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Biological nitrogen removal from wastewater by *Paracoccus denitrificans* ISTOD1: optimization of process parameters using response surface methodology

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ABSTRACT

In freshwater ecosystems, the vital biogenic constituent is nitrogen and the increasing nutrient load discharge from wastewaters has turned into a more grievous issue. A previously demonstrated bacterial strain *Paracoccus denitrificans* ISTOD1 was studied for the bioremoval of nitrogen in the form ammonia-N from synthetic wastewater media. A high concentration of NH_4^+-N of 100 mg/L was used to study the behavioral pattern of the bacterial strain during the utilization process. Pre-optimization experiments with different culture conditions for the strain ISTOD1 exhibited high ammonia removal efficiency of 83.9 %, 75.7% and 76.23% in presence of glucose as C-source, pH 7 and temperature of 30°C respectively. Optimization of process parameters for enhancing the heterotrophic nitrification and denitrification process in synthetic wastewater done using the Response Surface Methodology (RSM) showed 98.2% and 91.8% reduction in the concentration of ammonia and nitrate, respectively as compared to the pre-optimized conditions. Nitrogen balance analysis revealed that approximately 67 % of NH_4^+-N was removed as gas products and 32.4% was transformed into biomass. This study helps in developing a biodegradation treatment method under aerobic conditions suitable for purification of ammonia-rich wastewater and eliminating the by-product such as NO_3^-N involved in polluting the receiving waters.

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1. Introduction

One of the major environmental problems presently is the emerging nitrogen pollution caused by the continuous accumulation of nitrogenous compounds in the form of ammonia, nitrite and nitrate nitrogen in the surrounding environment. The increasing civilization and human activities has led to negative consequences leading to excessive amount of organic contaminants and nitrogenous wastewater which includes industrial, domestic, agricultural wastewater etc. being discharged into the surrounding fresh water bodies [Gao at al., 2014; Wang et al., 2016; Gupta and Thakur, 2015]. The presence of uncontrolled discharges of wastewater containing high concentration of aqueous ammonia leads oxygen depletion, increase occurrence of pathogenic microorganisms, is toxic to the fish and nitrate damages their immune system as a result it ultimately affects the human health through bioaccumulation [Huang et al., 2017; Grguric et al., 2000]. Therefore, there is an urgent need for a stringent research so that the nitrogen-containing wastewater could be effectively purified before discharging.

Physical, chemical and biological approaches help in the conventional removal of nitrogen from both wastewater and natural water. However, biological treatment approach has proved to be more advantageous compared to others due to its ease of implementation, being eco as well as pocket friendly. Biological nitrogen removal in wastewater is usually carried out by the nitrification-denitrification processes in a two-stage treatment process. Recently, researchers are focusing on the applicability of a single-stage treatment approach though simultaneous nitrification and denitrification (SND). Microbes that are capable of conducting heterotrophic oxidation of NH_4^+-N and reducing the nitrification products, NO_2^- and NO_3^- completely under aerobic conditions are gaining attention and have been reported for high ammonia removal efficiency such as *Paracoccus denitrificans* ISTOD1 [Medhi et al., 2017], *Diaphorobacter* sp. [Ge et al., 2015], *Agrobacterium* sp. LAD9 [Chen and Ni, 2012], *Acinetobacter* sp. HA2 [Yao et al. 2013], *Vibrio diabolicus* SF16 [Duan et al., 2015], *Bacillus sp. YX-6* [Song et al., 2011].

The major factors affecting the heterotrophic nitrification–aerobic denitrification process are carbon source, C:N ratio, temperature, initial pH, shaking speed and dissolved oxygen (DO) concentration. Previous studies have shown that different species of bacteria have their own distinct response to these parameters [Chen and Ni, 2012; Niel et al., 1992]. However, if the influence strength of these factors and their interactions on the heterotrophic nitrification–aerobic denitrification process are systematically evaluated, it will help in better understanding of the mechanism. Therefore, to evaluate valuable parameters which affect a process, Response surface methodology (RSM), a combination of mathematical and statistical methods for the design of experiments assists with the main objective of optimizing the process. It is widely used as an effective statistical method in various fields [Asadi and Zilouei, 2017].

This present study investigates a novel bacterium *Paracoccus denitrificans* ISTOD1 isolated from the wastewater samples of a municipal sewage treatment plant showing characteristic nitrogen removal pathway. The objective of the study was to optimize the physico-chemical conditions required to remove ammonia and nitrate nitrogen from synthetic

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wastewater. In previous studies, people have paid more attention to the conditions, like temperature, pH, C:N ratio but reports on duration are limited. Herein, the factors affecting the performance of strain ISTOD1 in estimating the maximum nutrient degradation were comprehensively evaluated based on the RSM analysis, and the possible nitrogen removal pathway was explored through the nitrogen mass balance analysis.

2. Material and methods

2.1. Microorganism and culture condition

A previously reported potent bacterial strain *Paracoccus denitrificans* ISTOD1 (gene bank accession number – KX417305) isolated from influent waste water samples collected from Okhla Sewage Treatment Plant (OK STP) located at JasolaVihar, New Delhi, India (28° 32'52.9069° N and 77°16'36.7867°E) was used for the study of heterotrophic nitrification and aerobic denitrification [Medhi et al., 2017]. The bacterial strain was grown in Luria Bertani (LB) medium composed of (g/L): tryptone, 10; yeast extract, 5; NaCl, 10 for 24 h, was harvested by centrifugation at 8000 rpm for 10 min and washed twice with sterile distilled water. 2% of the cultured pellet was resuspended in 50 ml of synthetic basal medium (SBM) for inoculation. The composition of SBM (g/L) was as follows: $(NH_4)_2SO_4$; glucose; NaCl, 4; Na2HPO4.12H2O; 21.5, of KH2PO4, 0.9 and 3% v/v trace elements solution. The trace elements solution was prepared as accordingly described in Medhi et al., [2017]. The prepared media had a pH of 7.3. The concentrations of $(NH_4)_2SO_4$ as the sole nitrogen source and glucose as carbon source were adjusted maintaining at a C:N ratio (w/w) of 10, incubated under aerobic conditions at 30 °C and 150 rpm.

2.2. Selection of conditions for pre-optimization

2.2.1. Selection of ammonium concentration

The influence of the ammonium concentration on the nitrification and denitrification capacity of *P. denitrificans* ISTOD1 was assessed in SBM. Initial NH_4^+-N concentrations were adjusted to 50, 100 and 350 mg/L NH_4^+-N using $(NH_4)_2SO_4$. During incubation, the cultures were sampled periodically to determine optical density (OD_{600}) and the levels of NH_4^+-N at regular intervals for 48 h. The percent removal of ammonium determined the best concentration to be taken for further analyses. The culture conditions were as described above. All the tests were conducted in triplicates.

2.2.2. Selection of carbon source

Strain ISTOD1 was cultured in SBM described above with the addition of the following carbon compounds: glucose (2% w/v), sucrose (2% w/v) and molasses (2% v/v) as carbon sources. Medium was supplemented with filter sterilized glucose and sucrose. 10% molasses as a final concentration from 5% liquor (stock) was prepared by diluting with distilled water and autoclaved. The solution was then left overnight for settling and 2% clarified molasses was used. The amount of each carbon source was adjusted to give a C:N ratio of 10 with a constant concentration of NH_4^+-N. 2% of the bacterial culture from LB medium was inoculated in SBM and cultured at 30°C, 150 rpm. Aliquots were collected periodically for chemical analysis and cell growth. All the tests were performed in triplicates.

2.2.3. Estimation of pH and temperature range

The strain ISTOD1 was inoculated into the LB medium and cultured at 30°C for 16-18 h until the value of OD_{600} reached 1.0. The culture was centrifuged at 8000 rpm at 10 min and the washed pellet (1% w/v) was then resuspended in 50 mL of SBM as inoculum containing 100 mg/L of NH_4^+-N. The cultures were incubated at pH of 4, 7 and 10 whereas temperature ranges were 15°C, 30°C and 40°C for 48 h. The samples were collected at a 4 h interval to detect the cell growth and the remaining ammonium concentration for evaluating the ammonium removal efficiency at both the parameters. All the tests were conducted in triplicates.

2.3. Analytical methods

The growth of the isolate was measured by Varian Carry 100 Bio spectrophotometer at a wavelength of 600 nm. Ammonia was determined by the Phenate method at a wavelength of 640 nm and nitrite was determined by N-(1-naphthyl)-ethylene diamine photometry method at a wavelength of 543 nm, as per standard methods given in APHA [2012]. Nitrate was measured by Phenol Disulphonic Acid (PDA) method at a wavelength of 410 nm [CPCB, 2010]. TN amount was calculated by adding concentrations of ammonia-N, nitrate-N and nitrite-N. Intracellular nitrogen content was calculated by subtracting the TN of inoculated medium following centrifugation (4°C, 15 min, 3600g) from the TN of non-centrifuged medium [Yang et al., 2011].Percent reduction was calculated by the formula $(C_i-C_f)/C_i * 100$ where, C_i is the initial concentration and C_f is the final concentration. All the statistical analysis in this work was analyzed by Microsoft excel and Sigma plot SPSS11.0 software.

2.4. Box-behnken design for optimization of the environmental factors influencing nitrification and denitrification process

Response Surface Methodology (RSM) was used to investigate the effects of initial C:N ratio, pH and duration on the activity of heterotrophic nitrification–aerobic denitrification by the strain *P.denitrificans* ISTOD1. 2% of the bacterial culture from LB medium was inoculated in 100 ml of SBM in 250 ml flasks and incubated at 30°C, 150 rpm. The amount of carbon source was changed to adjust the C:N ratios in the SBM by maintaining a constant ammonia nitrogen concentration at 104.34 mg/L.

2.4.1. Experimental design

The levels of the three independent variables (C:N ratio, pH, duration) were defined according to Box– Behnken design (BBD). 17 experiments were required to assess the effects of the three independent variables for the procedure (Table 1), each at three different concentration levels of low (-1), medium (0) and high (+1) on two responses, ammonia removal (mg/L) and nitrate removal (mg/L) for simultaneous nitrification and denitrification. The experimental design matrix derived from the Box–Behnken model with the coded levels (minimum, medium and maximum) and actual values of the three variables chosen for these 17 different sets of experiment [Gupta and Thakur, 2016]. A genuine replicate was done to estimate the experimental error. The statistical experimental designs and graphical analysis were performed with the help of Design Expert 11 software (Stat-Ease Inc., Minneapolis, USA).

2.5. Nitrogen balance analysis

For Nitrogen testing, the optimized conditions (pH, C:N ratio, duration) obtained in the RSM analysis were used for the experiment. The nitrogen balance and removal efficiency for heterotrophic nitrification and aerobic denitrification was observed in 250 ml flasks. Strain ISTOD1 was inoculated in 100 ml of SBM having 100 mg/L NH_4^+-N as fixed amount of initial nitrogen concentration. The flask was incubated at 30°C, 150 rpm. All tests were carried out in triplicates. Aliquots of 2 ml were collected periodically for the detection of ammonia nitrogen, nitrite, nitrate and intracellular N**.**

3. Results and discussion

3.1. Strain selection

P.denitrificans ISTOD1 colony morphology after incubating for 24 h at 30°C on LB plates were small circular shaped, opaque with smooth edges, creamy white color. Gram staining confirmed it as a gram-negative strain. The described potent heterotrophic nitrification- aerobic denitrification strain ISTOD1 was amplified and sequenced using 16S rDNA gene and then was submitted to GenBank database with an accession number – KX417305, an indigenous bacterium isolated from the influent wastewater samples of OK STP, was used for the optimization studies for nitrogen removal. *Paracoccus denitrificans* has been previously studied as a model organism for the study of denitrification [Baker et al., 1998] after its pioneered discovery of aerobic denitrification ability to perform single stage nitrogen removal process [Robertson and Kuenen, 1984]. This denitrifier strain has been well reported for nitrification-denitrification process aerobically as well as anaerobically [Medhi et al., 2017]. Keeping in view of the above literatures, *P. denitrificans* ISTOD1, being a potent bacterial strain was selected for study of bioremoval of nutrient (nitrogen) present in wastewater.

3.2. Pre-optimization conditions

3.2.1. Influence of ammonium concentration

The ability of heterotrophic organisms to oxidise ammonia as a sole nitrogen source has been generally linked to aerobic denitrification. *P.denitrificans* ISTOD1 demonstrated an ability to remove ammonium at a concentration range of 100 to 350 mg/L [Medhi et al., 2017]. In this study, 50 mg/L NH_4^+-N was also included to observe the ammonium removal efficiency at lower concentration range. Ammonium removal efficiency was observed at all the three (48.7 ± 0.5, 104.80 ± 1.67, 351.68 ± 4.69 mg/L NH_4^+-N) different concentrations. The strain exhibited the maximum removal efficiencies of 80%, 86% and 76% at the end of 48 h under the three concentrations respectively as depicted in

Table 1: Experimental runs suggested by BBD model for optimization of 3 factors for minimum generation 2 responses

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A: C:N ratio	B: Duration	C: pH	Conc. of ammonia	Conc. of nitrate
			h		mg/L	mg/L
3	1	10	168	7	40.82	1.02
17	2	55	96	7	0.4	1.47
10	3	55	168	4	29.71	16.7
1	4	10	24	7	4.5	2.31
6	5	100	96	4	28.61	12.11
14	6	55	96	7	0.42	1.45
16	7	55	96	7	0.42	1.46
9	8	55	24	4	15.83	10.03
15	9	55	96	7	0.46	1.42
12	10	55	168	10	13.57	10.52
11	11	55	24	10	15.94	10.03
5	12	10	96	4	30.94	5.12
2	13	100	24	7	15.37	16.83
7	14	10	96	10	32.63	3.74
13	15	55	96	7	0.4	1.45
8	16	100	96	10	33.21	8.37
4	17	100	168	7	0.62	3.84

Fig.1a. Among the three concentrations, the heterotrophic nitrification was demonstrated best using 100 mg/L and therefore, this concentration was fixed as nitrogen source in further analysis as well as for optimization analysis. Previous studies have indicated that several strains were capable of removing a single concentration of ammonia. Ammonia removal efficiency of 75% and 80% by *Paracoccus versutus* LYM and *Serratia marcescens* W5 was observed using 100 mg/L NH_4^+-N [Shi et al., 2013; Wang et al., 2016]. As comparison to other studies the strain ISTOD1 appears to be a better strain of ammonium oxidation.

3.2.2. Influence of carbon sources

Carbon sources considerably affect the growth and rate of hetrotrophic nitrification and aerobic denitrification. Experiments were conducted to determine the effect of glucose, sucrose and molasses as sole carbon sources on growth and NH_4^+-N removal efficiency by the strain ISTOD1 as shown in Fig. 1b. Glucose and sucrose accelerated the growth of the bacterial strain and thus OD_{600} reached the peaks at 24 h, slowly rising till 48 h until it came to a stationary phase after 72 h. In presence of molasses, there was a steady growth but at 24 h the OD_{600} reached 0.72 as compared to glucose and sucrose where the OD_{600} reached 1.88 and 1.63 respectively. In correlation with the growth, ammonia was also reduced simultaneously. Accordingly, the NH_4^+-N removal efficiency within 24 was 50.4%, 31.8% and 44.1% whereas within 48 h 83.9%, 47.5% and 78.4% was respectively in presence of glucose, sucrose and molasses. As the results indicated, the strain ISTOD1 tends to choose glucose followed by molasses and sucrose. Glucose was also used for reporting aerobic nitrification- denitrification by *P. retgerri* YL [Zhao et al., 2010]. In our study, the possible reason of preferring glucose by strain ISTOD1 might be explained that glucose being a monosaccharide (simple molecular structure) can be easily utilized by the bacteria and the reducibility of glucose stimulate the heterotrophic nitrification-aerobic denitrification process. Unlike from strain ISTOD1, heterotrophic nitrogen removal strains like *A. faecalis* NR showed a NH_4^+-N removal efficiency of 98.9% with citrate followed by 94.9% with glucose as the carbon source and *Bacillus cereus* GS-5 showed 94.4% NH_4^+-N removal efficiency with acetate and 64% with glucose as the carbon source [Zhao et al., 2017; Rout et al., 2017]. It was also reported that glucose requires enzymatic conversion before entering into metabolism, unlike citrate or acetate directly enters metabolism without any modifications [Guo et al., 2016]. These results imply that the use of organic carbon significantly affects the growth and substrate removal efficiency and the type of carbon source and bacteria both influences the bacterial performance.

3.2.3 Effect of pH and temperature in ammonium removal

The influence of the other two experimental parameters, pH and temperature are depicted in Fig. 1c. The growth as well as nutrient removal ability of the strain ISTOD1 at various pH (4, 7, 10) and temperature (15°C, 30°C, 45°C) were investigated. Growth was observed in the entire pH range with maximum growth obtained at pH 7 followed by pH 10 and then pH 4. At pH 7, the cell growth value with OD_{600} was found to be 0.8 at 24 h steadily increases till OD_{600} of 1.16 after 48 h of incubation, whereas in pH 10 the growth was slow and at 48 h OD_{600} reached 0.26 from 0.06. In the case of pH 4, the growth was minimal showing OD_{600} of 0.15 which indicates that acidic conditions inhibited the bacterial proliferation. The nutrient removal efficiency of the strain followed exactly the same trend exhibiting 75.7%, 37% and 17.8% at pH 7, 10 and 4 respectively. Previous studies have reported maximum nutrient removal in neutral or slightly alkaline condition since it usually facilitates the heterotrophic nitrification process by making more free ammonia available in the medium and moreover, acidic or alkaline conditions are harmful to the microbes [Huang et al., 2017; Rout et al., 2017]. Maximum heterotrophic nitrogen removal was also shown by *P. retgerri* YL as well as *P.stutzeri* T1 after 96 h incubation at pH 7 [Zhao et al., 2010; Guo et al., 2013]. Taking into account the results, pH 7 was fixed as the optimum parameter strain ISTOD1 and was selected for further optimization experiments.

Fig. 1d shows the effect of temperature on the growth adaptability and NH_4^+-N removal efficiency of strain ISTOD1. The selected bacterial strain was able to grow and remove ammonium successfully at a broad range of temperature. It was also observed in this study that the microbial growth was also affected by the temperature. Maximum cell density at 24 h reached an OD_{600} of 0.039 at 15°C, 0.464 at 30°C and 0.048 at 45°C while at 48 h reached an OD_{600} of 0.06 at 15°C, 1.30 at 30°C and 0.089 at 45°C. Even after that, a steady growth was obtained from strain ISTOD1 till 120 h showing adaptability to 15°C and 45°C (data not shown). Along with cell growth the NH_4^+-N removal efficiency also correlated well with temperature. The NH_4^+-N removal percentage increased from 5.46% at 15°C to 76.23% at 30°C after 48 h of nutrient removal. Further increasing in temperature to 45°C resulted in a remarkable decrease in the ammonium removal efficiency showing removal percentage of only 9.31%. Most of the studies were comparable to the results obtained in the present study and have reported strains having an optimum temperature of 30°C- 37°C, such as *Bacillus* N13 at 30°C [Huang et al., 2017], *Pseudomonas fluorescens* wsw-1001 at 30°C [Zhang et al., 2015], *Bacillus cereus* GS-5 at 35°C [Rout et al., 2017] and strain ISTOD1 from this

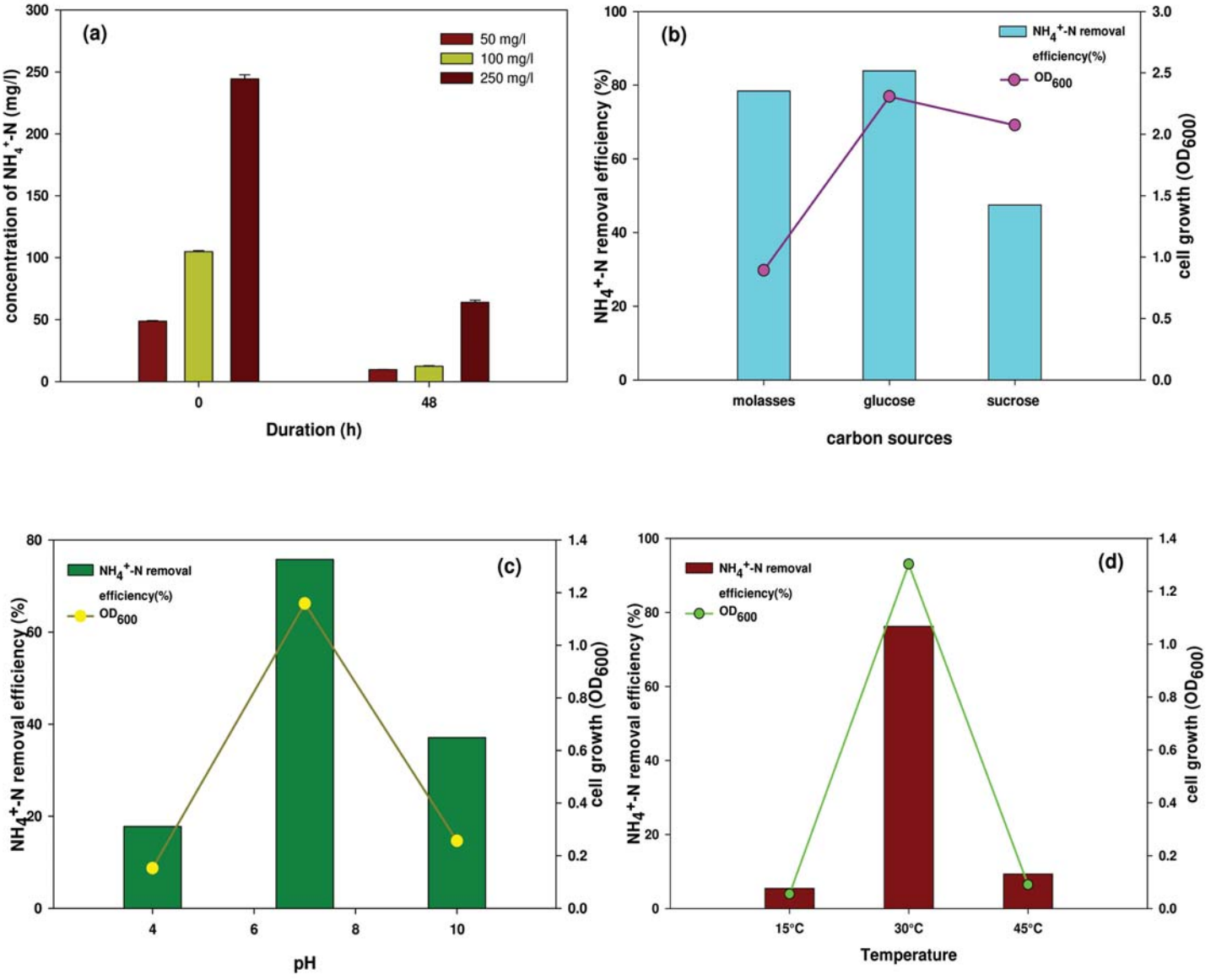

Fig. 1. Pre-optimized conditions for ammonia-N removal by *P.denitrificans* ISTOD1 under (a) initial ammonium concentration (b) presence of carbon sources (c) pH (d) temperature

study. Current studies have described that heterotrophic nitrification and aerobic denitrification are mainly carried out in mesophilic conditions [Wang et al., 2016] and moreover nitrification process is temperature sensitive likely due to the alterations in enzyme activity of ammonia monooxygenase which is responsible for heterotrophic nitrification [Huang et al., 2017]. In natural water environments, the temperature is rarely higher than 30°C and therefore it was set up as the optimum range for further research.

3.3. Process optimization by RSM

Response surface methodology (RSM) was used to enhance the treatment performance for nitrogen removal (ammonia and nitrate) by *P.denitrificans* ISTOD1. The factors C:N ratio, pH and duration were optimized and the quadratic response matrix were created using Box-Behnken design (BBD), which is one of the designs in RSM (Table 1). The relationship between these three factors and two responses (concentration of ammonia (mg/L) and concentration of nitrate (mg/L)) were derived with the help of quadratic equations. The regression equation coefficients were evaluated and the data fitted to a second-order polynomial equation for ammonia and nitrate removal of the SBM culture supernatant. The quadratic equations derived from BBD were used to describe the relationship between the two responses in terms of coded factors and nitrogen concentrations as suggested by software were as follows:

$$\text{Conc. Of Ammonia(mg/L)} = +0.4200 - 3.89^{*} A + 4.14^{*} B - 1.22^{*} C - 12.77^{*} AB + 0.7275^{*} AC - 4.06^{*} BC + 13.75^{*} A^{2} + 1.16^{*} B^{2} + 17.18^{*} C^{2} \quad (1)$$

$$\text{Conc. Of Nitrate (mg/L)} = +1.45 + 3.62^{*} A - 0.8900^{*} B - 1.41^{*} C - 2.92^{*} AB - 0.5900^{*} AC - 1.55^{*} BC + 0.0325^{*} A^{2} + 4.52^{*} B^{2} + 5.85^{*} C^{2} \quad (2)$$

Where, A = C:N ratio, B = Duration and C = pH

Analysis of variance (ANOVA) was used to define the adequacy of the model. The following values were obtained for the two responses viz., concentration of ammonia and nitrate, respectively: Model F-value (14.52 and 5.25), ''R-Squared'' (0.9492 and 0.8710) and CV % (31.75 and 46.79). The values indicated that the quadratic model was reproducible and in agreement with the experimental values (Fig. 2). The p-values are less than 0.05 for both the responses which establishes the significance of the model.

3.3.1. Statistical analysis of factors on responses

The comparison of the three factors in the coded equations (1 and 2)

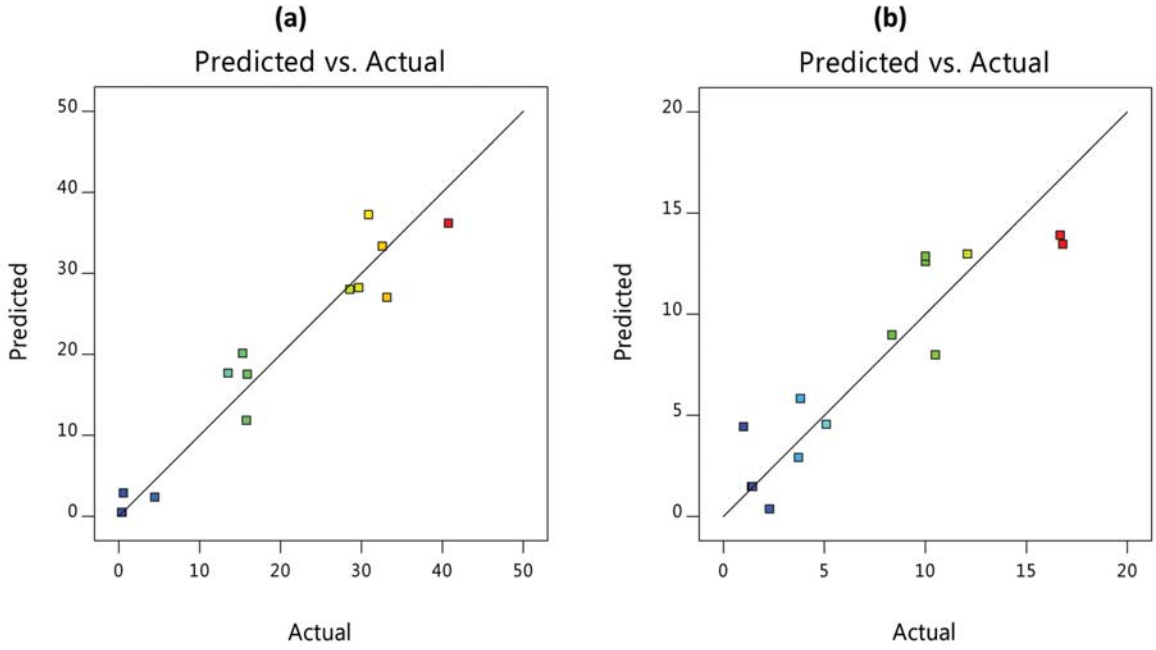

Fig.2. Graphical representation of the actual value obtained from experiments as compared to those predicted by the BBD model for responses: (a) concentration of ammonia and (b) concentration of nitrate

indicates that C:N ratio and pH were negatively correlated while duration showed positive correlation to the concentration of ammonia. In case of concentration of nitrate, C:N ratio was positively correlated while duration and pH were negatively correlated. A higher C:N ratio promotes growth of heterotrophic nitrifying bacteria resulting into higher assimilation of ammonia and its conversion to nitrate which ultimately gets removed by denitrifcation process from the culture medium.

The effect of interactions of the three factors on the two responses was graphically represented through three-dimensional (3-D) plots as suggested by the model. In case of ammonia the graphs revealed a marked influence of low and high values of all the three factors where the positive correlation of pH and C:N ratio with concentration of ammonia was represented by a hyperbolic sheet on the 3-D plot (Fig. 3c) exhibiting a depression towards the mid value of both the factors. The influence of duration with C:N ratio and pH was represented by a parabolic curve (Fig. 3a and 3e) where ammonium concentration varied linearly with duration in both cases, having much higher values towards extreme ends (-1 and +1) when interacting with C:N ratio as compared to its interaction with pH. In case of response 2, the curves depict a parabolic influence of pH and duration whereas a linear influence of C:N ratio. Therefore, the combined effect of pH and duration on the concentration of nitrate was represented by a similar hyperbolic sheet as in Fig. 3f while the interaction of C:N ratio with pH and duration showed minimum values along the mid-range (Fig. 3b and 3-D). It was observed that neutral range of pH, lower mid range of C:N ratio and mid range of duration resulted in maximum removal of ammonia and nitrate concentrations.

According to previous studies that the nitrogen removal performance of some heterotrophic nitrifying and denitrifying bacteria are hampered in nitrification medium with a low and high C:N ratio as they require optimum concentrations of organic compounds to assimilate ammonia [Joo et al., 2006; Huang et al., 2017; Taylor et al., 2009]. The linearly varying positive effect of duration on concentration of ammonia could possibly be due to the release of ammonium ions into the culture medium as a result of dead and decay of bacterial cells with exhaustion of available nutrients [Medhi et al., 2017]. The negatively varying effect of duration on concentration of nitrate could be due to an efficient aerobic denitrification process. Some studies have reported that the activity of the enzymes involved in nitrification-denitrification process declines at very low and high pH [Guo et al., 2016], which explains the negative effect of pH on both the responses.

3.3.2. Validation results

After analyzing the individual and interactive effects of the three factors, the optimized conditions predicted by the model (desirability value 0.977) were as follows: pH of 6.9, C:N ratio of 31.3 and duration of 53.3 h (Fig. 4). Validation experiments were set in triplicates with the above predicted optimal conditions resulted in 98.2% and 91.8% reduction in the concentration of NH_4^+-N and NO_3^-N, respectively as compared to their values (12.43 and 8.84) under pre-optimized conditions as depicted in Table 2. The results thus establish the efficiency of bacteria in performing heterotrophic nitrification- aerobic denitrification process and applicability

Table 2: Concentration of ammonia (mg/L) and concentration of nitrate (mg/L) before and after optimization of process parameters

Variable	Optimization condition		Conc. of ammonia(mg/L)			Conc. of nitrate (mg/L)		
	Before	After	Optimization			Optimization		
			Before	After		Before	After	
				Predicted	Experimental		Predicted	Experimental
C:N ratio	10	31.3						
Duration (h)	48	53.3	12.43	0.2378	0.2197	8.84	0.7595	0.7251
pH	7.3	6.9						

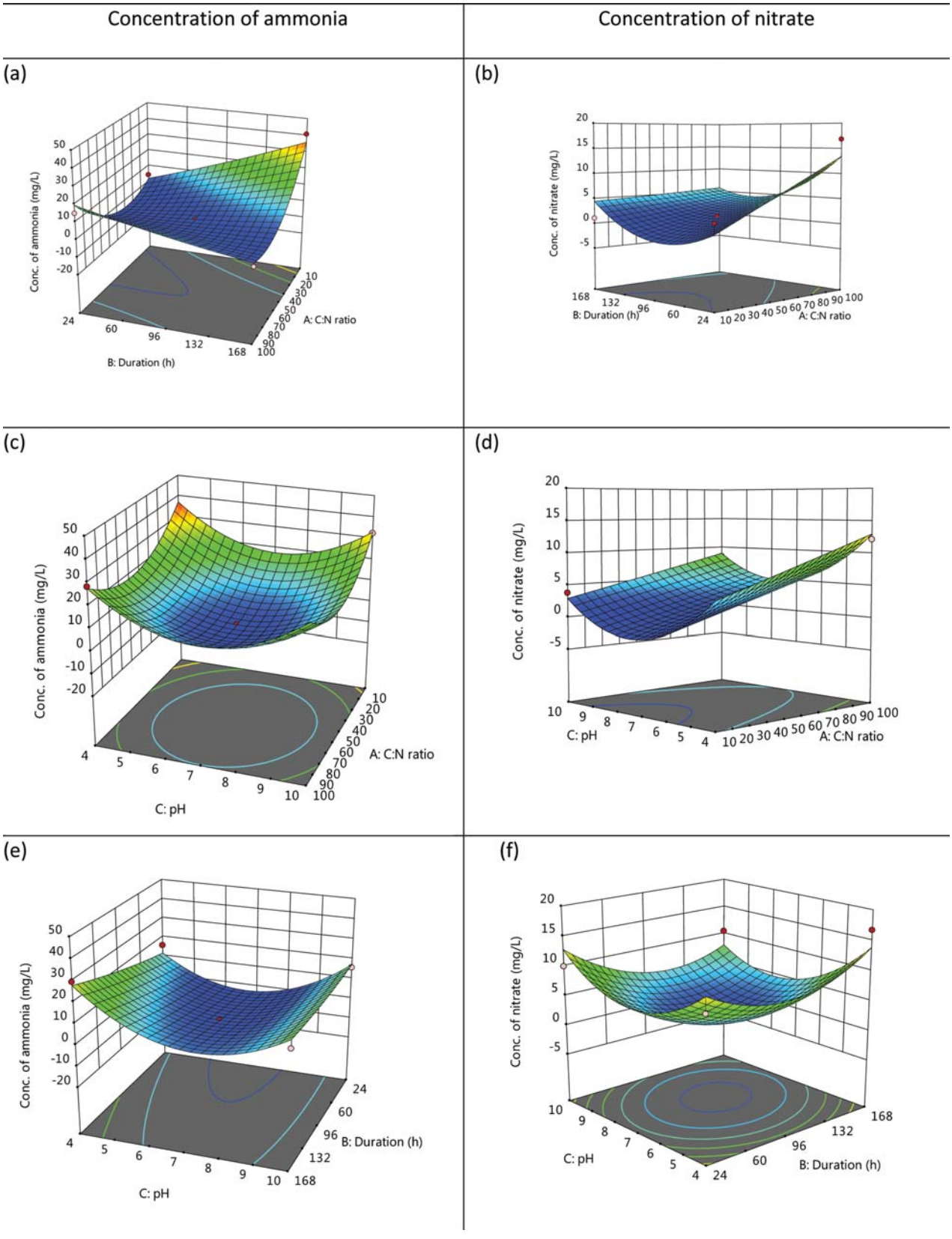

Fig.3. 3-D response surface graphs showing interaction among the three factors on: concentration of ammonia (a, c, e) and concentration of nitrate (b, d, f).

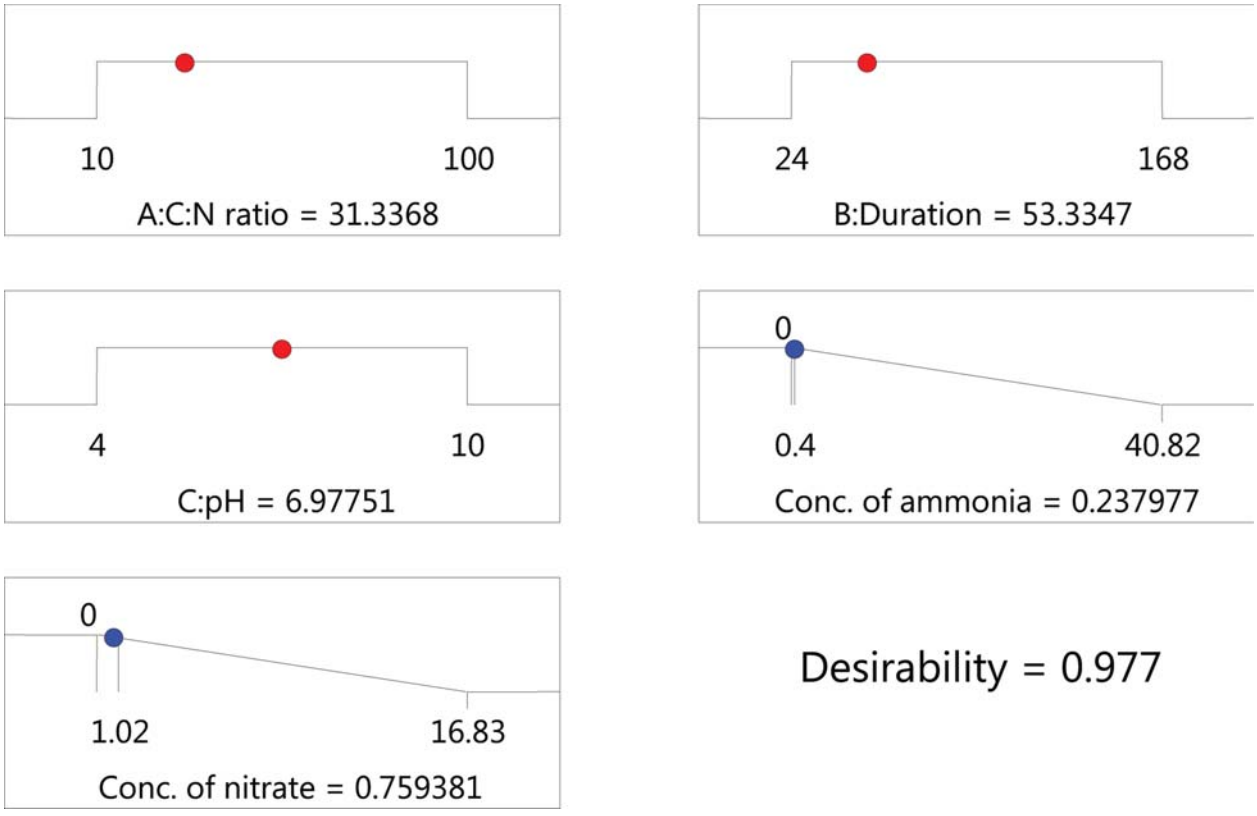

Fig.4. Validation ramps obtained by the BBD model showing the predicted optimized values of factors for minimum response generation.

and efficiency of the model in the process optimization. Chen and Ni [2012] demonstrated the maximal ammonium removal was obtained after analyzing with RSM using the optimal conditions for pH, temperature, C:N ratio and shaking speed. Zheng et al. [2017] also reported maximum ammonia removal rate of 0.59 ± 0.04 mg/L when strain HITLi7^T was cultured in medium with optimized trace elements solution obtained from RSM analysis.

3.4. Nitrogen balance during heterotrophic nitrification–aerobic denitrification process

To study the nitrogen transformation, the balance of total nitrogen in the heterotrophic nitrification–aerobic denitrification process carried out by the strain ISTOD1 was measured and evaluated after the validation experiment was conducted using the optimized parameters obtained by RSM from both the media and the cells. All data were average values in the experimental period. The removal of NH_4^+-N using glucose as carbon source for bacterial growth took place simultaneously, indicating it was a true heterotrophic process. Table 3 depicts the nitrogen balance profile of the degradation of NH_4^+-N taken as the sole N-source and the total nitrogen (TN). The initial NH_4^+-N concentration was 99.31 mg/L. With the decrease of ammonia nitrogen, the accumulations of nitrate occurred but the concentrations were very low, while nitrite was undetectable showing its prominent nitrogen removal ability. At the end of cultivating for 53 h, NH_4^+-N was calculated to 0.28 mg/L showing 99.72% of ammonia being removed accompanied with the trace amount accumulations of nitrate and a portion of NH_4^+-N converted to form intracellular N. After comparing the initial and final TN concentration, 67% of the initial nitrogen was removed from SBM probably dissimilated by strain ISTOD1 to form gas products or gaseous nitrogen. While, on the other hand, 32.4% of nitrogen was assimilated into the biomass in the heterotrophic nitrification–aerobic denitrification process by *P.denitrificans* ISTOD1. Considering the measurement errors caused by different analytic methods, the nitrogen was balanced. Similar studies reported that *S. marcescens* W5 was able to convert 51.06% of NH_4^+-N [Wang et al., 2016], 84.4 % for

Table 3: Nitrogen balance by strain ISTOD1 after utilizing NH_4^+-N as the sole nitrogen source.

Initial	Final amount of N (mg/L)				N lost(mg/L)
NH_4^+-N (mg/L)	NH_4^+-N	NO_2^-N	NO_3^-N	Intracellular	
99.3086 ± 0.114	0.2197 ± 0.30	0.0	0.7251± 0.02	32.1445 ± 1.88	66.3201 ± 0.2

Vibrio sp. Y1-5 [Li et al., 2017], while *Acinetobacter calcoaceticus* HNR is able to convert 40.23% of NH_4^+-N to N_2 [Zhao et al., 2010], about 60% for *Pseudomonas stutzeri* strain T1 [Guo et al., 2013]. Compared to them, strain ISTOD1 also had efficient nitrogen removal abilities.

4. Conclusion

The present study focuses on the bioremoval ability of nitrogen by *P.denitrificans* ISTOD1, isolated from wastewater samples. Almost all NH_4^+-N and NO_3^-N was removed under aerobic condition. The preference of glucose followed by molasses and sucrose exhibited the strain to be a heterotrophic bacterium. The work highlighted the potency of bacterial strain in effectively utilizing ammonia from wastewaters containing a high C:N ratio and also demonstrated the efficiency of BBD model in optimization of process parameters. The nitrogen balance, as a consequence revealed that a part of nitrogen was assimilated into the bacterial biomass whereas another part dissimilated into gaseous products. All results implied that the strain ISTOD1 had a promising prospect in the present day scenario in utilization and removal of nitrogen for enhancing wastewater treatment practices.

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Conflict of intertest

The authors declare that they have no competing interests

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