An Experimental Investigation of Nitrogen Removal via Heterotrophic Nitrification and Aerobic Denitrification (HNAD) Pathway

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Submitted by

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DECLARATION

I hereby declare that the work presented in the dissertation entitled “An Experimental Investigation of Nitrogen Removal via Heterotrophic Nitrification and Aerobic Denitrification (HNAD) pathway” in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET) Deemed to be University, Patiala, is a genuine record of my own work during the period from January 2021 to June 2023, under the supervision of Dr. Bunushree Behera, Department of Biotechnology, TIET, Patiala, Punjab. The information included in this thesis has not been submitted to another college or institution in India or overseas for the award of any degree.

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CERTIFICATE

This is certified that the thesis entitled An Experimental Investigation of Nitrogen Removal via Heterotrophic Nitrification and Aerobic Denitrification (HNAD) pathway submitted by Neha Thakur (Registration No. 302101034), a postgraduate student of the Department of Biotechnology in partial fulfilment for the award of the degree of Masters of Science at Thapar Institute of Engineering and Technology, Patiala, Punjab 147004, 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 or institute.

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LIST OF SYMBOLS AND ABBREVIATIONS

mg/L	Milligrams per Liter
C/N	Mass of carbon to Mass of nitrogen
N	Nitrogen
C	Carbon
N/A	Not Available
°C	Degree Celsius
rpm	Rotation per Minutes
%	Percent
mL	Milliliter
g	Gram
mL/min	Milliliter per Minute
min	Minute
nm	Nanometer
mg	Milligram
L	Liter
g/L	Grams per liter
µL	Microliter
OD	Optical density

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ABSTRACT

Nitrogen removal from wastewater is critical due to increasing eutrophication concerns and its associated ecological hazards. Heterotrophic nitrification aerobic denitrification (HNAD) bacteria offer a feasible single reactor system for managing diverse nitrogenous contaminants, which are present in wastewater. However, the process scale-up is often limited due to the inability of strains in tolerating the changes in temperature and other operational conditions. There is a huge need for bioprospecting novel strains capable of sustaining at real-time conditions. Thus, the present study aimed to isolate and identify native HNAD strains from the sewage treatment plant of Thapar Institute of Engineering and Technology, Patiala through biochemical and molecular characterization. Initial visualization and microscopic identification showed the bacterial strains to form opaque, red, and yellow colonies with characteristic rod-shaped and gram-positive nature. Phylogenetic analysis through 16S rRNA sequencing identified the strains as *Rhodococcus ruber* and *Cellulosimicrobium cellulans*. Shake flask experiments showed a significant growth rate in simultaneous nitrification and denitrification (SND) medium with doubling time of consortia 131 min.

Keywords: Eutrophication; HNAD microbes; Nitrogen; Phylogenetic analysis; Wastewater

Chapter 1: Introduction

1.1 Background of the study

Recent years have seen a lot of research focused on the presence of nutrients mainly nitrogen in wastewater. due to its potential to have harmful effects on eutrophication and aquatic life that absorbs it **(Yao *et al.*, 2013).** As a result, the removal of nutrients from wastewater has lately assumed a key position in wastewater treatment facilities. Contrary to other approaches, the biological treatment process for nutrient removal is commonly employed since it is simple to use, more effective, requires less maintenance, and has other advantages as well. **(Qu *et al.*, 2015**). Controlling nitrogen pollution in wastewater treatment is complex. Fertilizers containing nitrogen as a component and domestic and commercial wastewater discharge are the primary nitrogen sources in wastewater (**Xia *et al.*, 2020**). Nitrogen in wastewater cause many environmental problems. Nitrogen in wastewater leads to eutrophication. Eutrophication can seriously impact the ecosystem and people's health by killing aquatic plants and animals and degrading water quality (**Lang *et al.*, 2020**). Thus, it is essential to improve wastewater treatment technology to remove nitrogen. Alternating conditions, such as aerobic-anoxic-anaerobic, etc., are used in the traditional biological nitrogen removal method. It makes the treatment procedure more complex, uses more energy, and costs more. (**Zheng *et al.*, 2014**)

The anaerobic denitrification and aerobic nitrification processes make up the conventional biological nitrogen removal method. Ammonia-oxidizing bacteria and archaea first convert ammonia to nitrite during the process of nitrification, and subsequently NOB convert nitrite to nitrate (**Xi *et al.*, 2022**). Prior to being converted into NO, N_2O, and N_2 by heterotrophic bacteria during denitrification, nitrate first undergoes a transformation into nitrite (**Xi *et al*.**, **2022).** These two processes must be carried out in two reactors, during which the control of process parameters and the addition of raw materials are carried out independently due to the distinct needs for dissolved oxygen (DO) and organic matter. This results in the conventional process's dual economically disadvantages, namely high building costs and ongoing maintenance expenses (**Lei *et al.*, 2016; Ouyang *et al.*, 2020**). This conventional belief has been disproved by the identification of simultaneous HNAD bacteria (**Robertson *et al.*, 1988**). This single-stage nitrogen removal method was developed in response to the pioneering discovery of the aerobic denitrification capability of *Paracoccus denitrificans*, which allows the bacterium to take in both oxygen and nitrate at the same time. The model predicts that *Paracoccus denitrificans* can do heterotrophic nitrification, which uses organic carbon sources, and aerobic denitrification, which refers to the microbe's ability to do so. Due to the simultaneously nitrification and denitrification capacities of HNAD bacteria, the A single reactor is capable of handling a two-stage reaction. Comparatively to the traditional approach, SND technology can significantly reduce operating expenses and boost denitrification efficiency. The use of SND has drawn much interest due to the recent rise in discoveries using SND bacteria. Studies on the mechanism of biological nitrogen removal and the nitrogen cycle show that simultaneous nitrification and denitrification reactions can happen spontaneously and are frequent in the environment (**Jetten *et al.*, 2008).** *Acinetobacter sp. GA* and *Pseudomonas sp. GP* were co-cultured by **Guo *et al.* (2018),** and they discovered that under an initial nitrogen concentration of 100 mg/L, the co-culture system considerably increased the efficiency of total nitrogen (TN) removal. Similar to this, in maricultural wastewater, the bacterial consortia *Marinomonas* and *Halomonas* demonstrated improved NH_4^+-N removal efficiency and more stable cell number than the single strain **(Huang *et al.*, 2021**). The complicated and unstable environment is probably the reason why there are still restrictions on the stable and useful use of the co-strains that have already been established. For its practical implementation, study into the underlying mechanisms of improved nitrogen removal efficacy is also essential.

Chapter 2: Literature Review

Recent years have seen significant advancements in the treatment of wastewater using SNAD, or simultaneous nitrification and denitrification. "SNAD" refers to the simultaneous enzymatic processes of nitrification, which converts ammonium into nitrate, and denitrification, which transforms nitrate to nitrogen gas. Since nitrogen, a common pollutant in home and industrial wastewater, is eliminated during this procedure, it is of utmost importance in the treatment of wastewater. Numerous bacteria have been shown to be able to do nitrification and denitrification simultaneously (SNAD). These include *Acinetobacter calcoaceticus* (**Zhao *et al.*, 2010),** *Rhodococcus sp.* (**Zheng *et al.*, 2022**), *Marinobacter sp.* (**Zheng *et al.*, 2022**), and *Klebsiella pneumonia* **(Padhi *et al.*, 2013).** These microorganisms have the ability to concurrently nitrate ammonium to produce nitrate and denitrate to produce nitrogen gas. These bacteria' identification and characterisation have shed important light on their capabilities and prospective uses in wastewater treatment systems.

A noteworthy milestone in developing one-stage nitrogen removal procedures was the discovery of *Paracoccus denitrificans* capacity for aerobic denitrification. This bacterium exhibits the unique ability to simultaneously respire oxygen and nitrate, thus enabling efficient nitrogen removal. An influential study conducted by **Chen *et al*. (2012)** investigated the effectiveness of *Rhodococcus sp. CPZ*24 in eliminating ammonium nitrogen. Within a 20-hour period, this microorganism successfully eradicated 50 mg/L of ammonium nitrogen and accomplished a 67% reduction in nitrate nitrogen. Another study shed light on the extraordinary capabilities of a recently isolated bacterium known as *Ochrobactrum anthropi HND19.* This bacterium demonstrated exceptional performance in heterotrophic nitrification and aerobic denitrification, achieving an impressive removal efficiency of approximately 98.6% for NH_4^+-N and 97.6% for NO_3^--N. *Ochrobactrum anthropi HND19* exhibited rapid growth compared to autotrophic microorganisms, exhibited tolerance towards higher ammonium loadings, and effectively carried out SND within a single reactor **(Xia *et al.*, 2020).** However, individual strains' growth and nitrogen removal capabilities are susceptible to environmental variations **(Huang *et al.*, 2021).** Study conducted by **Ke *et al.*, (2021),** characterized an isolated HNAD strain, ZJB20129, which exhibited excellent nitrogen removal capacity in culture media. However, observed limitations when applying this strain to actual wastewater treatment. To overcome these limitations, constructing artificial microbial communities is a promising approach due to community systems' greater metabolic diversity and more robust metabolic capacities than single strains **(Li *et al.*, 2020).** Additionally, species interactions within microbial communities have various biological processes including mutualism, the "cheating effect," and quorum sensing **(Zhang *et al.*, 2019a),** which can enhance the resilience of microbial communities to environmental stressors.

High-throughput genome sequencing has emerged as a feasible strategy for better understanding the active microflora community involved in wastewater treatment, particularly for HNAD bacteria **(Li *et al.*, 2020). Guo *et al.* (2018)** demonstrated that co-culturing *Acinetobacter sp. GA and Pseudomonas sp. GP* significantly improved total nitrogen (TN) removal efficiency in a co-culture system with an initial 100 mg/L nitrogen concentration. Similarly, the bacterial consortia *Marinomonas and Halomonas* exhibited enhanced NH_4^+-N removal efficiency and more stable cell quantities than single strains in maricultural wastewater **(Huang *et al.*, 2021)**. Further investigation into the collaborative mechanisms of HNAD co-strains was conducted. **Zhang *et al.* (2019b)** discovered that cooperation between *Enterobacter sp. Z1 and Klebsiella sp. Z2* promoted the expression of nitrogen removal-related enzymes. In a fungus-bacterium consortium of *Penicillium citrinum WXP-2* and *Citrobacter freundii WXP-9,* nitrogen removal was enhanced through accelerated electron transfer **(Chen *et al.*, 2021).**

However, the stable and efficient application of currently constructed co-strains still needs to be improved, likely due to the complex and variable nature of the environment. Furthermore, exploring the underlying mechanisms behind these co-strains' improved nitrogen removal performance is of great value to facilitate their practical implementation. **Table [1]** shows few of the isolated SND species and their source of origin.

Table 1. Isolated SND bacterial species

S no.	**Species**	**Origin of microbes**	**References**
1.	*Pseudomonas tolaasii Y-11*	Paddy soil	**He *et al.* (2016)**
2.	*Klebsiella pneumoniae y6*	Seawater	**Wang *et al.* (2020*)***
3.	*Vibrio diabolicus SF16*	Marine sediments	**Duan *et al.* (2015)**
4.	*Rhodococcus sp. CPZ24*	Swine wastewater	**Chen *et al.* (2012)**
5.	*Cupriavidus sp. S1*	Coking plant wastewater	**Sun *et al.* (2016)**
6.	*Zobellella taiwanensis DN-7*	Landfill leachate	**Lei *et al.* (2016)**
7.	*Acinetobacter junii YB*	Laboratory-scale SBR	**Yang *et al*. (2015)**
8.	*Paracoccus versutus LYM*	Seabed sludge	**Zhang *et al.* (2015)**
9.	*Zoogloea sp. N299*	Drinking water reservoir	**Huang *et al.* (2015)**
10.	*Bacillus methylotrophicus L7*	Laboratory-scale MBR	**Yao *et al.* (2013)**

The majority of current denitrification research focuses on studying single, pure strains. In batch experiments, certain strains showed potential denitrification capacities, and research has been done to improve treatment effectiveness in reactors (**Wang *et al.*, 2020; Jia *et al.*, 2020**). These results have not yet, nonetheless, been applied in real world situations. Inadequate bacterial resources and ignorance of the metabolic characteristics of HNADM (High Nitrate- and Nitrite-Removal Activity) strains are the main causes of this. It is imperative to find more

HNADM strains and learn more about the parameters affecting their growth and metabolism in order to make it easier to construct large-scale application systems. **Table [2]** illustrates few of the isolated bacterial strains and their nitrogen removal efficiency.

Table 2. Nitrogen removal efficiency of different bacterial strains

S. No	Bacterial strains	Nitrogen removal efficiency	References
1	*Bacillus cereus* GS-5 (Tatapani, Odisha, India)	NH_4^+-N: 96% NO_3^--N: 95% NO_2^--N: 84%	**Rout *et al.* (2017)**
2	*Enterobacter cloacae* HW-15 (River in Southwestern hubei province, China)	NH_4^+-N: 99% NO_3^--N: 88% NO_2^--N: 59%	**Wan *et al.* (2017)**
3	*Pseudomonas mendocina* X49 Sewage treatment Plant, Beijing, China	NH_4^+-N: 26.39 mg L^{-1} h^{-1} NO_3^--N: 4.54 mg L^{-1} h^{-1} NO_2^--N: 6.25 mg L^{-1} h^{-}	**Xie *et al.* (2021)**
4	*Thauera* sp. SND5 ((WWTP) in Beijing, China)	NH_4^+-N: 2.85 mg L^{-1} h^{-1} NO_3^--N: 1.98 mg L^{-1} h^{-1} NO_2^--N: 2.42 mg L^{-1} h^{-1}	**Wang *et al.* (2020)**
5	*Acinetobacter* sp. GA and *Pseudomonas* sp. GP (Sewage treatment plant, Hangzhou, China)	*Acinetobacter* Total organic carbon: 25.17% Total dissolved nitrogen: 30.18% *Pseudomonas* TOC: 11.76% TDN: 7.13% Co-culture TOC and TDN: 93.67% and 91.97% respectively	**Guo *et al.* (2018)**

According to previous studies **(Tan *et al.*, 2020; Zhu *et al.*, 2020**), a number of variables, including the carbon supply, pH, C/N ratio, dissolved oxygen (DO), temperature, metal ions, and salinity, have a substantial impact on HNAD microbes. A detailed examination of these elements will be provided in the sections that follow.

2.1. Factors affecting the nitrogen removal efficiency of HNAD strains

2.1.1. pH

The biological nitrogen removal processes are significantly impacted by the medium's pH. Weakly alkaline conditions favour nitrification, whereas low pH inhibits anaerobic ammonium oxidation (ANB) (**Zhang *et al.*, 2020a, 2020b; Zhang *et al.*, 2012**). Low pH causes heterotrophic nitrification rates to slow down, however fungi and acidophilic bacteria are more resistant to acidic environments than other heterotrophic nitrifying microorganisms (**Li *et al.*, 2018; Liu *et al.*, 2017; Yang *et al.*, 2017).**

Studies have demonstrated that the acid tolerance of different HNADM strains varies, with some strains able to thrive in the pH range of 3 to 9. Additionally, through adapting, many HNADMs can widen their pH tolerable range **(Zeng *et al.*, 2020).** HNADMs have a broad range of acid adaptation, which raises the possibility of their practical use in the treatment of acid wastewater. The ideal pH range, however, is between 6 and 9, and neutral to mildly alkaline settings are typically more suited for the growth of most HNADMs **(Nancharaiah *et al.*, 2018).**

Notably, the ideal pH range for HNAD microbial growth and effectiveness at removing nitrogen may vary. For instance, the pH range between 6.0 and 6.5 is ideal for the growth of the strain *Bacillus* MS 30, whereas the pH range between 7.5 and 8 shows the highest ammonia oxidation efficiency **(Zhang *et al.*, 2012**).

2.1.2. Carbon source effect

Contrary to autotrophic nitrification, heterotrophic nitrification depends on organic carbon to improve ammonium removal and cell proliferation **(Joo *et al.*, 2005; Ren *et al.*, 2014**). In biological water treatment systems, catabolism and anabolism are important cellular mechanisms that provide energy and nourishment for bacterial metabolism and synthesis **(Chen *et al.,* 2011; Nancharaiah *et al.,* 2018).**

The metabolic processes, microbial enzyme activity, bacterial growth, and nitrogen degradation are all impacted by the degradability, chemical composition, and molecular weight of the carbon source (**Zhang *et al.,* 2014; Zhao *et al.,* 2017**). For instance, *Acinetobacter Yii YB*, a strain capable of HNAD, is unable to grow in the absence of organic carbon or remove ammonium. When given organic carbon in the culture conditions, the strain does, nevertheless, show a little amount of development **(Yang *et al.,* 2015).** This implies that organic matter and ammonium are metabolised simultaneously, possibly as a result of particular mechanisms for electron transfer and charge separation during heterotrophic nitrification.

2.1.3. C/N ratio

For heterotrophic nitrogen-removing and ammonium-oxidizing microorganisms (HNADMs), lower C/N ratios are often associated with lower denitrification and ammonia nitrogen removal capacities **(Chen *et al.,* 2011; Li *et al.,* 2015; Ren *et al.,* 2014**). On the other hand, exceeding the ideal C/N ratio causes a modest reduction in the capacity for both ammonia oxidation and total nitrogen removal **(Chen *et al.,* 2012).**

The impact of C/N ratios on HNADMs has been studied in reactor studies. To increase NO3-N penetration, Wang et al. (2020) added a mixed culture of *Acidovorax sp. YH02* and *Delftia sp. YH01* to a reactor. When the C/N ratio was 8, 2000 mg/L of NO3-N could be eliminated satisfactorily. **Jia *et al.* (2020)** used *Stenotrophomonas maltophilia DQ01* to test the effects of various C/N ratios on nitrogen removal in a moving bed biofilm reactor. At a C/N ratio of 7.5, they successfully attained a considerable HNAD efficiency of 94.21% and a total nitrogen removal rate of 94.43% **(Jia *et al.,* 2020)**

2.1.4. Temperature

Temperature plays a critical role in both nitrification and denitrification processes, with significant sensitivity observed. Nitrification and denitrification are highly When the temperature falls below 10°C, inhibition occurs. **(Caballero *et al.*, 2012).** Most HNADMs (heterotrophic nitrogen-removing and ammonium-oxidizing microorganisms) exhibit optimal activity between 25 and 37 °C. However, extreme temperatures, whether high or low, can strongly impede the metabolic functions of HNADMs **(Liu *et al.*, 2019).** For instance, the HNADMs strain *Providencia rettgeri* YL has a much lower efficiency of ammonia nitrogen removal at temperatures over 40 °C or below 10 °C, giving less than 10% removal efficiency **(Huang *et al*., 2013)** Despite the impact of temperature on HNADMs, these microorganisms can still exhibit nitrification and denitrification capabilities under extreme temperature conditions. Isolated strains like *Microbacter-SFA13 and Acinetobacter-Y16* have demonstrated the ability to carry out these processes at temperatures as low as 5 °C, indicating their resilience to cold environments **(Zhang *et al.*, 2012; Huang *et al.*, 2013).** The presence of HNADMs across various temperature ranges underscores their potential contributions to nitrogen cycling in different environments, particularly in wastewater treatment in cold regions.

2.1.5. Dissolved oxygen

The processes of ammonia oxidation and denitrification both depend heavily on dissolved oxygen (DO). Anaerobic denitrifying bacteria are often unable to grow and function properly in the presence of DO. HNADMs may, nevertheless, carry out denitrification in an aerobic environment. Although DO concentration influences HNADMs, anaerobic denitrifying bacteria often have a more significant effect **(Joo *et al.*, 2005; Zhao *et al.*, 2012; Ren *et al.*, 2014).** Various HNADMs may have different optimal DO levels for denitrification **(Robertson *et al.*, 1989),** *Acinetobacter* sp. ND7, *Cupriavidus* sp. S1, *and Alcaligenes faecalis* are three

HNAD bacteria that have demonstrated great adaptation to DO changes **(Sun *et al.*, 2016; Joo *et al.*, 2005; Xia *et al.*, 2020)**. However, the denitrification efficiency of HNADMs often declines as DO rises. However, there are several exceptions. For instance, *Alcaligenes faecalis* strain TUD, an isolated HNAD bacterium, could still perform nitrification and denitrification even at higher DO concentrations **(Xia *et al.*, 2020).** Furthermore, *Pseudomonas stutzeri* YG-24's capacity for denitrification seems to be less impacted by DO **(Li *et al.*, 2015)**

Chapter 3

Research gap and objective

There are several critical areas where study is lacking in the process of nitrogen removal from wastewater by HNAD. First, in order to improve the effectiveness and stability of HNAD, it is necessary to optimise the process variables and operational settings. For maintaining long-term performance, it is also essential to comprehend the dynamics of the microbial population participating in HNAD and its reaction to various wastewater compositions and conditions. To maximise nitrogen removal, research on the use and availability of organic carbon sources as well as their effects on HNAD is required. Furthermore, tackling issues with nitrite build-up and nitrous oxide emissions in HNAD systems is crucial for the long-term viability of both the environment and cooperate operations. Finally, research should concentrate on scaling up HNAD and creating plans for its useful application in large-scale wastewater treatment facilities. Closing these knowledge gaps will help to improve HNAD for effective and long-lasting nitrogen removal from wastewater

Objectives

1. To isolate, identify and characterize the bacterial stain from sewage wastewater treatment plant.
2. Screening of bacteria on different carbon sources.

Chapter 4: Materials and methods

4.1. Wastewater characterization

4.1.1 COD (chemical oxygen demand) measurement:

In COD measurement, a 10 mL sample is mixed with 5 mL of 0.041M $K_2Cr_2O_7$ solution, 15 mL of digestion reagent ($Ag_2SO_4 + H_2SO_4$), and 1 mL of $HgSO_4$. The mixture is refluxed for two hours at 150°C. The samples are titrated using a standard ferrous ammonium sulphate (FAS) solution after cooling to room temperature. The formula is used to determine the COD value.:

COD (mg O2 /L) = (B - A) × M × 8000 / Volume of sample

Where:

- B is the mL of FAS used for the sample
- A is the mL of FAS used for the blank
- M is the molarity of FAS
- Volume of sample is the volume of the original sample

If the samples were diluted, the final COD value is multiplied by the dilution factor (df).

4.1.2 DO (dissolved oxygen) and BOD (biochemical oxygen demand) measurement:

For BOD measurement, the samples are diluted with dilution water. Each sample requires 1 liter of dilution water. The dilution water is prepared by adding $FeCl_2$, $CaCl_2$, $MgSO_4$, and phosphate buffer to aerated tap water in a specific ratio.

The diluted sample is divided into three DO bottles labelled for day zero, day three, and day five BOD calculations. These bottles are placed in a BOD incubator at 20°C.

For the day zero bottles, KI (Potassium Iodide), $MnSO_4$ (Manganese (II) sulfate), and H_2SO_4 (Sulfuric acid) are added, and the reagents are mixed. A volume of 201 mL of the sample is transferred to an Erlenmeyer flask. The sample is titrated against 0.025 M sodium thiophosphate using a few drops of 2% starch indicator. The volume of sodium thiosulfate used in the titration provides the dissolved oxygen (DO) value for the sample.

The BOD value is calculated using the formula:

BOD = (Titrant used on 1st day for Blank - Titrant used on 5th day) - (Titrant used on 1st day for sample - Titrant used on 5th day)

4.1.3 pH measurement:

pH measurement is done using a pH meter and buffer solutions. Two standard buffer solutions with established pH values are used to calibrate the electrodes of the pH metre. Then, the water sample is tested by immersing the electrodes in the sample and allowing a stabilization time. Once a constant reading is obtained, the pH value is recorded. pH measurement is important for assessing the acidity or basicity of a solution, including water samples.

4.1.4 Electrical conductivity measurement:

To measure electrical conductivity (EC), an EC meter is calibrated using standard solutions with known conductivity. The electrodes are immersed in the water sample, and the reading is allowed to stabilize. Once a steady reading is obtained, it is recorded. Electrical conductivity provides information about the solution's ability to carry an electric current and is useful for water quality assessment and industrial processes.

4.1.5 TDS and TSS measurement:

For TDS (Total Dissolved Solids) measurement, a TDS meter is used. The meter is immersed in the water sample, and the reading is taken after stabilization. TDS measurement provides information about the quantity of dissolved solids in the water.

For TSS (Total Suspended Solids) measurement, a filter is wetted, and a predetermined sample volume is measured. The sample is filtered, and the volume filtered is recorded. TSS measurement provides information about the quantity of suspended solids in the water.

Both TDS and TSS measurements are important for assessing water quality and understanding the composition of solids in the water sample.

4.2 Preparation of media and dilutions from wastewater

Nutrient agar (NA) and nutrient broth (NB) were used to promote bacterial growth. for preparing bacterial culture plates and suspension cultures, respectively. 1.3 g of NB was dissolved in 100 ml of distilled water. The same concentration was employed for a 100 ml NA media, adding an equal amount of agar to promote solidification. For isolating specific HNAD bacteria, Simultaneously, nitrification denitrification (SND) media was prepared by dissolving 0.472 g of $(NH_4)_2SO_4$, 0.722 g of KNO_3, 1.3 g of CH_3COONa, 1.53 g of BM and 2 mL of a trace element solution were combined with 1 L of distilled water **(Rout *et al.*, 2017).**

Dilutions with distilled water were made using wastewater that was collected from the sewage treatment plant inlet at TIET, Patiala. A 250 ml flask was used to prepare NA media, which was then sterilized using an autoclave. Culture plates were prepared by pouring 20 ml of the media into each plate, allowing them to solidify and labelling them accordingly. A 10^{-2} dilution was labelled as 1 ml of wastewater diluted with 9 ml of distilled water. To make a 10^{-3} dilution, 1 ml of the 10^{-2} dilution was added to 9 ml of distilled water. This process was repeated to create dilutions up to 10^{-8}. 1 ml from the 10^{-6} dilution was inoculated onto a culture plate using a spreader, and the same procedure was followed for the 10^{-7} and 10^{-8} dilutions. Duplicates of all plates were made to increase the likelihood of obtaining diverse bacterial colonies. A control plate was also prepared by inoculating only distilled water and spreading it. Clingfilm was used to seal the plates, which were then incubated for 24-48 hours at 37 °C. The plates were checked for different bacterial colonies after the incubation period.

4.3 Sub-culturing

Sub-culturing was carried out using the quadrant streaking method to isolate specific bacterial strains and obtain purified colonies. This was done on SND media for isolating specific bacteria, which can do both nitrification and denitrification. Initially, a colony was picked from the culture plate containing various bacterial colonies derived from wastewater dilutions using a sterilized inoculation loop. The loop was heat sterilized to streak the colony circularly on a culture plate quadrant. Subsequent streaking was performed by extending parallel lines from one quadrant to the next, ensuring the passage of cells. This process was repeated for each different bacterial colony to be isolated. After incubation, bacterial colonies were streaked again using the quadrant streaking to create separate culture plates for different bacterial isolates. For another 24 hours, the plates were incubated at 37 °C. A third streaking was performed from the previously incubated plates to ensure pure strain isolation, then a 24-hour incubation period at 37 °C. Nutrient broth cultures were prepared and autoclaved. After incubation, a colony from the third streaking culture plate was inoculated into the liquid culture. This process was repeated for each different bacterial colony. For 24 hours, the liquid cultures were cultured in a shaker incubator at 120 rpm and 37 °C. Subsequently, an inoculation loop was heat sterilized, dipped into the liquid cultures, and streaked onto agar plates using the quadrant streaking method. This step was repeated for each individual bacterial culture. After sealing, the plates were placed in an incubator at 37 ℃ for 24 hours.

4.4 Gram staining of the isolated bacterial colonies

A thick peptidoglycan layer's presence or absence is used by the gram staining technique to distinguish between gram-positive and gram-negative bacteria., resulting in purple-colored bacterial colonies for gram-positive and red or pink-colored bacterial colonies for gram-negative. This step also ensures that the isolated colonies are not contaminated with another bacterial species **(Hussey *et al.*, 2005).**

In the procedure, a clean glass slide was taken and wiped with alcohol to eliminate contaminants. A smear at the centre of the slide was made using a sterile inoculation loop, and the smear was carefully heat-fixed over a flame for approximately 30 s. Then, crystal violet was poured over the smear and allowed to rest for 1 minute before being washed off with 5 seconds with distilled water. Gram's iodine was then poured all over the slide., with a waiting time of 1 min, and subsequently washed off with distilled water in a gentle and indirect stream for 5 seconds. Decolorizer was added dropwise, with a wait time of 15 seconds, to remove excess color. Next, the slide was flooded with safranin for 30-45 seconds and washed off with distilled water. After allowing the slide to dry, a cover slip was applied over the smear. The glass slide was observed under a light microscope, initially at 10x magnification and then at 40x magnification. Notes were taken to record the morphology and stain color of the bacteria for identification purposes. Immersion oil was used before applying the coverslip for microscopy at 100x magnification.

4.5 Biochemical characterization of isolated bacteria

In order to better characterise the strains, a variety of tests were carried out using the HilMViC biochemical kit. The following tests were performed (**Bhutia *et al.*, 2021)** Urease test, Phenylalanine Deamination, Nitrate Reduction, H2S production, Citrate utilisation, Glucose utilisation, Adonitol utilisation, Arabinose utilisation, Lactose utilisation, Mannitol utilisation, Sorbitol utilisation, and Adonitol utilisation. The 16s rRNA gene sequence served as the basis for the molecular analysis. Using the HiPurA bacterial DNA purification spin column kit (MB505-250PR, HiMedia, India), genomic DNA was extracted from the pure culture.

4.6 Molecular characterization of isolated strains

Using the universal prokaryotic primers F27 5'-'AGAGTTTGATCMTGGCTCAG -3' and 1492R 5'-'GGTTACCTTGTTACGACTT -3', the 16S rDNA gene sequence was amplified by PCR. The Applied Biosystems Veriti Thermo Cycler was used to carry out the PCR amplification. Exonuclease 1 and a shrimp alkaline phosphatase purification kit from New England Biolabs, India, were used to purify the PCR products. Applied Biosystems' BigDye Terminator v.3.1 cycle sequencing kit was used to sequence the amplified area. Using the Basic Local Alignment Search Tool and "rRNA/ITS datasets," the other microbial sequences located in the National Centre for Biotechnology Information database were compared with the 16S rDNA sequence. Additional research was performed using MEGA11 software, which also produced a phylogenetic tree using the neighbour-joining tree approach and a bootstrap value of 1000. (**Abdulhakeem *et al.*, 2023)**

4.7 Screening of consortia on different carbon sources

For the screening to assess the performance of the bacteria's growth, the bacteria were cultivated on three different carbon sources. Dextrose, sucrose, and sodium citrate were the carbon sources that were used. 100 ml of SND media were mixed with a 5 ml inoculum of the bacteria, and the combination was then incubated at 37 °C with constant shaking at 120 rpm. Every six hours, the effectiveness of removing ammonium and nitrate as well as bacterial growth was assessed.

4.9 HNAD mediated nitrogen removal performance of the strains

100 mL of selective media was inoculated with 5 mL of the microbe's active pre-culture.

The control will be used as the corresponding media without inoculation. The samples will be taken from the flasks at intervals of two hours to measure the optical density (OD) at 600 nm for microbial growth. After that, they were centrifuged at a speed of seven thousand revolutions per minute for ten minutes to obtain supernatant, which will be used to detect ammonium and nitrate nitrogen. The following equations (Eq. 1 and 2) were used to calculate removal rates (RR) and removal efficiencies (RE):

$$RE(\%)= ((C_{o-} C_t)/C_o) 100 \quad (1)$$

$$RR(mg\ L^{-1}\ h^{-1})= (C_{o-} C_t)/t \quad (2)$$

where, C_o and C_o (mg L^{-1}) are the starting concentration and the concentration of $NH_4{}^+$-N, $NO_3{}^-$N at time t, t being the length of time the microbial strain has been treated.

Chapter 5

Results and discussion

5.1 Wastewater characterization

The results obtained from analysing wastewater parameters, which encompassed the measurement of BOD, COD, TSS, TDS, pH, and electric conductivity, are presented **Table [3]**. The table serves as a concise summary of the numerical values obtained for each parameter, allowing for easy comparison and interpretation of the wastewater characteristics. These results play an important role in understanding the quality and composition of the wastewater under investigation and provide valuable insights for further analysis and decision-making in wastewater treatment and management processes.

Table 3. Different parameters/characteristics of wastewater streams

S no.	**Parameters**	**Inlet**	**Outlet**
1.	BOD (mg/l)	279 ± 9	190.5 ± 12.5
2.	COD (mg/l)	464 ± 22.63	320 ± 0
3.	DO (mg/l)	_N/A	_N/A
4.	TDS (in ppm) at 22℃	847.2 ± 0.71	785.4 ± 0.28
5.	TSS (mg/ml)	0.029 ± 0.004	0.122 ± 0.003
6.	Electrical Conductivity (µS)	1558 ± 1.41	1430.5 ± 0.71
7.	pH	8.485 ± 0.45	7.85 ± 0.07

5.2. Isolation and preliminary study of isolated bacterial strain from wastewater

Two distinct bacterial colonies were separated and identified based on their physical characteristics from the culture plates made from wastewater. The colonies were assigned the labels R and Y. The bacterial colonies Y and R **Fig. [1a and b]** were selected due to their contrasting morphological features. Colony R appeared reddish, while colony Y exhibited a yellowish, but it was opaque.

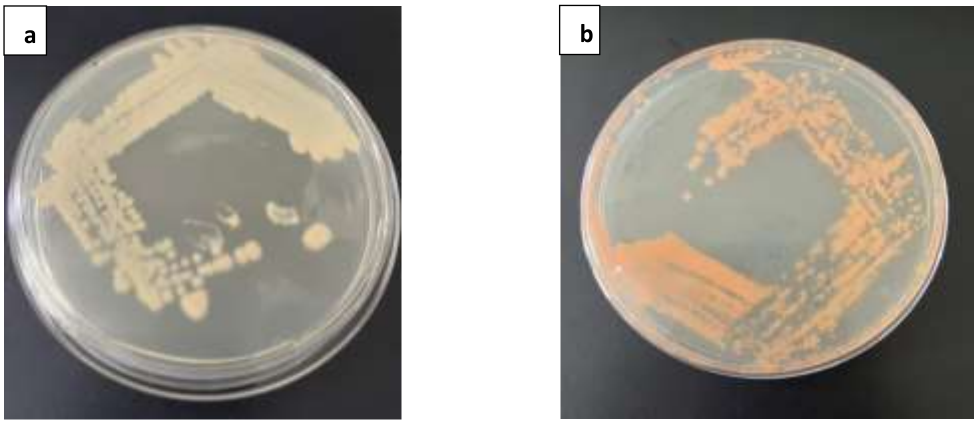

Fig. 1: Plates showing colony isolates a). Yellow and b). Red colonies

5.3 Morphological features

The morphological features of both Red and Yellow strain are mentioned in **Table [4].**

Table 4. Different morphological features of strains

S No.	Analysis	Red strain	Yellow strain
1.	Shape	Rod	Coccoid
2.	Colony Appearance	Round	Circular
3.	Margin	Entire	Entire
4.	Surface	Smooth	Sticky
5.	Opacity	Opaque	Opaque

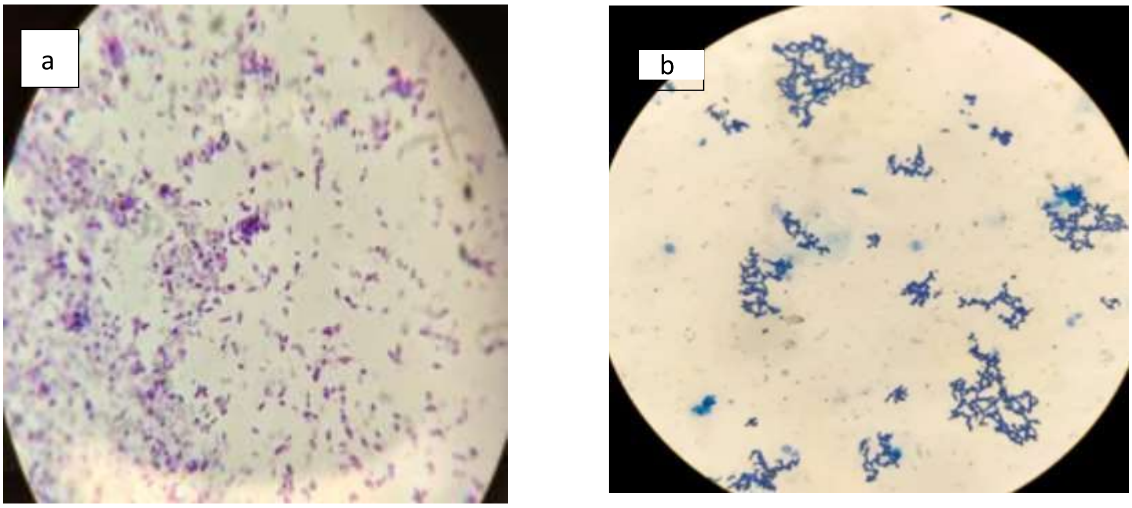

Fig 2: a) Gram staining image of Y strain b) Gram staining image of R strain

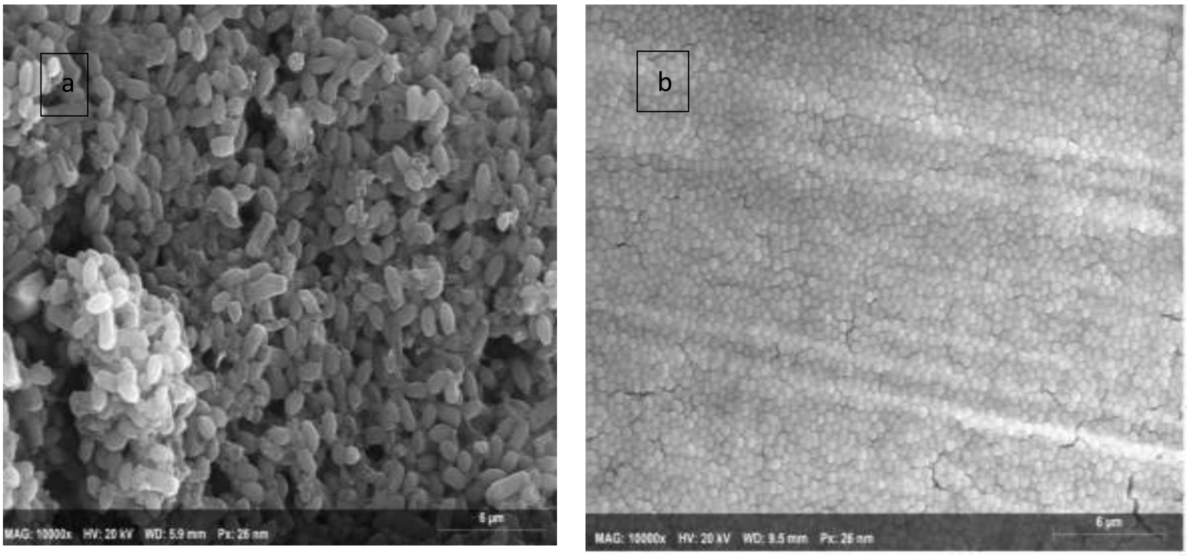

Fig. 3: Sem images of a.) *Rhodococcus ruber* strain 524 b.) *Cellulosimicrobium cellulans* strain 184 at magnification 10000x

5.4. Biochemical characterization

The biochemical characterization of the pure isolates was conducted using the HiMViC (Himedia) biochemical test kit. Studies have shown that the HiMViC tests are effective in differentiating various gram-positive bacterial species based on their metabolic properties (**Bhutia *et. al.,* 2021**). All biochemical tests were conducted in accordance with the manufacturer's instructions. The results, demonstrated in **Table [5],** indicate that the yellowcoloured isolate demonstrated nitrate reduction and carbohydrate utilization capabilities. Similarly, the red-coloured isolate exhibited amino acid utilization, nitrate reduction, and carbohydrate utilization abilities. The observation of nitrate reduction ability in both strains further strengthens the experimental evidence supporting denitrification in this study. **Venkateswarulu *et al.* (2023)** used biochemical tests to characterize the bacterial strain for uricase production. *Bacillus sp.*, described by **Rout *et al.* (2017)** with HNAD ability, was biochemically characterized and reported to be positive for starch and casein hydrolysis, catalase, urease, and Voges-Prauskauer tests.

Table 5. Biochemical characterization of red and yellow isolates

S no.	Biochemical test	Red colony	Yellow colony
1	Citrate utilization	Negative	Negative
2	Ornithine utilization	Positive	Negative
3	Lysine utilization	Positive	Negative
4	Nitrate Reduction	Positive	Positive
5	Urease	Negative	Negative
6	Phenylalanine Deamination	Negative	Negative
7	Sorbitol	Negative	Negative
8	Arabinose	Positive	Negative
9	Lactose	Negative	Positive
10	Adonitol	Negative	Positive
11	Glucose	Negative	Positive
12	H_2S Production	Negative	Negative

5.5 Molecular characterization

Using 16s rRNA gene sequencing, the two isolated bacteria were molecularly characterised. For both strains, the universal primers 27F and 1492R were used to extract and amplified the DNA. The phylogenetic tree obtained from the product of PCR amplification resulted from agarose gel electrophoresis of both strains is shown in **Fig. [5 and 7].** Single amplified bands were observed and size was estimated using 1 kb DNA ladder from Geneilabs. Using BLASTN homology searches, NCBI's results showed that strain 524 was closely related to *Rhodococcus* species. and strain184 was closely related to *Cellulosimicrobium* species. Strain524 was found to have 99.93% homology with *Rhodococcus ruber* (Accession no. NR_026185.1) using BLASTN search. Using MEGA11 software, multiple sequence alignment was performed with MUSCLE algorithm and phylogenetic tree was prepared with bootstrap phylogeny test and maximum composite likelihood substitution model. Phylogenetic tree **Fig. [4]** further shows that strain 524 together with *Rhodococcus electrodiphilus* (Accession no. NR_179575.1) formed a distinct linkage in the tree, with 99.86% homology. Strain 184 was found to have 99.59% homology with *Cellulosimicrobium cellulans (*Accession no. NR_119095*)* after BLASTN search. Similar to strain524, phylogenetic tree **Fig. [6]** was prepared and strain184 with *Cellulosimicrobium aquatile (*Accession no. NR 146008.1) showed closest linkage in evolutionary relationship with 99.45% homology. Phylogenetic tree has been commonly used in detecting homology between different evolutionary-related microbial species (**Pradhan *et al.*, 2022**). **Rout *et al.* (2017)** showed a close linkage of the identified *Bacillus cereus* GS-5 strain for heterotrophic nitrification and aerobic denitrification (HNAD) with *Bacillus cereus* PNA-2 using phylogenetic analysis. 16s rRNA sequencing and maximum likelihood analysis resulted in the identification of *Chryseobacterium haifense* from abbatoir wastewater with capacity of simultaneous heterotrophic nitrification and aerobic denitrification (**Kundu *et al.*, 2014**).

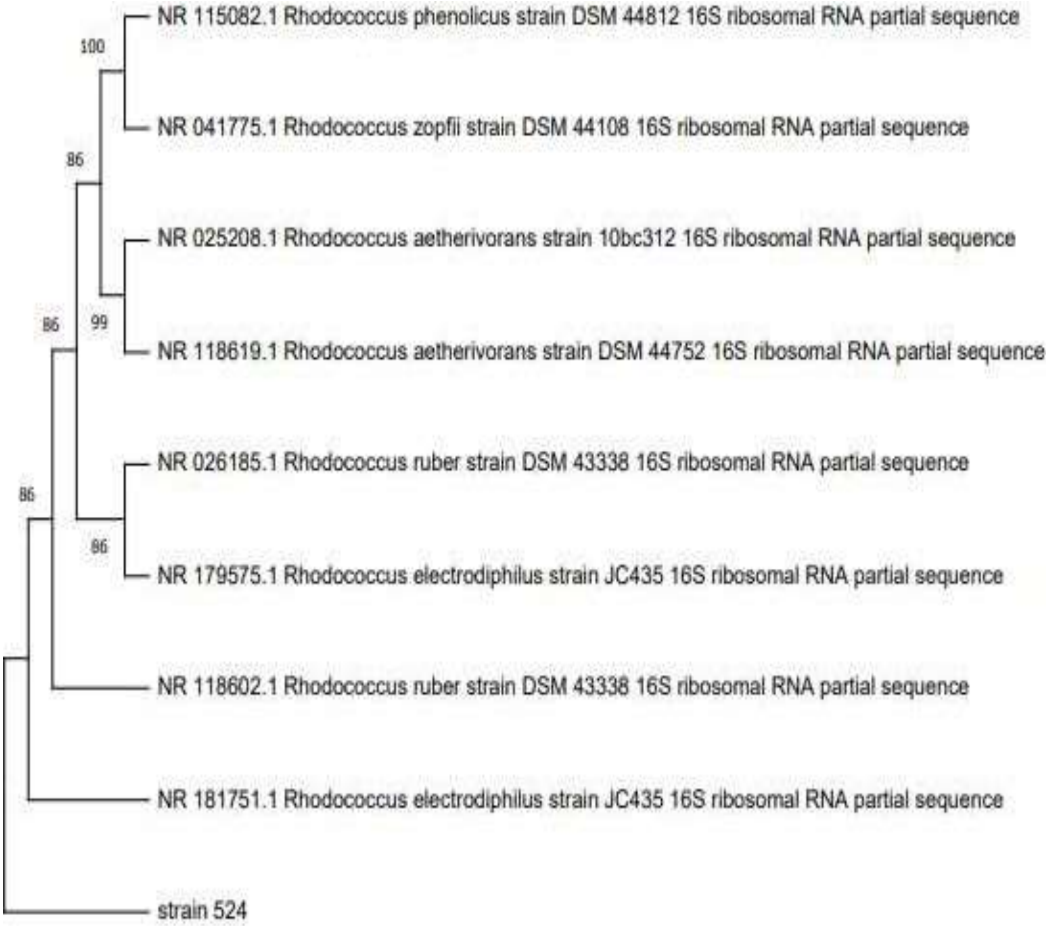

Fig. 4: Phylogenetic tree for *Rhodococcus ruber* strain 524

B. 16s rRNA PCR Product:

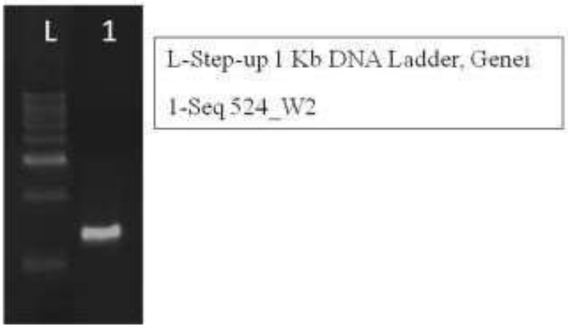

Fig. 5: Electrophoresis gel image of *Rhodococcus ruber* strain 524

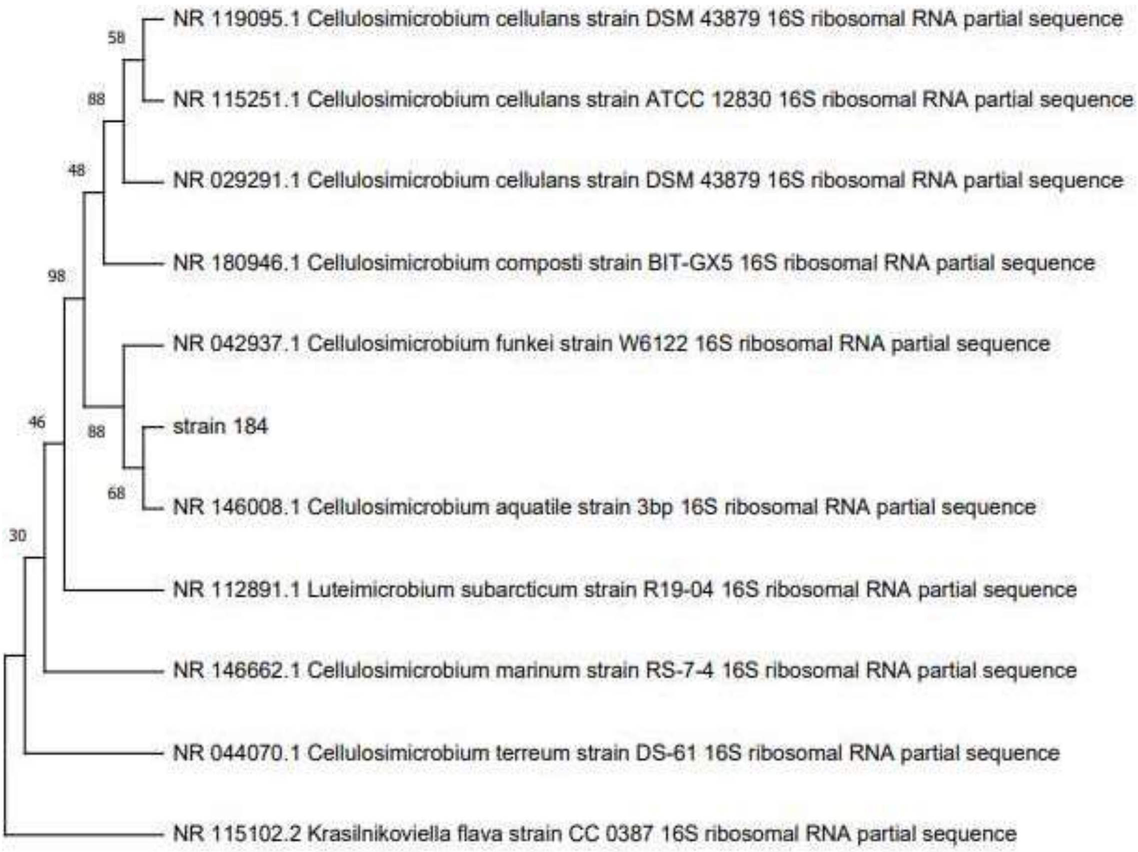

Fig. 6: Phylogenetic tree of *Cellulosimicrobium cellulans* strain 184

B. 16s rRNA PCR Product:

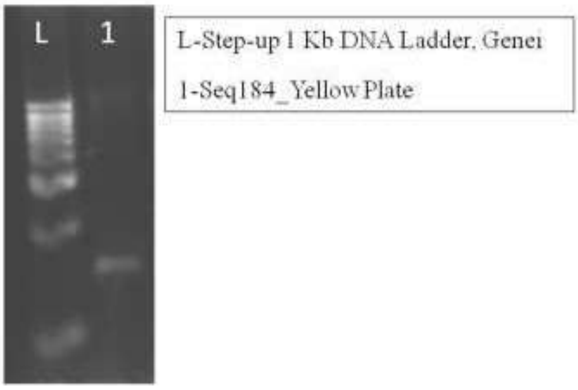

Fig. 7: Electrophoresis gel image of *Cellulosimicrobium cellulans* strain 184

5.6 Growth and nitrogen removal efficiency

The experiment involved inoculating 10 ml of inoculum into 100 ml SND media. Optical density (OD) at 600 nm, nitrate concentration **(Cataldo *et al.*, 1975)** and ammonium concentrations **(Squire *et al* ., 1966)** were measured every 2 hours to assess removal efficiency under optimal conditions. The results of the experiment are shown in **Fig. [8-10].**

The results indicate that the maximum removal rate for ammonium 93.5 ± 2.5% and nitrate nitrogen 64.87 ± 4.87% was achieved. The highest removal efficiency for ammonium nitrogen and nitrate nitrogen was observed between 6 to 8 h, indicating the elimination of both ammonium and nitrate nitrogen compounds during the experimental period.

Rout *et al.* (2017) found in a different study that the isolated bacteria demonstrated greater efficiency in denitrification when both nitrate nitrogen and ammonium nitrogen were present in the SND media simultaneously. This result supports the current experiment.

Furthermore, according to a study by **Wei *et al.* (2020),** the maximum removal rates for both ammonium and nitrate nitrogen happened between 6 and 8 h. This finding aligns with the results obtained in the present experiment.

The experiment successfully removed nitrogen compounds and observed the highest removal efficiency for ammonium nitrogen between 6 to 8 h. Previous studies have also reported similar findings regarding the efficiency of denitrification with nitrate nitrogen and ammonium nitrogen in SND media, as well as the highest removal rates occurring between 6 to 8 h.

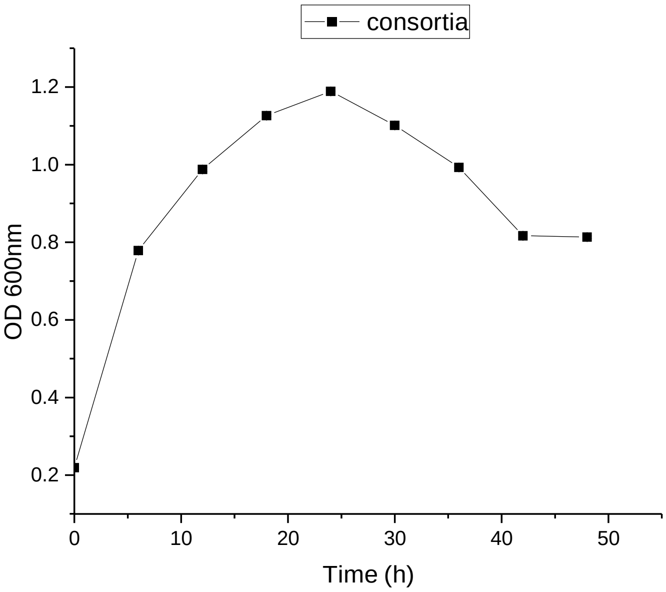

Fig. 8: Growth curve of consortium consisting of *Rhodococcus ruber* and *Cellulosimicrobium cellulans*

Fig. 9: Picture of consortia on a SND media plate consisting of *Rhodococcus ruber* and *Cellulosimicrobium cellulan*

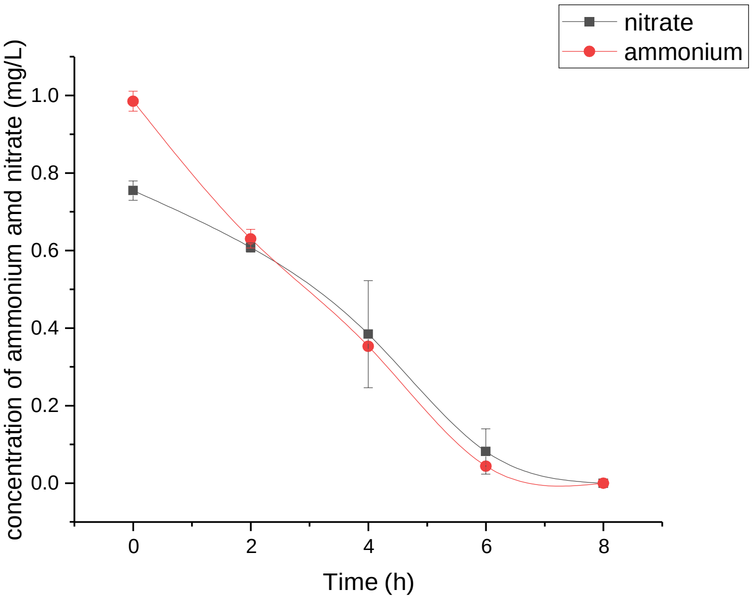

Fig. 10: Ammonium and nitrate removal efficiency of consortia

5.7 Screening of consortia in different carbon sources

Carbon sources are crucial in supplying energy and electrons for nitrogen removal in a co-culture system (**Wang *et al.*, 2021**). In an experiment, a consortium of bacteria was cultured in an SND medium with different exogenous sole carbon sources: glucose, sucrose, and sodium citrate. The respective OD600 values were measured for each carbon source. Interestingly, the strain's capacities for ammonium and nitrate removal varied depending on the carbon source used, with dextrose (glucose) demonstrating the highest effectiveness, followed by sucrose and sodium citrate. This indicates that the strain exhibited superior nutrient removal activity when cultivated in a defined medium. The results of the consortia growth and their nitrate and ammonium removal efficiency in different carbon sources has been shown in **Fig [11-13].**

Various exogenous single carbon sources were added to a growth medium in a study by **Rout *et al.* (2017)** to test the *Bacillus cereus GS-5* strain. The order of effectiveness for carbon sources was sodium acetate, glucose, Nutrient Broth (NB), sodium succinate, and sodium citrate, respectively. However, when assessing the strain's nitrogen removal capacity in the presence of various carbon sources, in the following order: glucose, NB, sodium acetate, sodium succinate, and sodium citrate. The most effective carbon source for removing nitrogen, according to their research, is sodium acetate. In a co-culture system, the kind of carbon source is essential for nitrogen removal. The experiment found that the best carbon source for removing nitrogen was dextrose (glucose). Sodium acetate was shown to be the most effective carbon source for the *Bacillus cereus GS-5* strain in the study by **(Rout *et al.*, 2017).**

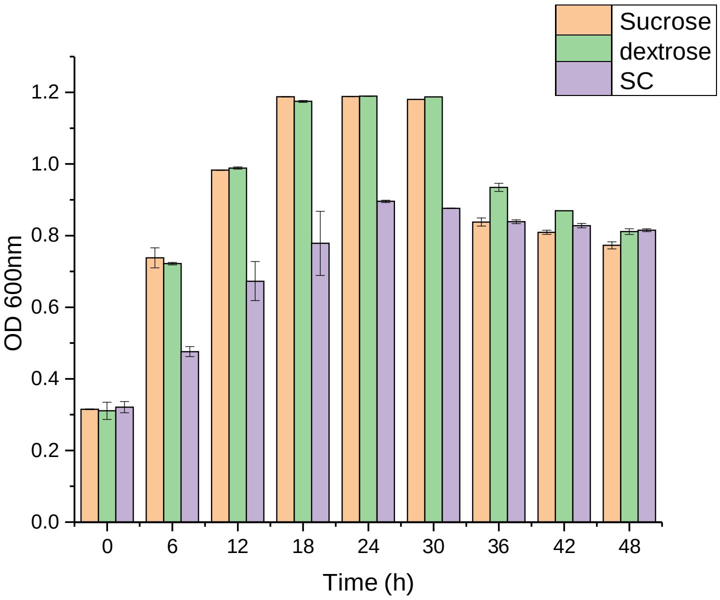

Fig. 11: Growth curve of consortia in different carbon sources

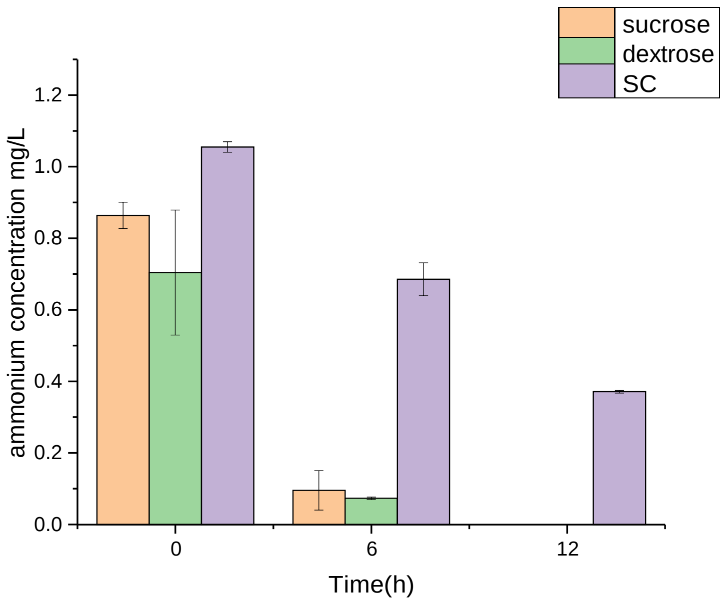

Fig. 12: Ammonium removal efficiency in different carbon sources

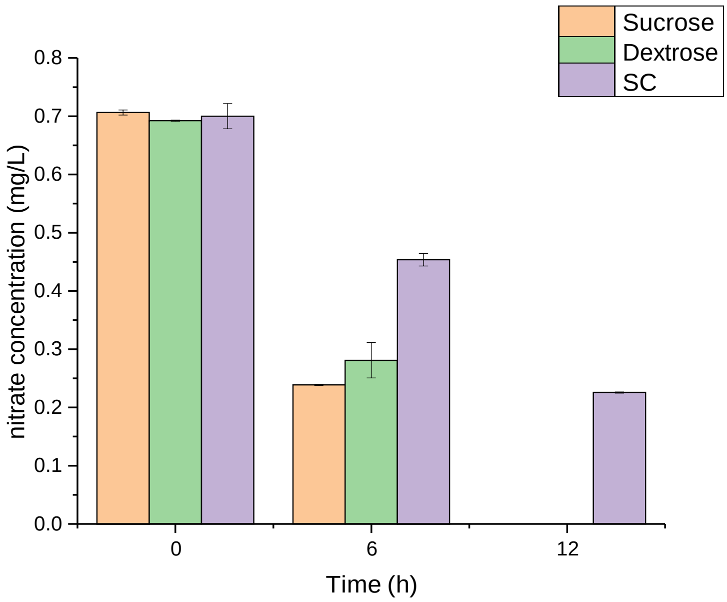

Fig. 13: Nitrate removal efficiency with different carbon source

Chapter - 6

6.1 Conclusion

In conclusion, this pioneering study successfully demonstrated bacterial consortia's remarkable HNAD capabilities. These consortia are made up of two strains, which was taken from the wastewater treatment plant at TIET, Patiala, and exhibited the unique ability to perform HNAD processes. The findings of this study hold significant promise for the elinination of ammonium and nitrate from wastewater.

The heterotrophic nitrification ability of consortia allows to convert ammonium to nitrate, while their aerobic denitrification ability facilitates the transformation of nitrate to nitrogen gas. These dual processes are vital in reducing ammonium and nitrate levels in wastewater, preserving environmental quality, and safeguarding aquatic ecosystems. Identifying strains *Rhodococcus ruber strain524* and *Cellulosimicrobium cellulans strain184* as highly effective agents for ammonium and nitrate removal provides valuable insights for developing advanced wastewater treatment strategies.

6.2 Future prospectus

The potential application of consortia in large-scale treatment systems holds great promise for achieving efficient and sustainable wastewater management. By harnessing the capabilities of consortia, it is possible to improve water quality and enhance environmental protection. Continued research and implementation of these consortia in practical wastewater treatment systems can contribute to developing effective solutions for addressing the challenges associated with wastewater pollution. Ultimately, these efforts can lead to substantial improvements in water quality and contribute to preserving our ecosystems for future generations.

References

1. He, T., Li, Z., Sun, Q., Xu, Y., & Ye, Q. (2016). Heterotrophic nitrification and aerobic denitrification by Pseudomonas tolaasii Y-11 without nitrite accumulation during nitrogen conversion. *Bioresource Technology*, *200*, 493-499.
2. Wang, H., Li, J., Wang, B., & Chen, G. (2020). Deciphering pollutants removal mechanisms and genetic responses to ampicillin stress in simultaneous heterotrophic nitrification and aerobic denitrification (SHNAD) process treating seawater-based wastewater. *Bioresource Technology*, *315*, 123827.
3. Duan, J., Fang, H., Su, B., Chen, J., & Lin, J. (2015). Characterization of a halophilic heterotrophic nitrification–aerobic denitrification bacterium and its application on treatment of saline wastewater. *Bioresource technology*, *179*, 421-428.
4. Chen, P., Li, J., Li, Q. X., Wang, Y., Li, S., Ren, T., & Wang, L. (2012). Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium Rhodococcus sp. CPZ24. *Bioresource technology*, *116*, 266-270.
5. Sun, Z., Lv, Y., Liu, Y., & Ren, R. (2016). Removal of nitrogen by heterotrophic nitrification-aerobic denitrification of a novel metal resistant bacterium Cupriavidus sp. S1. *Bioresource Technology*, *220*, 142-150.
6. Yang, L., Ren, Y. X., Liang, X., Zhao, S. Q., Wang, J. P., & Xia, Z. H. (2015). Nitrogen removal characteristics of a heterotrophic nitrifier Acinetobacter junii YB and its potential application for the treatment of high-strength nitrogenous wastewater. *Bioresource technology*, *193*, 227-233.
7. Zhang, Y., Shi, Z., Chen, M., Dong, X., & Zhou, J. (2015). Evaluation of simultaneous nitrification and denitrification under controlled conditions by an aerobic denitrifier culture. *Bioresource Technology*, *175*, 602-605.

8. Yao, Y. C., Zhang, Q. L., Liu, Y., & Liu, Z. P. (2013). Simultaneous removal of organic matter and nitrogen by a heterotrophic nitrifying–aerobic denitrifying bacterial strain in a membrane bioreactor. *Bioresource Technology*, *143*, 83-87.
9. Huang, T. L., Zhou, S. L., Zhang, H. H., Bai, S. Y., He, X. X., & Yang, X. (2015). Nitrogen removal characteristics of a newly isolated indigenous aerobic denitrifier from oligotrophic drinking water reservoir, Zoogloea sp. N299. *International Journal of Molecular Sciences*, *16*(5), 10038-10060.
10. Lei, Y., Wang, Y., Liu, H., Xi, C., & Song, L. (2016). A novel heterotrophic nitrifying and aerobic denitrifying bacterium, Zobellella taiwanensis DN-7, can remove high-strength ammonium. *Applied Microbiology and Biotechnology*, *100*, 4219-4229.
11. Chen, Q., & Ni, J. (2012). Ammonium removal by Agrobacterium sp. LAD9 capable of heterotrophic nitrification–aerobic denitrification. *Journal of bioscience and bioengineering*, *113*(5), 619-623.
12. Chen, P., Li, J., Li, Q. X., Wang, Y., Li, S., Ren, T., & Wang, L. (2012). Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium Rhodococcus sp. CPZ24. *Bioresource technology*, *116*, 266-270.
13. Joo, H. S., Hirai, M., & Shoda, M. (2005). Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by Alcaligenes faecalis No. 4. *Journal of Bioscience and Bioengineering*, *100*(2), 184-191.
14. Yang, X. P., Wang, S. M., Zhang, D. W., & Zhou, L. X. (2011). Isolation and nitrogen removal characteristics of an aerobic heterotrophic nitrifying–denitrifying bacterium, Bacillus subtilis A1. *Bioresource technology*, *102*(2), 854-862.
15. Zhang, Q. L., Liu, Y., Ai, G. M., Miao, L. L., Zheng, H. Y., & Liu, Z. P. (2012). The characteristics of a novel heterotrophic nitrification–aerobic denitrification bacterium, Bacillus methylotrophicus strain L7. *Bioresource technology*, *108*, 35-44.

16. Khardenavis, A. A., Kapley, A., & Purohit, H. J. (2007). Simultaneous nitrification and denitrification by diverse Diaphorobacter sp. *Applied Microbiology and Biotechnology*, *77*, 403-409.

17. Zhao, B., He, Y. L., Hughes, J., & Zhang, X. F. (2010). Heterotrophic nitrogen removal by a newly isolated Acinetobacter calcoaceticus HNR. *Bioresource technology*, *101*(14), 5194-5200.

18. Zheng, H. Y., Liu, Y., Gao, X. Y., Ai, G. M., Miao, L. L., & Liu, Z. P. (2012). Characterization of a marine origin aerobic nitrifying–denitrifying bacterium. *Journal of Bioscience and Bioengineering*, *114*(1), 33-37.

19. Padhi, S. K., Tripathy, S., Sen, R., Mahapatra, A. S., Mohanty, S., & Maiti, N. K. (2013). Characterisation of heterotrophic nitrifying and aerobic denitrifying Klebsiella pneumoniae CF-S9 strain for bioremediation of wastewater. *International Biodeterioration & Biodegradation*, *78*, 67-73.

20. Chen, C., Wang, Z., Zhao, M., Yuan, B., Yao, J., Chen, J., ... & Savitskaya, T. (2021). A fungus–bacterium co-culture synergistically promoted nitrogen removal by enhancing enzyme activity and electron transfer. *Science of The Total Environment*, *754*, 142109.

21. Zhang, Y., Xu, Z., Li, J., Liu, D., Yuan, Y., Chen, Z., & Wang, G. (2019). Cooperation between two strains of Enterobacter and Klebsiella in the simultaneous nitrogen removal and phosphate accumulation processes. *Bioresource technology*, *291*, 121854.

22. Huang, F., Pan, L., He, Z., Zhang, M., & Zhang, M. (2021). Heterotrophic nitrification-aerobic denitrification characteristics and antibiotic resistance of two bacterial consortia from Marinomonas and Halomonas with effective nitrogen removal in mariculture wastewater. *Journal of Environmental Management*, *279*, 111786.

23. Xia, Y., Zhang, M., Tsang, D. C., Geng, N., Lu, D., Zhu, L., ... & Ok, Y. S. (2020). Recent advances in control technologies for non-point source pollution with nitrogen and phosphorous

from agricultural runoff: current practices and future prospects. *Applied Biological Chemistry*, *63*(1), 1-13.

24. Chen, P., Li, J., Li, Q. X., Wang, Y., Li, S., Ren, T., & Wang, L. (2012). Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium Rhodococcus sp. CPZ24. *Bioresource technology*, *116*, 266-270.
25. Zhang, M., Li, A., Yao, Q., Wu, Q., & Zhu, H. (2020). Nitrogen removal characteristics of a versatile heterotrophic nitrifying-aerobic denitrifying bacterium, Pseudomonas bauzanensis DN13-1, isolated from deep-sea sediment. *Bioresource Technology*, *305*, 122626.
26. Zhang, Y., Dai, S., Huang, X., Zhao, Y., Zhao, J., Cheng, Y., ... & Zhang, J. (2020). pH-induced changes in fungal abundance and composition affects soil heterotrophic nitrification after 30 days of artificial pH manipulation. *Geoderma*, *366*, 114255.
27. Zhang, Q. L., Liu, Y., Ai, G. M., Miao, L. L., Zheng, H. Y., & Liu, Z. P. (2012). The characteristics of a novel heterotrophic nitrification–aerobic denitrification bacterium, Bacillus methylotrophicus strain L7. *Bioresource technology*, *108*, 35-44.
28. Li, Y., Chapman, S. J., Nicol, G. W., & Yao, H. (2018). Nitrification and nitrifiers in acidic soils. *Soil Biology and Biochemistry*, *116*, 290-301.
29. Liu, H., Lu, Q., Wang, Q., Liu, W., Wei, Q., Ren, H., ... & Ruan, R. (2017). Isolation of a bacterial strain, Acinetobacter sp. from centrate wastewater and study of its cooperation with algae in nutrients removal. *Bioresource technology*, *235*, 59-69.
30. Yang, Y., Liu, Y., Yang, T., & Lv, Y. (2017). Characterization of a microbial consortium capable of heterotrophic nitrifying under wide C/N range and its potential application in phenolic and coking wastewater. *Biochemical Engineering Journal*, *120*, 33-40.
31. Zhang, Y., Dai, S., Huang, X., Zhao, Y., Zhao, J., Cheng, Y., ... & Zhang, J. (2020). pH-induced changes in fungal abundance and composition affects soil heterotrophic nitrification after 30 days of artificial pH manipulation. *Geoderma*, *366*, 114255.

32. Nancharaiah, Y. V., & Reddy, G. K. K. (2018). Aerobic granular sludge technology: mechanisms of granulation and biotechnological applications. *Bioresource technology*, *247*, 1128-1143.

33. Joo, H. S., Hirai, M., & Shoda, M. (2005). Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by Alcaligenes faecalis No. 4. *Journal of Bioscience and Bioengineering*, *100*(2), 184-191.

34. Ren, Y. X., Yang, L., & Liang, X. (2014). The characteristics of a novel heterotrophic nitrifying and aerobic denitrifying bacterium, Acinetobacter junii YB. *Bioresource Technology*, *171*, 1-9.

35. Chen, Q., & Ni, J. (2011). Heterotrophic nitrification–aerobic denitrification by novel isolated bacteria. *Journal of Industrial Microbiology and Biotechnology*, *38*(9), 1305-1310.

36. Zhang, J., Sun, W., Zhong, W., & Cai, Z. (2014). The substrate is an important factor in controlling the significance of heterotrophic nitrification in acidic forest soils. *Soil Biology and Biochemistry*, *76*, 143-148.

37. Zhao, B., Tian, M., An, Q., Ye, J., & Guo, J. S. (2017). Characteristics of a heterotrophic nitrogen removal bacterium and its potential application on treatment of ammonium-rich wastewater. *Bioresource technology*, *226*, 46-54.

38. Yang, L., Ren, Y., Liang, X., Zhao, S., Wang, J., Xia, Z., (2015). Nitrogen removal characteristics of a heterotrophic nitrifier Acinetobacter junii YB and its potential application for the treatment of high-strength nitrogenous wastewater. *Bioresource technology*, 193, 227–233.

39. Itokawa, H., Hanaki, K., Matsuo, T.,(2001). Nitrous oxide production in high-loading biological nitrogen removal process under low cod/n ratio condition. *Water Research*. 35, 657–664.

40. Silva, L. C. F., Lima, H. S., de Oliveira Mendes, T. A., Sartoratto, A., de Paula Sousa, M., de Souza, R. S., ... & da Silva, C. C. (2019). Heterotrophic nitrifying/aerobic denitrifying bacteria: Ammonium removal under different physical-chemical conditions and molecular characterization. *Journal of environmental management*, *248*, 109294.
41. Chen, Q., & Ni, J. (2011). Heterotrophic nitrification–aerobic denitrification by novel isolated bacteria. *Journal of Industrial Microbiology and Biotechnology*, *38*(9), 1305-1310.
42. Li, C., Yang, J., Wang, X., Wang, E., Li, B., He, R., Yuan, H., (2015). Removal of nitrogen by heterotrophic nitrification–aerobic denitrification of a phosphate accumulating bacterium Pseudomonas stutzeri YG-24. *Bioresource. Technology*. 182, 18–25.
43. Chen, Q., & Ni, J. (2012). Ammonium removal by Agrobacterium sp. LAD9 capable of heterotrophic nitrification–aerobic denitrification. *Journal of bioscience and bioengineering*, *113*(5), 619-623.
44. Rodriguez-Caballero, A., Hallin, S., Påhlson, C., Odlare, M., & Dahlquist, E. (2012). Ammonia oxidizing bacterial community composition and process performance in wastewater treatment plants under low temperature conditions. *Water Science and Technology*, *65*(2), 197-204.
45. Liu, Y., Ai, G. M., Wu, M. R., Li, S. S., Miao, L. L., & Liu, Z. P. (2019). Photobacterium sp. NNA4, an efficient hydroxylamine-transforming heterotrophic nitrifier/aerobic denitrifier. *Journal of bioscience and bioengineering*, *128*(1), 64-71.
46. Huang, X., Li, W., Zhang, D., Qin, W., (2013). Ammonium removal by a novel oligotrophic acinetobacter sp. Y16 capable of heterotrophic nitrification–aerobic denitrification at low temperature. *Bioresource technology*. 146, 44–50
47. Zhao, B., An, Q., He, Y.L., Guo, J.S., (2012). N2O and N2 production during heterotrophic nitrification by Alcaligenes faecalis strain NR. *Bioresource technology*. 116, 379–385

48. Robertson, L.A., Cornelisse, R., De Vos, P., Hadioetomo, R., Kuenen, J.G., (1989). Aerobic denitrification in various heterotrophic nitrifiers. Antonie Van Leeuwenhoek 56, 289–299

49. Sun, Z., Lv, Y., Liu, Y., Ren, R., 2016. Removal of nitrogen by heterotrophic nitrification aerobic denitrification of a novel metal resistant bacterium Cupriavidus sp. S1. *Bioresource technology*. 220, 142–150

50. Xia, L., Li, X., Fan, W., Wang, J., (2020). Heterotrophic nitrification and aerobic denitrification by a novel Acinetobacter sp. ND7 isolated from municipal activated sludge. *Bioresource technology*. 301, 122749.

51. Huang, H. K., & Tseng, S. K. (2001). Nitrate reduction by *Citrobacter diversus* under aerobic environment. *Applied Microbiology and Biotechnology, 55*, 90–94.

52. Wang, H., Chen, N., Feng, C., & Deng, Y. (2021). Insights into heterotrophic denitrification diversity in wastewater treatment systems: Progress and future prospects based on different carbon sources. *Science of the Total Environment*, *780*, 146521.

53. Smith, A. C., & Hussey, M. A. (2005). Gram staining protocols. *Washington, DC: American Society of Microbiology*.

54. Cataldo, D. A., Maroon, M., Schrader, L. E., & Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Communications in soil science and plant analysis*, *6*(1), 71-80.

55. Horn, D. B., & Squire, C. R. (1966). The estimation of ammonia using the indophenol blue reaction. *Clinica Chimica Acta*, *14*(2), 185-194.

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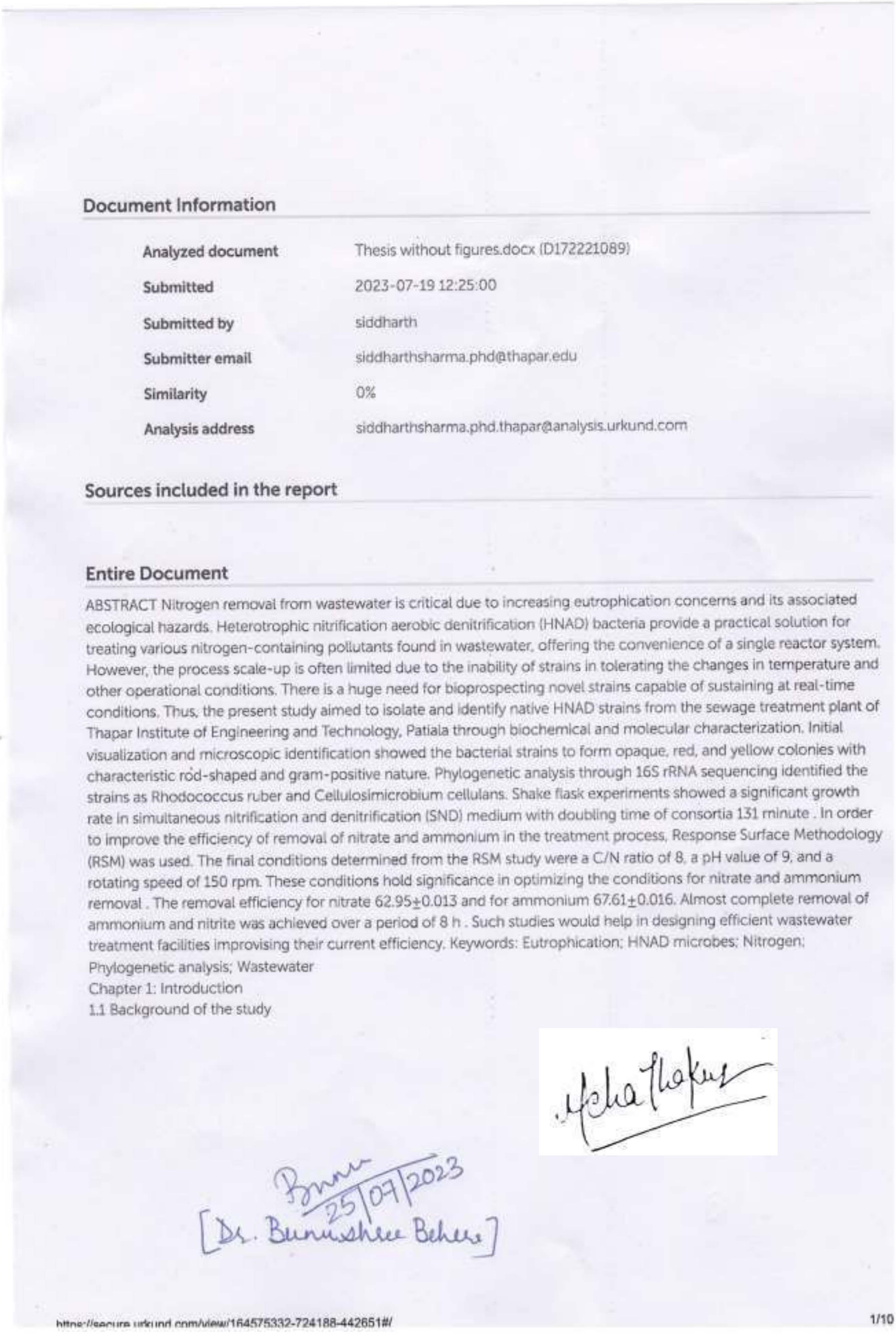

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ABSTRACT Nitrogen removal from wastewater is critical due to increasing eutrophication concerns and its associated ecological hazards. Heterotrophic nitrification aerobic denitrification (HNAD) bacteria provide a practical solution for treating various nitrogen-containing pollutants found in wastewater, offering the convenience of a single reactor system. However, the process scale-up is often limited due to the inability of strains in tolerating the changes in temperature and other operational conditions. There is a huge need for bioprospecting novel strains capable of sustaining at real-time conditions. Thus, the present study aimed to isolate and identify native HNAD strains from the sewage treatment plant of Thapar Institute of Engineering and Technology, Patiala through biochemical and molecular characterization. Initial visualization and microscopic identification showed the bacterial strains to form opaque, red, and yellow colonies with characteristic rod-shaped and gram-positive nature. Phylogenetic analysis through 16S rRNA sequencing identified the strains as Rhodococcus ruber and Cellulosimicrobium cellulans. Shake flask experiments showed a significant growth rate in simultaneous nitrification and denitrification (SND) medium with doubling time of consortia 131 minute . In order to improve the efficiency of removal of nitrate and ammonium in the treatment process, Response Surface Methodology (RSM) was used. The final conditions determined from the RSM study were a C/N ratio of 8, a pH value of 9, and a rotating speed of 150 rpm. These conditions hold significance in optimizing the conditions for nitrate and ammonium removal . The removal efficiency for nitrate 62.95±0.013 and for ammonium 67.61±0.016. Almost complete removal of ammonium and nitrite was achieved over a period of 8 h . Such studies would help in designing efficient wastewater treatment facilities improvising their current efficiency. Keywords: Eutrophication; HNAD microbes; Nitrogen; Phylogenetic analysis; Wastewater

Chapter 1: Introduction

1.1 Background of the study

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