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Analyzing Simultaneous Heterotrophic Nitrification and Aerobic Denitrification Potential of a Newly Isolated Bacterium, Bacillus cereus strain GS5

SND by Newly Isolated Bacillus cereus GS5 strain

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***Abstract*-The present study aims in investigating simultaneous heterotrophic nitrification and aerobic denitrification (SND) ability of a newly isolated bacterium. The bacterial strain GS5 was isolated from a laboratory scale denitrifying bioreactor and characterized. Based on phenotypic and phylogenetic characteristics as derived from partial 16S rRNA gene sequence, the isolate was identified to be *Bacillus cereus* GS5 strain (Gen Bank accession no. KM212993). The strain had exhibited the capability of utilizing ammonia, nitrate and nitrite as sole nitrogen sources in presence of external carbon sources, thus heterotrophic in nature. Considering, initial concentration of ammonium as 50 mg/L, 92.02% was removed after 24h incubation with little accumulation of the intermediates such as hydroxylamine, nitrite, and nitrate. Similarly, the new isolate had a high denitrifying ability, from initial 100 mg/L nitrate, 98.26% was removed in a 24h period under aerobic conditions, with reduced level of nitrite accumulation (0.613 mg/L). Thereby, confirming aerobic denitrification. Gas production as a result of denitrification process was indicated by the presence of gas bubbles in the Durham tubes and on the surface of the microbial culture broth. The results illustrated that the optimum aerobic denitrification conditions for strain GS5 were, a C/N ratio of 3.5, incubation temperature of 35 °C, an initial pH of 7, and rotation speed of 125 rpm (dissolved oxygen 2.75-3 mg/L). Therefore, the new isolate *Bacillus cereus* GS5 strain, with aerobic heterotrophic nitrification-denitrification ability, can be used in full scale treatment systems for facilitating simultaneous nitrification and denitrification (SND) in order to remove nitrogen from contaminated water.**

Keywords*– *Bacillus cereus GS5 strain, Heterotrophic nitrification, Aerobic denitrification, Simultaneous nitrification and denitrification (SND)

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I. *Introduction*

Nitrogen being a component of DNA, RNA and protein of living beings, plays the role of a key nutrient in their growth and developments. But excess release of nitrogen to the natural receiving aquatic ecosystems may contribute much to eutrophication, methemoglobinemia or "blue baby syndrome" in infants, miscarriages in case of pregnant women, acute poisoning in cattle and the formation of carcinogenic compounds such as nitrosoamines and nitrosoamides [1,2]. Therefore, nitrogen removal is of great concern in wastewater treatment process.

Many physical, chemical and biological methods were being practiced to remove nitrogen from wastewater in recent pasts. But the removal of nitrogen from wastewater by nitrifiers and denitrifiers was adjudged to be the most efficient and low cost method. The conventional biological treatment processes are achieved through nitrification, the oxidation of NH_3 to NO^{3-} via NO^{2-} by autotrophic nitrifiers and denitrification, the heterotrophic conversion of NO^{3-} to N_2 [3] by anoxic denitrifiers. But the disadvantages of this system lie in the following points: (i) the rate of nitrification is slow, and (ii) the autotrophs are vulnerable to high loads of ammonium and organic matter. Hence, their applications are restricted when treating high-strength ammonium wastewater. As an alternative to the conventional method, simultaneous nitrification and denitrification (SND) has been intensively studied in recent times [4]. In SND approach nitrification and denitrification could occur concurrently in one reactor under identical operating conditions. The SND process could have been possible due to discovery of novel microbes capable of performing heterotrophic nitrification and aerobic denitrification. Some of the recent studies have reported the existence of some special bacteria (*Bacillus subtilis* A1, *Acinetobacter calcoaceticus* HNR etc.) that are capable of performing nitrification under aerobic conditions, hence, called aerobic denitrifiers and nitrification by utilizing organic carbon sources, so termed as heterotrophic nitrifiers [5,6]. As compare to the conventional mechanism, nitrogen removal by heterotrophic nitrification and aerobic denitrification appears more promising with several advantages like (i) the utilization of organic substrates and the tolerance to oxygen, (ii) the process of denitrification could balance the change of pH in the reactor, avoiding the acidification caused by nitrification, (iii) the diversity of substrates and products of heterotrophic

nitrification can sustain the mixed culture and expanded the application scope [7,8,9]. In this way the discoveries of heterotrophic nitrification and aerobic denitrification bacteria broke through the limitation of traditional conception, and provided a new pathway for biological technology of denitrification.

The SND is a somewhat new process and so as the finding of heterotrophic nitrification and aerobic denitrification potential of some specific microbes. However, the isolation of the microbes exhibiting heterotrophic nitrification and aerobic denitrification are reported rarely. In this work, a novel heterotrophic nitrifying and aerobic denitrifying bacterium, strain GS, was isolated from a lab scale denitrifying reactor used for the treatment of nitrate rich aqueous solution. The isolated microbe's aerobic denitrification and heterotrophic nitrification ability were explored.

II. Materials and methods

A. Bacterial isolation and identification

In order to isolate the bacterial strain, 6 month old mature bio-film sample from the laboratory scale denitrifying reactor was collected and suspended in sterile saline solution. The suspension was centrifuged; re-suspended and appropriate dilution was plated using selective media. The plates were incubated for a specific time period. Single colonies were picked from the plates; sub cultured in liquid broth and subsequently streaked to get isolated colonies of the desired microbe as shown in Fig.1a. After repeated streaking and ensuring the purity, GS5 strain was isolated.

DNA was isolated from the bacterial culture, using DNA extraction kit (Merck Bioscience, India). Quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed (Fig. 1b). Fragment of 16S rDNA gene was amplified by PCR using specific forward and reverse primers. The PCR amplicon was purified and processed to get a consensus sequence of 1356 bp16S rDNA gene. The gene sequence was used to carry out BLAST in NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Phylogenetic tree was constructed using MEGA 5.

Figure 1.(a) Agar plate showing isolate bacterial colony, (b) 1.2% Agarose gel showing single 1.5 kb of 16S rDNA amplicon

B. Culture medium and heterotrophic nitrification and aerobic denitrification performance

For isolation and evaluation of heterotrophic nitrification and aerobic denitrification ability, synthetic mediums such as nitrate broth, nitrite broth and ammonium broth have been used throughout the study. The respective broths have been prepared by mixing appropriate amount of $NaNO_3$, $NaNO_2$ and NH_4Cl with Basal Medium (BM) and trace element solution. The active culture of strain (taken from exponential growth phase) was used as inoculums (2%) in 100 mL of nitrate, nitrite and ammonium broth taken in 250 mL Erlenmeyerflasks. Theflasks were incubated aerobically with rotational speed of 125 rpm at 35°C. The respective medium without inoculation was used as the control. The samples were taken from the flasks at 2 h interval to determine the optical density (OD) at 600 nm for microbial growth measurement, and the respective concentrations of nitrate, nitrite and ammonium to estimate the denitrification and nitrification efficiency

C. Analytical methods

The samples taken after every 2h interval were centrifuged at 10000 rpm for 15 minutes prior to analysis. Chemical oxygen demand (COD), Ammoniacal nitrogen (NH_4^+-N), Nitrate nitrogen (NO_3^--N), Volatile fatty acid (VFA), Dissolved oxygen (DO) and pH were determined respectively, by colorimetric method (5220-D), Ion Selective Electrode (ISE) method, 4500-NH_3, 4500-NO_3^-, Orion 5-Star DO probe and HACH digital pH meter as per the procedure mentioned in the standard methods for the examination of water and wastewater, APHA 1999 [10].

III. Results and discussions

A. Identification of GS5 strain

Six numbers of purified and isolated colonies from selective agar media were examined for nitrifying and denitrifying abilities by periodic monitoring of ammonium, nitrate and nitrite concentrations. Out of the considered isolates GS 5 strain was found to be the most effective exhibiting excellent nitrification and denitrification activities. The colonies in agar plate were pale yellow, circular shaped with semitransparent, wet and smooth-surfaces. By using sequencs of PCR amplicon of 1356 bp16S rDNA gene homology comparision was done with the NCBI genbank database through BLAST analysis. The BLAST results indicated that the strain GS 5 was closely related to *Bacillus cereus* strain JC175 (Accession no. KJ534423.1) showing 99% 16S rRNA gene sequence similarity. The phylogenetic tree (Fig. 2) was constructed using neighbor-joining method with a boot strap value of 1000 replications to explore the evolutionary history of the isolated microbe. Evolutionary analyses were conducted in MEGA5 tool. Table 1 shows sequences producing significant alignment with that of the test micro organism. Nucleotide sequence of the isolates was

submitted to GenBank nucleotide sequence databases and the GenBank accession number for the same is KM212993.

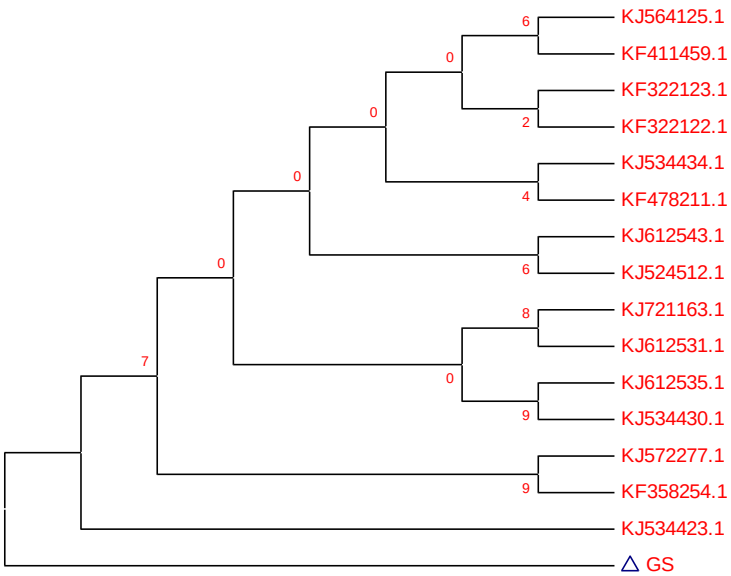

Figure 2. Phylogenetic tree based on comparison of partial 16S rRNA gene sequence using MEGA 5.0.

Table 1. Sequence Producing Significant Alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KJ721163.1	Bacillus cereus strain DB13136	2494	2494	100%	0.0	99%
KJ612543.1	Bacillus cereus strain EM17	2494	2494	100%	0.0	99%
KJ612535.1	Bacillus cereus strain EM9	2494	2494	100%	0.0	99%
KJ612531.1	Bacillus cereus strain EM4	2494	2494	100%	0.0	99%
KJ564125.1	Bacillus cereus strain LD22	2494	2494	100%	0.0	99%
KJ572277.1	Bacillus cereus strain L-3	2494	2494	100%	0.0	99%
KJ524512.1	Bacillus subtilis strain BF14	2494	2494	100%	0.0	99%
KJ534434.1	Bacillus cereus strain LD147	2494	2494	100%	0.0	99%
KJ534430.1	Bacillus cereus strain JD15	2494	2494	100%	0.0	99%
KJ534423.1	Bacillus cereus strain JC175	2494	2494	100%	0.0	99%
KF411459.1	Bacillus cereus strain LP-10	2494	2494	100%	0.0	99%
KF478211.1	Bacillus cereus strain PGSI2	2494	2494	100%	0.0	99%
KF358254.1	Bacillus cereus strain L10	2494	2494	100%	0.0	99%
KF322123.1	Bacillus cereus strain NBAII B5	2494	2494	100%	0.0	99%
KF322122.1	Bacillus cereus strain NBAII B4	2494	2494	100%	0.0	99%

B. Growth and COD removal

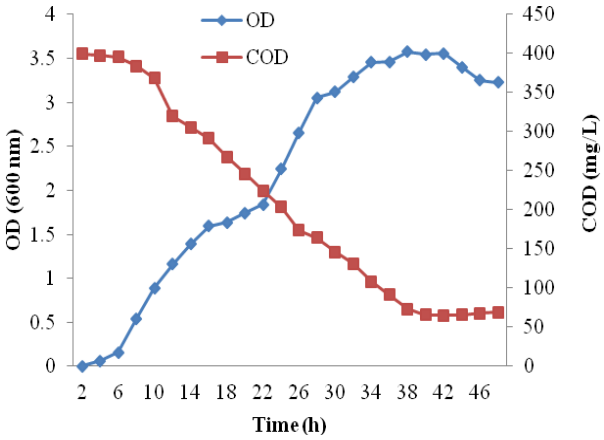

Figure 3. Growth profile and COD utilization of Bacillus cereus GS5

The growth and COD utilization characteristics of *Bacillus cereus* GS5 was investigated in shaking cultures, as shown in Fig. 3. The growth curve shows that, the lag phase lasted for approximately 6h following which isolate GS5 grew exponentially for 34h. Highest biomass production was observed around 40h following which the microorganism entered into the stationary phase of growth and finally declination in growth starts from 42h onwards. But in our subsequent experiments, we have taken into account 24h incubation time. The COD utilization result demonstrated that during the growth period, maximum COD utilization rate was found to be 83.56%, starting from 400 mg/L. Simultaneous COD utilization and growth of the microbe indicated about the heterotrophic nature of the microbe. Similar kind of the results were obtained by Kundu et al. [11], who observed 95.5% COD removal after 48h incubation time by R31 strain. In the following section nitrification ability of the microbe will be discussed. In this section heterotrophic nature has already been discussed. So combining both the section, heterotrophic nitrification ability of the isolate can be justified.

C. Evaluation of heterotrophic nitrification

Fig. 3 shows the profile of ammonium nitrogen utilization by GS5 strain with relation to time. The shake culture experiment was performed by taking ammonium broth medium, containing glucose as sole carbon source and maintaining COD/NH_4^+-N approximately 3.5. After an incubation time of 24h 92.02% of ammonium was consumed starting from 50 mg/L, with simultaneous utilization of COD, thereby demonstrating the heterotrophic nitrification ability of GS-5. It was observed that the initial ammonium utilization rate was slow and rapid utilization started only after onset of exponential growth phase. Maximum utilization was attained within 18 h, after which the utilization declined. The subsequent formation of nitrite and nitrate also demonstrated in the figure. Maximum nitrite accumulation corresponds to approximately 41% ammonium removal where as for nitrate

maximum accumulation corresponds to approximately 63% of ammonium utilization. The corresponding nitrite released during ammonia degradation progressively decreased and became almost undetectable after 20h. Similarly accumulated nitrate concentration decreased after reaching a peak value. This observable fact advocates that both nitrification and denitrification were occurring simultaneously and consistent with the previous findings [11, 12]. In order to confirm the denitrification potential of the isolate, further experiments were done considering $NaNO_3$ and $NaNO_2$ as sole nitrogen source in aerobic condition and the same has been explained in the following section.

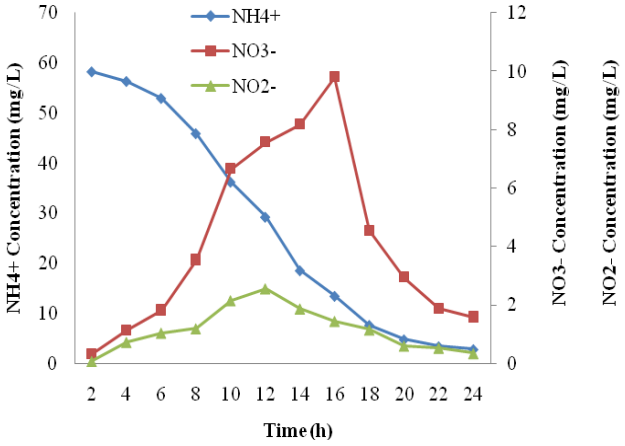

Figure 4. Nitrification ability of strain GS5 taking NH_4Cl as sole nitrogen source

D. *Evaluation of aerobic denitrification*

The aerobic denitrification potential of the strain GS-5, was evaluated under shake culture condition in nitrate and nitrite broth where DO was maintained at 2.75 mg/L. In the respective broth $NaNO_3$ and $NaNO_2$ were used as sole nitrogen source and glucose as sole carbon source in the basal medium.

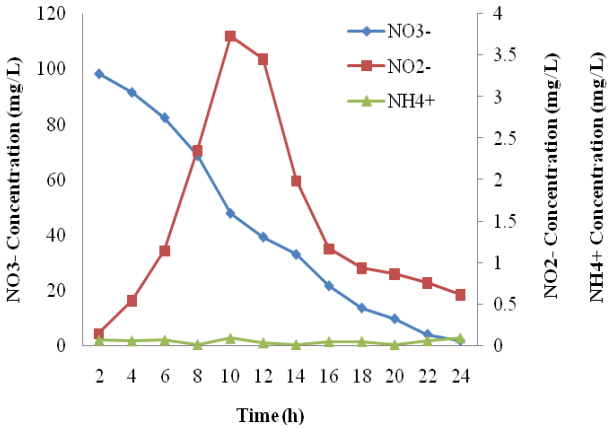

Figure 5. Denitrification ability of strain GS5 taking $NaNO_3$ as sole nitrogen source

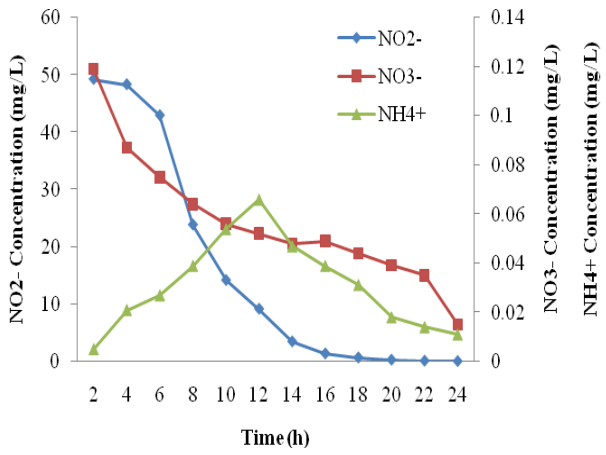

Figure 6. Denitrification ability of strain GS5 taking $NaNO_2$ as sole nitrogen source

Fig. 5 shows the nitrate reduction ability of stain GS5 under an aerobic condition where as Fig. 6 represents the nitrite reduction ability of the isolate. As per the Fig. 5, a noteworthy decrease of nitrate concentration was observed in 20h of incubation. Approximately 98% of the nitrate was removed in 24h. Significant accumulation of nitrite started from 6h with a maximum of 3.728 mg /L at 10h. However, the nitrite concentrations started decreasing after 12h and reduced to 0.613 mg/L after 24h. the results demonstrated that when $NaNO_3$ was used as the sole nitrogen source to the basal medium it was reduced via NO_2^-.

From Fig. 6 it is clear that nitrite reduction took place considerably after the onset of lag phase i.e., 8h of growth period. After 18h the concentration of nitrite was almost negligible (0.762 mg/L) and the final concentration after 24 h was reported to be 0.133 mg/L. Throughout the experimental period intermediate concentration was found to be negligible. It was observed that using sodium nitrite as the sole nitrogen source resulted in its removal without formation of nitrate ion.

The inference from the above two paragraphs imply that the isolated strain GS5 could utilize nitrite and nitrate induced by ammonia and denitrification occurs aerobically via NO_2^- pathway. Aerobic dentrifiers utilize NO_2^- by intracellular assimilation or by extracellular reduction pathways [12]. The current study contemplated that the denitrification of both nitrate and nitrite ion could occur aerobically and consistent with the findings of co-respiration of O_2, NO_3^- and NO_2^-[12, 13].

This study verified that ammonium, nitrate and nitrite could all be used by the strain GS5 under aerobic condition. This may be attributed to the presence of nitrate reductase, nitrite reductase (nir) and hydroxyl amine oxidase in cell periplasm [12]. The transformation of NO_2^- to NO or N_2O chiefly regulated by expression of nir enzyme [14]. Similarly, the presence of two key enzymes such as hydroxyl amine oxidase (hao) and nitrate reductase (nar) can be confirmed by PCR amplification of hao and nar genes [12], thereby providing the added substantiation of heterotrophic nitrification and aerobic denitrification by *Bacillus cereus* GS5 strain.

IV. Conclusions

In this manuscript a bacterium isolated from a mature bio-film of a lab scale denitrifying bioreactor was identified as *Bacillus cereus* GS5 by the help of physiochemical features and phylogenetic analysis. The bacterium was capable of utilizing ammonia, nitrate and nitrite used as sole nitrogen sources in basal medium in presence of glucose as the sole external carbon sources. The simulataneous stabilization of COD and ammonium nitrogen confirms heterotrophic nature of the isolate. Taking initial concentration of ammonium as 50 mg/L, 92.02% was removed after 24h incubation with little or no accumulation of the intermediates such as hydroxylamine, nitrite and nitrate. Similarly, from initial nitrate concentration of 100 mg/L, 98.26% was removed in a 24h period under aerobic conditions, with reduced level of nitrite accumulation (0.613 mg/L). But using sodium nitrite as the sole nitrogen source resulted in its removal (from initial concentration of 50 mg/L to final concentration of 0.133 mg/L within 24h) without formation of nitrate under aerobic condition. Thus, inferred that the isolated strain GS5 could utilize both nitrite and nitrate as sole nitrogen source and denitrification occurs aerobically via NO_2^- pathway. The results illustrated that the optimum aerobic denitrification conditions for strain GS5 were, a C/N ratio of 3.5, incubation temperature of 35 °C, an initial pH of 7, and rotation speed of 125 rpm (dissolved oxygen 2.75-3 mg/L). Therefore, it is suggested study that the new isolate *Bacillus cereus* GS5 strain, with aerobic heterotrophic nitrification and aerobic denitrification ability, can be used in full scale treatment systems for facilitating simultaneous nitrification and denitrification (SND) in order to remove nitrogen from contaminated water.

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In this work a novel microbe was isolated from a lab scale denitrifying bio-reactor. The microbe was identified to be *Bacillus cereus* GS5 from phylogenetic analysis. The microbe exhibited the phenomenon of heterotrophic nitrification and aerobic denitrification. This feature of the microbe can be explored extensively and the microbe can be used in wastewater treatment systems meant for nitrogen removal from contaminated water. In simultaneous nitrification and denitrification (SND) approach nitrification and denitrification could occur concurrently in one reactor under identical operating conditions, thereby avoiding the complexity of conventional biological nitrogen removal approach. The simultaneous utilization of organic substrates, degradation of ammonium, utilization of nitrate and nitrite as sole nitrogen sources and the tolerance to oxygen are some of the unique features of the microbe.