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Chapter 19

NITRATE REMOVAL FROM SYNTHETIC HIGH NITRATE WASTE BY A DENITRIFYING BACTERIUM

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ABSTRACT

The work aims towards isolating organisms capable of treating high nitrate wastewater and optimizing the process for maximum denitrification rate. A denitrifying bacterium strain, isolated from the wastewater of a fertilizer denitrification plant (FDP), was screened from a total of 160 isolated cultures based on its high nitrate removal efficiency. Biochemical tests and 16S rDNA sequence analysis showed the bacterium genus to be *Pseudomonas* and close to *aeruginosa* species. The culture on acclimatization to high strength nitrate waste [10000 ppm NO_3 (2258 ppm NO_3-N)] in a sequence batch reactor, showed complete degradation in a time period of just 1.75 h. The specific nitrate and nitrite degradation rate of the process using the acclimatized culture was further increased by 54.4 % and 15 % respectively on optimizing the process using orthogonal array method. The applicability of this isolate for high rate denitrification process was investigated in a 4 L reactor and the two important enzymes involved in the first two steps of denitrification process, NaR and NiR were assayed. This provided an *invitro* index of the ability of the cells to reduce nitrate and nitrite. The reactor was run successfully for 2 months without any change in the activity.

Keywords**:** Denitrification; Sequence Batch Reactor; Orthogonal array; NaR; NiR

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1.   INTRODUCTION

The high nitrogen level in wastewater has become a growing concern, which has increased the necessity to develop efficient N-removal techniques. High nitrate wastes (> 1000 ppm NO_3-N) are usually generated by fertilizer, metal finishing and nuclear industry (Glass and Silverstein, 1998; Glass and Silverstein, 1999) whose treatment has become a challenge for these industries. When such wastewaters are released into water streams, they cause various ill effects like methehemoglobinemia in infants and is also suspected to cause cancer (Forman et al., 1985). In USA the permissible level for nitrate in drinking water is 10 ppm NO_3-N (USEPA, 1987). Biological denitrification which is the reduction of oxidized nitrogen compounds like nitrate or nitrite to gaseous nitrogen compounds is the most important and widely used method to treat nitrate wastes as it enables the transformation of nitrogen compounds into harmless nitrogen gas. This process is performed by various chemorganotrophic, lithoautotrophic and phototrophic bacteria and some fungi (Shoun and Tanimoto, 1991; Zumft, 1997) especially under oxygen reduced or anoxic conditions (Focht and Chang, 1975). Denitrifiers can be naturally found in sediments, surface waters, soils, as well as in municipal and industrial wastes. The universality and common distribution of denitrifiers is related to their species complexity and different physiological requirements.

Focusing on biological nitrate removal, sequence batch reactor (SBR) is described as a very effective alternative to conventional activated sludge systems. SBR technology is based on the assumption that the microorganisms are exposed periodically to defined process conditions and this is effectively achieved in a fed batch system in which exposure time, frequency of exposure and amplitude of the respective concentration can be set independently of any inflow condition (Wilderer et al., 2001). From the process-engineering point of view, the composition and the metabolic properties of the microbial composition in the SBR system comes to a steady state by the enforcement of controlled short-term unsteady state conditions.

Biological denitrification of wastewater is usually a slow process and lasts several days. Efforts have been made to increase the nitrate removal rate. Adapted bacterial cultures from various industrial wastewater treatment plants have been proved to be very useful and observation suggests that cells need to be adapted to nitrate for full denitrifying ability (Hiraishi et al., 1995). Along with adaptation of the cultures, optimization of pH, temperature and carbon: nitrogen may also enable high rate of denitrification (Glass and Silvertsein, 1998; Nair et al., 2007).

Thus, if optimal conditions for denitrification processes can be defined, isolated cultures may be employed for N-waste removal in controlled conditions.

So far studies on denitrification of high nitrate waste using activated sludge (Glass and Silvertsein, 1998; Foglar et al., 2005; Dhamole et al., 2007; Nair et al., 2007) have been carried out, however not many studies have been carried out on treatment of high nitrate wastes (>7500 ppm NO_3) using pure cultures. The aim of this study was to determine the denitrification activity of a bacterium RS152, isolated from a FDP wastewater and its efficiency to denitrify high nitrate waste (10000 ppm NO_3). The identification of the strain was carried out using the ID 32 GN system (BioMerieux) and further confirmed using the 16S rDNA sequencing method. The studies were extended further to optimize the denitrification process for maximum denitrification rate using the orthogonal array method and by testing the isolate in bench scale experiments to establish optimal operational conditions for the treatment of wastewater. Simultaneously, the activity of the two important enzymes involved in the first two steps of denitrification process, nitrate reductase (NaR) and nitrite reductase (NiR) were also evaluated.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals were purchased from M/S Hi-Media Limited, Mumbai, India and were of the highest purity available.

2.2 Isolation of denitrifiers

The arbitrarily named strain RS152 was isolated from the wastewater sample collected from a fertilizer industry. Soil and sludge samples were enriched in succinate nitrate medium (Disodium succinate hexahydrate 11 g; $NaNO_3$ 100 g; peptone 4 g in 1000 ml of deionized water, pH 7.2). The medium composition was modified by increasing the nitrate concentration, as our aim was to find an isolate capable of denitrifying high nitrate waste. Sludge sample (one ml) was inoculated in the media and incubated for 1 week under anaerobic conditions at $30^{o}C$ (Gamble et al., 1997). Loopful from the above enriched media was then directly inoculated into high concentration nitrate broth (100 g $NaNO_3$ l^{-1}) incubated for 1 week under anaerobic conditions at $30^{o}C$ and the tubes that showed growth were plated onto the same nitrate concentration agar plates. They were incubated anaerobically in anaerobic jars containing gas paks at $30^{o}C$ for 48 hr. Colonies of bacteria showing different morphological features were picked and isolated in pure culture.

2.3 Screening of denitrifiers

Different screening criteria's were used to categorize the isolated cultures and obtain the desired strain. The initial categorization was based on the study of their cultural characteristics. Cultures with similar cultural characteristics were grouped together and only one of the lot was selected. These cultures were then screened using the second categorization studies, which was based on their natural capabilities (without adaptation) to degrade nitrate. For degradation studies synthetic waste was used which had the following composition, Na_2HPO_4 7 g l^{-1}, K_2HPO_4 1.5 g l^{-1}, $MgSO_4$ 0.1 g l^{-1}, NaCl 0.3g l^{-1} and trace element solution 2ml l^{-1}. Trace element solution consisted of $CaCl_2$ 5.54 g l^{-1}, $FeSO_4$ $7H_2O$ 5.0 g l^{-1}, $MnCl_2$. H_2O 5.06 1 g l^{-1}, $ZnSO_4$ $7H_2O$ 2.2 g l^{-1}, $CuSO_4$ $5H_2O$ 1.51 g l^{-1}, $CoCl_2$ H_2O 1.61 g l^{-1}, EDTA 50 g l^{-1}, $(NH_4)_6MO_7O_{24}$ H_2O 1.1 g l^{-1} (Dhamole et al., 2007). Sodium acetate was used as a carbon source and sodium nitrate was used as nitrogen source. For screening out isolates with natural degradation capacity a nitrate concentration of 112 ppm NO_3-N (500 ppm NO_3) was used. The isolates which showed complete degradation at a lower time period were selected and were further screened. In this study the nitrate as well as the nitrite and ammonia levels were estimated. This screening was important, as a true denitrifier is the one, which doesn't accumulate nitrite or ammonia (Tiedge, 1994). The isolates which did not show any accumulation of NO_2 and NH_3 were screened out and were compared amongst each other to pick out the best desired isolate. This study was carried out in 250 ml flasks with 150 ml synthetic waste. The nitrate source used was sodium nitrate (1129 ppm NO_3-N) and carbon source was sodium acetate. 3 gL^{-1} MLSS culture was inoculated into the flasks and incubated at 30^oC under anaerobic conditions. Samples were withdrawn at regular time intervals from each of the flasks and the nitrate, nitrite and ammonia content was estimated.

2.4 Identification of isolated bacterium

Identification of the denitrifying bacteria was performed according to the ID 32 GN system (BioMerieux) for identification of nonfermenting rods. Identification was further confirmed using 16S rDNA sequencing of PCR products of extracted genomic DNA (Oyaizu, 1992). The bacterial DNA was amplified by polymerase chain reaction using thermal cycler (Eppendorf., Hamburg, Germany). The 50 μl reaction mixture contained the following compounds: 2 μl DNA template, 30 pmol each of the primers 5'-AAG GAG GTG ATC CAG CCG CA- 3', and 5'-AGA GTT TGA TCC TGG CTC AG- 3', 3 μl dNTP mix (10 mM), 4 μl of 10x PCR buffer (100 mM Tris (pH 9.0), 15 mM $MgCl_2$, 500 mM KCl), 1U of Taq polymerase. The amplification program of the PCR consisted of the following steps: denaturing for 1 min at 94^oC, annealing for 1 min at 56^oC and elongation

for 2 min at 72°C, followed by a final synthesis step of 5 min at 72°C. The PCR products (10μl) were analyzed on 1.5 % agarose gels and visualized by UV excitation after staining with ethidium bromide (0.5 mgL^{-1}). The PCR products were subsequently purified using Genei Quick PCR Purification kit. Nucleotide sequences were obtained by a sequencer (ABI PRISM model 377, Applied Biosystems, Foster city, CA). The partial 16S rDNA sequences (500- 800 bp) were compared with known sequences of 16S rDNA in the National Center for Biotechnology Information (NCBI) Genbank by using the Basic Local Alignment Search Tool (BLAST) algorithm (Aenson et al., 1999). The partial sequence of the 16S rRNA gene of the strain RS152 (822bp) was aligned with the sequences of other organisms described in the Fig.3, using CLUSTAL W (Thompson et al., 1994). Using the Kimura 2-parameter model developed by Kimura (1980), the pair wise evolutionary distances were calculated. The phylogenic tree was constructed using a tree-making algorithm (Neighbour Joining). This was carried out using the TREECON 3.1 program (Van de Peer and De Wachter, 1994). All the materials required for above studies were obtained from Bangalore Genei, Bangalore, India.

2.5   Acclimatization of isolate to high strength nitrate waste

To acclimatize the isolate RS152 to high strength nitrate concentration by a stepwise acclimatization procedure to increasing nitrate concentration was carried out in a SBR (Dhamole et al., 2007). A SBR was fabricated in the laboratory from borosil glass material having a total working volume of 1 L capacity. The reactor was operated in sequencing batch mode at a temperature of 37°C ± 2°C under anoxic conditions. Anoxic conditions were maintained by purging nitrogen gas from one of the pipes into the medium for 5 min and sealing the mouth of the reactor with parafilm. The total cycle period of 24 h consisting of 1.5 h of withdrawal and centrifugation phase, 0.5 h of filling phase and 22 h of reaction phase was employed throughout. All of these processes were carried out under aseptic conditions. The medium was under circulation using a magnetic stirrer. Reaction was initially started by inoculating 3 gL^{-1} MLSS of pure culture (obtained by growing in enrichment media) into 1 L synthetic nitrate waste (1125 mgL^{-1} NO$_3$- N). The C: N mole ratio was 2:1. After 24 h of reaction period, 500 ml of the media was withdrawn and centrifuged. The biomass was inoculated back into the reactor and 500 ml of sterile synthetic nitrate waste was filled into the reactor to make the total volume of 1 L. Nitrate, nitrite and ammonia of the decanted fluid were analyzed everyday. The isolate biomass was acclimatized to each nitrate concentration (224 ppm NO$_3$-N, 1128 ppm NO$_3$-N and 2258 ppm NO$_3$-N) for 15 days each.
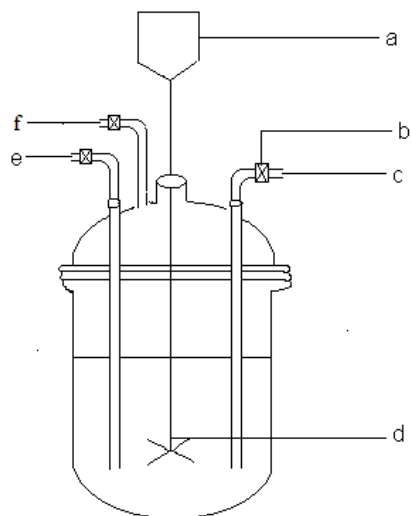
Fig. 1 A   Fig.1 B

Figure 1. 4 L stirred tank reactor. A: Schematic representation of the reactor a. Overhead agitator. b. Valve. c. Inlet for purging sterile N_2 gas. d. Impellar. e. Sampling port. f. vent to remove air during purging of N_2 gas. B: Photograph of the working bioreactor. Denitrification of 1129 ppm NO_3-N synthetic nitrate waste by the isolate was carried out for a period of 1 month.

2.6   Optimization of denitrification process

Using "MINITAB 13.30" software, the L25 (5^3) orthogonal array design was developed and analyzed. The design of L25 (5^3) orthogonal array and the different parameters (pH, temperature and carbon: nitrogen) that were optimized in the present study has been shown in Table 1. A 4 L reactor with 2 L working volume at a concentration of 1129 ppm NO_3-N and an acclimatized biomass concentration of 3 gL^{-1} MLSS was used to carry out all the 25 runs. The reactor was run under aseptic conditions and anoxic conditions were provided by purging sterile nitrogen gas from one of the vents for 5 min and sealing the mouth of the reactor with parafilm. The medium was kept under constant agitation using a stirrer at 140 rpm.

2.7   Application of the isolate in a bioreactor

A stirred tank reactor of 4 L capacity was fabricated in the laboratory from Borosil glass material. A schematic representation of the SBR is given in the Fig.1. The acclimatized biomass (3 gL^{-1} MLSS) was inoculated in the reactor. A headspace of 2 L was provided to prevent any solid loss generally caused because of foaming. An overhead stirrer with four-blade glass propeller was used for mixing. The stirrer speed was kept high (200 $\pm$ 50 rpm) enough to maintain a

Table 1. L25 (5^3) orthogonal array design for denitrification process using isolate RS152

(A) Temperature	(B) Carbon: Nitrogen	(C) pH	Total degradation time (min)
1	1	1	95
1	2	2	85
1	3	3	75
1	4	4	80
1	5	5	85
2	1	2	90
2	2	3	70
2	3	4	80
2	4	5	85
2	5	1	85
3	1	3	90
3	2	4	75
3	3	5	80
3	4	1	85
3	5	2	70
4	1	4	95
4	2	5	85
4	3	1	90
4	4	2	75
4	5	3	70
5	1	5	90
5	2	1	85
5	3	2	75
5	4	3	75
5	5	4	90

Level	(A) Temperature	(B) Carbon: Nitrogen	(C) pH
1	Room Temperature	1.90	6.5
2	32ºC	2	7.5
3	37ºC	2.25	8.5
4	42ºC	2.5	9.5
5	47ºC	2.75	10.5

homogenous suspension of biomass. Denitrification studies were carried out according to optimized conditions studied above. The reactor was operated as a batch system under anaerobic conditions. Samples were collected at regular time intervals using sterile syringe. These samples were analyzed for nitrate, nitrite, NaR and NiR enzyme.

2.8   Analytical methods

The nitrate and nitrite concentrations in the samples were analyzed using DIONEX Ion chromatograph fitted with an IC Pak anion column AS11 (2 x 250 mm) column. NaOH (12 mM) was used as eluent. Ammonia was estimated using Nesslers method (APHA, 1997). All the samples were centrifuged and filtered before analysis. Dilutions were carried out using deionized water. The NaR and NiR enzyme was assayed according to Kenji et al. (1981). The protein was determined by the Millers method (Miller, 1959). Mixed liquor suspended solids (MLSS) were determined by following standard methods (APHA, 1997). Anaerobic conditions in liquid medium were maintained by purging the medium with N_2 gas and sealing with an airtight rubber cork. Anaerobic conditions in solid medium were provided by incubating the agar plates in anaerobic jars (BBL Gas Pak systems) with gas paks.

2.9   Nucleotide sequence accession numbers

The 16S rRNA gene sequence from pure culture of RS152 has been deposited in the NCBI nucleotide sequence database under accession number DQ361030.

3.   RESULTS

3.1   Isolation and screening

To isolate denitrifiers, sludge sample from a fertilizer industry and soil samples were enriched in succinate nitrate broth and the enriched samples were inoculated onto high nitrate concentration agar plates and incubated under anaerobic conditions. About 160 isolates capable of growing in high nitrate (2258 ppm NO_3-N) were obtained. To screen out the desired denitrifier from 160 cultures, their cultural characteristics were studied. 95 isolates having different characteristics were selected. These 95 isolates were brought down to 16 isolates, which were capable of degrading nitrate without accumulation of nitrite or ammonia and were thus termed as true denitrifiers (organisms which utilize nitrite further to produce either NO or N_2O or N_2 gas). From a total of 16 isolates, the numbers were brought down to 4 best isolates, which were capable of degrading about 90 % of 10000 ppm nitrate (2258 ppm NO_3-N). Measurements were made during the anaerobic growth on nitrate to compare the denitrification properties of these four cultures. The results (Fig. 2) clearly indicated that the isolate RS152 degraded both nitrate and nitrite effectively as compared to isolates RS16, RS17 and RS18 with only 4 ppm of residual NO_3-N and 23 ppm NO_2-N which eventually degraded in a period of 105 min. There was no accumulation of ammonia in any

of the cultures showing that denitrification was the route taken by all the isolates. For the isolates, RS16, RS17 and RS18 the residual nitrate were 79.3 ppm NO_3-N, 108 ppm NO_3-N and 121 ppm NO_3-N respectively. Thus the isolate RS152, which was capable of denitrifying high nitrate waste completely at a higher rate, was found to be superior to other isolates.
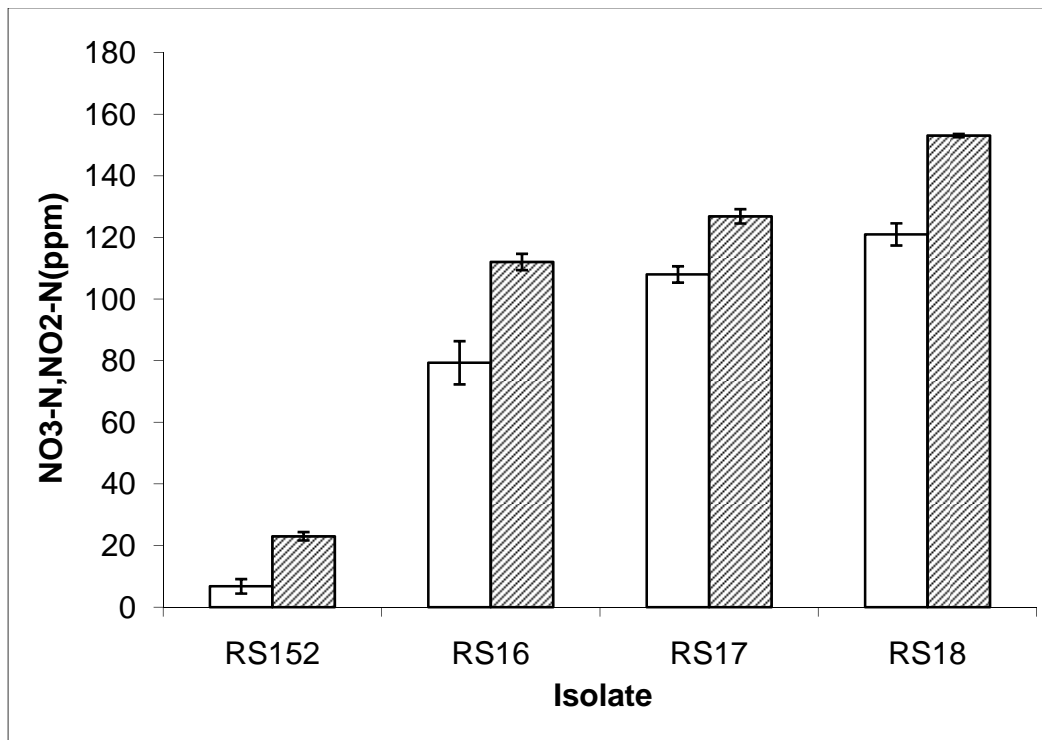
Figure 2. Comparison of the four cultures based on their nitrate degradation capability in 96 h. Medium: Synthetic nitrate waste; Nitrogen: Sodium nitrate (1129 ppm NO_3-N); Carbon: Sodium acetate; C: N: 2:1; Anaerobic condition: Purging of N_2 gas; Working volume: 150 mL; pH: 7; Temperature: 30^oC; Agitation: 100 rpm; MLSS: 3 gL^{-1}. Error bars indicate standard deviation of triplicate samples.

3.2   Identification and Biochemical characterization

The results of identification of the strain using the ID 32 GN system are shown in Table 2. The strain RS152 could grow on substrates like, N- Acetylglusosamine, D- Ribose, Itaconic acid, Sodium malonate, Sodium acetate, Lactic acid, L- Alanine, Potassium 5- Ketogluconate, Glygogen, D- Melibiose, L- Fucose, D- Sorbitol, L- Arabinose, Propionic acid, Trisodium citrate, L- Histidine, Potassium 2- Ketogluconate, 3- Hydroxybutryric acid, 4- Hydroxybutyric acid and L- proline. These results when compared with *Pseudomonas aeruginosa* showed 96.7 % identity. Similarly, comparative 16S rDNA gene sequence analysis

showed affiliation of the isolate RS152 to the genus *Pseudomonas* as seen in Figure 3. The strain RS152 showed 100 % similarity to *Pseudomaonas aeruginosa* strain DQ095913.1. Thus the genotypic studies further confirmed that the strain RS152 belonged to genus *Pseudomonas* and strain *aeruginosa*.

Table 2. Phenotypic characteristics of strain RS152 using the ID 32 GN system

Tests	Substrates	Qty (mg/cupule)	RS152 strain
RHA	L-RHAmnose	0.68	ND
NAG	N-Acetyl-Glucosamine	0.68	+
RIB	D-RIBose	0.70	+
INO	INOsitol	0.70	-
SAC	D-SACcharose	0.66	-
MAL	D-MALtose	0.70	-
ITA	ITAconic acid	0.23	+
SUB	SUBeric acid	0.35	-
MNT	SodiumMaloNaTe	1.20	+
ACE	SodiumACEtate	0.55	+
LAT	LacTic acid	0.32	+
ALA	L-ALAnine	0.68	+
5KG	Potassium 5-LetoGluconate	0.90	+
GLYG	GLYcogen	0.64	+
mOBE	3-hydOxyBEnzoic acid	0.23	-
SER	L-SERine	0.80	-
MAN	D-MANitol	0.68	-
GLU	D-GLUcose	0.78	-
SAL	SALicin	0.52	-
MEL	D-MELibiose	0.66	+
FUC	L-FUCose	0.64	+
SOR	D-SORbitol	0.68	+
ARA	L-ARabinose	0.70	+
PROP	PROPionic acid	0.29	+
CAP	CARic acid	0.11	-
VALT	VALeric acid	0.25	-
CIT	TrisodiumCITrate	0.57	ND
HIS	L-HIStidine	0.80	+
2KG	Potassium 2-Ketogluconate	0.98	+
3OBU	3-hydOXYBEnzoic acid	0.30	+
pOBE	4-hydOXYBEnzoic acid	0.23	+
PRO	L-PROline	0.52	+

3.3   Acclimatization studies

To acclimatize the isolate to high nitrate concentrations, the reactor was initially inoculated with 3 gL^{-1} MLSS .The isolate was acclimatized to increasing concentrations of nitrate (224 ppm NO_3-N, 1129 ppm NO_3-N and 2258 ppm NO_3-N) for 15 days each in a stepwise process. This process has been successfully

carried out in our laboratory using sludge (Dhamole et al., 2007; Nair et al., 2007).
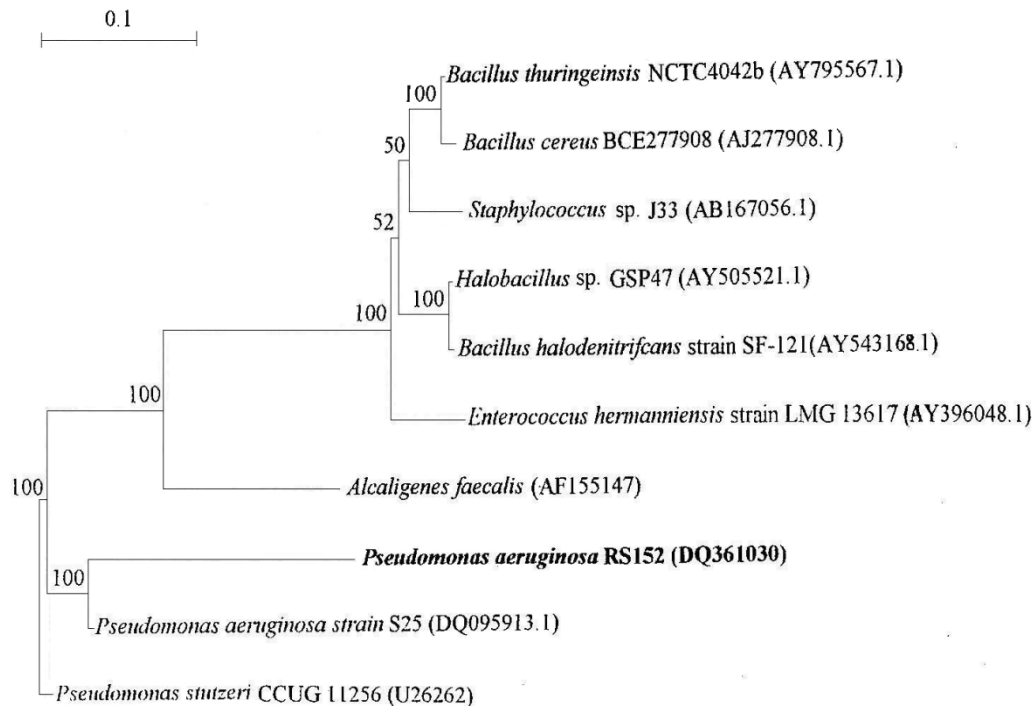
Figure 3. Phylogenic relationships of isolate RS152 and other organisms on the basis of 16S rDNA sequences. Bootstrap values (%) are given at the nodes. Scale bar, 10 inferred nucleotide changes per 100 nucleotides.

In the first stage, the isolate was acclimatized to 224 ppm NO_3-N synthetic nitrate waste for 15 days as described above. The nitrate and the nitrite profile for 224 ppm NO_3-N influent synthetic nitrate waste, which after dilution with the recycled culture and media becomes half, is depicted in Fig. 4. It is seen that 224 ppm NO_3-N synthetic nitrate waste was completely degraded by the isolate in a period of 1 h. Nitrite accumulation was low, because of the low nitrate concentration and high degradation rate of nitrate and nitrite (Dhamole et al., 2007). After a period of 15 days the same biomass was used in the second stage of acclimatization to 1129 ppm NO_3-N synthetic nitrate waste. Here the influent nitrate concentration was increased to 1129 ppm NO_3-N and the acclimatization was carried out in the same manner for another 15 days. Fig. 4 also shows the nitrate and nitrite profile during the degradation of 1129 ppm NO_3-N. Reduction

of entire nitrate was observed in 1.25 h, with a slight increase in build up of nitrite, which eventually got degraded in 1.25 h.

Acclimatization studies were further carried out for 2258 ppm NO_3-N for a period of 15 days using the same biomass. It can be seen from Fig. 4 that the nitrate was degraded completely to nitrite in a period of 1.5 h, with a build up of nitrite, which eventually got degraded in a period of 1.75 h. The reactor was operated for a period of 45 days at different nitrate concentrations and the acclimatized culture was used for optimization and enzymic studies.
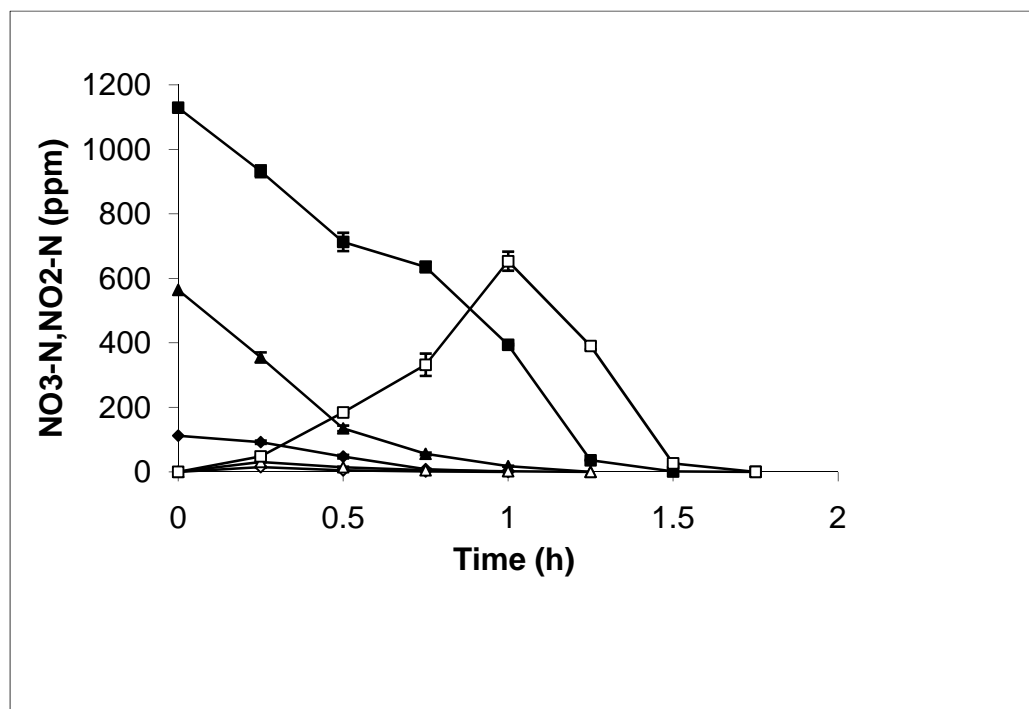

Figure 4. Nitrate and nitrite profile during denitrification of 224 ppm NO_3-N influent synthetic nitrate waste (actual concentration in the reactor = 112 ppm NO_3-N), 1129 ppm NO_3-N influent synthetic nitrate waste (actual concentration in the reactor = 564 ppm NO_3-N) and 2258 ppm NO_3-N influent synthetic nitrate waste (actual concentration in the reactor = 1129 ppm NO_3-N). MLSS for all concentrations = 3 gL^{-1}. Error bars indicate standard deviation of triplicate samples.

3.4   Orthogonal matrix method

The importance of parameters like pH, temperature and carbon to nitrogen ratio was determined using the orthogonal matrix method to get an optimum value for highest denitrification rate in a stirred tank reactor. L25 (5^3) orthogonal array was selected for this system with 3 different parameters. Temperature, Carbon: Nitrogen and pH are the variables optimized in the present study. Table 1

indicates the experimental set of L25 (5^3) orthogonal array for denitrification of synthetic nitrate waste using isolate RS152, along with the different variables and their factors used and the experimental results. These results were analyzed using MINITAB 13.30 software. The response table for means (smaller is better) obtained by analyzing the data for orthogonal array is shown in Table 3. It can be observed that the delta values of pH and carbon: nitrogen is higher than that of temperature. The main effect plots for the system, which shows the optimum levels of each factor obtained by statistical analysis, is shown in Fig. 5. It can also be observed that for each of the three variables at five levels, one level increases the mean compared to the other level. This difference is the main effect, i.e temperature at level 2, carbon: nitrogen at level 2, 3, 4 and 5 and pH at level 2. Thus the temperature at 37°C, carbon: nitrogen of 2:1 and above and a pH of 8.5 were the optimum levels for highest denitrification rate using the isolate strain RS152.

Table 3: Response table for mean

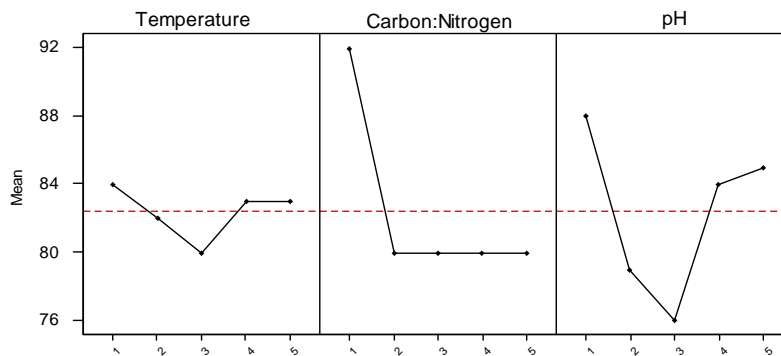| Level	(A) Temperature	(B) Carbon: Nitrogen	(C) pH
1	84	92	88
2	82	80	79
3	80	80	76
4	83	80	84
5	83	80	85
Delta	4	12	12

Main Effects Plot for Means

Figure 5. Main effect plot for denitrification by isolate RS152

3.5 Bioreactor studies

The denitrification activity of the isolate strain RS152 was observed in a stirred tank reactor of 4 L capacity fabricated in our laboratory. The reactor was run using the optimal conditions calculated from the orthogonal method and the specific denitrification rates were analyzed.

Biological denitrification can be depicted as

$$NO_3 \xrightarrow{K_{NO3}} NO_2 \xrightarrow{K_{NO2}} N_2$$

Where, K_{NO3} and K_{NO2} are zero order coefficients. Zero order data fit before and after optimization are shown in Fig. 6 and Fig. 7 respectively. The specific denitrification rate before optimization was found to be $K^1_{NO3} = 263.5$ mg NO_3–N g^{-1} MLSS h^{-1}, $K^1_{NO2} = 157.6$ mg NO_2–N g^{-1}MLSS h^{-1}, while after optimization it was found to be $K^2_{NO3} = 388.2$ mg NO_3–N g^{-1} MLSS h^{-1}, $K^2_{NO2} = 182.2$ mg NO_3–N g^{-1} MLSS h^{-1}. Specific rate of nitrate reduction was found to increase by 54.4 % on optimization, while the specific rate of nitrite reduction was found to increase by 15 % after optimization, thereby confirming the findings.
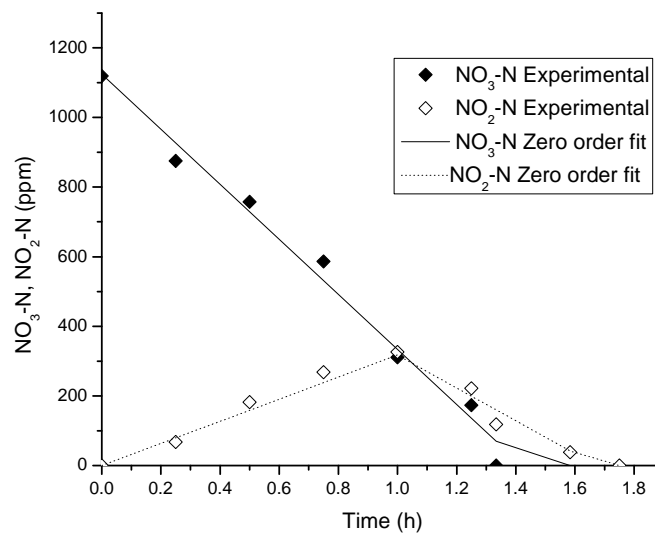

Figure 6. Experimental and zero order data fit for 1129 ppm NO_3-N synthetic nitrate waste before optimization. Medium: Synthetic nitrate waste; Nitrogen source: Sodium nitrate (1129 ppm NO_3-N), Carbon source: Sodium acetate; C/N: 2:1, pH: 7; Temperature: 30°C; Working volume: 2 L; Agitation: 140 ± 50 rpm; K^1NO_3= 263.5 mg NO_3–N g^{-1} MLSS h^{-1}; K^1_{NO2}= 157.6 mg NO_3–N g^{-1} MLSS h^{-1}; MLSS= 3 gL^{-1}.
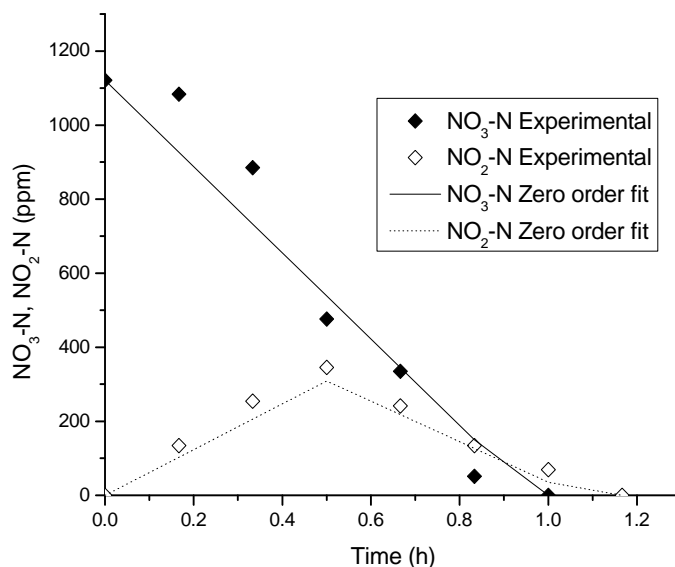
Figure 7. Experimental and zero order data fit for 1129 ppm NO_3-N synthetic nitrate waste after optimization. Medium: Synthetic nitrate waste; Nitrogen source: Sodium nitrate (1129 ppm NO_3-N), Carbon source: Sodium acetate; C/N: 2:1, pH: 8.5; Temperature: 37°C; Working volume: 2 L; Agitation: 140 ± 50 rpm; K^2NO_3= 388.2 mg NO_3–N g^{-1} MLSS h^{-1}; K^2NO_2= 182.2 mg NO_2–N g^{-1} MLSS h^{-1}; MLSS= 3 gL^{-1}.

3.6   Enzyme studies

In vitro enzymatic activity of the isolate was carried out to understand the microbial metabolism physiology of high nitrate waste. Samples were assayed for NaR and NiR enzymes during the denitrification process of 1129 ppm NO_3-N in the stirred tank reactor. The enzyme assays show that the isolate strain RS152 contain activities of the denitrifying enzymes nitrate reductase and nitrite reductase, both of which are induced on cultivation with nitrate. On comparison with the nitrate and nitrite profiles (Fig. 7), the NaR and NiR specific activity also shows similar trend. As seen in Fig. 8, the NaR specific activity (1601 micromoles h^{-1} mg^{-1} protein) was found to be the maximum at a time period of 0.5 h, which eventually decreases, with the degradation of nitrate. Similarly the NiR specific activity (937 micromoles h^{-1} mg^{-1} protein) was found to be maximum at a time period of 0.6 h, which also reduces, with degradation of nitrite.
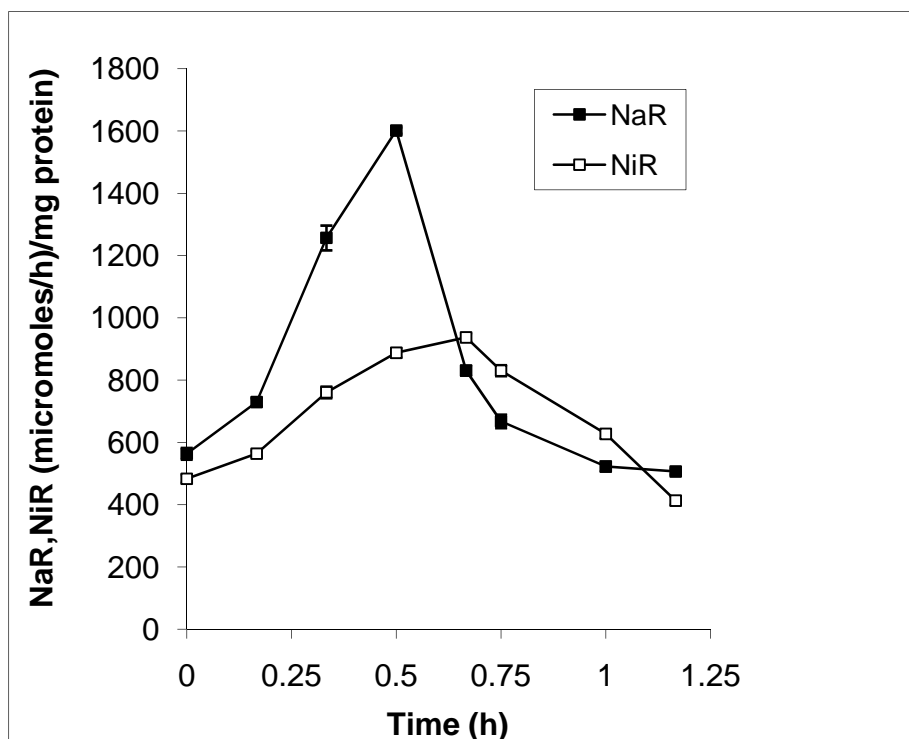
Figure 8. Nitrate reductase (NaR) and nitrite reductase (NiR) profile during the denitrification of 1129 ppm NO_3-N synthetic nitrate waste. Medium: Synthetic nitrate waste; Nitrogen source: Sodium nitrate (1129 ppm NO_3-N); Carbon source: Sodium acetate; Carbon: Nitrogen: 2:1, pH: 8.5; Temperature: 37°C; Working volume: 2 L; Agitation: 140 ± 50 rpm; MLSS: 3 gL^{-1}.

4.   DISCUSSION

Denitrification of high nitrate waste is still a challenge for industries producing nitrate wastes as high as 1000 ppm NO_3-N. Though treatments of such wastes have so far been reported using microbial consortia (Glass and Silverstein 1999; Foglar et al., 2005), very few studies have been carried out on nitrate waste treatment using an isolate, especially wastes containing high nitrate concentrations. In this study, to isolate a culture capable of denitrifying high nitrate, extensive screening of 160 cultures isolated from different habitats was carried out. Four denitrifiers capable of degrading about 90 % of 2258 ppm NO_3-N without any accumulation of NO_2 and NH_3 were isolated and compared amongst themselves. The results clearly indicated that the isolate RS152 degraded nitrate completely and more effectively as compared to isolates RS16, RS17 and RS18. The complete removal of nitrate as high as 2258 ppm NO_3 in a time period of just 105 min shows that this strain could play a substantial role in nitrogen

removal in sludge systems, and therefore was considered for further studies. This study showed that the isolated culture had the potential to be utilized in high rate denitrification processes which generally takes hours to degrade even low nitrate levels (< 100 ppm NO_3-N). Though denitrification using microbial consortia is a much known process as compared to isolate, these studies are important as (i) it gives a clear picture of the mechanism of the denitrification process which is obscure when a consortia is used. (ii) Accumulation of biomass which is a common problem faced during reactor studies using consortia can be avoided as isolate biomass can be used in low concentrations as compared to consortia.

Identification studies of the isolate RS152 using both biochemical characterization and 16S rRNA gene sequence analysis, indicated that the strain RS152 is closely related to *Pseudomonas aeruginosa* strain. Though biochemical studies have been proved to be successful in identifying the strains to the species level, using 16S rRNA gene sequence is a classic approach to detect and identify isolates accurately. The phylogenetic tree indicated that the strain RS152 forms a closest clade with *Pseudomonas aeruginosa* strain S25 with bootstrap resampling values of 100 %. The ID 32 GN system further confirmed the results by showing 96.7 % identity to *Pseudomonas aeruginosa*. On the basis of combined phenotypic and genotypic data, the strain RS152 belongs to the genus *Pseudomonas aeruginosa* which is also the most frequently isolated denitrifier from natural ecosystems (Gamble et al., 1977).

One of the methods used to biologically treat high nitrate wastes is by sequential adaptation to nitrate waste (Dhamole et al., 2007). Periodic exposure of microorganisms to defined process condition is found to be effective and this has developed into the SBR technology (Wilderer et al., 2001). Similar strategy was used to, wherein the isolate RS152 was acclimatized to high strength nitrate waste (224 ppm NO_3-N, 1129 ppm NO_3-N and 2258 ppm NO_3-N) in a stepwise manner for 15 days each in a SBR. The nitrate wastes were completely degraded without any accumulation of nitrite. The inoculum level remaining the same throughout the period of 45 days, during which the reactor was run, showed that the reactor was stable and efficiently denitrifying high nitrate wastes. This acclimatized culture was used for further studies on optimization of the denitrification process.

The denitrification rate, which is usually slow in any system, can be enhanced by optimizing various environmental conditions in which organisms degrade the nitrate. If optimal conditions for these processes can be defined, this isolate can be employed for high N waste removal in controlled conditions. Parameters like temperature, C:N, pH etc are some of the factors which contribute significantly to the denitrifying efficiency of the system. The Orthogonal array method, which has been used in various fermentation processes (Xu et al., 2003) to improve media for primary and secondary metabolite production, has been applied in wastewater

treatment systems to optimize conditions to get highest possible degradation rate (Nair et al., 2007). In the present study it was observed that the delta values for pH and C:N was the same while for temperature it was low. The magnitude of order of delta values shows the effect of the variables on the response characteristic of interest and the delta values being higher for pH and C:N ratio as compared to temperature, the most important factors controlling the denitrification activity of the isolate was pH at 8.5 and C:N of 2:1.

Using the optimum levels (temperature at 37^oC, carbon: nitrogen of 2:1 and a pH of 8.5), the specific denitrification rates were compared before and after the optimization process. For biological denitrification, zero order kinetics has been reported with respect to nitrate and nitrite (Moore and Schroeder 1971; Glass and Silverstein 1999; Foglar et al., 2005). The denitrification rate of the isolate RS152 was maximized by determining the most suitable denitrifying conditions. Also the specific denitrification rates of the isolate RS152 on comparison with that of a microbial consortium used in our previous studies (Dhamole et al., 2007; Nair et al., 2007), showed that the isolate showed a much higher denitrifying activity than microbial consortium thereby proving to be better. The specific denitrification rates for the isolate after optimization was found to be $K^2_{NO3} = 388.2$ mg NO_3–N g^{-1} MLSS h^{-1}, $K^2_{NO2} = 182.2$ mg NO_2–N g^{-1}MLSS h^{-1}, while the specific denitrification rates for microbial consortium after optimization was found to be $K^2_{NO3} = 32.2$ mg NO_3–N g^{-1} MLSS h^{-1}, $K^2_{NO2} = 16.3$ mg NO_2–N g^{-1} MLSS h^{-1}.

Many studies have been carried out in understanding the physiology of microbial metabolism of inorganic nitrogen compounds. Estimation of nitrate incorporation rates using stable isotope ^{15}N as a tracer are used for carrying out metabolic studies (Dugdale and Wilkerson 1986). But these studies often have problems with the loss of ^{15}N label. Berges and Harrison (1995) have shown that the measurement of enzyme activities is a good index of a biological rate. During enzymological studies of organisms, variability in enzyme immunochemical cross reactivity, efficiency and regulation within functional groups are often studied to a great extent (Korner 1993; Ka et al., 1997). Such results suggest that the regulation of ecosystem processes that are mediated by microbes may infact be affected by, and reflect, the community composition of functional groups. In the above studies it can be observed that (Fig. 8) the NaR and NiR specific activity show a similar profile as the nitrate and nitrite profiles (Fig. 7). The NaR activity was also found to be more (1601 micromoles/ h/ mg protein) as compared to NiR activity (937 micromoles/ h/ mg protein). This explains the rate of nitrate degradation to be faster as compared to nitrite reduction. The quantitative determination of the two enzymes nitrate reductase (NaR) and nitrite reductase (NiR), therefore gives an insight in understanding the metabolic activity during

the whole process of denitrification by the isolate which was not complete with the nitrate and nitrite degradation profiles.

5.   CONCLUSIONS

Denitrification of high strength nitrate wastes by pure cultures is usually slow and lasts even several days. The isolate RS152 was found to be highly efficient in degrading nitrate wastes as high as 10000 ppm NO_3 (2258 ppm NO_3-N) in a time period of 1.75 h only, which to the best of authors knowledge is not reported for any isolate so far. Also comparative studies on the specific denitrification rates of the isolate RS152 and microbial consortium used in our previous studies as well as in other literatures showed that the isolate showed higher denitrifying activity thereby proving to be better (Glass and Silverstein 1999; Foglar et al., 2005; Dhamole et al., 2007; Nair et al., 2007). It can thus be applied for an efficient and economical high rate denitrification processes.

6.   APPENDIX

SBR = Sequence batch reactor
NaR = Nitrate reductase
NiR = Nitrite reductase
K^1_{NO3} = Specific rate of nitrate reduction before optimization (mg NO_3–N g^{-1} MLSS h^{-1})
K^1_{NO2} = Specific rate of nitrite reduction before optimization (mg NO_2–N g^{-1} MLSS h^{-1})
K^2_{NO3} = Specific rate of nitrate reduction after optimization (mg NO_3–N g^{-1} MLSS h^{-1})
K^2_{NO2} = Specific rate of nitrite reduction after optimization (mg NO_2–N g^{-1} MLSS h^{-1})

7.   REFERENCES

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