/L)	100	100	480	1600	880
3.	TOC (mg/L)	52.5	75	375	675	1200
4.	TKN (mg/L)	N/A	0.1848	N/A	N/A	N/A
5.	BOD (mg/L)	14	48	219	160	185

4.3 Assessment of heterotrophic denitrification efficiency

Each set of experiments were conducted in triplicates except the column mode of operation and were compared with control condition, without solid carbon sources. The heterotrophic denitrification performance was evaluated in term of nitrate removal efficiency (RE) as per the following equation: Removal Efficiency (%) = $(C_0 - C_e)/C_0 \times 100$

C_0 and C_e = influent and effluent nitrate concentrations (mg/L), respectively.

4.3.1 Denitrification assessment under column mode of operation

The continuous mode of denitrification experiment was continued over a period of 40-45 days. The results obtained are presented in Figure 4.2. As it can be observed from the figure, in the first ten days, denitrifying efficiency of the denitrifying column was very low. With the inlet's concentration of 75 ppm and effluent being 46.7 and 33 mg/L on day 7 and day 10 respectively, denitrification during the initial period is slow for SPD. On day 20, a maximum denitrification efficiency of 96.13% was achieved. This demonstrates that continuous operation improves nitrate removal because the stable biofilm establishment inside the column. A considerably higher nitrate concentration in effluent was seen whenever the process stopped. However, after the first 10 days, an efficiency of more than 90% was achieved in maintaining nitrate concentrations lower than 10 mg/L throughout the following 41 days. The poor denitrification efficiency at the beginning might be attributed to the immature biofilm or unstable biofilm of the heterotrophic denitrifying biomass. However, with passage of time, the higher denitrification efficiency observed in the column is because of the establishment of the stable denitrifying biofilm inside the column.

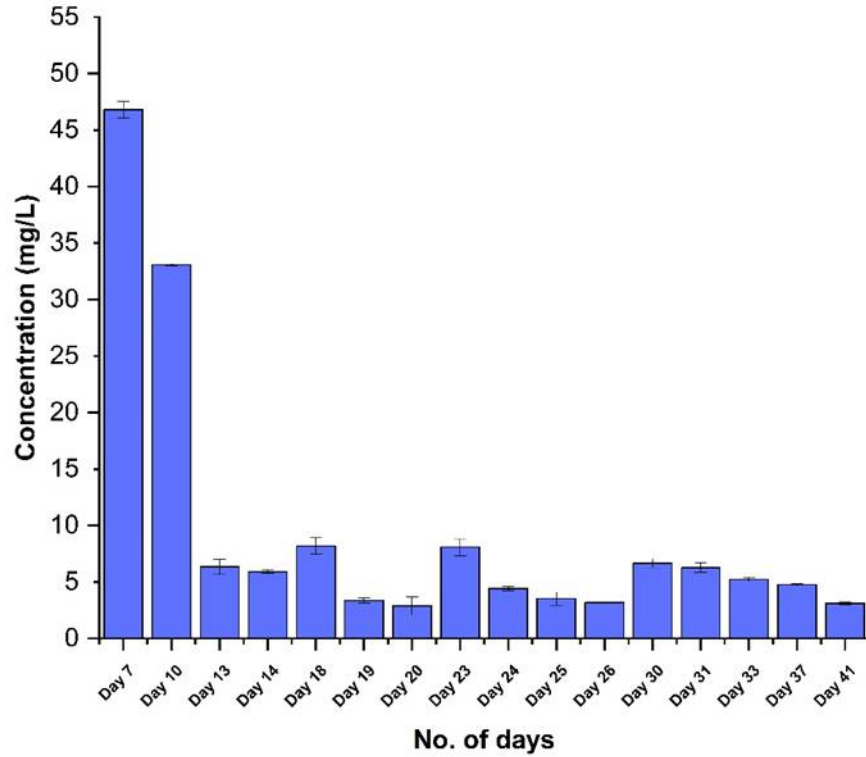


Figure 4.2: Variation of Nitrate concentrate in column's effluent

4.3.2 Denitrification assessment under batch mode of operation

Batch mode of denitrification experiments were carried under anoxic conditions in wash bottles and under aerobic (micro-aerophilic) condition in Erlenmeyer flasks. Under anoxic condition, WS demonstrated the best sustained nitrate removal capability among the three waste organic solids, with a maximum efficiency of 72.6%. All of the biomass exhibited a consistent pattern of denitrification as depicted in the Figure 4.3. After day 13, the addition of nitrate solution causes the nitrate concentration to rise. The highest levels of efficiency that CS and RS could reach were 60.3% and 58.6%, respectively. Because the breakdown of the carbon source gives the nitrate solution a dark appearance on the final day of tests, greater OD values were observed. Since RS degrades more quickly than the other two agricultural waste, a greater OD value was seen, which corresponded to a higher concentration.

4.3.3 Denitrification in flasks

The preliminary denitrification studies were conducted in Erlenmeyer flasks. Nitrate concentrations in the flasks were monitored for 95 days, primarily at intervals of five days. On

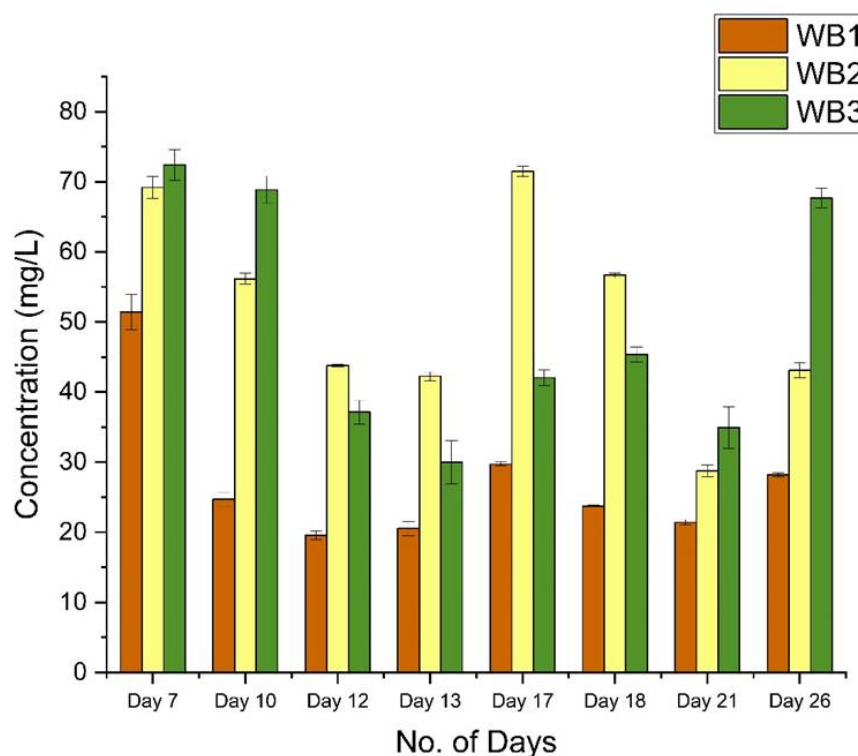


Figure 4.3: Variation of nitrate concentrate in wash bottles

the days indicated by an asterisk (*), 75 ppm of nitrate solution was added because these flasks were operated in batch mode. All the waste solid carbon sources effectively reduced nitrate concentration in the first 25 days; RS had the highest efficiency of 94.9% and brought nitrate concentration down to 3.8 mg/L. But after that, it gradually lost effectiveness at removing nitrate. This demonstrates that RS releases a significant amount of carbon in initial days only, which promotes rapid bacterial growth. In contrast to RS, WS is a delayed carbon release waste organic source, ensuring long-term carbon availability to heterotrophic microorganisms. Due to this, flasks 1 and 3 maintained removal efficiencies that were significantly greater than RS's, at 75.2% and 68.6%, respectively (Figure 4.4 and 4.5). The patterns of denitrification for tender CS were relatively comparable to WS, although its efficiency was in the middle of RS and WS's in the early days. For the first 15 days, flask with all three substrates removed nitrate more effectively than the flask with just two substrates (WS+CS), but after that, the trend reversed. This can be explained by the carbon release characteristics of the three waste organic solids. As was already stated, WS and CS acted as delayed release carbon sources, but RS acted as a rapid release carbon source. Because WS and CS were present in higher concentration in flask 5 for the remaining days and bacteria probably only relied on them after the carbon supply from RS was exhausted, flask 5

eliminated nitrate more effectively than flask 6 throughout the remaining days. When batch modes of identical SCS were examined, one with the higher concentration was more effective because denitrifying bacteria had more carbon available to them. Because of this, flasks 3 and 4 both attained their maximum efficiency of WS (91.8%) and CS (89.9%) which was higher than those of flask 1 and 2 respectively as demonstrated in Figure 4.4.

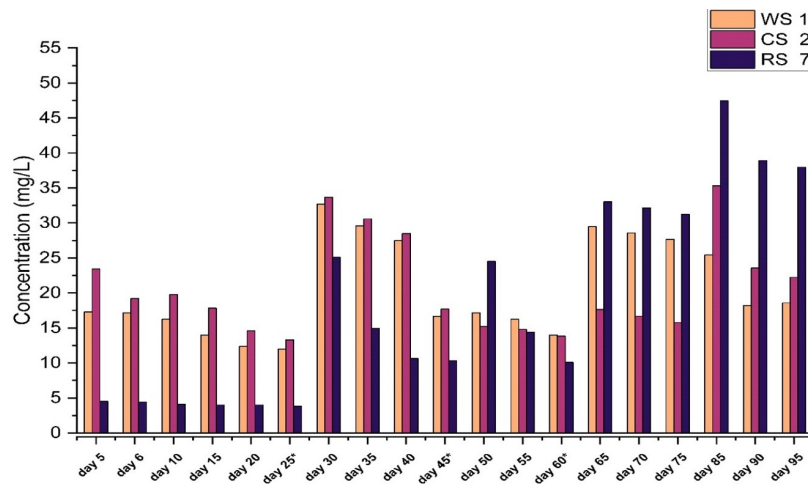


Figure 4.4: Variation of nitrate concentrate in flask 1,2 and 7

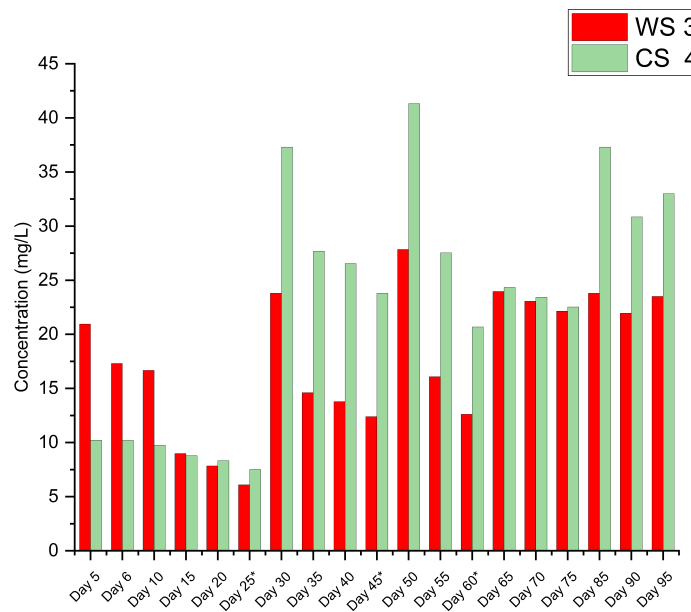


Figure 4.5: Variation of nitrate concentrate in flask 3 and 4

Nitrate content rose for all SCS upon the addition of nitrate feed to the flask, but the bacteria present in them gradually carried out denitrification this pattern was seen over the course of the 95 days. OD values of flasks increased over the last 15 days in a manner comparable to those of wash bottles, although there was no obvious trend. However, the increase in values might be attributed to the degradation of biomass and the colour they impart to solution, which is detected in spectrophotometer. OD values were especially high for flasks 5 and 6, presence of larger amount of carbon sources lead to more degradation.

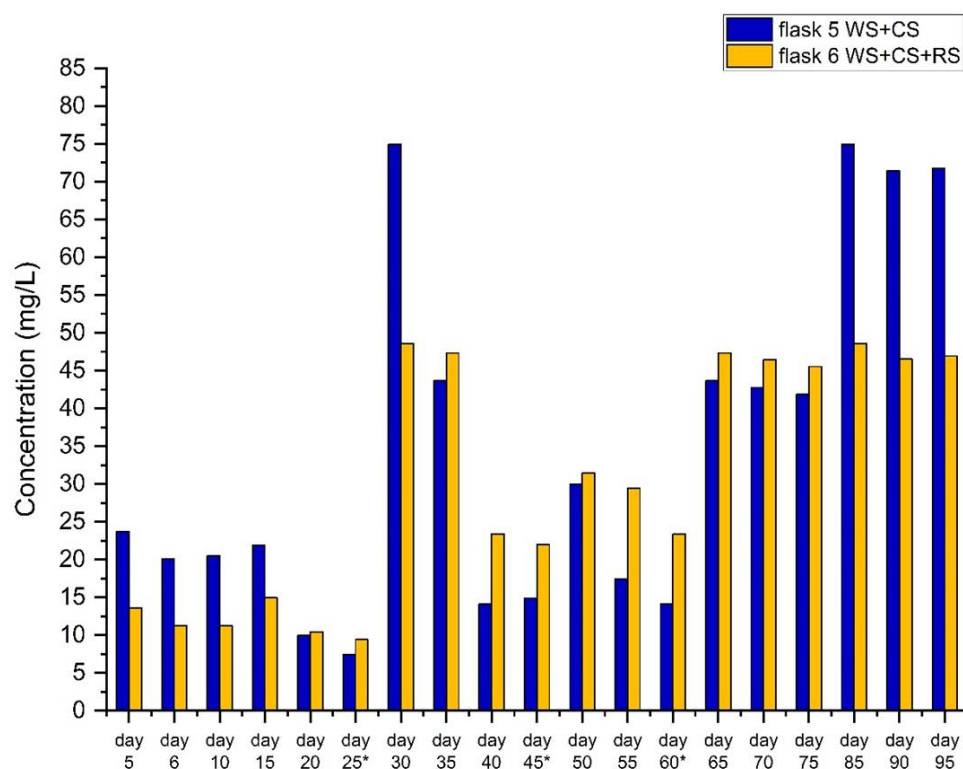


Figure 4.6: Variation of nitrate concentrate in flask 5 and 6

4.4 Characterization of waste organic solids before and after denitrification

The waste organic solid samples before and after denitrification are subjected to scanning electron microscope analysis for monitoring the biodegradation mediated surface changes. The development of biofilm is significantly impacted by WOSS's surface properties. Analysing the variations between a carbon source's surface before and after denitrification reveals information about the SCS's degradation, porosity, microbial proliferation, and evenness. SEM

images demonstrate the alteration in the morphologies of both the agricultural waste of before and after the denitrification process. Broken cellulose and pores in cell walls were visible in treated WS's images. In such damaged WS cells, further magnification revealed the presence of microorganisms as visible in Figure 4.7 and 4.8. Untreated RS's smooth surface signifies lignin coverage, while a sample from a column's rough surface denotes the breakdown of lignin walls, which enhances the porosity of RS fibers. This roughness increases the RS's outer surface area, which aids in bacterial biofilm formation.

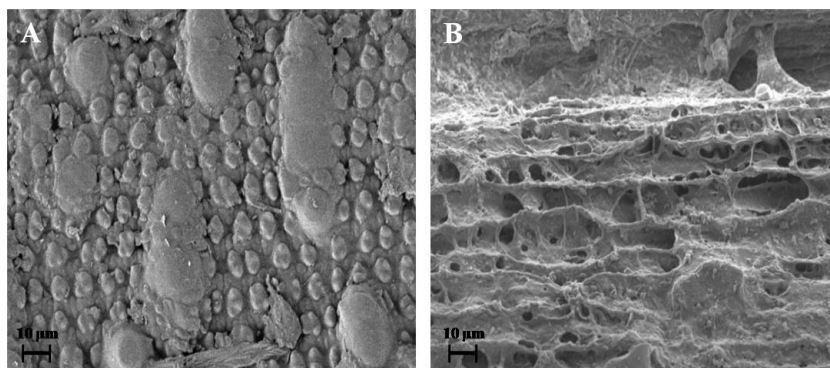


Figure 4.7: SEM images of RS before and after denitrification

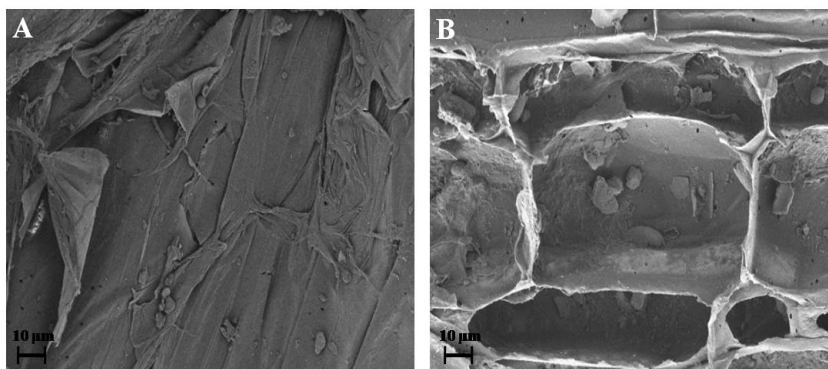


Figure 4.8: SEM images of WS before and after denitrification

4.5 EDS (Energy Dispersive Spectroscopy) characterisation

The elemental composition of the waste organic solids before and after denitrification was analysed using Energy dispersive spectroscopy or Electron dispersive X-ray spectroscopy (EDS). The EDS analysis results of RS and WS are presented in Figure 4.9 and elemental

composition percentages are summarized in Table 4.2. The EDS results revealed that the carbon content increased in both RS and WS samples after denitrification as compared to the samples before denitrification. The carbon content of RSR and WSR were measured to be 24.63 and 43.19%, respectively whereas the corresponding carbon content of RSC and WSC were observed to be 45.69 and 49.8%. The observed trend is quite obvious since the formation of biofilm on the surface of waste organic sources contributed to the overall carbon content of the substrates. This increased carbon content of the RSC and WSC is further supporting the biofilm formation as evidenced from the SEM images.

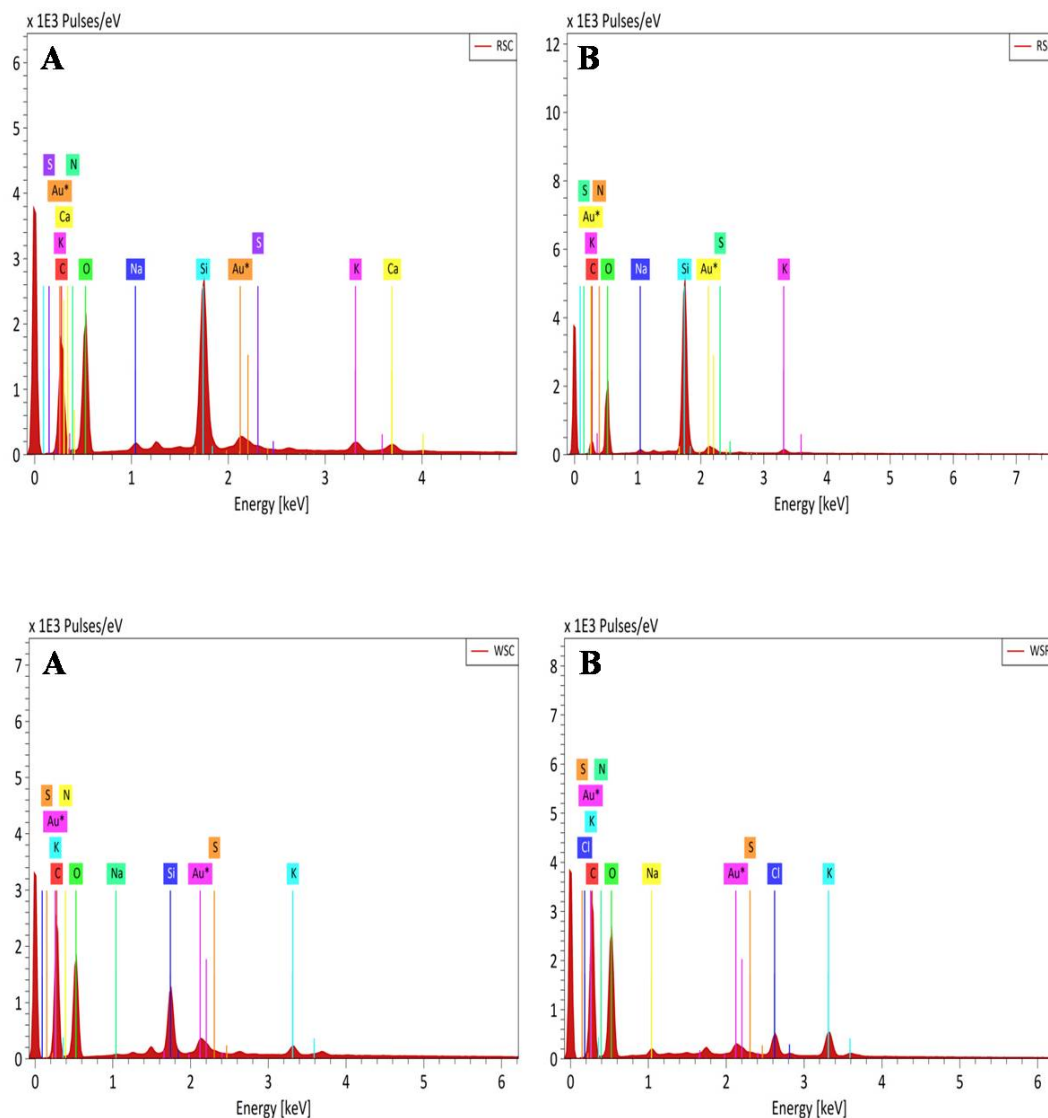


Figure 4.9: EDS spectra of solid organic waste before and after denitrification (a) RS (b) WS

Table 4.2: **Elemental composition of WOSS before and after denitrification**

S.No.	Element	At.No.	Mass %			
			RSR	RSC	WSR	WSC
1.	C	6	24.63	45.69	43.19	49.80
2.	O	8	44.99	40.14	46.21	39.79
3.	Na	11	0.64	0.74	0.18	0.07
4.	Si	14	21.37	8.39	3.36	3.72
5.	K	19	1.24	0.84	4.10	0.59
6.	N	7	1.85	0.00	2.68	2.89
7.	S	16	0.09	0.24	0.28	0.19

4.6 Fourier transform infrared (FTIR) spectroscopy analysis

The changes in functional groups, their bending, stretching, etc., patterns were compared through FTIR analysis of the waste organic solids before and after denitrification. The obtained results are presented in Figure 4.10. The diverse molecular bending and stretching of chemical groups is represented by different FTIR peaks. In WS the peaks between 2,900 and 2850 cm^{-1} represent aliphatic groups, difference in intensities of these peaks indicate either pure or amorphous cellulose. Peak at 1640 cm^{-1} has different absorption intensity which corresponds to different concentration of carboxylates. Peaks around 1500 cm^{-1} indicate lignin content.

O-H stretching of hydrogen bonds is represented by a broad band of tiny peaks between 3200 and 3400 cm^{-1} . stretching of C-N bonds has been attributed to the peak near 2920 cm^{-1} . These bands signify modifications to the cellulose structure. Peak 1445 cm^{-1} is associated with CH₂ bending. In RS, lignin is seen towards the peaks of 1300 cm^{-1} . Sharp peaks around 1000 cm^{-1} imply glycosidic interactions. The peak around 1000 cm^{-1} is assigned to C-O-C pyranose ring skeletal (Chen et al., 2011). Peak at 1630 cm^{-1} indicates formation of carboxylic acids, esters, and a significant rise in that peak is observed on treated RS. These FTIR results show that the denitrifying bacteria in the column are responsible for utilizing the lignin and hemicellulose from the RS and WS fibres.

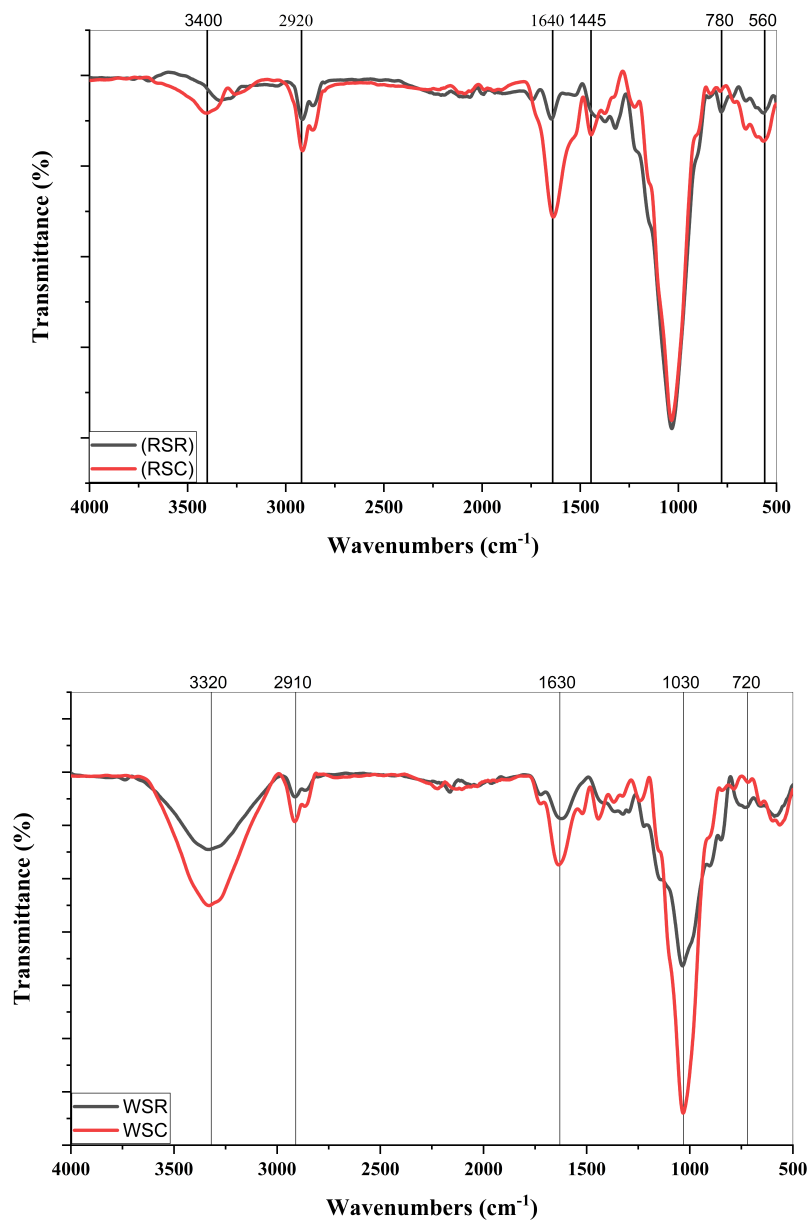


Figure 4.10: FTIR spectra of solid organic waste before and after denitrification (a) RS (b) WS

4.7 XRD (X-Ray Diffraction) analysis

The change in crystallinity or amorphous nature of the waste organic solids before and after denitrification was monitored via X-ray diffraction. The X-ray diffractograms of RSR, RSC, WSR, and WSC are presented in the Figure 4.11. The diffractograms of RSR and RSC

exhibited two major reflections at 2 around 22 and 35 °C. Similarly, the X-ray diffractograms of WSR and WSC also exhibited two major reflections at 2 around 22 and 35 °C. The peak at 22 °C indicated the higher exposure of cellulose in both the samples whereas the broad hump appeared around 35 °C might be due to amorphous cellulose. The variations in the crystal structures of the waste organic solids might be attributed to the microbial enzymatic mediated biodegradation of the lignocellulosic biomass. This finding further supports the utilization of the waste organic solids as carbon sources for heterotrophic denitrification.

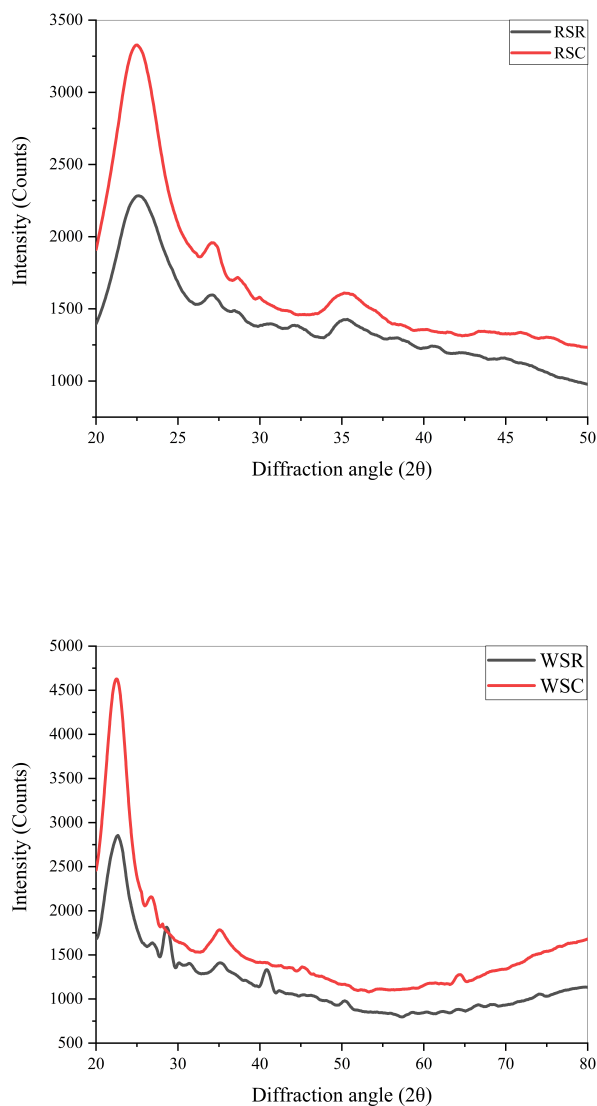


Figure 4.11: XRD spectra of solid organic waste before and after denitrification (a) RS (b) WS

4.8 Isolation and characterization of denitrifying microorganisms

At the end of the column mode of operation, partially degraded waste organic solid samples were collected from the column for analysis of denitrifying microbial biomass. The developed biofilms were detached from the spent RSC and WSC and suspended in sterile saline solution. After a couple centrifugation and re-suspension round of the sample, appropriate dilution was plated using selective denitrifying media. The plates were incubated for 24-48 h. Two colonies exhibiting best denitrification activity were picked, sub cultured in liquid broth and subsequently streaked to isolate pure colonies of the desired microbe as shown in Figure 4.12. The identification of the strains through 16S rDNA gene homology approach is under progress as the samples are out sourced to National Collection of Industrial Microorganisms (NCIM), Pune.

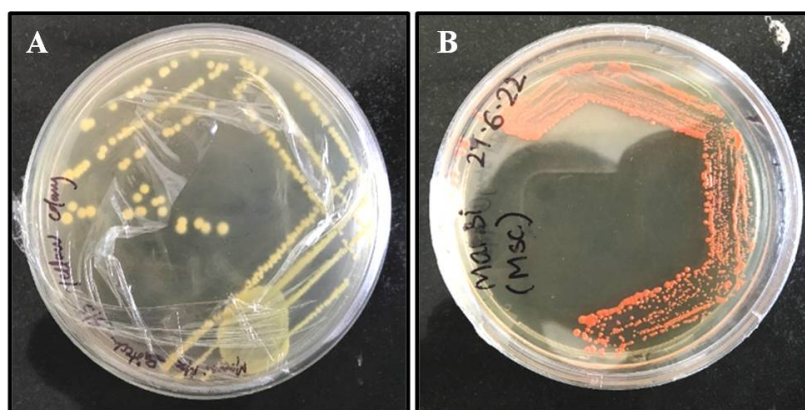


Figure 4.12: Isolation of bacterial colonies from denitrifying column

The biochemical characterization of the pure isolates was done by biochemical test kit, HiMViC (Himedia). All the biochemical tests were performed following the manufacturer's instructions. Testing for the generation of hydrogen sulphide, methyl red, citric acid consumption, and carbohydrate fermentation were performed. The results are presented in Table 4.3. As shown in the table the yellow colour isolate exhibited nitrate reduction and carbohydrate utilization ability. Likewise, the red colour isolate exhibited amino acid utilization, nitrate reduction, and carbohydrate utilization ability. The nitrate reduction ability of both the strain further supports the experimental evidences of denitrification of this study.

Table 4.3: Biochemical analysis of red and yellow bacterial colonies

S.No.	Test	Yellow colony	Red colony
1	Citrate utilization	-	-
2	Lysine utilization	-	+
3	Ornithine utilization	-	+
4	Urease	-	-
5	Phenylalanine Deamination	-	-
6	Nitrate Reduction	+	+
7	H ₂ S production	-	-
8	Glucose	-	-
9	Adonitol	-	-
10	Lactose	-	-
11	Arabinose	-	+
12	Sorbitol	-	-

Chapter 5

Conclusions and future perspective

5.1 Conclusion

The present work validated the potential application of the selected waste organic solids, WS, RS, and CS as promising carbon sources and biofilm carrier for heterotrophic denitrification process. The denitrifying efficiency of the organic substrates were verified both in batch and continuous mode of experiments using synthetic nitrate solution. On comparing the three organic waste's efficiency in batch and continuous modes, denitrification performance was observed to be better in the column due to the presence of more biomass, stable biofilm formation, and the presence of both difficult and easy to degrade carbon sources, which ensure the immobilized microbes the access to both fast and slow released carbon. The existence of mixed waste organic solids, a favourable C/N ratio, and the development of a stable biofilm of denitrifying bacteria mandated nitrate removal in both early and later days, contributed to the experiment's longevity in the column, with denitrification efficiency of 96.13%. SEM, FTIR, EDS and XRD findings demonstrated the biodegradation of the lignocellulosic biomass. Study of the surface of the carbon source gives indication of established biofilm, an uneven and rough surface of WOSS can safeguard the adhesion of biofilms and provide it room to grow further. Higher magnification SEM imaging revealed the presence of microbial colonies inside the WS cells with torn cell walls. Microbial analysis of the column after the completion of experimental period revealed the involvement of limited consortium of active denitrifiers (yellow and red isolates), the identification of which are under progress. Biochemical analysis revealed the nitrate reduction potential of both red and yellow isolates, thereby confirming the experimental evidences of denitrification of this study. Thus, this study demonstrated a promising alternative utilization of low or no cost carbon source for the remediation of wastewater.

5.2 Future perspective

However, the focus of this work was predominantly on finding novel and efficient resources that can be used as substrates for heterotrophic denitrification. There is an obvious need to carry forward further experiments using real wastewater to address the existing issues related to the longevity and economics of carbon sources. The microbial biofilm plays a vital role in denitrifying columns. So, characterizing the developed biofilm and subsequently studying the biofilm dynamics and important relationship pertinent to heterotrophic denitrification could be established. The reusability of the partially degraded organic wastes after the completion of the denitrification process can be targeted through composting and subsequent usage as organic fertilizers in agricultural applications.

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ABSTRACT Wastewater treatment is used to preserve the quality of scarce freshwater resources and improve their usability. Solid-phase denitrification is a method that has the potential to improve nitrate removal from wastewater. Being heterotrophic by nature, the source of organic carbon determines how well the denitrification process proceeds because both a lack of and an abundance of organic carbon cause disruptions in the heterotrophic denitrifiers' usual metabolism. So, choosing the right carbon source is crucial for the best denitrification process. Therefore, the purpose of this study is to evaluate a few locally available waste organic solids as potential carbon source for heterotrophic denitrifiers. The primary research variables included in this project were the influent flow rate, influent nitrate concentration, and amount of carbon source and its effect on denitrification rate. Batch and continuous mode of experiments were used to thoroughly study and assess the denitrification potential of agricultural wastes, namely rice straw (RS), wheat straw (WS), tender coconut shells (CS) and mix of the three. Maximum denitrification efficiency of 96.13% was achieved in continuous mode of operation, surpassing the efficiency of each individual carbon source of 72.6% (WS), 60.3% (CS), and 58.6% (RS). When the physical features of the organic wastes were examined using SEM, FTIR, and EDS, the findings showed significant morphological changes in samples after denitrification as compared to original samples. The nitrate reduction ability of the microbial strains isolated from the denitrifying column as confirmed by biochemical characterization validated the experimental evidences of this study. These results provide a theoretical framework for effectively using agricultural waste as a solid carbon source and improving denitrification efficiency in wastewater treatments.

Keywords: Solid phase denitrification, Heterotrophic denitrification, Wheat straw, Rice straw, Waste organic solids
CHAPTER 1: INTRODUCTION

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