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Prediction of denitrification capacity of alkalotolerant bacterial isolates from soil – An artificial neural network model

OLJA LJ. ŠOVLJANSKI[1], ANA M. TOMIĆ[1]*, LATO L. PEZO[2], ALEKSANDRA S. RANITOVIĆ[1] and SINIŠA L. MARKOV[1]

[1]*University of Novi Sad, Faculty of Technology Novi Sad, Bulevar cara Lazara 1, Novi Sad, Serbia and* [2]*University of Belgrade, Institute of General and Physical Chemistry, Studenski trg 12/V, Belgrade, Serbia*

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Abstract: In the past decades, the bioremediation process based on denitrification by aerobic heterotrophic bacteria was extensively studied for different engineering approaches. Besides the fact that only non-pathogenic and non-biofilm forming bacteria must be used, it is very important to isolate bacteria or a group of bacteria in nature with the capacity to remove completely nitrate without accumulation of nitrogen oxides or ammonia as intermediates. In this article, the denitrification capacity of 43 bacterial strains isolated from slightly alkaline and calcite soils along the Danube River were investigated by artificial neural network (ANN) modelling. According to the obtained results, an ANN model was developed for the prediction of denitrification capacity of bacterial soil strains based on six signification denitrification indicators: biomass and N_2 gas production, nitrate and nitrite concentration as well as nitrite and ammonia formation. The ANN model showed a reasonably good predictive capability of the outputs (overall R^2 for prediction was 0.958). In addition, the experimental verification of the ANN in laboratory testing indicated that the ANN could predict the denitrification capacity of soil bacteria during the denitrification process in laboratory conditions.

Keywords: nitrogen cycle; denitrification capacity; denitrifying soil bacteria; experimental verification.

INTRODUCTION

Besides the fact that the nitrogen cycle occurs spontaneously in nature as an essential process in biological systems, nitrogen is often the limiting factor and functional relationships within the nitrogen cycle have changed significantly over time.[1] In the past decades, frequent application of nitrogen-based pesticides and fertilizers in agriculture and urban areas, as well as the uncontrolled release of insufficiently treated wastewater, have multiplied the nitrogen concentration in

* Corresponding author. E-mail: anav@uns.ac.rs
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ecosystems. Furthermore, the anthropogenic influence has transcended the biogenic effect of microorganisms in the nitrogen cycle and the deposition of nitrogen component in nature has become a large ecological problem (*e.g.*, eutrophication, deterioration of different materials, health problems, *etc.*).[2] Nitrification and denitrification are major oxidative changes in the nitrogen cycle where microorganisms catalyze the transformations of nitrogen and maintain balance in all ecosystems.[3] To remove nitrogen oxides from nature, one of the sustainable solutions may be a bioremediation technique based on of denitrification which is a highly specific and rapid bioprocess for removing nitrate and nitrite.[4] In bioremediation processes based on denitrification, denitrifying bacteria isolated from contaminated areas and/or denitrifiers which are previously isolated from other locality and marked as effective bioagents can be used for complete nitrate reduction.[5] In addition, it is very important to find a group of bacteria with the capability to completely remove nitrate without accumulation of other nitrogen oxides or ammonia as an intermediate, which is significant for use in bioremediation processes.[2] Denitrification can be defined as the metabolic activity of heterotrophic aerobic bacteria that can reduce the complete amount of nitrates present in a matrix.[1] Between 10 and 15 % of the microbial population in soil, water and sediment can reduce nitrogen oxides.[6] According to the denitrification capacity, members of *Pseudomonas* and *Bacillus* genus are dominant, while *Pseudomonas stutzeri* is marked as a model organism for aerobic denitrification.[7] Moreover, the efficiency of the denitrifying bacteria isolated from different sites was confirmed on the laboratory scale[2,8] as well as in-field application under different conditions.[9–11]

In order to identify the denitrification capacity of newly isolated bacterial strains from the soil and to reveal complex, non-linear relationships in multivariate data, a prediction model was developed in the form of artificial neural networks (ANN).[12,13] ANNs are widely used and accepted as good modelling tools that can rapidly provide empirical solutions to the problems, according to a set of experimental data.[14]

The denitrification capacity of 43 soil bacterial strains was determined by the predictive ANN modelling technique. For the first time, six significant responses of the denitrification process marked as denitrification indicators (biomass and N_2 gas production, nitrate and nitrite concentration as well as nitrite and ammonia formation) were used for developing an ANN model for prediction of denitrification capacity of soil bacteria based on two outputs: the bacterial strain and the incubation time. Within 72 h of the incubation period, all differences between the bacterial strains during the targeted process were compared with the bacterium *P. stutzeri* ATCC 17588, which is well known as a model microorganism for the complete denitrification process.

EXPERIMENTAL

Microorganisms

A total number of 43 bacterial strains (labelled as I_1–I_3, II_1–II_{10}, III_1–III_{20} and IV_1–IV_{10}) previously isolated from slightly alkaline and calcite areas along the Danube River[15] were observed during the denitrification process under laboratory conditions. According to Šovljanski *et al*. (2019),[15] selected bacterial strains, determined as alkalophilic and sporogenic soil bacteria, have wide biokinetic zones for temperature and pH growth value, which is important for bioremediation processes where a specifically adapted bacterial community is required. The denitrifying bacterium *Pseudomonas stutzeri* ATCC 17588 was used as a referent strain for the complete denitrification process, because of its efficiency for the total reduction of nitrogen oxides to molecular nitrogen.[16] All bacterial strains were stored in Nutrient Broth (HiMedia, Mumbai, India) with the addition of glycerol (Lachner, Neratovice, Czech Republic) and kept in a deep-freezer (Snijders Labs, Tilburg, The Netherlands) at −70 °C.

Experiment settings

In order to determine the denitrification capacity, all bacterial strains, as well as the reference were grown overnight on TSA plates (Tryptone Soya Agar, HiMedia, Mumbai India) at 30 °C. The denitrification process was monitored during 72 h at 37 °C through several parameters relevant to the nitrate reduction process: biomass and N_2 gas production, nitrate and nitrite concentration, as well as nitrite and ammonia formation.[2] Additionally, these parameters marked as denitrification indicators the responses of which were used for further statistical analysis. Inoculation of 3 ml nitrate broth (DifcoTM Nitrate Broth, Becton, Dickinson and Company, France) was performed with the addition of 0.2 ml freshly prepared bacterial suspension (approx. concentration 3×10^6 CFU ml^{-1}). Biomass production was monitored through the occurrence of turbidity in the inoculated broths (using McFarland standards), while N_2 gas production was detected by Durham tubes. For detection of the presence of nitrites in the cultivation media, the Griess test was performed, while for semi quantification of nitrate and nitrite concentration, a nitrate and nitrite test strip kit (Quantofix® Nitrate Nitrite, Macherey–Nagel, Duren, Germany) was applied. In order to check ammonia formation, qualitative analyze by the Nessler reagent was performed. The detection limit for the Griess test is 1–2 μM nitrate ions,[17] while for Nessler reagent, it is 1.17 μM ammonia.[18] All analysis was performed every 24 h of the incubation period for all 43 bacterial isolates and for the reference strain *P. stutzeri* ATCC 17588.

Statistical analysis

Mathematical modelling was performed using Statistica 10.0 software (StatSoft Inc., 2010). The independent variables used for the modelling of ANNs were the bacterial strain and the incubation time, while the output variables were previously mentioned denitrification indicators: biomass and N_2 gas formation, nitrates and nitrites concentration (using semi-quantitative methods) and nitrite and ammonia formation (using the qualitative methods for proofing the nitrate/ammonia content).

ANN modelling

A multi-layer perceptron model (MLP), consisting of three layers (input, hidden, and output layer), in predictive model construction was employed. MLPs are widely applied to manage learning problems involving a set of input–output data and used to establish the dependencies between the input and output data. The ANN model is known for its high capability for approximating nonlinear functions.[19,20] In order to improve the behaviour of the ANN

model, both inputs and outputs were normalized before the calculation (min–max normalization strategy was applied, according to the Statistica's default). The data were repeatedly presented to the network during the calculation.[21,22] A series of different ANN topologies were used, in which the number of hidden neurons varied from 1 to 15, and the training process of the network was run 100,000 times with random initial values of weights and biases. The experimental database for the ANN was randomly divided into training (70 % of experimental data), cross-validation (15 %), and testing data (15 %). The cross-validation set was used to test the performance of the network, while training was in progress as an indicator of the level of generalization and the time at which the network has begun to over-train. The testing data set was used to examine the network generalization capability. The Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm was used as an iterative method for solving nonlinear problems, to speed up the convergence and for optimization, during ANN modelling. Coefficients associated with the hidden and the output layers (weights and biases) were grouped in matrices W_1 and B_1; W_2 and B_2, respectively. The obtained neural network can be written using matrix notation:[23-25]

$$Y = f_1(W_2 f_2(W_1 X + B_1) + B_2) \quad (1)$$

where: Y is the matrix of the output variables, f_1 and f_2 are transfer functions in the hidden and output layers, respectively, and X is the matrix of input variables.

The accuracy of the models

The numerical verification of the developed model was tested using commonly used indicators, such as coefficient of determination (R^2), reduced chi-square (χ^2), mean bias error (*MBE*), root mean square error (*RMSE*) and mean percentage error (*MPE*). These parameters were calculated according to Pavlić *et al.*[21]

Global sensitivity analysis

Yoon's interpretation method was used to determine the relative influence of the bacterial strain and the incubation time on the output denitrification indicators.[26] This method was applied based on the weight coefficients of the developed ANN.

Experimental verification of ANN in laboratory testing

In order to demonstrate the accuracy of the ANN model for the output variables prediction of the bacterial strains, the denitrification capacity was tested at three-selected time points (24, 48 and 72 h) during the incubation time. For experimental verification of the ANN model under laboratory conditions, one bacterial strain from all isolation locations (I–IV) was chosen as a group representative.

RESULTS AND DISCUSSION

In order to determine the denitrification capacity of 43 bacterial strains from soils, statistical analysis was conducted (Tables I and II, Fig. 1, Tables III and ,IV and Tables S-I and S-II of the Supplementary material to this paper). In order to register a difference between the bacterial strains from the soil, the complete denitrifying bacteria, *Pseudomonas stutzeri* ATCC 17588 was chosen as the reference. The reference bacterium can reduce a high level of nitrogen oxides within 72 h.[2] The large number of bacteria that are involved in the nitrogen cycle do not have the possibility for total reduction of nitrogen oxide. These incomplete denitrifiers can reduce nitrate to nitrite or ammonia, but not to nitrogen gas,[2] and

therefore is very important to determine the denitrification capacity of bacterial strains from nature. Vidaković *et al.*[2] reported that six significant parameters, named denitrification indicators in this paper, can specify the denitrification capacity of bacterial strains. Accordingly, the denitrification indicators were analysed as responses during the incubation period of the denitrification process and the gained results were compared with those of *P. stutzeri* ATCC 17588.

Although reference strain *P. stutzeri* ATCC 17588 reduced all the nitrate within the incubation period, which could be noticed by all tested indicators, this strain had only one positive response within the first 24 h of the incubation period – formation nitrate.[2] After the initial lag phase, *P. stutzeri* ATCC 17588 showed high efficiency in the transformation of nitrate into nitrogen gas.[2] On the other hand, the bacterial strains from soil followed different denitrification patterns and to analyze the complex data for all 43 tested bacterial isolates, descriptive statistics of the collected data were determined and the results are presented in Table I.

TABLE I. Descriptive statistics of the denitrification process during the experiment; Min. – minimum, Max. – maximum, *SD* – standard deviation

Statistics value	Denitrification indicator					
	Biomass production (McFarland No.)	N_2 gas production (+/–)	Concentration, mg/L		Formation (+/–)	
			Nitrate	Nitrite	Nitrite	Ammonia
Min.	0.000	0.000	50.000	0.000	0.000	0.000
Max.	3.000	1.000	500.000	80.000	1.000	1.000
Average	1.605	0.708	340.000	42.915	0.676	0.023
SD	0.879	0.457	136.739	30.776	0.465	0.151

ANN model

The prediction denitrification capacity of the bacterial strains (I_1–I_3, II_1–II_{10}, III_1–III_{20} and IV_1–IV_{10}) during the incubation period was obtained by an artificial neural network model (ANN). The obtained optimal neural network model showed good predictive capability to fit accurately the experimental data and can be used to anticipate the outputs for a broad range of the input data. The optimal number of neurons in the hidden layer for output variables was 9 (network MLP 45–9–6) to obtain high values of R^2 (during the training cycle R^2 was 0.958) and low error values (sum of squares – *SOS*, Table II).

TABLE II. ANN summary for the observed results; Train. – training cycle of ANN calculation; Test. – testing cycle; Valid. – validation cycle; MLP – multi-layer perceptron model; BFGS – Broyden–Fletcher–Goldfarb–Shanno

Network name	Performance			Error			Training algorithm	Error function	Hidden activation	Output activation
MLP	Train.	Test.	Valid.	Train.	Test.	Valid.	Train.	Test.	Valid.	Train.
45–9–6	0.958	0.851	0.902	532.986	663.023	497.707	0.958	0.851	0.902	532.986

The elements of the matrix W_1 and vector B_1 (presented in the bias row) are given in Table S-I of the Supplementary presents and the elements of matrix W_2 and vector B_2 (bias) for the hidden layer, used for calculation in Eq. (1), are present in Table S-II.

The predicted values were very close to the desired values in most cases, in terms of the R^2 value, for the ANN models. The *SOS* value obtained with the ANN models are of the same order of magnitude as the experimental errors for all six parameters of the denitrification process for the 43 bacterial isolates during the incubation time.

The ANN model predicted the denitrification capacity of the bacterial strains based on the denitrification indicators reasonably well for a broad range of the process variables (as seen in Fig. 1, where the experimentally measured and ANN model predicted values are presented).

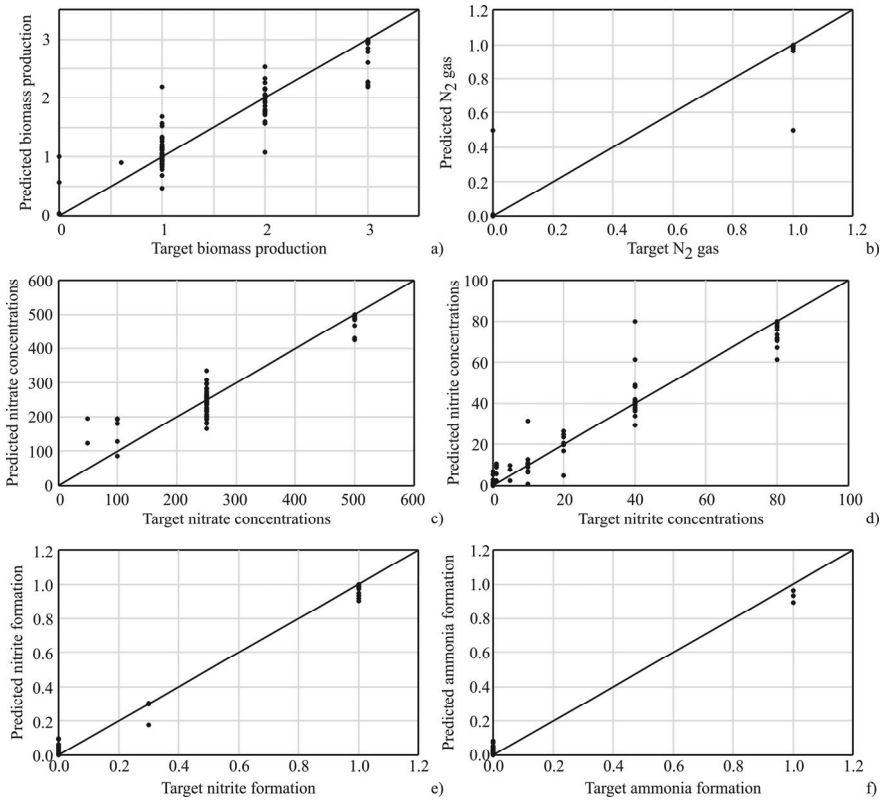

Fig. 1. Experimentally obtained and the ANN model predicted denitrification indicators for all the tested bacterial strains.

The quality of the model fit was tested, and the residual analysis of the developed model was tested using the aforementioned statistical indicators (R^2,

χ^2, *MBE*, *RMSE* and *MPE*), and the results were presented in Table III. According to these results, the ANN model had an insignificant lack of fit tests, which means the model satisfactorily predicted denitrification capacity for all the tested strains during the denitrification process. A high R^2 is indicative that the variation was accounted for and that the data fitted the proposed model satisfactorily.[27–29]

TABLE III. The "goodness of fit" tests for the developed ANN model; χ^2 – reduced chi-square; *MBE* – mean bias error; *RMSE* – root mean square error; *MPE* – mean percentage error; *R*2 – coefficient of determination; Skew – skewness; Kurt – kurtosis; Mean – mean of the residuals; *SD* – standard deviation of the residuals; Var – variance of the residuals

Variable	χ^2	*RMSE*	*MBE*	*MPE*	R^2	Skew	Kurt	Mean	*SD*	Var
Biomass production	0.088	0.292	0.009	11.172	0.889	–0.225	4.022	0.009	0.293	0.086
N_2 gas production	0.004	0.062	0.001	0.454	0.981	–0.034	63.966	0.001	0.062	0.004
Nitrate concentration	1060.2	32.06	0.999	11.39	0.945	–1.030	4.409	0.999	32.165	1034.571
Nitrite concentration	39.486	6.186	0.066	46.760	0.959	–2.225	15.127	0.066	6.210	38.563
Nitrite formation	0.001	0.031	0.003	0.649	0.995	0.231	4.689	0.003	0.031	0.001
Ammonia formation	0.001	0.022	0.001	0.159	0.980	0.139	5.105	0.001	0.022	0.001

The obtained ANN models are complex (474 weights-biases coefficients) because of the high nonlinearity of the developed system. The values R^2 between the experimental and outputs of the ANN model were respectively: 0.889; 0.981; 0.945; 0.959; 0.995 and 0.980, for the biomass and N_2 gas production, nitrate and nitrite concentration, as well as nitrite and ammonia formation. The ANN model had an insignificant lack of fit tests, which means the model satisfactorily predicted the denitrification capacity during the denitrification process for all the tested bacterial strains based on the following denitrification capacity. A high R^2 is indicative that the proposed model satisfactorily explained the experimental results.

Global sensitivity analysis – Yoon's interpretation method

In this section, the influence of two input variables (the bacterial strain and incubation time), on the predicted denitrification indicators was studied. According to the Yoon method, the bacterial strain was the most influential parameter with an approximate relative importance of 60.4 %, while the influence of time was 39.6 %.

Experimental verification of ANN in laboratory testing

In order to test the accuracy of the developed ANN models, experimental verification of the model was performed under laboratory conditions. For verification of ANN models in laboratory testing, two previously untested incubation time points (36 and 60 h) were used for checking the denitrification indicators by the methods already mentioned in this paper (see *Experimental*). The gained experimental results for the chosen bacterial isolates I_3, II_6, III_{11} and IV_1 were compared with predicted results. During the isolation and selection procedure, these bacterial isolates I_3, II_6, III_{11} and IV_1 (as representative of each isolation group) defined as preeminent alkalophilic and sporogenic strains with wide temperature and pH biokinetic zone.[15] According to the obtained results, as shown in Table IV, a few minor differences between the experimental and predicted values were noticed, and these samples were marked with an asterix. Only three aberrations for isolates II_6 and III_1 were noticed within 36-hours incubation period, while the other two isolates followed the predicted patterns for the same sampling time point. In addition, all isolates showed expected denitrification capacity at the second tested sampling time point (60 h). Regarding the incubation time that was explored during the denitrification experiment, the comparative results indicate that the ANN can predict the denitrification capacity of the bacterial strains isolated from soils under laboratory conditions.

TABLE IV. Evaluation of the denitrification capacity and bacterial behaviour of the ANN prediction

Incubation time, h	Selected bacterial strain	Denitrification indicator					
		Biomass production (McFarland No.)	N_2 gas production (+/–)	Concentration, mg/L		Formation (+/–)	
				Nitrate	Nitrite	Nitrite	Ammonia
36 (predicted)	I_3	3.000	1.000	500.000	79.539	1.000	0.009
	II_6	0.298	1.000	329.211	46.545	0.589	0.008
	III_1	2.999	1.000	500.000	79.828	1.000	0.000
	IV_1	3.000	1.000	500.000	79.989	0.128	0.005
36 (experimental)	I_3	3	1	500	80	1	0
	II_6	0	1	250[a]	40	0[a]	0
	III_1	2[a]	1	500	80	1	0
	IV_1	3	1	500	80	0	0
60 (predicted)	I_3	3.000	1.000	500.000	80.000	1.000	0.010
	II_6	0.017	0.832	64.527	1.854	0.012	0.000
	III_1	2.992	1.000	499.999	71.656	1.000	0.006
	IV_1	3.000	1.000	489.410	79.479	1.000	0.261
60 (experimental)	I_3	3	1	500	80	1	0
	II_6	0	1	50	1	0	0
	III_1	3	1	500	80	1	0
	IV_1	3	1	500	80	1	0

[a]The experimental value is different relative to the predicted value

CONCLUSIONS

The developed ANN empirical model for 43 bacterial strains isolated from soil gave a good fit to experimental data and was able to predict successfully the denitrification capacity based on six significant denitrification indicators: biomass and N_2 gas production, nitrate and nitrite concentration, as well as nitrite and ammonia formation. In spite of the high number of different bacterial strains, the artificial neural network models showed a reasonably good predictive capability (during the training cycle R^2 was 0.958). Furthermore, the ANN experimental verification under laboratory conditions showed that the selected mathematical model could predicate denitrification capacity during the targeted reduction process for all tested bacterial strains. The developed mathematical models provided adequate precision for a practical study under laboratory conditions and scale-up processes for a wide range of laboratory and industrial applications, where using denitrifying bacteria isolated from soil represents the effective solution of nitrogen accumulation remediation.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/index, or from the corresponding author on request.

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И З В О Д

ПРЕДВИЂАЊЕ СПОСОБНОСТИ ДЕНИТРИФИКАЦИЈЕ АЛКАЛОТОЛЕРАНТНИХ БАКТЕРИЈСКИХ ИЗОЛАТА ИЗ ЗЕМЉИШТА – МОДЕЛ ВЕШТАЧКЕ НЕУРОНСКЕ МРЕЖЕ

ОЉА Љ. ШОВЉАНСКИ[1], АНА М. ТОМИЋ[1], ЛАТО Л. ПЕЗО[2], АЛЕКСАНДРА С. РАНИТОВИЋ[1] и СИНИША М. МАРКОВ[1]

[1]*Универзитет у Новом Саду, Технолошки факултет Нови Сад, Булевар цара Лазара 1, Нови Сад и* [2]*Универзитет у Београду, Институт за општу и физичку хемију, Студентски трг 12/V, Београд*

У протеклим деценијама, процес биоремедијације на бази денитрификације посредством аеробних хетеротрофних бактерија је опсежно проучаван за различите инжењерске приступе. Поред чињенице да треба користити само непатогене бактерије које не формирају биофилмове, од посебног је значаја изоловати и окарактерисати бактерију или групу бактерија које имају могућност потпуног уклањања нитрата без накупљања азотних оксида или амонијака као међупроизвода. У овом раду су истражени капацитет денитрификације 43 изолата из благо алкалних, калцитних земљишта дуж тока реке Дунав. На основу добијених резултата је развијен емпиријски модел вештачке неуронске мреже за предвиђање капацитета денитрификације бактеријских сојева на основу шест значајних индикатора процеса денитрификације: детекције продукције биомасе и молекулског азота у виду гаса, концентрације нитрата и нитрита, као и формирања нитрита и амонијака у току инкубационог периода. Модел вештачке неуронске мреже је показао прилично добру способност предвиђања излаза (укупни R^2 за предвиђање је 0,958). Поред тога, експериментална верификација неуронске мреже у лабораторијским условима је указала да се употребом ове математичке методе може предвидети капацитет

циљане реакције код бактеријских сојева изолованих из земљишта у лабораторијским условима.

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