Application of central composite design approach for optimization nitrate removal from aqueous solution by immobilized *Pseudomonas putida*

Marwa E. El-Sesy and Sabah S. Ibrahim

ABSTRACT

High nitrate concentration is a dangerous pollutant in the environment. Immobilization for the optimum denitrifying bacterial strain isolated from collected wastewater samples was suggested for bioremediation of excessive nitrate concentration from aqueous solutions and its denitrification activity under different pH, nitrate concentration, bacterial beads, temperature and sodium alginate concentration conditions was explored. The active isolate was identified as *Pseudomonas putida* MT364822.1 by 16S rRNA analysis. Nitrate bioremediation process was optimized by applying response surface methodology based on central composite design approach. Nitrate uptake was significantly affected by variables of study (*P*-value <0.05). Maximum removal of nitrate (91.1%) was achieved at pH 7, nitrate concentration 400 mg/L, immobilized bacterial beads 3.0 g/L, temperature 35 °C and sodium alginate concentration 2.5% as optimal variable values. For application, immobilized *P. putida* MT364822.1 removed 82.2% of nitrate from raw fish farm effluent. Storage and reusability experiments showed that the immobilized strain stronger and more stable than the pure strain. The results suggested that immobilized *P. putida* MT364822.1 is a highly promising and suitable microorganism for use in the bio-removal of nitrate, and the central composite design was more effective in optimizing variables to achieve the best nitrate removal efficiency.

Key words | bioremediation, central composite design, immobilized bacteria and denitrification, nitrate contamination, wastewater

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HIGHLIGHTS

- Bioremediation of the excessive nitrate concentration from aqueous solutions using bacterial isolates from wastewaters.
- Immobilization for the best denitrifying bacterial strain isolated during the study.
- Applying response surface methodology based on central composite design approach for optimization.
- An application using immobilized denitrifying bacteria in nitrate bio-removal from raw fish farm effluent was studied.

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INTRODUCTION

Nitrate is considered as one of the major nutrients that many living organisms require for physiological processes. High concentrations of nitrate are a great problem to the environment as well as to public health. Surface water may contain high levels of nitrate due to excessive and random use of fertilizers in agriculture, which is used to increase the quality of soil and results in leakage of nitrate from soil to surface and ground water. Nitrate pollution may also be due to other sources like industrial effluent, which has a high concentration of nitrate, domestic wastewater, municipal sewage, canals septic tanks, sewage dumping grounds and animal feedlots (Wakida & Lerner 2006; Krishnaswamy *et al.* 2009; Roy *et al.* 2012; De Filippis *et al.* 2013). High levels of nitrate were detected in groundwater as reported by Filintas *et al.* (2008). The presence of high concentrations of nitrate in drinking water can cause public health problems like stomach cancer in infants (Jin *et al.* 2004).

Burghate & Ingole (2017) demonstrated three technologies that showed some capability for nitrate removal including ion exchange, reverse osmosis and biological denitrification. Jafari *et al.* (2015) mentioned that biological denitrification is considered to be the most appropriate technology compared to other techniques for treatment of nitrate contaminated water. Several studies (Eckford & Fedorak 2002; Shao *et al.* 2011; Lim *et al.* 2017) reported that biological denitrification process is traditionally extolled to be the most economical processes, in addition to being environmentally sound, and is the most promising application being studied for nitrogen removal from wastewaters. King *et al.* (2012) explained that denitrifying bacteria can be used to reduce nitrate to harmless nitrogen gas (N_2). Kunapongkiti *et al.* (2019) found that the denitrification process can be promoted using cell immobilization. Nitrate reduction is explained by the following equation:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Cell immobilization is a technique that allows microorganisms to grow in a solid matrix and successfully limits the mobility of the cells using synthetic or natural polymers, which can be used as biocatalysts instead of free cells (Bouabidi *et al.* 2019). Immobilization gives an easier purification: enhance cell residence time, substrate loading rate and separation procedures of solids from liquids (Stolarzewicz *et al.* 2011; Kunapongkiti *et al.* 2019).

Immobilized living cells have been reported to be very effective in bioremediation (Abdullateef *et al.* 2020). Kumar & Saramma (2012) reported that some studies have increasingly focused on the use of immobilized microorganisms as a valid method that avoids the harvesting problem. From previous studies, the heterotrophic nitrification and aerobic denitrification were identified for nitrogen removal process via microbial entrapment with sodium alginate gel. The immobilized cells gave a higher removal percentage than free living cells (Yan *et al.* 2019).

Maximum nitrate removal percentage is influenced by several factors including pH, incubation time, nitrate concentration and bacterial inoculum. Several statistical experimental designs may be used as useful techniques to improve optimizing the process variables. Screening designs, such as fractional factorial and Plackett–Burman, may be used for pre-formulation evaluations.

Response surface methodology (RSM) is used only when a few significant factors are involved in optimization process. RSM, a special type of experimental design, can be described as a collection of both mathematical and statistical techniques for designing, improving, optimizing, and formulating product designs where several variables potentially affect characteristics of products designs. RSM represents the most useful method for analyzing data and is an effective statistical technique for modelling the optimization of multiple parameters that have an effect on response surface (Baş & Boyacı 2007; Mansouri *et al.* 2012; Tripathy & Murthy 2012). Useful practices in response surface designs include central composite design (CCD), Box–Behnken design, and two- and three-level factorial design (Palamakula *et al.* 2004).

A comparison study by Rakić *et al.*(2014) of four experimental design types in response surface designs (two-level full factorial design, CCD, Box–Behnken design and three-level factorial design) were reported for the determination of fluconazole and its impurities. The study reported that both central composite and three-level full factorial design models provided satisfactory statistical parameters. A study by Acharya *et al.* (2018) represented that the CCD and Box–Behnken are the most important surface-response methods for designing the test. Another studies stated that CCD is the most common experimental design used in process optimization studies under RSM and is widely used to create second-order surface-response models (Chen *et al.* 2012; Moradi *et al.* 2016; Asadzadeh *et al.* 2018).

In this study, the feasibility of using immobilized *Pseudomonas putida* for nitrate removal from aqueous solution was estimated using the CCD in RSM for optimizing different important variables for removal efficiency. Samples were collected from Sabal Drain and also from fresh effluent fish farm at Al-Menoufiya and were collected during October 2019 before being sent to the laboratory for analysis.

METHODOLOGY

Sampling

Wastewater samples were collected in triplicates from Sabal Drain, which is the largest drain in Al-Menoufiya, Egypt. It extends for more than 62 km and discharges its contents into the Rosetta branch of the River Nile (Abdel-Khalek *et al.* 2018), as shown in Figure 1, in special containers for the testing of parameters according to *Standard Methods for the Examination of Water and Wastewater* (APHA 2012).

Pre-enrichment cultivation medium

To isolate bacteria capable of reducing nitrate (NO_3^-) in aqueous solutions, about 10 mL of collected wastewater sample was first individually grown in a 250 mL Erlenmeyer flask containing sterilized 100 mL of a modified minimum denitrification medium (DM) according to Kesserű *et al.* (2003): 1.8 g K_2HPO_4, 1.08 g KH_2PO_4, 0.1 g $MgSO_4$ $7H_2O$, 0.5 g NH_4Cl, 0.1 g KCl, 0.5 g/L $NaNO_3$, and 0.05 $CaCl_2$ $2H_2O$ g/L. The enrichment was incubated for 24 h at 35 °C to isolate bacteria that can survive in high concentrations of nitrate. The inoculum was used for further removal studies. The obtained bacterial consortia survived harmful conditions in the presence of high levels of nitrate in the solutions and were used as inoculum for further bioremoval studies. One mL of consortium was spread on DM agar plates and amended with 0.5 g/L of nitrate concentration before incubating for 24 h at 35 °C. The predominated bacterial colonies were selected for the rest of the research. The strains were maintained on slants of nutrient agar.

Nitrate reduction test

Nitrate tests from Angar *et al.* (2016) were followed to select the bacterial strain with the nitrate reductase enzyme, which reduces nitrate (NO_3^-) to harmless nitrogen gas (N_2) and then becomes denitrifying bacteria. A heavy inoculum of test organism was inoculated in sterilized nitrate broth tubes fitted with Durham tubes containing peptone 5 g/L, meat extract 3 g/L, potassium nitrate 1 g/L and with a final pH of 7.0 ± 0.2. It was incubated at 35 °C for 24 h. After incubation, nitrogen was checked in the Durham tubes before adding reagents. If there was no nitrogen gas, two reagents were added: three drops of 1% of nitrate reagent A (sulfanilic acid) and one drop of 0.02% nitrate reagent B (alpha-naphthylamine). The development of a

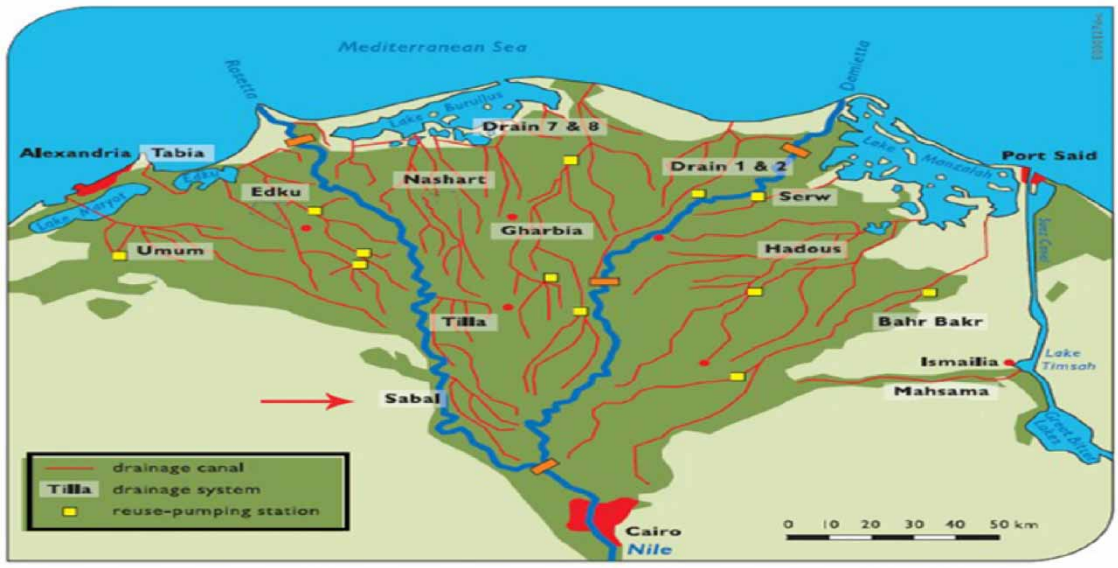

Figure 1 | Map of the studied area showing Sabal Drain.

red or purple colour within 5 min was considered a positive reaction for nitrate reduction to nitrite. Negative tests were further confirmed with the addition of zinc dust, where zinc can reduce the nitrate to nitrite. Failure of colour development was recorded as a positive test for nitrate reduction beyond nitrite to gaseous or non-gaseous nitrogen compounds. The development of the red colour after the addition of zinc particles indicated the presence of nitrate in the culture medium.

Immobilization of active strains

The immobilization technique for isolated nitrate-reducing bacterial strains was used. First, about 2% of sodium alginate was dissolved in saline 0.85% at pH 7 and then autoclaved for 5 min. About 5 mL of bacterial culture cotaining 10^6 colony-forming units (CFU) per millilitre was centrifuged at 2,000 rpm for 10 min. The supernatant was discarded and the cells were re-suspended in 5 mL of saline solution (0.85%). About 5 mL of sodium alginate was mixed with 5 mL of bacterial suspension and was shaken manually for 20 min. Round beads, approximately 3 mm in diameter, were obtained by dropping the alginate bacterial mixture through a sterilized syringe into a sterile 250 mL Erlenmeyer flask containing 50 mL of cold sterile 2% calcium chloride, at room temperature in sterile conditions. The formed beads were allowed to harden in calcium chloride for 1 h. The beads were washed several times in sterile saline solutions (0.85%) to remove any remains of calcium chloride and were later stored at 4 °C in autoclaved distilled water (Kumar & Saramma 2012).

Genetic identification of nitrate-reducing bacteria

For identification of the bacterial strain, the genomic DNA was extracted from bacterial culture grown in nutrient broth and was centrifuged at 3,000 rpm for 15 min. Extraction of genomic DNA was carried out using the PrepMan™ ultra sample preparation reagent (PN 4322547). The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the universal bacterial specific primer performed in a 20 μL reaction containing 10 μL Premix Taq, 1.5 μL of DNA template, 0.5 μL forward primer, 0.5 μL reverse primers and 7.5 μL double-distilled water. The PCR programming used for the initial step was 95 °C for 10 min, 30 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s and the final step was at 72 °C for 10 min. The amplified PCR product was detected in the samples by running on 1.5% agarose gel and sequencing as the 16S rRNA amplicon. Resulting gene sequences were subjected to BLAST search tool (Altschul *et al.* 1997) in the NCBI gene database and submitted to GenBank to obtain the accession number. Alignment with the similar sequences was carried out using ClustalW (Thomas *et al.* 2013). Finally, MEGA X software (https://www.megasoftware.net) was used to build the phylogenetic tree by the neighbour-joining method, with 1,000 bootstrap replicates and the maximum composite likelihood model (Kumar *et al.* 2008; Kumari *et al.* 2016).

Preliminary experiment for nitrate removal ability of active strain

Experiments were carried out by adding five different concentrations of nitrate (200, 400, 600, 800 and 1,000 mg/L) separately in 500 mL Erlenmeyer flasks containing sterilized 100 mL of DM at a range of temperatures from 10 to 45 °C. The effects of pH, ranging from 3.0 to 9.0 were tested. Then, 1 g/L of immobilized active bacterial beads was added and the effects of different sodium alginate concentrations were examined, ranging from 0.5 to 2.5%. All these tests were conducted in duplicate. The residual nitrate was evaluated by ion chromatography ICS-5,000, and the percentage of nitrate removal was calculated from the difference between the initial concentration of nitrate and the residual nitrate concentration in the solution. The calculation can be described by the following equation, Equation (1):

$$\mathrm{NR}\% = \frac{(C_i - C)}{C_i} \times 100 \quad (1)$$

where NR% is nitrate removal percentage, C_i and C represents initial and final concentration of the nitrate (mg/L), respectively in mineral salt medium (DM) after incubation for 24 h (Ghanim 2013; He *et al.* 2018; Massoudinejad *et al.* 2018).

Optimization of bioremediation process by CCD

RSM based on CCD is a mathematical and statistical technique which is useful for designing experiments, analyzing the interaction between independent variables and creating the optimum synergy of this response before checking the response patterns (Khuri & Mukhopadhyay 2010).

Experimental design

Five independent variables factors, i.e. pH, temperature, nitrate concentration, bacterial inoculum concentration and sodium alginate concentration, were studied at five levels: $-\alpha$(minimum), -1(low), 0(central), $+1$(high) and $+\alpha$(maximum), where these levels were selected based on preliminary study results, as well as previous studies (Tong *et al.* 2014; Asadzadeh *et al.* 2018). The factors and levels are shown in Table 1.

The CCD model was designed using the statistical software Minitab 18. A total of 32 experiments were found to be sufficient to analyze data and determine the interaction of five independent variables through five levels of nitrate removal efficiency. After all experiments, ion chromatography system was used to determine residual nitrate and the nitrate bioremediation was calculated from Equation (1). The experimental data were subjected to multiple regression analyses, and the mathematical relationship between the nitrate removal (response values) and five independent variables was expressed by the second-order polynomial model according to Equation (2):

$$Y = \beta_o + \sum^{k} \beta_i X_i + \sum^{k} \beta_{ii} X_i^2 + \sum^{k} \sum^{k} \beta_{Ij} X_i X_J + \epsilon \quad (2)$$

where Y is the predicted nitrate removal percentage (response), k is the number of studied independent factors, X_i and X_J are the uncoded independent variables, β_o is the constant coefficient, β_i, β_{ii} and β_{ij} are the linear coefficient, quadratic coefficient and interaction coefficient, respectively, and ε is the random error (Lin & Wu 1996).

After statistical modelling and regression analysis, analysis of variance (ANOVA) was used with the significance level P-value ($P \leq 0.05$), F-value and the coefficient R^2 to define the significance of each factor in the fitted equations and to evaluate the goodness of fit in each case (Asadzadeh *et al.* 2018). Minitab was used to produce three-dimensional (3D) response surface plots as a graphical representation for the regression equation for the optimization of reaction conditions. It was considered as the most useful approach in revealing the conditions of the reaction system. They were plotted based on the simultaneous interaction effect of the two-factor levels on the responses by plotting the removal percentage (response) on the Z-axis and two independent variables on the X-axis and Y-axis, and keeping the other variables at the coded zero point (Sarrai *et al.* 2016).

Evaluation of percentage nitrate removal from fish farm effluent by immobilized denitrifying bacterial strain

Fresh effluent samples from an open fish farm located at Al-Menoufiya governorate were collected. The collected raw effluent samples were immediately transported to the environmental laboratory and analyzed for water quality parameters.

Collected samples were treated by immobilized denitrifying strain, using the suggested optimum condition by RSM based on CCD. Nitrate concentration was measured after biotreatment in the collected samples, and the nitrate bioremediation percentage was calculated using Equation (1). When the raw effluent served as the control, all experiments were performed in triplicate.

The free and immobilized strain was stored at 4 °C in saline solution for 2 months and used for treating the collected fish farm samples. The storage, reusability activity and bioremediation percentage was determined every week.

Table 1 | Independent variables factors and the levels of the variables for designing the central composite

			Coded actual levels				
Independent variables factors	**Unit**	**codes**	**Minimum $-\alpha$**	**Low level (−1)**	**Medium level (0)**	**High level (1)**	**Maximum $+\alpha$**
Bacterial inoculum concentration	g/L	A	1	1.5	2	2.5	3.0
Sodium alginate concentration	–	B	0.5%	1.0%	1.5%	2.0%	2.5%
Nitrate concentration	mg/L	C	200	400	600	800	1,000
pH	–	D	3.0	5.0	7.0	8.0	9.0
Temperature	°C	E	10	15	25	35	45

α value = 2.00 for CCD in the case of five independent variables.

RESULT AND DISCUSSION

Isolation of nitrate removal bacteria

The consortium from the collected wastewater sample was spread after enrichment separately on DM agar amended with 500 mg/L of nitrate. On the basis of morphology, nine different colonies were obtained. Further selection for these colonies was made on the basis of nitrate reduction test. Nitrate test with isolated strains is shown in Figure 2. The strong nitrate reduction ability appeared with isolate named (S6), which reduced nitrate to nitrogen gas, and was therefore selected for their nitrate removal efficiency for further studies. Isolate with name (S2a) reduced nitrate to nitrite, and the solution changed to a pink colour, as shown in Figure 2. The other strains gave no change in colour when reagents A, B or the zinc powder were added, suggesting that nitrate reduction did not occurred. Positive results appear red, representing nitrate reduction to nitrite ($NO_3 \rightarrow NO_2$). Negative results showed no colour change and the test needed to be completed by adding zinc dust. After the addition of zinc dust, a positive result showed appear no colour, suggesting nitrate reduction nitrite to gaseous or non-gaseous nitrogen compounds. A negative results shows red colour and suggests no nitrate reduction (Marietou *et al.* 2009).

Immobilization for the active nitrate removal bacteria

The final bacterial beads appeared after they were washed several times in distilled water to remove any calcium chloride residue, as shown in Figure 3.

Identification of strain

Partial sequence of isolate S6 based on 16S rRNA gene sequence with (1,527 bp) was obtained and submitted to the GenBank. The accession number MT364822.1 was obtained. Analysis using BLAST indicated that strain *P. putida* MT364822.1 (http://www.ncbi.nlm.nih.gov/MT364822.1), with excellent nitrate removal ability isolate during the study, was closely related to *Pseudomonas sp*. There are several studies about denitrifying species such as *Pseudomonas sp*., which can be isolated from canals, ponds, soils and activated sludge, that have the ability to utilize nitrate (Kesserű *et al.* 2003; Wu *et al.* 2012b). A phylogenetic tree was constructed on the basis of the gene sequences 16S rRNA of the isolate, with other reference sequences shown in Figure 4, and the result showed that *P. putida* MT364822.1 (S6) belongs to *P. putida* species.

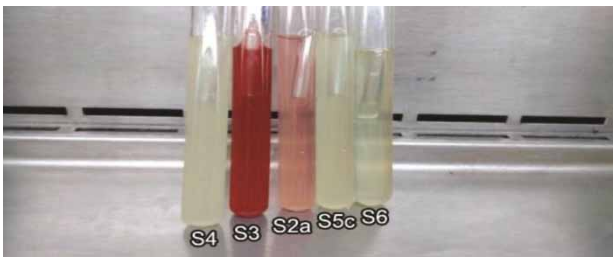

Figure 2 | Photo showing nitrate test after adding reagents (a) and (b).

Figure 3 | Immobilized active nitrate removal bacteria beads (digital camera).

CCD modeling

RSM based on CCD was employed for studying interaction effects between five independent variables to optimize the nitrate bioremediation process. The variables were tested at five different levels $-\alpha$(minimum), -1(low), 0(central), $+1$(high) and $+\alpha$(maximum) acquired from preliminary experiments in this study, as shown in Table 1.

According to the experimental CCD design, a total of 32 runs were carried out, and the results of the statistical plan and response procedures are summarized in Table 2. Results showed that the efficacy of nitrate removal in five different levels of the five variables was different. According to the results of the experimental CCD study, the highest and lowest percentages of nitrate removal by immobilized *P. putida* MT364822.1 strain, were 90.94% and 57.81%, respectively, and were obtained in experiment run no. 26 and 9, respectively. This may be attributed to the change in variables, which results in different interactions that affect the removal efficiency, allowing them to become an effective tool for optimizing the removal process. In addition, high accuracy of the model was estimated from a

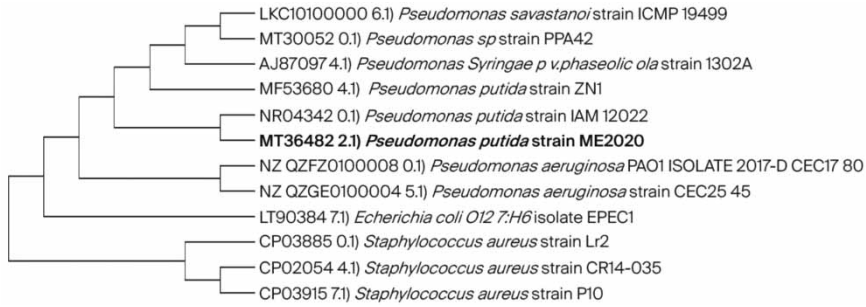

Figure 4 | Phylogenetic tree based on the 16S rRNA gene sequence of *P. putida* S6 (MT364822.1) and other reference sequences.

Table 2 | CCD for five variables with experimented coded and predicted response (nitrate removal %)

						Nitrate removal %	
No.	**A**	**B**	**C**	**D**	**E**	**Experimented**	**Predicted**
1	0.00	−1.00	0.00	−2.00	1.00	73.71	72.66
2	0.00	0.00	0.00	0.00	−1.00	70.41	71.83
3	−2.00	0.00	1.00	0.00	2.00	69.22	70.09
4	0.00	0.00	0.00	0.00	1.00	81.66	80.39
5	1.00	1.00	0.00	0.00	−1.00	73.45	74.06
6	1.00	0.00	0.00	2.00	1.00	83.96	82.88
7	1.00	0.00	0.00	−1.00	0.00	74.61	73.87
8	−1.00	0.00	0.00	1.00	0.00	82.53	81.77
9	0.00	−1.00	1.00	0.00	−1.00	56.55	57.81
10	1.00	−1.00	0.00	0.00	−1.00	65.33	64.77
11	2.00	0.00	1.00	0.00	0.00	87.86	88.11
12	0.00	−1.00	0.00	1.00	1.00	65.73	66.21
13	−1.00	1.00	0.00	0.00	0.00	75.14	76.33
14	0.00	0.00	1.00	−1.00	1.00	75.81	74.79
15	1.00	0.00	−2.00	0.00	0.00	86.60	87.10
16	0.00	1.00	0.00	−1.00	−2.00	72.82	73.55
17	0.00	0.00	1.00	1.00	0.00	78.01	77.31
18	−1.00	−1.00	0.00	0.00	1.00	60.47	61.55
19	0.00	0.00	−1.00	−1.00	0.00	77.84	78.33
20	0.00	−2.00	−1.00	0.00	1.00	68.30	69.40
21	0.00	0.00	−1.00	1.00	0.00	86.11	87.35
22	0.00	0.00	0.00	0.00	1.00	72.59	73.00
23	0.00	1.00	2.00	0.00	−1.00	77.66	76.41
24	−1.00	0.00	−1.00	0.00	0.00	84.32	83.44
25	0.00	2.00	−1.00	0.00	−1.00	75.77	74.71
26	2.00	1.00	0.00	0.00	1.00	90.77	90.94
27	−1.00	0.00	0.00	−1.00	1.00	75.96	74.73
28	−1.00	0.00	−1.00	0.00	0.00	84.32	83.88
29	0.00	2.00	−1.00	0.00	−1.00	75.77	76.37
30	0.00	1.00	0.00	1.00	1.00	89.77	90.10
31	−2.00	0.00	0.00	−2.00	1.00	75.96	76.44
32	2.00	−2.00	0.00	0.00	−2.00	85.77	86.36

small difference between the actual and predicted efficiency in estimating nitrate removal efficiency (response variable).

Second-order polynomial equation and a quadratic model were used for express the relationship between independent variables and response values. Accordingly, the regression model was well-fitted and the efficacy of nitrate removal by immobilized *P. putida* MT364822.1 strain for the significant variables was obtained from the following regression Equation (2):

$$\begin{aligned}\text{Removal}\,\% = {} & 66.61 + 1.708\,A + 9.49\,B - 8.61\,C + 6.16\,D \\ & + 3.03\,E + 2.55\,A{*}A + 0.24\,B{*}B + 2.595\,C{*}C \\ & + 4.157\,D{*}D + 7.87\,E{*}E + 2.28\,A{*}B \\ & + 6.44\,A{*}C - 5.46\,A{*}D + 5.55\,A{*}E \\ & + 6.07\,B{*}C - 5.48\,B{*}D + 7.30\,B{*}E \\ & + 3.73\,C{*}D + 0.20\,C{*}E - 9.88\,D{*}E\end{aligned}$$

In the above equation, A is the immobilized bacterial beads (mg), B is sodium alginate concentration (%), C is nitrate concentration (mg/L), D is the pH and E is the temperature (°C).

The second-order polynomial equation is widely used in RSM because it is very flexible, it can take on a wide variety of functional forms, so it will work well as an approximation to the true response surface; it is easy to estimate the parameters (β) in the second-order model and there is evidence that second-order models work well in solving real response surface problems (Carley *et al.* 2004).

Model validation and statistical analysis

ANOVA analysis

The results of experiments were subjected to variance analysis (ANOVA) to validate the model and variance analysis. The significance of each coefficient in the model was determined using the F-test and probability of error (*P*-values) for ANOVA at a 95% confidence. Experimental design was significant when *P*-value <0.05 while *P*-values >0.1 indicated that the model terms were not significant (Moradi *et al.* 2016). The resulting ANOVA on this model are listed in Table 3. Based on results of *P*-values, the model was highly significant, as shown in the (0.000) *P*-value in the regression. Moreover, first-order effects of all five variables (A, B, C, D and E) were significant for responses process. The *P*-values were larger than (0.05) for the model interaction, B*B (0.825), A*B (0.316), C*D (0.057) and C*E (0.925), which represented the insignificance of these terms in nitrate removal efficiency by immobilized *P. putida* MT364822.1.

Lack-of-Fit test coefficient was created to determine whether the selected model was sufficient to describe the observed data or whether a more complicated model should be used. The test would be significant if the model was not well-fitted (Wu *et al.* 2012a). The high *P*-value for lack-of-Fit in this model (0.762) was not significant, thus implying that the model was valid and significant to demonstrate the correlation between the factor variables and response process (Jafari *et al.* 2014). The F-value of the model was 13.69, which indicated the significance of the model. Also, the fitting of selected models to the experimental data was tested by calculating the determination regression coefficient (R^2). The high value of determination regression coefficient ($R^2 = 0.9614$) represented a good correlation between the experimental values and predicted response, as shown in Figure 2. The model will be stronger when the determination regression coefficient (R^2) becomes nearer to one (Jaafari & Yaghmaeian 2019). The significance of the model was also confirmed by the adjusted R^2 and predicted R^2. The adjusted regression coefficient (R^2) for the removal efficiency model (0.8912) was in reasonable agreement with determination regression coefficient R^2 value. The predicted regression coefficient (R^2) was estimated 0.7831 and revealed that the predicted response could be well calculated by the model. Therefore, there was a satisfactory match between the adjusted regression coefficient of the experiment and the predicted regression coefficient of the model in this study.

Interpretation of residual graphs

Normal probability plot of the residuals is a graphical plotting technique for assessing the normal distribution of data (Chambers *et al.* 1983). If the points on the plot fall fairly close to the straight line, then the data are normally distributed, and some scatter is to be expected (Pokhrel & Viraraghavan 2006). Figure 5(a) plots the residuals values against the predicted response. Residuals appeared scattered randomly around zero, suggesting that errors have a constant variance. Predicted versus experimental values were plotted and are shown in Figure 5(b) where shows a good correlation and agreement between the experimental values and predicted response in which all the points are close to the diagonal line. Figure 5(c) shows normal probability plot of residual values. Experimental points appear arranged reasonably, suggesting normal distribution. In Figure 5(d) the residuals are plotted

Table 3 | ANOVA for the quadratic polynomial mode of CCD results for nitrate removal by immobilized *P. putida*

Source	DF	Sum of square	Mean of square	F-value	*P*-value	Significant
Model	20	2,057.84	102.892	13.69	0.000	Significant
Linear	5	709.76	141.953	18.89	0.000	Significant
A	1	30.46	30.464	4.05	0.049	Significant
B	1	582.68	582.683	77.53	0.000	Significant
C	1	482.84	482.845	64.25	0.000	Significant
D	1	154.30	154.296	20.53	0.001	Significant
E	1	57.07	57.066	7.59	0.019	Significant
Square	5	383.68	76.737	10.21	0.001	Significant
A*A	1	46.47	46.468	6.18	0.030	Significant
B*B	1	0.39	0.388	0.05	0.825	Insignificant
C*C	1	85.51	85.511	11.38	0.006	Significant
D*D	1	141.85	141.852	18.87	0.001	Significant
E*E	1	139.15	139.147	18.51	0.001	Significant
2-Way interaction	10	966.59	96.659	12.86	0.000	Significant
A*B	1	8.31	8.306	1.11	0.316	Insignificant
A*C	1	184.42	184.415	24.54	0.000	Significant
A*D	1	120.27	120.267	16.00	0.002	Significant
A*E	1	41.13	41.135	5.47	0.039	Significant
B*C	1	114.02	114.020	15.17	0.002	Significant
B*D	1	39.75	39.753	5.29	0.042	Significant
B*E	1	119.39	119.393	15.89	0.002	Significant
C*D	1	33.91	33.909	4.51	0.057	Insignificant
C*E	1	0.07	0.069	0.01	0.925	Insignificant
D*E	1	115.76	115.759	15.40	0.002	Significant
Error	11	82.67	7.515			
Lack-of-fit	7	41.04	5.863	0.56	0.762	
Pure error	4	41.63	10.408			
Total	31	2,140.51				
R^2		0.9614				
Adjusted R^2		0.8912				
Predicted R^2		0.7831				

*$P < 0.01$ highly significant; $0.01 < P < 0.05$ significant; $P > 0.05$ not significant.

against the order of runs used in the design. Residuals seem to be scattered randomly around zero. Except for two point (runs number 4 and 22 having a residual value of 4.9 and −5 respectively), all other points were found to fall in the critical alpha value ranges of +2 to −2. A histogram of the residuals plots used to demonstrate the distribution of the residuals for all observations are shown in Figure 5(e). Errors were distributed normally with zero and this shows a symmetrical histogram (Ranjan *et al.* 2009). A Pareto chart ranks the effect of effective variables on nitrate removal process (response) as in Figure 5(f). As shown in Figure 5(f), sodium alginate concentration and nitrate concentration are the most important parameters for nitrate removal efficiency, followed by pH, temperature and finally the bacterial inoculum, where A is the bacterial beads volume, B is the sodium alginate concentration, C is the nitrate concentration, D is the pH and E is the temperature.

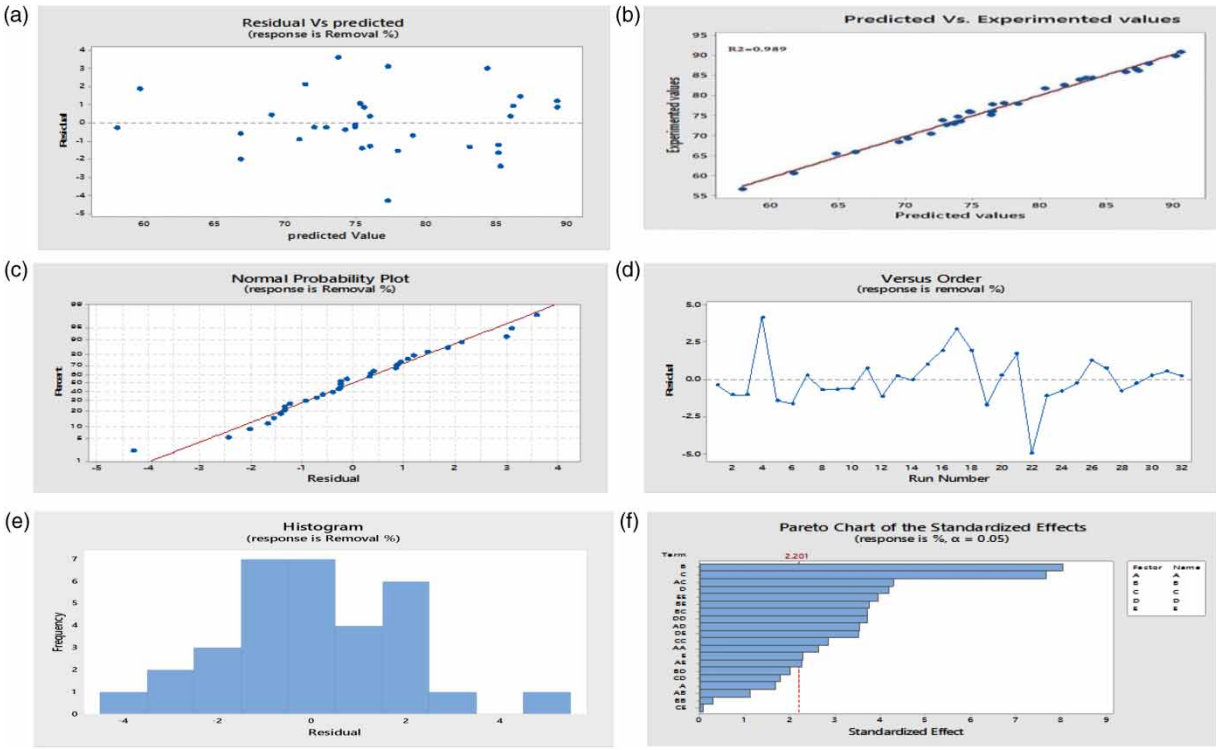

Figure 5 | Residual graphs of experimental data against the predicted values for nitrate removal using immobilized *P. putida* by RSM-based CCD. (a) residual vs. predicted, (b) predicted vs. expermintal values, (c) normal plot of residual, (d) residual vs. order of the data, (e) histogram of the residuals, (f) Pareto chart.

Three-dimension graphical plot and interaction effects of variables on nitrate removal

To demonstrate the interaction effects between variables gained by solving second-order polynomial equation, three-dimensional (3D) plots were created (Asadzadeh *et al.* 2018) and shown in Figure (6), where (a) bacterial beads volume and sodium alginate; (b) bacterial inoculum and nitrate concentration; (c) pH and bacterial concentration; (d) temperature and bacterial inoculum concentration; (e) sodium alginate concentration and nitrate concentration; (f) pH and sodium alginate concentration; (g) sodium alginate and temperature; (h) nitrate concentration and pH; (i) temperature and nitrate concentration; and (j) pH and temperature of 35° in efficacy of nitrate removal by immobilized *P. putida* MT364822.1.

The independent variable interactions are illustrated by 3D surface plots, derived from the quadratic polynomial model, as shown in Figure 6(a)–6(j).

The effect of bacterial beads volume. The study was performed using different volumes of immobilized bacterial cells, the results of which are shown in Figure 6(a)–6(c). Figure 6(a) shows the interaction between bacterial inoculum and sodium alginate concentration on the nitrate removal rate. As expected, there is an increase in the nitrate removal rate with an increase of bacterial beads. The maximum nitrate removal rate was achieved by increasing the bacterial beads range to 3 g. These results are in agreement with previous studies that have reported that the removal rate was dependent on bacterial beads volume and maximum nitrate removal, obtained by increasing bacterial concentration (Shawky *et al.* 2015). Also, it was observed that higher nitrate removal was achieved when sodium alginate concentration was 2%. Figure 6(b) illustrates the interaction of bacterial beads volume and nitrate concentration on the removal process. As shown in the 3D plots, increasing nitrate concentration up to 600 mg/L gradually decreases the removal process. 3D plots in Figure 6(c) show the combined effect of pH and bacterial concentration. The removal rate was about 90.9% when increasing pH to 7.0 and increasing the bacterial concentration balls to 3 g.

The effect of sodium alginate concentration. Figure 6(e) shows the interaction effect of the sodium alginate concentration and nitrate concentration on the removal rate. As the sodium alginate increases from 1.5 to 2%, the beads

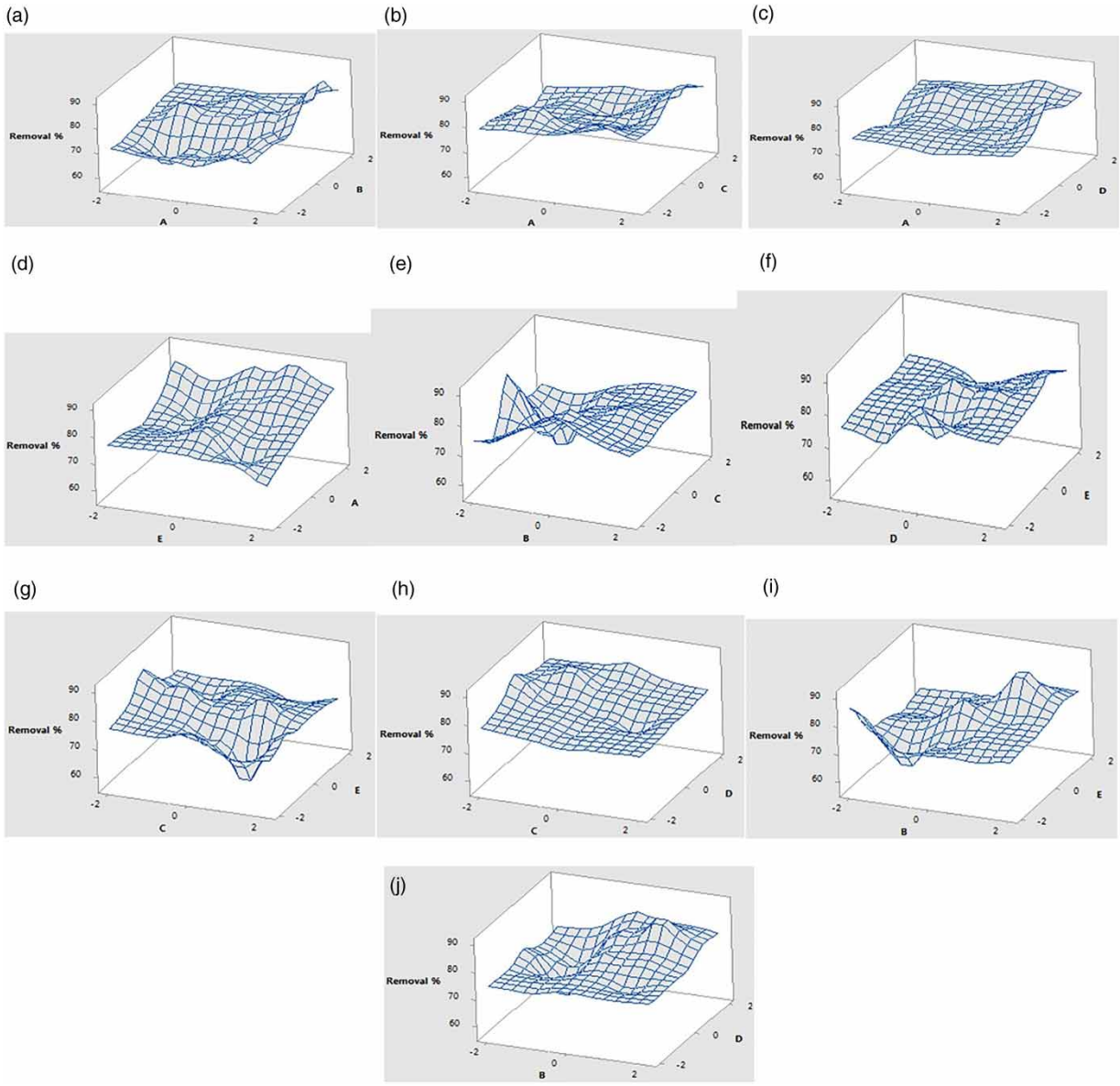

Figure 6 | Graphical interaction between variables by 3D responses surfaces.

showed a better transparency and hardness, and so the removal efficiency of bacterial isolate increased, with the highest removal efficiency at 87.86% and 90.7%. A study by Dong *et al.* (2017) demonstrated that as sodium alginate increased from 0.8% to 1.5%, the removal efficiency increased because the immobilized balls were transparent and soft, which affected positively on the removal efficiency. However, when the concentrations were higher than 2%, the immobilized balls were opaque, hard, and trailing and negatively affected the removal efficiency. Results and studies suggest that the concentration of sodium alginate affects the hardness of immobilized cells. The strength of immobilized cells increases as the concentration of sodium alginate increases. However if the concentration was too high, immobilized cells would not grow, and if the concentration was too low, immobilized cells would break.

Figure 6(g) depicts the effects of sodium alginate and temperature. From the curve, it could be found that the pattern of response increased with a sodium alginate increase from 1.0 to 2.0% and temperature increase from 25 to 35 °C.

The effect of nitrate concentration. The denitrification abilities of microorganisms can be enhanced by gradually exposing them to higher concentrations of nitrate.

Adaptation of a microbial community towards toxic or recalcitrant compounds is useful in improving the rate of bioremediation process (Dafale *et al.* 2010). As shown in Figure 6(h), nitrate concentration of 600 mg/L showed gradual decrease denitification ability of *P. putida* MT364822.1, with a nitrate removal of 56%. There was also an increase in the response process with an increase in initial nitrate concentration from 200 to 400 mg/L. Similar results were reported by Chen *et al.* (2015), which reported that the denitrification rate increased with initial nitrate concentration (100–350) mg/L and decreased at high nitrate loading (550–600) mg/L. Finally, nitrate removal efficiency was found to be inhibited at high nitrate loading (800–1,000 mg/L). In addition, Figure 6(i) shows the mutual effect of temperature and nitrate concentration on nitrate removal, with the highest efficiency at 400 mg/L and temperature of 35 °C.

The effect of temperature. Temperature is another influential important environmental factor for bacterial growth and their survival (He *et al.* 2018). The effect of simultaneous variations of pH and temperature on nitrate removal was studied, with the highest efficiency at pH 7 and temperature of 35 °C, as shown in Figure 3(j). It can be concluded from the 3D graphs in Figure 6(d) that increasing temperature leads to a linear increase in nitrate removal process in the model. A study by Munna *et al.* (2015) reported that *P. putida* is able to grow and conduct aerobic denitrification, with a nitrate removal of 82% at 35 °C, while growth declined at lower temperatures (25 °C). However, Wu *et al.* (2012b) evaluated that at 45 °C, the nitrate removal was almost completely reduced. At a temperature of 20 °C, the cell activity for nitrate removal was inhibited. Ji *et al.* (2015) reported that the optimum temperature for denitrification process was ranged from 25 to 37 °C, which is generally the optimum temperature for bacterial activity.

The effect of soluble pH. pH had a great effect on nitrate removal and also on bacterial growth, and is one of the leading factors that can influence bacterial ability in reducing efficiency (Zhang *et al.* 2011). Figure 6(f) shows the changing parameter of pH and sodium alginate concentration on nitrate removal yield. The optimum pH value is 7 and nitrate removal rate was gradually decreased when the pH increased to 9.0, with the removal percentage about 60.0%. These findings are confirmed by Cai *et al.* (2015) who concluded that neutral conditions enhanced denitrification rate. Glass & Silverstein (1998) also reported that optimum pH for denitrification was close to 8.0, while acidic environments (pH <5) inhibited denitrification and prevented the denitrification chain (Brady & Weil 2002). Burghate & Ingole (2017), however, reported that there was no significant effect of pH between 7.0 and 8.0 on the denitrification rate.

Prediction for optimum condition of nitrate removal. Based on the above findings and confirming the model's accuracy the optimum values for the independent variables and nitrate removal efficiency (responses) were obtained using the response optimizer in Minitab 18 program. Maximum efficacy of nitrate removal using immobilized *P. putida* MT364822.1 was predicted to be 91.1% when optimal conditions were pH 7, temperature 35 °C, nitrate concentration 400 mg/L, sodium alginate 2% and bacterial beads 3 g/L.

Analytical assessment of immobilized *P. putida* MT364822.1 for application in nitrate bio-removal from raw fish farm effluent

Water quality parameters of raw fish farm effluent are summarized in Table 4. The applicability of effective immobilized *P. putida* MT364822.1 and pure strain on nitrate removal from fish farm was estimated. Optimum conditions suggested by the CCD design were used. The pure strain had a faster removal rate of nitrate concentration than the immobilized *P. putida* MT364822.1 beads, which reached 89% and 82%, respectively, during the first 24 h of biotreatment (Figure 7).

Storage and reusability of immobilized *P. putida* MT364822.1

One of the main objectives of the immobilization is to extend the storage time of free cells. Therefore, storage stability and reusability of the immobilized system is very important for successful biotreatment and for the industrial application. As shown in Figure 8, the immobilized *P. putida* MT364822.1 was quite stable when stored at 4 °C for 8 weeks and showed the strength of the immobilized strain in repeated use for eight cycles, with retained activity up to 82%. However, the free strain was completely active for 2 weeks, after which the activity decreased about 19–55% after storage for 3–8 weeks. This enhanced stability of immobilized cells is probably a result of autolysis prevention by immobilization so the immobilized *P. putida* MT364822.1 had a longer removal of nitrate pollution (Yan *et al.* 2019). Various reports have discussed the high operational stability of immobilized strains (Lu *et al.* 2012).

Table 4 | Water quality parameters of raw fish farm effluent

Sample code	Fish farm	
Physicochemical parameters		
Temperature	°C	28
Odour	–	Fishy
Colour	–	Normal
Total suspended solids	mg/L	70.11
pH	–	7.4
Carbonate CO_3^{-2}	mg/L	0.00
Bicarbonate HCO_3^-	mg/L	433.9
Electrical conductivity (EC)	ms/cm	0.518
Total dissolved solids (TDS)	mg/L	238.62
Ammonia NH_3	mg/L	0.73
Major cations		
Calcium Ca^{+2}	mg/L	59
Magnesium Mg^{+2}	mg/L	69.42
Sodium Na^+	mg/L	57.68
Potassium K^+	mg/L	74
Major anions		
Chloride Cl^-	mg/L	18.8
Nitrite NO_2	mg/L	20
Nitrate NO_3^-	mg/L	68
Phosphate PO_4^{-3}	mg/L	0.15
Sulfate SO_4^{-2}	mg/L	8.15
Organic parameters		
Biological oxygen demand	mg/L	5
Chemical oxygen demand	mg/L	7
Dissolved oxygen	mg/L	3.5
Microbiological parameters		
Total bacterial count 37 °C	(CFU/mL)	9×10^2
Total bacterial count 22 °C	(CFU/mL)	38×10^3

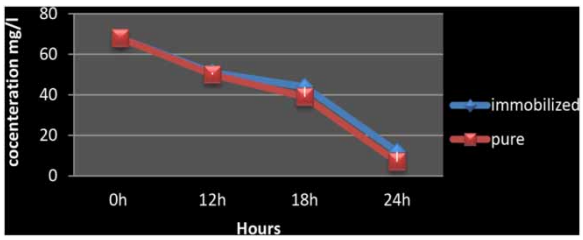

Figure 7 | Nitrate concentration (mg/L) from fish farm sample treated by effective immobilized *P. putida* MT364822.1 and pure strain.

Majeau *et al.* (2010) discussed that free strains are not stable under normal storage conditions, because when at room temperature or under refrigeration they lose their activity gradually over time. This instability is because protein may be denatured over time, but this can be resolved by immobilization (Leonowicz *et al.* 1988). Stability of the immobilized strain may be due to the improved stabilization of its active conformation and lower flexibility of the strain in the immobilized form (Lonappan *et al.* 2018).

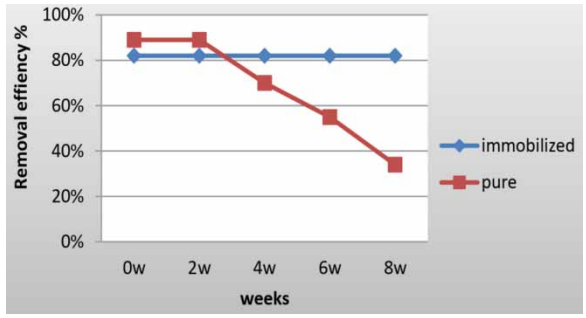

Figure 8 | Storage and reusability of immobilized *P. putida* MT364822.1 and free cells.

Mogharabi *et al.* (2012) studied the reusability of the entrapped strains and the results showed that 85% of the activity was retained after five successive cycles. Crestini *et al.* (2010) found that immobilized strains retained 68% of its activity for ten cycles. Reusability of the immobilized strains was suggested where the decrease in removal efficiency by immobilized strain during recycling could be due to detachment of the immobilized bacteria in the washing step (Bryjak *et al.* 2007; San *et al.* 2014).

CONCLUSION

The current study showed that the immobilization technique for certain species of bacteria was perfect for controlling nitrates in aqueous solution. The high removal efficiencies of nitrate by immobilized *P. putida* MT364822.1, which were isolated during the study from the Sabal Drain, represented the denitrification ability of this strain. RSM experimental design based on CCD was effective and was used to obtain the operational conditions for the maximum removal rates of nitrate by immobilized *P. putida* MT364822.1. In this study, the effective parameters in the CCD that supported optimal bacterial activity for nitrate removal in liquid medium included pH 7, temperature 35 °C, nitrate concentration 400 mg/L, bacterial beads volume 3 g/L and sodium alginate concentration 2%. Furthermore, the model was presented as 3D response surface plot to investigate the optimum zone of nitrate removal. Nitrate concentration removal from raw fish farm samples

over 24 h reached 89% and 82.2% by bacterial solution and immobilized *P. putida* MT364822.1 beads, respectively. The removal efficiency of the pure strain decreased after storage for 3–8 weeks. Storage and reusability experiments showed the highest operational stability of immobilized *P. putida* MT364822.1 for eight cycles with retained activity up to 82.2%. It is therefore suggested that immobilized *P. putida* MT364822.1 is an excellent bioremoval for nitrate in wastewater.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Abdel-Khalek, A. A., Elhaddad, E., Mamdouh, S. & Marie, M. A. S. 2018 The chronic exposure to discharges of Sabal drain induces oxidative stress and histopathological alterations in Oreochromis niloticus. *Bulletin of Environmental Contamination and Toxicology* **101** (1), 92–98.

Abdullateef, B., Garba, S. T. & Bello, H. S. 2020 Bioremediation potential of immobilized *Corynebacterium kutsceri* in the treatment of tannery industrial effluent from Challawa Industrial Estate, Kano State, Nigeria. *Journal of the Turkish Chemical Society Section A: Chemistry* **7** (2), 335–350.

Acharya, S., Sharma, S. K., Chauhan, G. & Shree, D. 2018 Statistical optimization of electrocoagulation process for removal of nitrates using response surface methodology. *Indian Chemical Engineer* **60** (3), 269–284.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

American Public Health Association (APHA), American Water Works Association & Water Environment Federation 2012 *Standard Methods for the Examination of Water and Wastewater*, 22nd edn. American Public Health Association, Washington, DC.

Angar, Y., Djelali, N. E. & Gana, S. K. 2016 Removal of nitrates in wastewaters. *Revista De Chimie* **67** (7), 1356–1359.

Asadzadeh, F., Maleki-Kaklar, M., Soiltanalinejad, N. & Shabani, F. 2018 Central composite design optimization of zinc removal from contaminated soil, using citric acid as biodegradable chelant. *Scientific Reports* **8** (1), 1–8.

Baş, D. & Boyacı, I. H. 2007 Modeling and optimization I: usability of response surface methodology. *Journal of Food Engineering* **78** (3), 836–845.

Bouabidi, Z. B., El-Naas, M. H. & Zhang, Z. 2019 Immobilization of microbial cells for the biotreatment of wastewater: a review. *Environmental Chemistry Letters* **17** (1), 241–257.

Brady, N. & Weil, R. 2002 *The Nature and Properties of Soils*, 13th edn. Prentice Hall International, New Jersey.

Bryjak, J., Kruczkiewicz, P., Rekuć, A. & Peczyńska-Czoch, W. 2007 Laccase immobilization on copolymer of butyl acrylate and ethylene glycol dimethacrylate. *Biochemical Engineering Journal* **35** (3), 325–332.

Burghate, S. & Ingole, N. 2017 Bio-removal of nitrate from wastewater by FBBR. *International Journal of Environment and Waste Management* **19** (4), 281–296.

Cai, C., Hu, S., Guo, J., Shi, Y., Xie, G. J. & Yuan, Z. 2015 Nitrate reduction by denitrifying anaerobic methane oxidizing microorganisms can reach a practically useful rate. *Water Research* **87**, 211–217.

Carley, K. M., Kamneva, N. Y. & Reminga, J. 2004 *Response Surface Methodology: CASOS Technical Report*. Center for Computational Analysis of Social and Organizational Systems, Carnegie Mellon University, Pittsburgh, PA.

Chambers, J. M., Cleveland, W. S., Kleiner, B. & Tukey, P. A. 1983 *Graphical Methods for Data Analysis*. Wadsworth & Brooks. Cole Statistics/Probability Series, Heidelberg, Germany.

Chen, Y., Zhao, L., Liu, B. & Zuo, S. 2012 Application of response surface methodology to optimize microwave-assisted extraction of polysaccharide from Tremella. *Physics Procedia* **24**, 429–433.

Chen, D., Yang, K. & Wang, H. 2015 High nitrate removal by autohydrogenotrophic bacteria in a biofilm-electrode reactor. *Desalination and Water Treatment* **55** (5), 1316–1324.

Crestini, C., Crucianelli, M., Orlandi, M. & Saladino, R. 2010 Oxidative strategies in lignin chemistry: a new environmental friendly approach for the functionalisation of lignin and lignocellulosic fibers. *Catalysis Today* **156** (1–2), 8–22.

Dafale, N., Wate, S., Meshram, S. & Neti, N. R. 2010 Bioremediation of wastewater containing azo dyes through sequential anaerobic–aerobic bioreactor system and its biodiversity. *Environmental Reviews* **18**, 21–36.

DebRoy, S., Das, S., Ghosh, S., Banerjee, S., Chaterjee, D., Bhattacharjee, A., Mukherjee, I. & Ray Chaudhuri, S. 2012 Isolation of nitrate and phosphate removing bacteria from various environmental sites. *OnLine Journal of Biological Sciences* **12**, 62–71. doi:10.3844/ojbsci.2012.62.71.

De Filippis, P., Di Palma, L., Scarsella, M. & Verdone, N. 2013 Biological denitrification of high-nitrate wastewaters: a comparison between three electron donors. *Chemical Engineering* **32**, 319–324.

Dong, Y., Zhang, Y. & Tu, B. 2017 Immobilization of ammonia-oxidizing bacteria by polyvinyl alcohol and sodium alginate. *Brazilian Journal of Microbiology* **48** (3), 515–521.

Eckford, R. & Fedorak, P. 2002 Planktonic nitrate-reducing bacteria and sulfate-reducing bacteria in some western Canadian oil field waters. *Journal of Industrial Microbiology and Biotechnology* **29** (2), 83–92.

Filintas, A., Dioudis, P., Stamatis, G., Hatzopoulos, J. & Karyotis, T. 2008 Environmental assessment of groundwater nitrate pollution from agricultural wastes and fertilizers in central Greece watersheds using remote sensing and GIS. *Paper presented at the Proc. of 3rd International Conference AQUA on: Water Science and Technology with Emphasis on Water & Climate*, 16–19 October, Athens, Greece.

Ghanim, A. 2013 Application of response surface methodology to optimize nitrate removal from wastewater by electrocoagulation. *International Journal of Scientific & Engineering Research* **4** (10), 1410–1416.

Glass, C. & Silverstein, J. 1998 Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Research* **32** (3), 831–839.

He, T., Ye, Q., Sun, Q., Cai, X., Ni, J., Li, Z. & Xie, D. 2018 Removal of nitrate in simulated water at low temperature by a novel psychrotrophic and aerobic bacterium, *Pseudomonas taiwanensis* strain. *Journal of BioMed Research International* **12** (1), 88–97.

Jaafari, J. & Yaghmaeian, K. 2019 Optimization of heavy metal biosorption onto freshwater algae (*Chlorella coloniales*) using response surface methodology (RSM). *Chemosphere* **217**, 447–455.

Jafari, A., Mahvi, A. H., Godini, H., Rezaee, R. & Hosseini, S. S. 2014 Process optimization for fluoride removal from water by *Moringa oleifera* seed extract. *Fluoride* **47** (2), 152–160.

Jafari, S. J., Moussavi, G. & Yaghmaeian, K. 2015 High-rate biological denitrification in the cyclic rotating-bed biological reactor: effect of COD/NO^{3-}, nitrate concentration and salinity and the phylogenetic analysis of denitrifiers. *Bioresource Technology* **197**, 482–488.

Ji, B., Yang, K., Zhu, L., Jiang, Y., Wang, H., Zhou, J. & Zhang, H. 2015 Aerobic denitrification: a review of important advances of the last 30 years. *Biotechnology and Bioprocess Engineering* **20** (4), 643–651.

Jin, Z., Chen, Y., Wang, F. & Ogura, N. 2004 Detection of nitrate sources in urban groundwater by isotopic and chemical indicators, Hangzhou City, China. *Environmental Geology* **45** (7), 1017–1024.

Kesserű, P., Kiss, I., Bihari, Z. & Polyák, B. 2003 Biological denitrification in a continuous-flow pilot bioreactor containing immobilized *Pseudomonas butanovora* cells. *Bioresource Technology* **87** (1), 75–80.

Khuri, A. I. & Mukhopadhyay, S. 2010 Response surface methodology. *Wiley Interdisciplinary Reviews: Computational Statistics* **2** (2), 128–149.

King, A., Jensen, V., Fogg, G. & Harter, T. 2012 Groundwater remediation and management for nitrate. *Technical Report* **5**, 30–40.

Krishnaswamy, U., Muthusamy, M. & Perumalsamy, L. 2009 Studies on the efficiency of the removal of phosphate using bacterial consortium for the biotreatment of phosphate wastewater. *European Journal of Applied Science* **1** (1), 6–15.

Kumar, S. S. & Saramma, A. 2012 Nitrate and phosphate uptake by immobilized cells of *Gloeocapsa gelatinosa*. *Journal of the Marine Biological Association of India* **54**, 119–122.

Kumar, S., Nei, M., Dudley, J. & Tamura, K. 2008 MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* **9** (4), 299–306.

Kumari, S., Rath, P., Sri, H. & Kumar, A. 2016 Chitosan from shrimp shell (*Crangon crangon*) and fish scales (*Labeo rohita*): extraction and characterization. *African Journal of Biotechnology* **15** (24), 1258–1268.

Kunapongkiti, P., Rongsayamanont, C., Nayramitsattha, P., Limpiyakorn, T., Kunapongkiti, P., Rongsayamanont, C. & Limpiyakorn, T. 2019 Application of cell immobilization technology to promote nitritation: a review. *Environmental Engineering Research* **25** (6), 807–818.

Leonowicz, A., Sarkar, J. M. & Bollag, J. M. 1988 Improvement in stability of an immobilized fungal laccase. *Applied Microbiology and Biotechnology* **29** (2), 129–135.

Lim, J. W., Beh, H. G., Ching, D. L. C., Ho, Y. C., Baloo, L., Bashir, M. J. & Wee, S. K. 2017 Central Composite Design (CCD) applied for statistical optimization of glucose and sucrose binary carbon mixture in enhancing the denitrification process. *Applied Water Science* **7** (7), 3719–3727.

Lin, S. H. & Wu, C. 1996 Electrochemical removal of nitrite and ammonia for aquaculture. *Water Research* **30** (3), 715–721.

Lonappan, L., Liu, Y., Rouissi, T., Pourcel, F., Brar, S. K., Verma, M. & Surampalli, R. Y. 2018 Covalent immobilization of laccase on citric acid functionalized micro-biochars derived from different feedstock and removal of diclofenac. *Chemical Engineering Journal* **351**, 985–994.

Lu, L., Xing, D. & Ren, N. 2012 Pyrosequencing reveals highly diverse microbial communities in microbial electrolysis cells involved in enhanced H_2 production from waste activated sludge. *Water Research* **46** (7), 2425–2434.

Majeau, J. A., Brar, S. K. & Tyagi, R. D. 2010 Laccases for removal of recalcitrant and emerging pollutants. *Bioresource Technology* **101** (7), 2331–2350.

Mansouri, Y., Zinatizadeh, A. A., Mohammadi, P., Irandoust, M., Akhbari, A. & Davoodi, R. 2012 Hydraulic characteristics analysis of an anaerobic rotatory biological contactor (AnRBC) using tracer experiments and response surface methodology (RSM). *Korean Journal of Chemical Engineering* **29** (7), 891–902.

Marietou, A., Griffiths, L. & Cole, J. 2009 Preferential reduction of the thermodynamically less favorable electron acceptor, sulfate, by a nitrate-reducing strain of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* 27774. *Journal of Bacteriology* **191** (3), 882–889.

Massoudinejad, M., Ghaderpoori, M., Shahsavani, A., Jafari, A., Kamarehie, B., Ghaderpoury, A. & Amini, M. M. 2018 Ethylenediamine-functionalized cubic ZIF-8 for arsenic adsorption from aqueous solution: modeling, isotherms, kinetics and thermodynamics. *Journal of Molecular Liquids* **255**, 263–268.

Mogharabi, M., Nassiri-Koopaei, N., Bozorgi-Koushalshahi, M., Nafissi-Varcheh, N., Bagherzadeh, G. & Faramarzi, M. A.

2012 Immobilization of laccase in alginate-gelatin mixed gel and decolorization of synthetic dyes. *Bioinorganic Chemistry and Applications* **15** (2), 90–110.

Moradi, M., Fazlzadehdavil, M., Pirsaheb, M., Mansouri, Y., Khosravi, T. & Sharafi, K. 2016 Response surface methodology (RSM) and its application for optimization of ammonium ions removal from aqueous solutions by pumice as a natural and low cost adsorbent. *Archives of Environmental Protection* **42** (2), 33–43.

Munna, M. S., Zeba, Z. & Noor, R. 2015 Influence of temperature on the growth of *Pseudomonas putida*. *Stamford Journal of Microbiology* **5** (1), 9–12.

Palamakula, A., Nutan, M. T. & Khan, M. A. 2004 Response surface methodology for optimization and characterization of limonene-based coenzyme Q10 self-nanoemulsified capsule dosage form. *AAPS PharmSciTech* **5** (4), 114–121.

Pokhrel, D. & Viraraghavan, T. 2006 Arsenic removal from aqueous solution by iron oxide-coated fungal biomass: a factorial design analysis. *Water, Air, and Soil Pollution* **173** (1–4), 195.

Rakić, T., Kasagić-Vujanović, I., Jovanović, M., Jančić-Stojanović, B. & Ivanović, D. 2014 Comparison of full factorial design, central composite design, and Box–Behnken design in chromatographic method development for the determination of fluconazole and its impurities. *Analytical Letters* **47** (8), 1334–1347.

Ranjan, D., Srivastava, P., Talat, M. & Hasan, S. 2009 Biosorption of Cr(VI) from water using biomass of Aeromonas hydrophila: central composite design for optimization of process variables. *Applied Biochemistry and Biotechnology* **158** (3), 524–539.

San, N. O., Celebioglu, A., Tümtaş, Y., Uyar, T. & Tekinay, T. 2014 Reusable bacteria immobilized electrospun nanofibrous webs for decolorization of methylene blue dye in wastewater treatment. *RSC Advances* **4** (61), 32249–32255.

Sarrai, A. E., Hanini, S., Merzouk, N. K., Tassalit, D., Szabó, T., Hernádi, K. & Nagy, L. 2016 Using central composite experimental design to optimize the degradation of tylosin from aqueous solution by photo-fenton reaction. *Materials* **9** (6), 428.

Shao, Y., Li, W., Yao, C., Qin, G. & Szewczyk, K. W. 2011 Influence of COD/N ratios on simultaneous removal of C and N compounds in biological wastewater treatment in sequencing fed-batch reactor and kinetic analysis. *Journal of Hazardous, Toxic, and Radioactive Waste* **15** (1), 42–47.

Shawky, H., Abdel, G. A., Bayomi, A. & Abdel, M. M. 2015 Removal of ammonia and phosphate from water resources using free and immobilized microalgae. *International Journal of Environment* **4** (3), 193–203.

Stolarzewicz, I., Białecka-Florjańczyk, E., Majewska, E. & Krzyczkowska, J. 2011 Immobilization of yeast on polymeric supports. *Chemical and Biochemical Engineering Quarterly* **25**, 135–144.

Thomas, V., Yallapu, M. M., Sreedhar, B. & Bajpai, S. K. 2013 Fabrication,characterization of chitosan/nanosilver film and its potential antibacterial application. *Journal of Biomaterials Science. Polymer Edition* **20**, 2129–2144.

Tong, S., Chen, N., Wang, H., Liu, H., Tao, C., Feng, C. & Zhao, J. 2014 Optimization of C/N and current density in a heterotrophic/biofilm-electrode autotrophic denitrification reactor (HAD-BER). *Bioresource Technology* **171**, 389–395.

Tripathy, S. K. & Murthy, Y. R. 2012 Modeling and optimization of spiral concentrator for separation of ultrafine chromite. *Powder Technology* **221**, 387–394.

Wakida, F. T. & Lerner, D. N. 2006 Potential nitrate leaching to groundwater from house building. *Hydrological Processes: An International Journal* **20** (9), 2077–2081.

Wu, J., Zhang, H., Oturan, N., Wang, Y., Chen, L. & Oturan, M. A. 2012a Application of response surface methodology to the removal of the antibiotic tetracycline by electrochemical process using carbon-felt cathode and DSA (Ti/RuO_2–IrO_2) anode. *Chemosphere* **87** (6), 614–620.

Wu, Z., Huang, S., Yang, Y., Xu, F., Zhang, Y. & Jiang, R. 2012b Isolation of an aerobic denitrifying bacterial strain from a biofilter for removal of nitrogen oxide. *Aerosol and Air Quality Research* **13** (3), 1126–1132.

Yan, Y., Fu, D. & Shi, J. 2019 Screening and immobilizing the denitrifying microbes in sediment for bioremediation. *Water* **11** (3), 614.

Zhang, J., Wu, P., Hao, B. & Yu, Z. 2011 Heterotrophic nitrification and aerobic denitrification by the bacterium *Pseudomonas stutzeri* YZN-001. *Bioresource Technology* **102** (21), 9866–9869.

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