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Design and optimization of parameters for continuous denitrification of water by methane using *Hyphomicrobium denitrificans* in a packed bed reactor

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ABSTRACT

In the present study, the continuous denitrification of water was carried out using *Hyphomicrobium denitrificans* coupled with methane in a packed bed reactor containing a mineral packing medium. The response surface methodology based on the Box–Behnken design was applied to examine the interaction effects of pH (6–8), temperature (20°C–30°C), and methane/air mixture flow rate (20–60 mL min^{-1}) on the nitrate removal (initial concentration of 100 mg L^{-1}) using the *Hyphomicrobium* coupled with the methane. Analysis of variance results indicated that the methane/air mixture flow rate had a maximum effect on the nitrate removal efficiency in comparison to the pH and temperature variations. The experimental design results indicated that the optimum values of pH 6.9, temperature 30°C, and flow rate 53 mL min^{-1} led to maximize the denitrification capacity of *Hyphomicrobium* in a packed bed reactor. The maximum experimental denitrification capacity was found to be 2.85 mg NO_3–N L^{-1} h^{-1} which was in good agreement with the predicted value by the Box–Behnken analysis (2.81 mg NO_3–N L^{-1} h^{-1}). The effluent nitrate concentration reached less than 50 mg L^{-1} in the nitrate flow rates of 1.4 and 2.5 mL min^{-1}. The obtained results demonstrated the high capability of *Hyphomicrobium denitrificans* coupled with methane for denitrification of water.

Keywords: Hyphomicrobium; Denitrification; Water treatment; Box–Behnken design; Packed bed reactor

1. Introduction

The discharge of high concentrations of nitrate (NO_3^-) into the environment affects the human's and animal's health [1,2]. Nitrate pollutions are widely observed in fertilizer, explosive, metal, and nuclear industries wastes [3]. The nitrate pollution of drinking water may lead the various health problems such as hypertension, gastrointestinal cancer, diabetes, spontaneous abortions, respiratory tract infections, and defects in central nervous [4]. The permissible level of nitrate in drinking water is reported as 50 mg NO_3 L^{-1} by the World Health Organization [5]. Therefore, various methods for denitrification of drinking water have been developed. These methods include the ion exchange [6,7], reverse osmosis [8], electro-dialysis [9,10], electrochemical [11,12] and biological denitrification [13–21]. Among them, the biological denitrification due to its high efficiency and low cost is almost preferred for nitrate removal in comparison to the other physicochemical processes. Biological denitrification of nitrate requires an external electron donor [22]. Nitrate is reduced to nitrite, nitric oxide (NO), nitrous oxide (N_2O) and di-nitrogen gas using anaerobic heterotrophic bacteria [23]. Several denitrificans provide partial denitrification reducing nitrate to NO and N_2O. Among them, *Hyphomicrobium* in the family of Hyphomicrobiaceae showed the high potential for the complete denitrification of NO_3^- to N_2 [24–26]. In the denitrification process of water, an external carbon source is commonly added to the process due to the low carbon/nitrogen ratio for the complete removal of nitrogen, [27].

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Different electron donors such as methanol, ethanol, methane, hydrogen, and acetate were studied for the denitrification process [27,28]. Methane as an inexpensive electron donor has been widely used for biological denitrification of drinking water or/and wastewater [29]. The methane oxidation coupled to the denitrification process could be categorized as aerobic methane oxidation coupled to denitrification (AME-D) and anaerobic methane oxidation coupled to denitrification (ANME-D) [29]. Based on stoichiometric Eq. (1), nitrate is reduced to dinitrogen, and methane is oxidized to carbon dioxide as follows [27]:

$$5CH_4 + 8NO_3^- \rightarrow 5CO_2 + 6H_2O + 4N_2 + 8OH^- \quad (1)$$

Most studies have been developed on the denitrification under AME-D in the presence of oxygen by methanotrophs. In this pathway, the methane oxidation to methanol is catalyzed via the methane monooxygenases [27]. In recent studies, researchers have focused on the denitrification with methane under anaerobic conditions in the presence of microbial consortium containing archaea and bacteria. In this method, denitrifiers reduce the nitrate to dinitrogen as $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$.

The denitrification with methane as an electron donor was studied in both batch- and fixed-bed reactors. Recently, the packed bed reactors have been employed for denitrification processes. *Hyphomicrobium* spp. are good candidates for denitrification systems supplied with methanol as a carbon source. Costa et al. [30] used the *Hyphomicrobium* coupled with methanol in a bioreactor for the denitrification process. In another study, Rissanen et al. [23] investigated the potential of *Hyphomicrobium* coupled with methanol for denitrification systems. Lemmer et al. [26] used the *Hyphomicrobium* at both aerobic and anoxic conditions with non-Cl-carbon sources. The denitrification capacities of *Hyphomicrobium*, *Hydrogenophaga*, and *Rhodobacter* have been studied by Tian et al. [31]. The methylotrophic bacteria including *Methyloversatilis* spp. and *Hyphomicrobium* spp. were also coupled with methanol for the denitrification process in a sequencing batch reactor. The efficiency of *Methylophilaceae* and *Hyphomicrobium* coupled with methanol for denitrification of municipal wastewater was investigated by Rissanen et al. [23]. In another study, Villemur et al. [32] used the *Hyphomicrobium* nitrativorans strain NL23 and *Methylophaga nitratireducenticrescens* strain JAM1 coupled with methanol for denitrifying of water. Cucaita et al. [33] also developed the denitrification capacity of *Hyphomicrobium* nitrativorans strain NL23 and *Methylophaga nitratireducenticrescens* strain JAM1 under marine conditions. However, there is little study about the interaction effect of parameters for the denitrification process by using *Hyphomicrobium* coupled with methane in a packed bed reactor.

Different parameters such as pH, time, flow rate, temperature, and the initial nitrate concentration could affect the continuous denitrification of drinking water using *Hyphomicrobium denitrificans* coupled with methane. Thus, the statistical experimental design methods could be applied to estimate the simultaneous effects of variables on the nitrate removal from water. In recent studies, both the Central-Composite Design [34] and Box–Behnken Design (BBD) [35,36] have been developed as experimental design techniques for optimization of variables and complete removal of nitrate. In the present study, the response surface methodology using BBD was used to evaluate the effect of various parameters including methane/air mixture flow rate, pH, and temperature using the *Hyphomicrobium* coupled with the methane in the presence of a packing medium on the denitrification of water.

2. Experimental

2.1. Materials

2.1.1. Microorganism

The methylotrophic strain *Hyphomicrobium* (DSM 1869) was purchased from DSMZ (Braunschweig, Germany). The bacterium was maintained at 4°C on nutrient agar slants containing 0.6% (v/v) methanol.

2.1.2. Mineral salt medium

The mineral compounds used for the initial inoculum development in this experiment were (per liter): $(NH_4)SO_4$, 1,000 mg; $NaH_2PO_4{\cdot}H_2O$, 500 mg; $MgSO_4{\cdot}7H_2O$, 200 mg; K_2HPO_4, 1,550 mg; Methylamine Hydrochloride, 3,400 mg, and Agar-Agar 15,000 mg. The trace element solution was (per liter): Na_2-EDTA, 50 mg; $ZnSO_4{\cdot}7H_2O$, 22 mg; $CaCl_2{\cdot}2H_2O$, 5.54 mg; $MnCl_2$ $4H_2O$, 5.06 mg; $FeSO_4$ $7H_2O$, 5 mg; $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 1.1 mg; $CoCl_2$ $6H_2O$, 1.61 mg and $CuSO_4{\cdot}5H_2O$, 1.57 mg. The final pH of mineral compounds was 7.2.

After growing the colonies of bacteria on the surface of the agar, colonies were picked from each sample and each colony was transferred into another mineral salt medium containing (per liter): $(NH_4)_2SO_4$, 1,750 mg; KH_2PO_4, 680 mg; $Na_2HPO_4{\cdot}7H_2O$, 6,140 mg; $MgSO_4{\cdot}7H_2O$, 100 mg; $FeSO_4{\cdot}7H_2O$ 20 mg; $CaCl_2{\cdot}2H_2O$ 20 mg; $MnSO_4{\cdot}7H_2O$ 5 mg; $ZnSO_4{\cdot}7H_2O$ 1.5 mg; $Na_2MoO_4{\cdot}2H_2O$ 0.04 mg; $CuSO_4{\cdot}5H_2O$ 0.04 mg; $CoCl_2{\cdot}6H_2O$ 0.6 mg; H_3BO_3 0.2 mg and 0.6% (v/v) methanol (Final stock solution). The organism was cultivated into the 250 mL Erlenmeyer flask containing 50 mL of the above medium at the temperature of 30°C for 72 h in an incubator.

2.2. Packed bed bioreactor

A laboratory-scale pack-bed bioreactor of a double-wall looped-glass tube of diameter 0.03 m and height 0.5 m was used. A schematic diagram of the bioreactor used in experiments is illustrated in Fig. 1. 210 mL cultivated medium and 21 mL inoculum solution were added into the reactor and sterilized before use. Filter-sterilized methanol was initially added to support bacterial growth. Then, the air and natural gas (mostly methane; 90%) were mixed and distributed through a perforated tube using a gas pump. The used pack-bed bioreactor was fed using a peristaltic pump (Provitec, AWG-5000; energy consumption of 0.012 kWh) and the effluent was discharged from overflow. The temperature of the bioreactor was controlled by a temperature loop controller (TLC). The pack-bed bioreactor was filled with a local scoria with 0.52 void ratio (provided

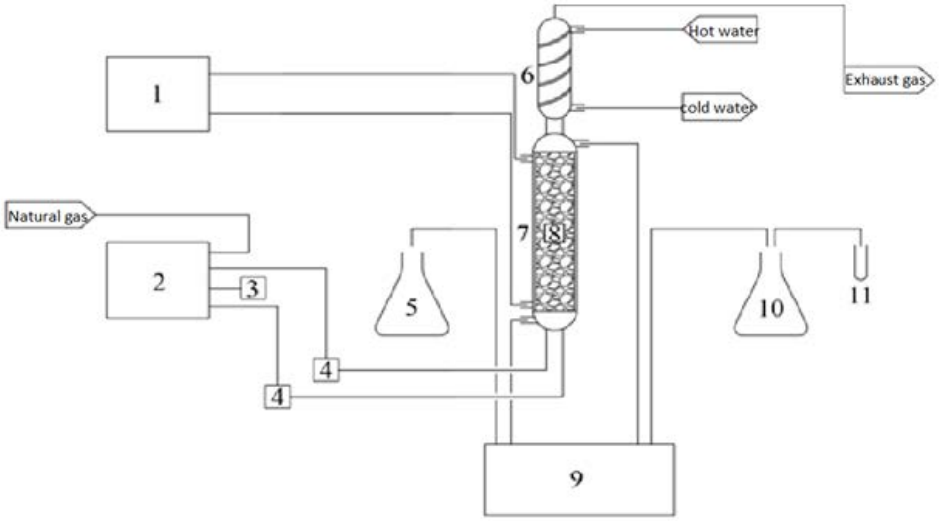

Fig. 1. Schematic packed bed reactor. 1. Thermae; 2. Double pump for sucking natural gas and air; 3. Air filter; 4. Gas and air flow meter; 5. Water contain nitrate; 6. Condenser; 7. Packed bed reactor; 8. Packing; 9. Two motor peristaltic pump for feeding and sucking water; 10. Denitrated water; 11. Sampling.

from Ghorveh mine, Sanandaj, Iran) (70%) as supporting material for microorganism's biofilm fixation.

2.3. Analytical methods

The initial NO_x^- concentration in the inflow water feeding and the effluent NO_x^- concentration in the reactor outlet was measured using Spectroquant® nitrate and nitrite test kits (Merck Millipore, Germany). The cell test kit has been used for total nitrogen photometric determination.

The removal efficiency (RE, %) and denitrification capacity (DC, mg NO_3–N L^{-1} h^{-1}) of the reactor are given as follows:

$$\mathrm{RE}(\%) = \left(\frac{C_{\mathrm{in}} - C_{\mathrm{out}}}{C_{\mathrm{in}}}\right) \times 100 \tag{2}$$

$$\mathrm{DC} = \frac{Q}{V}\left(C_{\mathrm{in}} - C_{\mathrm{out}}\right) \tag{3}$$

where C_{in} and C_{out} are the initial and the effluent NO_3–N concentration (mg L^{-1}); Q is the flow rate (L h^{-1}); and V is the reactor volume (L). All the tests were repeated in triplicate and the average values were reported as mean ± standard deviation. Since the DC values remained almost steady among all the various runs, suggesting an excellent capability of denitrification in the designed reactor.

2.4. Denitrification experiments

Based on Eq. (1) and theoretical evaluations, 16.49 g methane is needed for the removal of 100 mg NO_3^-. Therefore, 23.1 L methane could remove 100 mg NO_3^-. With respect to the methane/air mixture purity, and inlet flow rate of nitrate (1.4 mL min^{-1}), the air/methane mixture flow rate was estimated to be about 40 mL min^{-1} for removing 100 mg NO_3^-. Based on theoretical evaluations, the initial flow rate of air/methane mixture has been varied in the range of 20–60 mL min^{-1}. The initial pH of solution was found to be 6.8 ± 0.3. Therefore, the inlet pH of solution was varied between 5 and 9 to investigate the effect of acidic, and alkaline environments on the denitrification of water.

Fifteen experiments based on the BBD were carried out to investigate the interaction effect of key parameters of continuous denitrification process including the initial flow rate of air/methane mixture (20–60 mL min^{-1}), temperature (20°C–30°C) and inlet pH of solution (5–9) on the denitrification capacity (DC (mg L^{-1} h^{-1})) with the initial concentration of 100 mg L^{-1} nitrate using *Hyphomicrobium* coupled with methane in the presence of a mineral packing medium. The denitrification process time of 720 min was considered constant in the experiments which a slight enhancement in the final pH of solution ranging from 0.3 to 0.6 was observed. The experimental design and results of denitrification rate are presented in Table 1.

The short residence time for denitrification of water has an important role in industry reactors. The effect of inlet flow rate of nitrate (1.4, 2.5, 4, 5 and 7 mL min^{-1}) was investigated to select an optimized flow rate for a designed reactor on the denitrification of water.

The polynomial model for the denitrification capacity (mg L^{-1} h^{-1}) with respect to the denitrification process variables is expressed as follows:

$$\mathrm{DC}\left(\frac{\mathrm{mg}}{\mathrm{l}\cdot\mathrm{h}}\right) = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum_{i=1}^{3}\sum_{j=1}^{3}\beta_{ij} x_i x_j \tag{4}$$

where β_0, β_i, β_{ii}, and β_{ij} are the constant regression coefficient of the model. X_i, X_{ii} and X_{ij} represent the linear, quadratic and interactive terms of the encoded independent variables, respectively. The coefficient of determination (R^2) shows the accuracy of the full quadratic equation.

3. Results and discussion

3.1. Effect of pH, temperature and methane/air flow rate on the denitrification capacity

The effect of variables including pH, temperature, and methane/air mixture flow rate on the denitrification process using the *Hyphomicrobium* coupled with the methane in a packing medium at the center level of other parameters was investigated based on the experimental design which results are illustrated in Fig. 2. The optimum pH, temperature and mixture flow rate values were found to be 7°C, 30°C and 60 mL min^{-1}, respectively. The pH of the solution is an important parameter that affect on the behavior of denitrifiers during the denitrification process. As shown, the acidic (pH = 6) or alkaline (pH = 8) conditions had adverse effects on the growth of *Hyphomicrobium denitrificans* and subsequently, the denitrification capacity. The intramolecular electron transfer rate is decreased

Table 1
Experimental design for denitrification process and results

Run	pH	Temperature (°C)	Flow rate (mL min^{-1})	DC (mg L^{-1} h^{-1})	Fitted value by model (mg L^{-1} h^{-1})
1	6	20	40	2.09	2.10
2	8	20	40	1.85	1.88
3	6	30	40	2.47	2.44
4	8	30	40	2.39	2.38
5	6	25	20	1.25	1.27
6	8	25	20	1.11	1.10
7	6	25	60	2.52	2.52
8	8	25	60	2.42	2.40
9	7	20	20	1.09	1.06
10	7	30	20	1.62	1.63
11	7	20	60	2.48	2.47
12	7	30	60	2.74	2.76
13	7	25	40	2.50	2.47
14	7	25	40	2.45	2.47
15	7	25	40	2.48	2.47

DC – Denitrification capacity

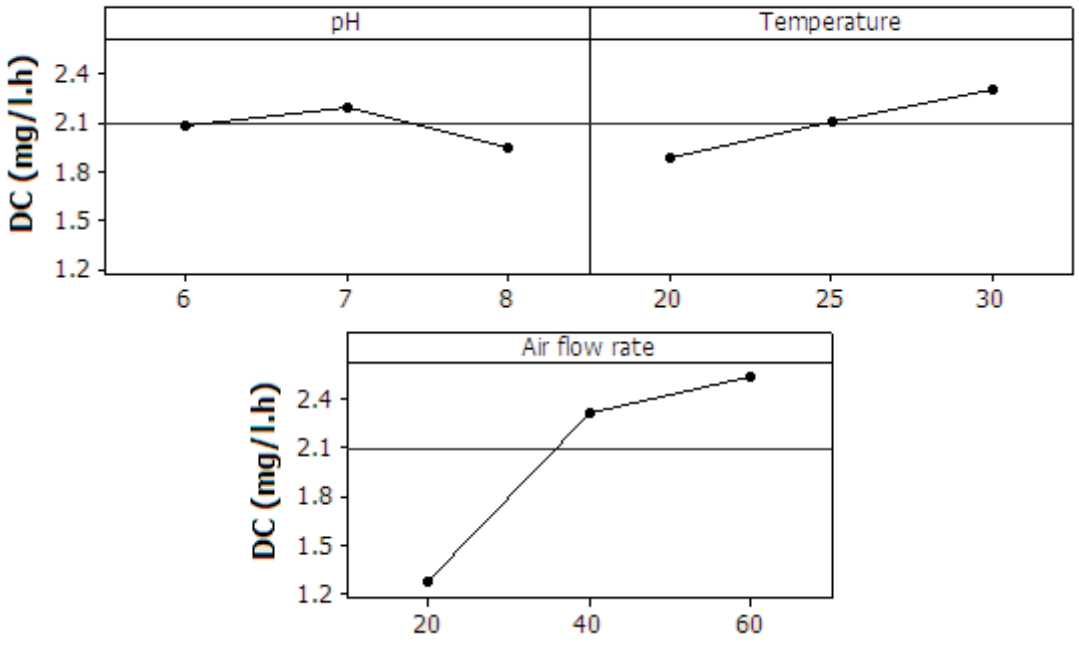

Fig. 2. Effect of pH, temperature, and methane/air mixture flow rate on the denitrification process.

sharply at pH of 8, which may be related to the inhibition of *Hyphomicrobium* activity and following the nitrate reduction rate [25]. Furthermore, the more accumulation of nitrate and following the more consumption of nitrate using *Hyphomicrobium denitrificans* at pH = 7 could be resulted in increasing DC. A slight increase of pH in effluent could be attributed to the alkalinity produced by the denitrifiers. Chen and Lin [37] observed the maximum denitrification at pH 7.

The temperature has an important role on the denitrification rate and design of denitrifying reactors. This effect is commonly described using an Arrhenius-type equation [38]. The *Hyphomicrobium denitrificans* was well grown at temperatures ranging from 20°C to 30°C [3]. As shown in Fig. 2, the nitrate removal efficiency was increased by rising temperature from 20°C to 30°C. This behavior may be attributed to increasing the activity of *Hyphomicrobium denitrificans* at higher temperatures. Similar trends were obtained by other researchers [3]. Payette et al. [39] indicated that the denitrification of water using *Hyphomicrobium denitrificans* coupled with methanol was significantly increased by about 53% and 62% with increasing temperatures from 23°C to 30°C and 36°C, respectively [39].

The carbon to nitrogen ratio has a critical factor for denitrification processes. The methane/air mixture flow rate (carbon/nitrogen ratio) must be enough to achieve a sufficient nitrate removal based on the generated methane in a wastewater treatment [28]. The influence of mixture flow rate on the DC is illustrated in Fig. 2. As shown, enhancement in flow rate resulted in a higher average denitrification rate and the DC was sharply increased when the methane/air mixture flow rate as an external carbon source was increased from 20 to 40 mL min^{-1}. The low difference in denitrification capacities at flow rates of 40 and 60 mL min^{-1} suggested that the higher flow rates than 40 mL min^{-1} did not play an important role in the denitrification process using *Hyphomicrobium* coupled with the methane.

3.2. Analysis of variance

An analysis of variance (ANOVA) was applied to describe the significance of variables (*F*- and *P*-values) and full quadratic response surface model (Table 2). *F*- and *P*-values (probability) with 95% confidence level were used to check the statistical significance of the quadratic model. As shown in Table 2, the linear terms, quadratic terms and two interaction terms exhibited the significant effect on the DC. The higher *F*-value of mixture flow rate in comparison to the *F*-values of pH and temperature demonstrated the highest influence of mixture flow rate on the denitrifiction process. By eliminating of insignificant terms (pH × Mixture flow rate; $p > 0.05$) from the full quadratic model (Table 3), the third Eq. (4) including a series of linear, quadratic and interaction terms for the DC was achieved as follows:

$$\mathrm{DC}\left(\frac{\mathrm{mg}}{\mathrm{l\cdot h}}\right) = -12.51 + 2.77x_1 + 0.13x_2 + 0.14x_3 - 0.21x_1^2 - 0.002x_2^2 - 0.001x_3^2 + 0.008x_1x_2 - 0.0007x_2x_3 \quad (5)$$

where x_1, x_2 and x_3 are pH, temperature (°C), and mixture flow rate (mL min^{-1}), respectively. The coefficient of determination (R^2) and lack of fit *p*-values for the modified model were found to be 0.993 and 1.55, respectively. After refining the model, the declined lack of fit value than that of 1.86 revealed that significant improvement was achieved by excluding non-significant term. The high value of R^2 (R^2 = 0.993) indicated a high reliability of the model in predicting the denitrification capacity.

3.3. Optimization of denitrification capacity

The optimization of nitrate removal efficiency and the complete denitrification are very important issues in

Table 2
ANOVA results for the denitrification capacity (mg L^{-1} h^{-1})

Source	DF	Seq. SS	*F*	*P*
Regression	9	4.49150	520.75	0.000
Linear	3	3.64323	1267.21	0.000
pH	1	0.03920	40.90	0.001
Temperature	1	0.36551	381.40	0.000
Mixture flow rate	1	3.23851	3379.32	0.000
Square	3	0.82325	286.35	0.000
pH × pH	1	0.17400	181.57	0.000
Temperature × Temperature	1	0.01311	13.68	0.014
Mixture flow rate × Mixture flow rate	1	0.69734	727.66	0.000
Interaction	3	0.02502	8.70	0.020
pH × Temperature	1	0.00640	6.68	0.049
pH × Mixture flow rate	1	0.00040	0.42	0.547
Temperature × Mixture flow rate	1	0.01822	19.02	0.007
Residual error	5	0.00479		
Lack-of-fit	3	0.00352	1.86	0.369
Pure error	2	0.00127		
Total	14	4.4926		

Table 3
ANOVA results for denitrification capacity (mg L^{-1} h^{-1}) after eliminating of insignificant parameters

Source	DF	Seq. SS	*F*	*P*
Regression	8	4.49110	648.79	0.000
Linear	3	3.64323	1403.49	0.000
pH	1	0.03920	45.30	0.001
Temperature	1	0.36551	422.42	0.000
Mixture flow rate	1	3.23851	3742.74	0.000
Square	3	0.82325	317.14	0.000
pH × pH	1	0.12337	201.09	0.000
Temperature × Temperature	1	0.00254	15.15	0.008
Mixture flow rate × Mixture flow rate	1	0.69734	805.91	0.000
Interaction	2	0.02462	14.23	0.005
pH × Temperature	1	0.00640	7.40	0.035
Temperature × Mixture flow rate	1	0.01822	21.06	0.004
Residual error	6	0.00519		
Lack-of-fit	4	0.00392	1.55	0.428
Pure error	2	0.00127		
Total	14	4.49629		

industry. By solving Eq. (5), the optimal encoded values of pH, temperature, and mixture flow rate were estimated to be 6.9°C, 30°C, and 53 mL min^{-1}, respectively. The optimum predicted value for denitrification capacity by the model was estimated to 2.81 mg NO_3–N L^{-1} h^{-1}. The experimental value for denitrification capacity in optimum conditions was found to be 2.85 mg NO_3–N L^{-1} h^{-1} which was in good agreement with the estimated value by the model.

3.4. Validation of model with experimental data

The linear relationship between the actual and predicted values of the denitrification capacity is illustrated in Fig. 3a. The high value of coefficient of determination (R^2 = 0.984) indicated that the experimental values of denitrification capacity exhibited a good agreement with the predicted values of the response by the linear regression model. A normal probability plot of residuals (difference between the model predicted denitrification capacity values and those derived experimentally) is shown in Fig. 3b. It was obvious that the data points on the plot reasonably overlapped the straight line which indicated that the errors were normally distributed. Furthermore, it was observed that the established quadratic model was adequate to estimate the denitrification capacity, as all the residuals were smaller than 5%.

3.5. Interaction effect of two variables

The interaction effect between the pair of variables at the center level of third variable on the denitrification capacity using *Hyphomicrobium denitrificans* is illustrated by the 3D response surface plots and counter plots (Fig. 4). As shown in Fig. 4a and b, the DC was increased by raising temperature from 20°C to 30°C and enhancement in pH values up to 7. At pH values higher than 7, DC was declined. The counter plots showed that the maximum DC (DC > 2.6 (mg L^{-1} h^{-1})) was occurred at temperatures higher than 28°C

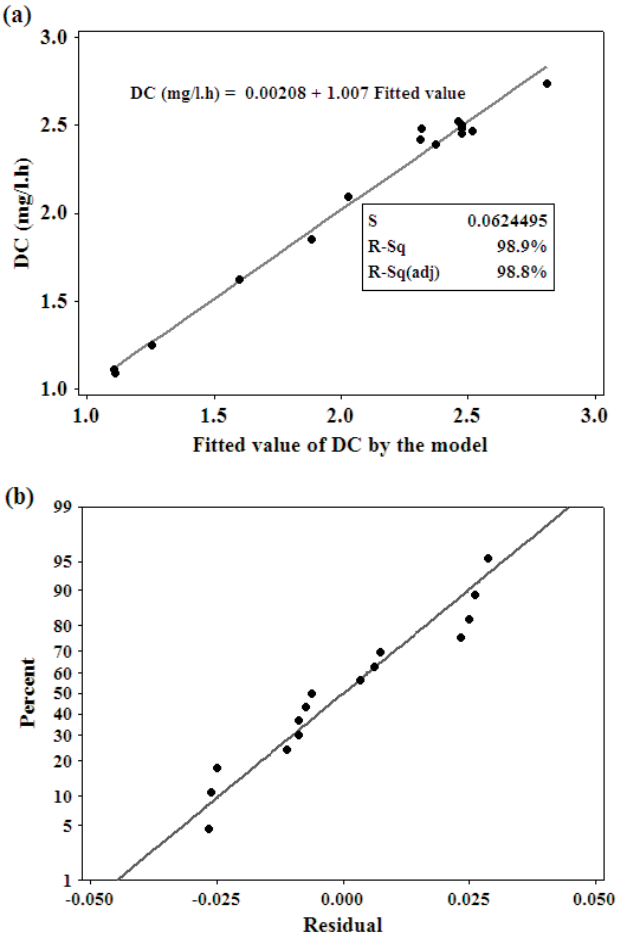

Fig. 3. (a) Plot of model predicted denitrification capacity against experimental denitrification capacity and (b) normal probability plot.

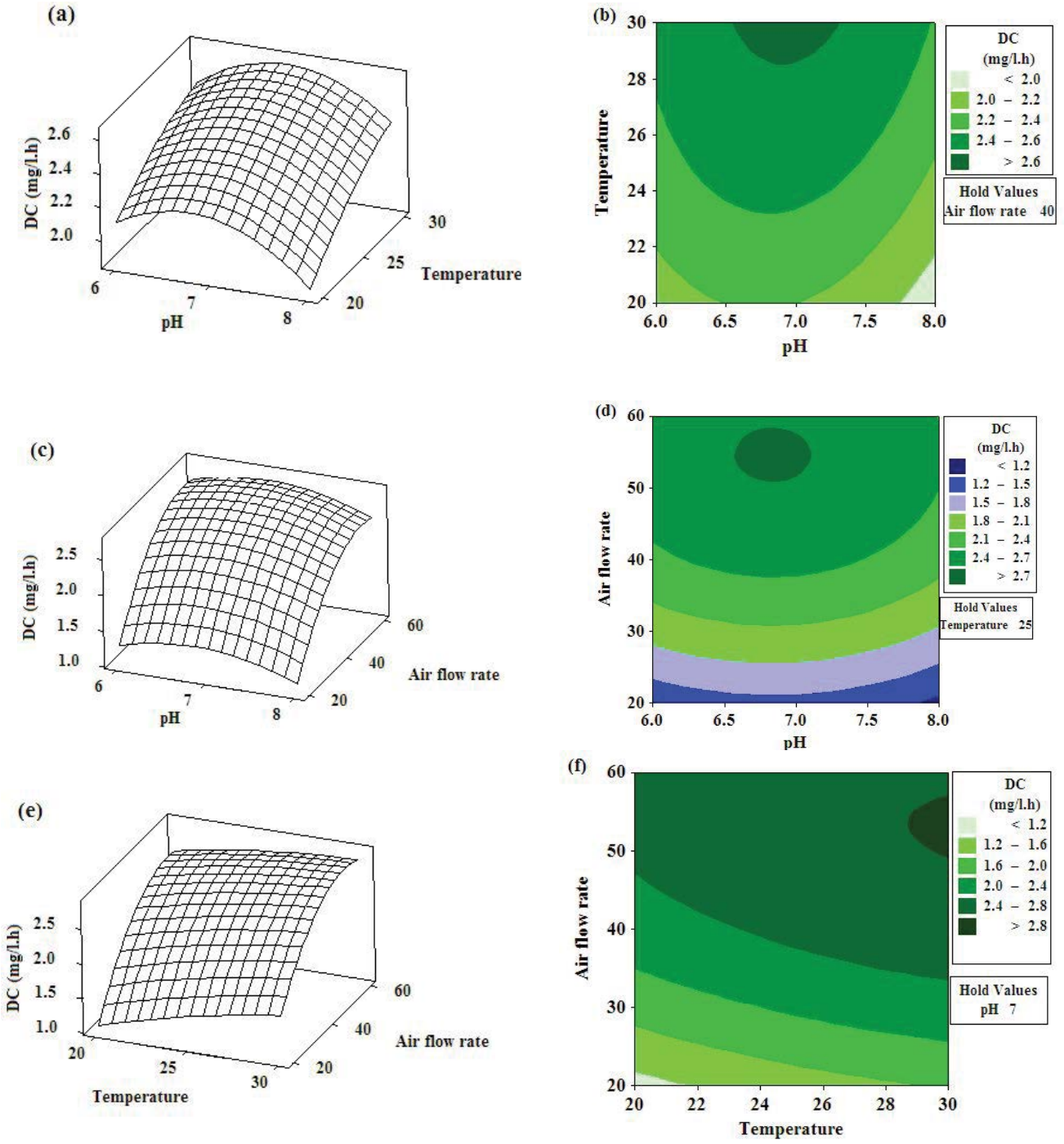

Fig. 4. Surface and counter plots of the response variable (denitrification capacity) for the different experimental variables (two factor-at-a-time). (a, b) pH and temperature, (c, d) pH and mixture flow rate, and (e, f) temperature and mixture flow rate.

and pH values ranging from 6.6 to 7.2. Fig. 4c and 4d shows the interaction effect of pH and methane/air mixture flow rate on the DC. As shown, the mixture flow rate had the maximum effect on the DC. The DC declined significantly at lower mixture flow rates. The DC values were estimated to be higher than 2.7 mg L^{-1} h^{-1} at mixture flow rates higher than 50 mL min^{-1}, and pH values ranging from 6.5 to 7. The simultaneous effect of temperature and mixture flow rate on the DC is illustrated in Fig. 4e and f. As shown, the higher DC values (DC > 2.8 mg L^{-1} h^{-1}) were obtained at pH 7, temperatures higher than 28°C and mixture flow rate higher than 50 mL min^{-1}.

3.6. Effect of nitrate inflow rate

The effect of inlet flow rate of nitrate (1.4, 2.5, 4, 5 and 7 mL min^{-1}) was investigated under the optimum conditions to obtain the maximum flow rate in which the

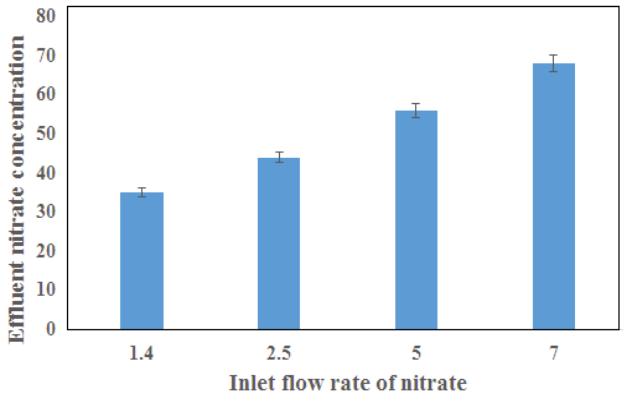

Fig. 5. Effect of inlet flow rate of nitrate on the effluent nitrate concentration.

nitrate concentration could decline to less than 50 mg L^{-1} under the shortest time. Since, the permissible level of nitrate in drinking water is 50 mg NO_3 L^{-1}; achieving effluent nitrate concentrations lower than 50 mg L^{-1} is inevitable. Fig. 5 shows the influence of nitrate inflow rate in the range of 1.4–7 mL min^{-1} using *Hyphomicrobium* coupled with methane in a packed bed reactor. As shown, the effluent nitrate concentration reached to less than 50 mg L^{-1} in the nitrate flow rates of 1.4 and 2.5 mL min^{-1}. The time was not enough for denitrification process at flow rates higher than 2.5 mL min^{-1}. Therefore, the inlet flow rate of 2.5 mL min^{-1} is selected as optimum value for denitrification of drinking water using *Hyphomicrobium denitrificans* in a designed packed bed reactor.

4. Conclusion

In this work, the *Hyphomicrobium denitrificans* was successfully coupled with the methane as an external carbon source in a packed bed reactor for denitrification of drinking water. The BBD was used to estimate the optimal conditions for denitrification process.

Three variables of denitrification process including pH, temperature, and methane/air mixture flow rate were considered at three levels in the denitrification of drinking water containing 100 mg L^{-1} nitrate. ANOVA results of BBD response revealed that the linear, quadratic and some interaction variables were found statically significant on the denitrification process. By optimizing of parameters (pH 6.9, temperature 30°C and mixture flow rate 53 mL min^{-1}), the maximum experimental denitrification capacity was found to be 2.85 mg NO_3–N L^{-1} h^{-1}. The effluent nitrate concentration reached to less than 50 mg L^{-1} under the inlet flow rate of 2.5 mL min^{-1}. The obtained results indicated the high capability of *Hyphomicrobium* coupled with methane for denitrifying of water to the lower permissible levels of nitrate in a packed bed reactor.

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