Annals of Biology **39** (2) : 175-184, 2023

Impact Score : 0.32 (Scopus)

Optimization of Octogen Degradation by a Consortium of Indigenous Bacteria Employing Box-Behnken Design

KIRTY SHARMA*, PRAVEEN SHARMA AND PRITAM SANGWAN[1]

Centre for Fire, Explosive and Environment Safety, Defence Research & Development Organization, New Delhi-110 054, India

**(e-mail : kirty.krt@gmail.com; ps.enbt@gmail.com; Mobile : 96435 94351)*

(Received: January 01, 2023; Accepted: February 13, 2023)

ABSTRACT

Biodegradation of Octogen (HMX) is a better as well as environment-friendly technique to reduce its adverse effects on the environment. Therefore, optimization of parameters of HMX degradation using a consortium of bacterial isolates derived from an explosive polluted area was conducted with the help of Response Surface Methodology (RSM). The study was carried out to reveal the relationship between dependent and independent variables during HMX degradation. Independent variables included the inoculum size, initial concentration of HMX and time period of sampling during the experimentation, and dependent variables included percentage reduction of HMX, optical density and nitrite released. All dependent and independent variables were analyzed employing Box-Behnken design of RSM. Results revealed that an initial concentration of 4 ppm HMX, time period of eight days and inoculation size of 4 ml was optimal for 82% removal of HMX. A high regression coefficient of 0.99 with a statistically significant fitted into the quadratic regression model for HMX reduction percentage was illustrated by the statistical evaluation employing analysis of variance (ANOVA).

Key words: Biodegradation, consortium, pollution, Box-Behnken design, HMX

INTRODUCTION

Octogen (HMX) is a high energetic material which is used as a major component in the production of various explosive formulations these days (Liu *et al.*, 2015). Octogen is a nitramine-based secondary explosive, and it is also called High Melting Explosive (HMX). Its production, storage and utilization for civil as well as defense purposes have led to the pollution of soil and water, which poses a severe danger to the biotic component of the environment. According to Johnson and Reddy (2015), HMX can adversely affect the liver, kidney, brain, heart and central nervous system in animals. HMX does not naturally occur in the environment. It is a recalcitrant compound that can persist in the environment for a prolonged period of time, resulting in pollution of air, water and soil. Explosive like HMX, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) 2, 4, 6 trinitrotoluene (TNT) have been used and produced extensively from the time of World War II. The increased demand and production of explosive compounds have procreated numerous explosive polluted areas over the globe making explosive contamination a worldwide problem. Strategies like incineration dumping at landfill sites and seas are usually practised to manage HMX-contaminated wastes. These conventional methods harm the environment and further deteriorate air, water and soil. Finding an environmentally benign remediation technique is thus necessary to address the pollution brought on by HMX contamination.

Microbial remediation is a sustainable and economical approach for the remediation of polluted sites. It has been observed that microbial treatments may be able to clean up areas that have been contaminated by industrial wastewater (Ahmad *et al.*, 2018; Zabermawi *et al.*, 2022). Several studies for microbial remediation of industrial waste like phenols, dyes and petroleum derivatives have been documented by various researchers (Shahi *et al.*, 2016; Omer *et al.*, 2017; Sepehr *et al.*, 2019; Bharathi *et al.* 2022). A number of investigations have also been conducted on the microorganisms' capacity for the explosive breakdown in both aerobic and anaerobic environments (Khan *et al.*, 2015; Liu *et al.*,

Presently : [1]Department of Environmental Science and Engineering, Guru Jambheshwar University of Science & Technology, Hisar-125 001 (Haryana), India.

2015). According to Khan *et al.* (2015), aerobic degradation of energetic materials offers various benefits over anaerobic degradation, including a higher rate of breakdown and less hazardous byproducts produced during the process. Sheehan *et al.* (2020) showed that rate of biodegradation of high explosive compounds by bacteria in aerobic environment is much faster in comparison to anaerobic bacteria and fungi by performing a meta-analysis. Nagar *et al.* (2018) and Meda *et al.* (2020) investigated HMX degradation by bacteria and found degradation of 87.7% in 15 days using *Bacillus toyonensis* and HMX degradation of 70% in 20 days using *Planomicrobium flavidum*, respectively. Moreover, the process of explosive degradation varies from species to species and needs to be optimized for application on a large scale.

A process with many factors influencing the outcomes can be improved and optimized using a statistical approach called Response Surface Methodology (RSM). This methodology utilizes lower-order polynomial equations and requires fewer experimental setups overall, which helps to cut down on the time and resources required for process optimization. A spherical, revolving RSM model called the Box-Behnken model, has at least three levels, and the independent variable is held constant at one of the three equidistant values. The Box-Behnken model's experimental combinations are equally spaced in middle and on each side of the process space. Use of RSM with Box-Behnken model for optimization of high explosives degradation by bacteria has been documented by Sangwan *et al.* (2015); Meda *et al.* (2020) and Sharma *et al.* (2021).

The current study was planned with the objective to optimise experimental parameters for HMX removal with the help of the Box-Behnken model of RSM in the aqueous phase utilizing a native bacterial consortium isolated from a polluted area. The experiment was performed under aerobic conditions. One of the most significant by-products produced during the breakdown of HMX is nitrite (Nagar *et al.*, 2018; Meda *et al.*, 2020). Therefore, the concentration of nitrite was also analyzed in this study. The HMX degradation potential of consortium containing bacterial isolates S5-TSB-17, MTCC No. 12883, S4-TSB-1 and MTCC No. 12854 were used in the present study which have not been explored so far. However, these isolates were previously used in the research to degrade RDX (Sharma *et al.*, 2021).

MATERIALS AND METHODS

HMX, with a purity of >99%, was provided by an explosive manufacturing facility in northern India for research purposes. The triple distilled water used in this study was purchased from Millipore. High-pressure liquid chromatography (HPLC) grade acetonitrile was bought from Sigma-Aldrich. Other additional chemicals utilized in the current study were analytical grade and were bought from reputable suppliers.

Samples of contaminated water and soil were taken from a polluted area close to an explosive manufacturing facility in Northern India according to standard protocol employing random systematic grid sampling, composite sampling and grab sampling techniques. The microbial strains from explosive contaminated samples were isolated and identified by the Council of Scientific & Industrial Research (CSIR), Institute of Microbial Technology (IMTech), Chandigarh, India and were provided in lyophilised form.

Trypticase Soy Agar (TSA) was used as a nutrient medium to prepare the culture plates on which the lyophilised isolates of bacteria were reactivated. TSA was prepared by adding 2.5 g dextrose, 5 ng NaCl, 2.5 g di-potassium phosphate, 3 g soya peptone, 17 g casein peptone and 15 g agar in one liter of demineralised water. The isolates of bacteria were sub-cultured in conical flasks containing Trypticase Soy Broth (TSB) as the nutrient medium. The conical flasks containing bacterial isolates were incubated at 32±2°C temperature, in an orbital shaker incubator at 120 rpm under aseptic environment. The bacterial cultures were thereafter acclimatised and maintained in another growth medium called Mineral Salt Medium (MSM) which had nutrients in a limited quantity. MSM1 and MSM2 were the two types of MSM prepared among which MSM1 was utilized to make bacterial isolates acclimatised to mineral salt medium. MSM2 was used as a growth media in the batch experiment in which HMX was substituted as the sole nitrogen source for bacterial growth. Composition of MSM1 was 1.74 g K_2HPO_4, 1.36 g KH_2PO_4, 0.061 g $MgSO_4 \cdot 7H_2O$, 0.053 g NH_4Cl, 0.59 g $C_4H_6O_4$, 0.92

g $C_3H_8O_3$, 0.900 g $C_6H_{12}O_6$, 0.278 mg $FeSO_4 \cdot 7H_2O$, 11.09 mg $CaCl_2 \cdot 2H_2O$, 0.015 mg $MnCl_2 \cdot 4H_2O$, 0.024 mg H_3BO_3, 0.0028 mg $ZnSO_4 \cdot 7H_2O$, 0.0024 mg $CuSO_4 \cdot 5H_2O$ and 0.0071 mg $CoCl_2 \cdot 6H_2O$ in 1L distilled water.

A consortium of isolates was prepared by conducting interaction studies between the microbes isolated from the explosive-contaminated area employing the following formula:

$$^nC_r = n!/(n-r)!(r)!$$

Where, n = total no. of microbial isolates, C = combination of microbial isolates, and r = no. of microbial isolates in each combination. Utilizing the above mentioned method, six combinations for consortium were created using four different bacterial strains. Every consortium combination had two strains of bacterial isolates. The bacterial consortia were developed by adding 2 ml of freshly made bacterial isolate culture broth in 100 ml autoclaved TSB growth media. The growth of both bacterial isolates in consortia broth was evaluated by observing Colony Forming Units (CFU) on TSA plates. For further study, isolate nos. S5-TSB-17 and S4-TSB-1 were employed because it was found that they were compatible with one another. After three days of incubation, uniform colonies of cream-white, *Paenibacillus aestuarii* and yellowish *Arthrobacter subterraneus* were present on culture plates. The consortium culture of *P. aestuarii* and *A. subterraneus* in MSM1 media having optical density (OD) 1.21 was used to inoculate HMX-containing MSM2.

A standard solution prepared in HPLC grade acetonitrile containing 100 mg/l HMX was filtered by passing through a 0.22 m filter to make it sterilized. The prepared stock solution was utilized to make the required 6, 4 and 2 mg/l HMX concentration. The bacterial consortium cultured in MSM1 was used in order to inoculate sterlized MSM2 with various concentrations of spiked HMX. The MSM2 inoculated with the consortium was kept at a temperature of 33±2°C with 120 rpm in an orbital shaker incubator. Different factors, such as contaminant concentration, inoculation size, and study duration, have a significant impact on the degradation process (Sangwan *et al.*, 2015; Meda *et al.*, 2020; Sharma *et al.*, 2021). Each factor or variable was required to be optimized for effective degradation studies. The Design-Expert software (Stat- Ease, Inc., Minneapolis, MN, USA), which utilized RSM as statistical tool, was used to plan experiments. RSM Box Behnken design was used for the optimization of process parameters for HMX biodegradation by a consortium of two bacterial isolates i.e. *P. aestuarii* and *A. subterraneus*. Various variable factors (independent variables) of this study are depicted in Table 1. Using the values of the independent variables, which were the HMX concentration of 2, 4 and 6 mg/l, the inoculation volume of 2, 4 and 6 ml, and the period of 4, 8 and 12 days, a total of 17 combinations were produced.

Table 1. Independent variables and their coded levels used for optimization for HMX

Variables	Range and levels (coded)		
	-1	0	+1
Initial HMX concentration (mg/l)	2	4	6
Inoculation size (ml)	2	4	6
Time duration (days)	4	8	12

The growth of bacterial consortia when they were introduced to various HMX concentrations was analyzed by observing the optical density of samples. One of the crucial by-products, Nitrite, was yielded during the biodegradation of HMX. The three response parameters–the HMX removal percentage, optical density and nitrite release were analyzed. The bacterial proliferation was estimated by monitoring the optical density of the samples. UV VIS spectrophotometer of Perkin Elmer was used to measure optical density at 600 nm. Hach DR1900 spectrophotometer was used to evaluate nitrite release by the diazotisation method. Perkin Elmer HPLC Flexar was used to analyze the concentration removal of HMX. The USEPA 8330A standard method was used to prepare the samples for HPLC analysis. A C18 column with dimensions of 3 m x 150 x 4.6 mm was used as the stationary phase, and the mobile phase consisted of a 50:50 v/v mixture of acetonitrile and triple-distilled water flowing at a rate of 1 ml/min. Detection of HMX in the samples was performed by a Photo Diode Array (PDA) detector.

Statistical estimation was carried out employing Design Expert 11 (Stat-Ease, Inc., Minneapolis, MN, USA) for the analysis of

variance (ANOVA) to evaluate the significance of each term in fitted equations and to analyze the goodness of fit. The two-way ANOVA was applied in the present study because factor variables were more than two. A second-order polynomial model in which interaction terms were applied to the exploratory results of the experiment can be written in the form of the following equation :

$$Y=a_o+\Sigma a_i x_i+\Sigma a_{ii} x^2_i+\Sigma a_{ij} x_i x_j$$

Where, Y = percent removal of HMX, a_o = offset term, a_i = first order main effect, a_{ii} = Second order main effect, a_{ij} = interaction effect, x_i and x_j = input factors.

To obtain relation with the exploratory variables and their responses, 3D plots were produced, and optimum variables were developed by the response surface methodology.

RESULTS AND DISCUSSION

Box-Behnken Design of RSM was used to produce different combinations of independent factors (Table 2) depicting independent factors and their responses which were generated with the help of Box-Behnken Design. The independent factors were tested for various parameters, each of which produced a unique set of responses. Different response parameters for HMX biodegradation were accommodated to a Box-Behnken design using experimental data. The response variables, including the HMX removal percentage, optical density and nitrite release were represented as 3D response graphs as a function of two independent variable combinations.

Growth of bacteria was assessed by monitoring optical density of the samples at different intervals of time. The optical density (OD) 3D surface plot as function of (a) Volume of suspensions (inoculation size) and initial HMX concentrations, (b) time duration and initial HMX concentration, (c) time duration and volume of suspension (Fig. 1). The maximum growth of the bacteria was obtained with the optical density of 0.27 in combination having 4 mg/l HMX concentration and 4 ml inoculation size in eight days time interval. The second highest bacterial growth, with an optical density 0.25, was observed in the combination having 6 mg/l HMX concentration with 4 ml volume of suspension, in a time interval of 12 days. The optical density, 0.11, was the lowest optical density measured at 4 mg/l HMX concentration with 2 ml inoculation size in 4 days time intervals. It was observed that the highest optical density observed in the sample was 1.45 times more than the lowest optical density. The optimum inoculation size and HMX concentration led to enhanced bacterial proliferation in the combination containing 4

Table 2. Box-Behnken Design Matrix for independent variables along with their observed response for HMX degradation

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
		A: Concentration (mg/l)	B: Volume of suspension (ml)	C: Time (days)	Optical density	Nitrite	% Removal of HMX
16	1	4	4	8	0.27	0.03	82
3	2	2	6	8	0.17	0.02	55
7	3	2	4	12	0.15	0.01	45
12	4	4	6	12	0.16	0.01	70
5	5	2	4	4	0.17	0.01	30
10	6	4	6	4	0.14	0.03	50
9	7	4	2	4	0.11	0.01	44.25
17	8	4	4	8	0.27	0.03	82
15	9	4	4	8	0.27	0.03	82
13	10	4	4	8	0.27	0.03	82
14	11	4	4	8	0.27	0.03	82
2	12	6	2	8	0.15	0.01	68.16
6	13	6	4	4	0.14	0.01	60
8	14	6	4	12	0.25	0.02	76.66
11	15	4	2	12	0.19	0.01	62.5
4	16	6	6	8	0.21	0.03	72.83
1	17	2	2	8	0.16	0.01	40

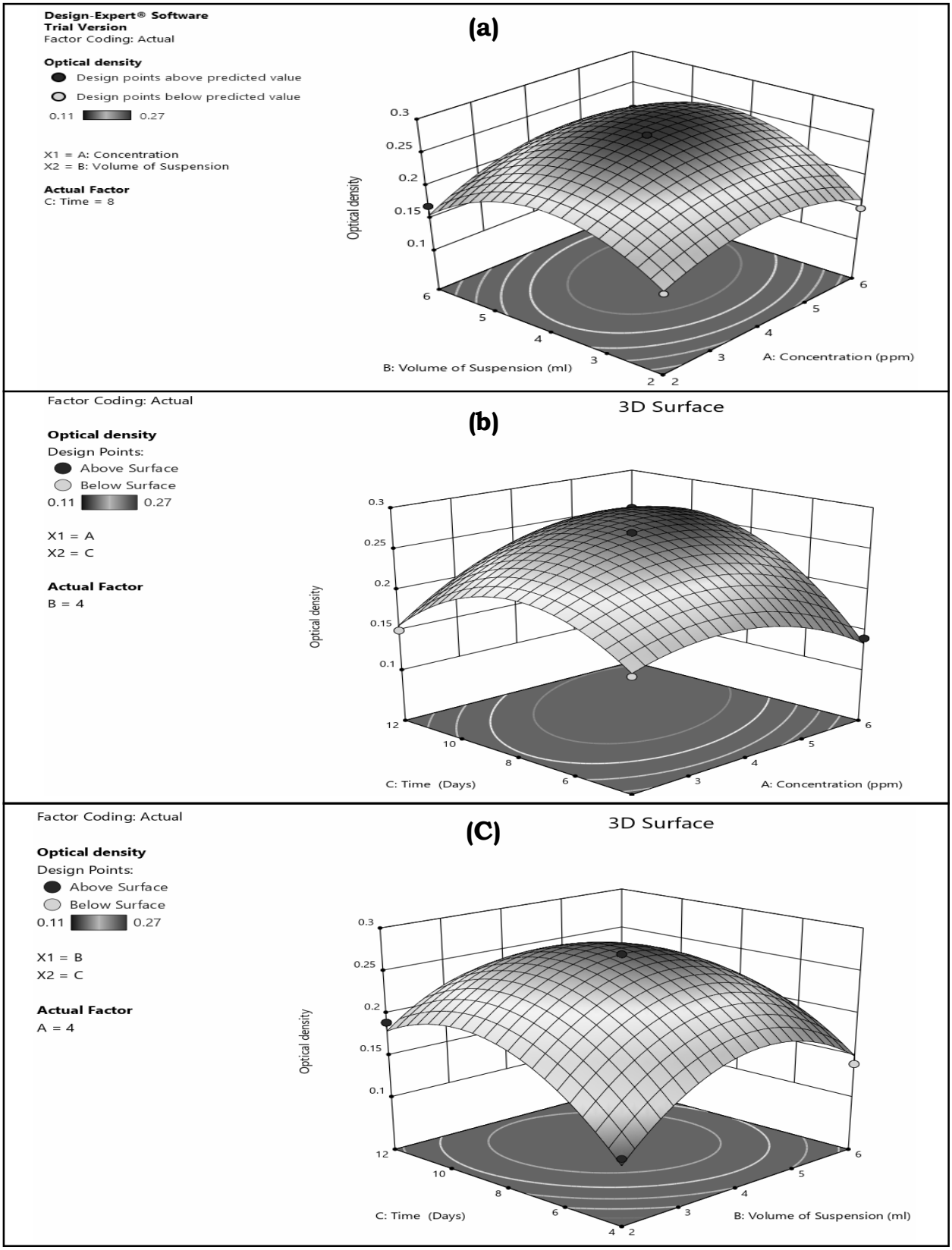

Fig. 1. 3D surface plot for optical density as a function of (a) volume of suspension (inoculation size) and initial HMX concentration, (b) time duration and initial HMX concentration and (c) time duration and volume of suspension.

mg/l HMX, 4 ml of inoculum suspension and time duration of eight days. The higher HMX concentration provided a sufficient amount of nitrogen source to the thriving microbes. Meda *et al.* (2020) found similar trends while investigating HMX biodegradation. After four days of inoculation, the consortium's bacteria became accustomed to the HMX in the MSM2 media and began using it as nitrogen source. It can be concluded that the bacterial consortium used in this study can tolerate HMX up to 6 mg/l concentration. However, at this concentration, the growth of bacteria was slow after first few days of inoculation.

It was observed that HMX degradation was affected by inoculation size, HMX concentration and time. Fig. 2 shows the 3D response graphs of percentage removal of HMX as functions of the (a) volume of suspensions (inoculation size) and initial HMX concentrations, (b) time durations and initial HMX concentrations and (c) time durations and volume of suspensions. It showed that the maximum amount of HMX degradation 82%, was attained at an initial 4 mg/l HMX concentration, with a 4 ml volume of suspension and 8-day incubation period, followed by 76.66% HMX removal at 6 mg/l initial HMX concentration, 4 ml volume of suspension and 12-day incubation period. Minimum degradation of 30% was found in a combination having 2 mg/l HMX, 4 ml inoculation size and four days time duration. At optimal conditions, the maximum percentage degradation of HMX was 1.73 times more than its minimum degradation percentage. A lesser time duration of four days with a minor initial concentration of just 2 mg/l HMX as a nitrogen source and bacterial abundance with inoculation size of 4 ml, resulted in the least percentage removal of HMX. In comparison to pure culture of isolates – *P. estuarii* and *A. subterraneus*, the same bacteria employed in the current investigation as a microbial consortium demonstrated greater stability and efficiency for HMX degradation.

Nitrite is among the significant by-products formed during the biodegradation of HMX (Nagar *et al.*, 2020). Fig. 3 illustrates the 3D surface graph of nitrite produced by a bacterial consortium as function of (a) inoculation size and initial HMX concentration, (b) time duration and initial HMX concentration and (c) time duration and volume of suspension. The highest nitrite release 0.03 mg/l was observed with three different combinations. The first set, which showed maximum nitrite concentration, had 4 mg/l initial HMX concentration along with 4 ml inoculation size, in eight days time period, and the second set of combination had 4 mg/l initial HMX concentration, 6 ml inoculation size and four days duration. Another set of combinations showing 0.03 mg/l nitrite concentration was 6 mg/l initial HMX concentration with 6 ml inoculation size in eight days. None of the combinations showed the highest nitrite concentration on 12 day of incubation. Reduced nitrite concentration might be a result of the nitrite ions' instability, their transformation to nitrate, or their utilization by the proliferating bacteria during the experimentation (Meda *et al.*, 2020). During the batch experiment, a predictable trajectory was not observed in the case of nitrite release. The nitrite in samples was generated by the consortium's aerobic breakdown of HMX. Nagar *et al.* (2018) showed comparable outcomes in their study.

Two-way ANOVA was employed to test the fitness and efficacy of the model and the coefficient of regression (R^2). ANOVA for the quadratic model is depicted in Table 3, the predicted regression model for every response is significant and valid as $P < 0.05$. For the three variables, HMX removal, nitrite released and optical density–ANOVA of higher significant values was observed with F-values of 87.58, 8.52 and 48.58, respectively.

The regression coefficient of model was 0.98, 0.91 and 0.99 for optical density, nitrite release and percentage removal of HMX, respectively. In case of percentage removal of HMX response, the predicted R^2 and adjusted R^2 were in reasonable agreement with each other. This demonstrated a strong correlation between experimental findings and model predictions, which also indicated the model's suitability for illuminating the true relationship between the selected factors. The final predictive equation in terms of coded factors with high levels of the factors coded as +1 and the low levels coded as -1 is listed below:

Optical density : $0.27+0.0125 \times A+0.00875 \times B+0.02375 \times C+0.0125 \times AB+0.0325 \times AC-0.015 \times BC-0.035 \times A^2 - 0.0625 \times B^2 - 0.0575 \times C^2$

% Removal of HMX : $82+13.4562 \times A+4.115 \times$

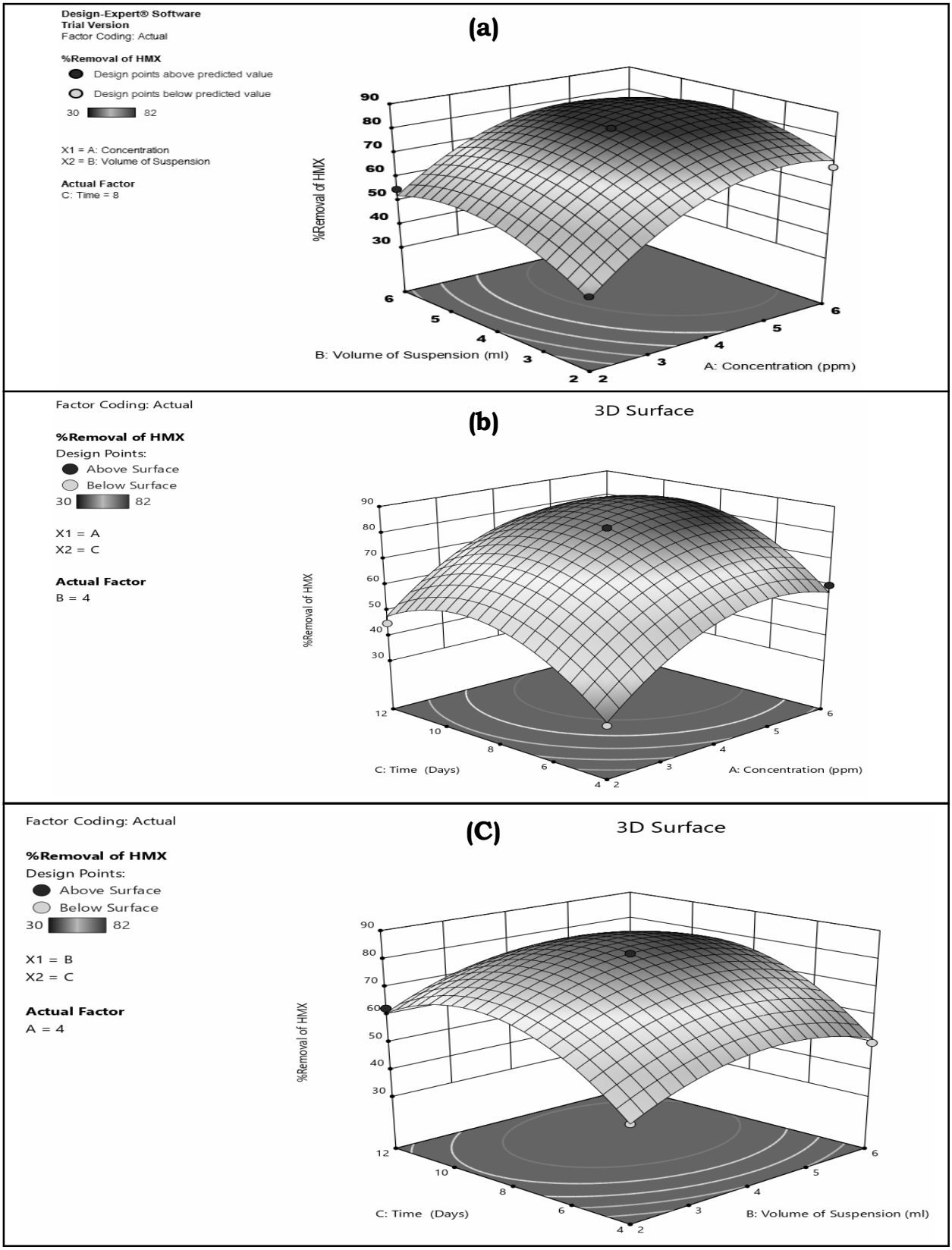

Fig. 2. 3D surface plot for the % removal of HMX by consortium as a function of (a) volume of suspension (inoculation size) and initial HMX concentration, (b) time duration and initial HMX concentration and (c) time duration and volume of suspension.

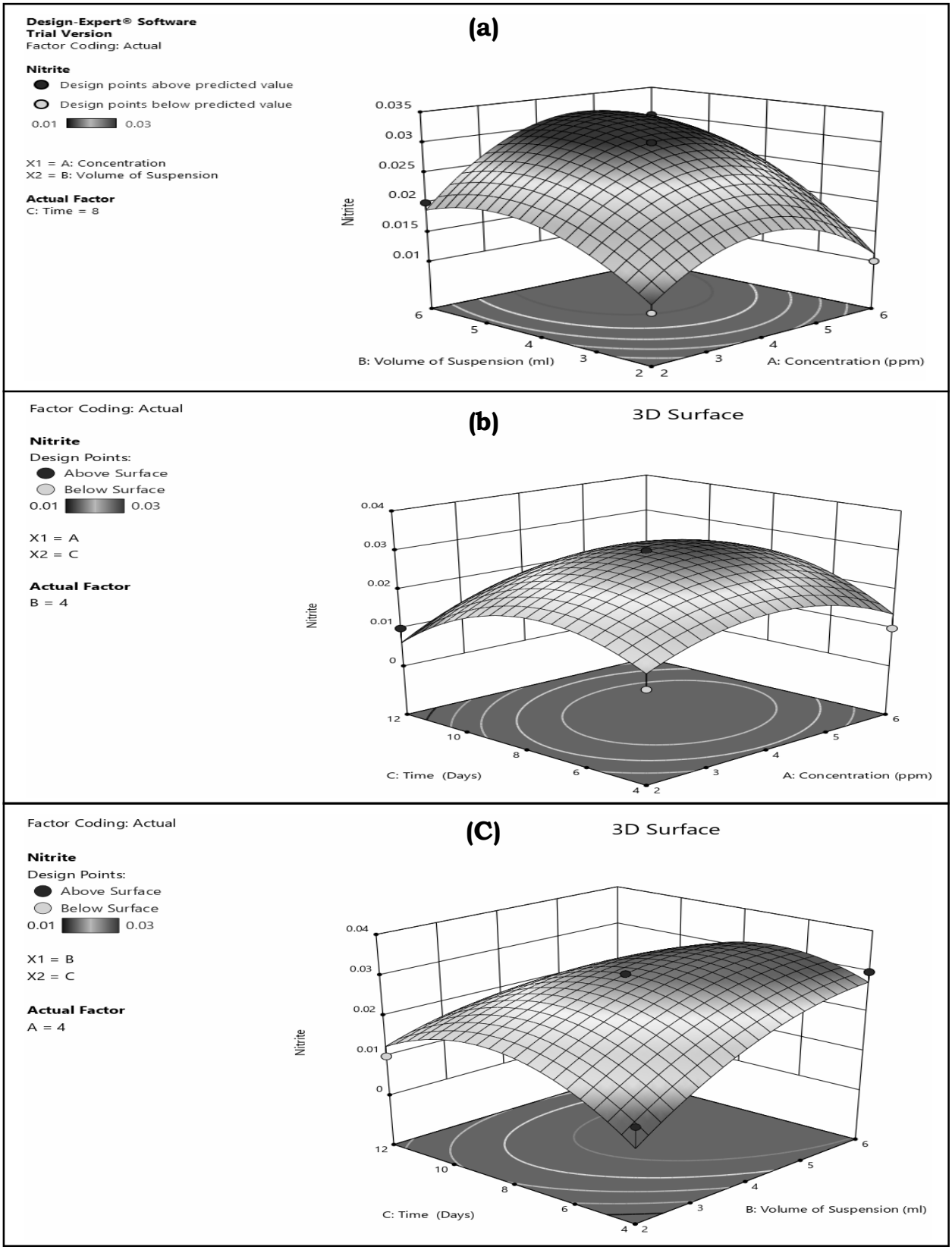

Fig. 3. 3D surface plot for nitrite released by consortium as a function of (a) volume of suspension (inoculation size) and initial HMX concentration, (b) time duration and initial HMX concentration and (c) time duration and volume of suspension.

Table 3. ANOVA for quadratic model for optical density, nitrite and % removal of HMX

Response variables	Source	Sum of squares	d. f.	Mean square	F-value	p-value		%CV	PRESS	R^2	Adj. R^2
Optical density	Model	0.0515	9	0.0057	48.58	< 0.0001	Significant	5.51	0.0132	0.98	0.96
	Lack of fit	0.0008	3	0.0003							
	Pure error	0.0000	4	0.0000							
	Correction total	0.0524	16								
Nitrite	Model	0.0014	9	0.0002	8.52	0.0050	Significant	21.77	0.002	0.91	0.80
	Lack of fit	0.0001	3	0.0000							
	Pure error	0.0000	4	0.0000							
	Correction total	0.0015	16								
% removal of HMX	Model	4647.83	9	516.43	87.58	< 0.0001	Significant	3.81	660.39	0.99	0.97
	Lack of fit	41.27	3	13.76							
	Pure error	0.0000	4	0.0000							
	Correction total	4689.10	16								

$B+8.73875 \times C - 2.5825 \times AB+0.415 \times AC+0.4375 \times BC-13.3875 \times A^2-9.615 \times B^2-15.6975 \times C^2$

Nitrite : $0.03+0.0025 \times A+0.00625 \times B - 0.00125 \times C+0.0025 \times AB+0.0025 \times AC - 0.005 \times BC - 0.0075 \times A^2 - 0.005 \times B^2 - 0.01 \times C^2$

Where, A=concentration of HMX, B=volume of suspension (inoculation size) and C=time duration in days.

Fig. 4 (a) illustrates the percentage of HMX removal predicted versus actual graph, and Fig. 4 (b) depicts graph of predicted versus actual values of optical density. The actual values and predicted values separated by standard deviation are shown in graphs presented in Fig. 4. The predicted values were the values estimated from the model, and the actual values analyzed response data from different sets of combinations. The proximity of the points in Fig. 4 (a) depicted a lower value of standard deviation for the percentage removal of HMX. In Fig. 4 (b), points on graph were scattered, which indicated a high value of standard deviation for optical density.

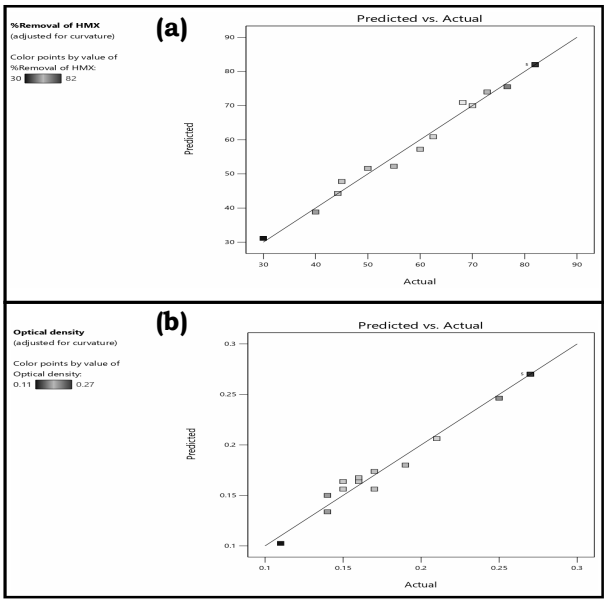

Fig. 4. Predicted vs. actual plot of responses (a) % removal of HMX and (b) optical density.

CONCLUSION

In the present study, Box-Behnken Model of Response Surface Methodology was employed for the optimization of process parameters of octogen degradation by the microbial consortium, which was derived from explosive contaminated area. The results showed that HMX degradation capability of the microbial consortium was affected by three factors, which included initial concentration of the contaminant, inoculation size and time interval of the sampling during the experimentation. It could be concluded that about 82% degradation of HMX can be attained by using consortium of *P. aestuarii* and *A. subterraneus* in aerobic conditions. Process variables affecting the experimental outputs can be optimized by employing RSM's Box-Behnken Design. The optimal conditions for degradation of HMX were identified as 4 mg/l initial HMX concentration, 4 ml inoculation size, and eight days time duration. The statistical analysis and proximity of exploratory results confirmed reliability of regression model. The results indicated that indigenous bacterial consortia employed in this study could biodegrade HMX present in the wastewater to a substantial level. These findings can help in lowering the costs of HMX remediation and also mitigating the issue of HMX-polluted wastewater in a sustainable way. Further detailed research is in progress to investigate the process of secondary metabolites produced

by bacterial consortia during the biodegradation of HMX and its applications on the field scale.

ACKNOWLEDGEMENTS

The authors are thankful to Sh. Rajiv Narang, OS & Director CFEES, Defence Research and Development Organization (DRDO), New Delhi for encouraging research and providing necessary facilities. The authors are also thankful to Dr. S. Mayilraj, Principal Scientist, IMTech, CSIR, and Chandigarh for isolation and identification of microbes. Ms. Kirty Sharma is thankful to Guru Jambheshwar University of Science & Technology, Hisar, Haryana, for providing University Research Fellowship.

FUNDING

This research was supported by the Defence Research and Development Organization (DRDO), India and Guru Jambheshwar University of Science & Technology, Hisar.

REFERENCES

Ahmad, M., Pataczek, L. and Hilger, T. H. (2018). Perspectives of microbial inoculation for sustainable development and environmental management. *Front. Microbiol.* **9**: 01-26.

Bharathi, D., Nandagopal, J. G. T., Ranjithkumar, R., Gupta, P. K. and Djearamane, S. (2022). Microbial approaches for sustainable remediation of dye-contaminated wastewater: A review. *Arch. Microbiol.* **204**: 169. *https://doi.org/10.1007/s00203-022-02767-3.*

Johnson, M. S. and Reddy, G. (2015). Wildlife toxicity assessment for high melting explosive (HMX). In: *Wildlife Toxicity Assessments for Chemicals of Military Concern,* Williams, M., Reddy, G., Quinn, M. and Johnson, M. S. (eds.). Elsevier, Amsterdam. pp. 087-105.

Khan, M. I., Yang, J., Yoo, B. and Park, J. (2015). Improved RDX detoxification with starch addition using a novel nitrogen-fixing aerobic microbial consortium from soil contaminated with explosives. *J. Hazard Mater.* **287**: 243-251.

Liu, Z., Lei, Z., Liu, Y. and He, Y. (2015). Anaerobic biodegradation of RDX and HMX with different co-substrates. *Chin. J. Chem. Eng* **23**: 704-709.

Meda, A., Sangwan, P. and Bala, K. (2020). Optimization of process parameters for degradation of HMX with *Bacillus toyonensis* using response surface methodology. *Int. J. Environ. Sci.* **17**: 4601-4610.

Nagar, S., Shaw, A. K., Anand, S,, Celin, S. M. and Rai, P. K. (2018). Aerobic biodegradation of HMX by *Planomicrobium flavidum. 3 Biotech* **8**: 455. *http://dx.doi.org/10.1007/s13205-018-1479-5.*

Nagar, S., Shaw, A. K., Anand, S., Celin, S. M. and Rai, P. K. (2020). Biodegradation of Octogen and Hexogen by *Pelomonas aquatica* strain WS2-R2A-65 under aerobic condition. *Environ. Technol.* **43**: 1003-1012.

Omer, F. S , Nalan, O. S. K., Asli, C., Turgay, T. and Tamer, U. (2017). Bacteria encapsulated electrospun nanofibrous webs for remediation of methylene blue dye in water. *Colloids Surf. B.* **152**: 245-251.

Sangwan, P., Celin, S. M. and Hooda, L. (2015). Response surface methodological approach for optimizing process variables for biodegradation of 2, 4, 6-trinitrotoluene using *Acinetobacter noscomialis*. *Eur. J. Adv. Eng. Technol.* **2**: 51-56.

Sepehr, S., Shahnavaz, B., Asoodeh, A. and Karrabi, M. (2019) Biodegradation of phenol by cold-tolerant bacteria isolated from alpine soils of Binaloud Mountains in Iran. *J. Environ. Sci. Health. A Tox Hazard Subst. Environ. Eng.* **54**: 367-379. https://doi.org/10.1080/10934529.2018.1553818.

Shahi, A., Aydin, S., Ince, B. and Ince, O. (2016) Reconstruction of bacterial community structure and variation for enhanced petroleum hydrocarbons degradation through biostimulation of oil contaminated soil. *Chem. Eng. J.* **306**: 60-66.

Sharma, K., Sharma, P., Celin, S.M., Rai P. K. and Sangwan, P. (2021). Degradation of high energetic material hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a microbial consortium using response surface methodological approach. *SN Appl. Sci.* **3**: 12. *https://doi.org/10.1007/s42452-020-04021-1.*

Sheehan, P. L., Sadovnik, R., Kukor, J. J. and Bennett, J. W. (2020). Meta-analysis of RDX biotransformation rate by bacteria and fungi. *Int. Biodeterior. Biodegradation* **146**: 01-13.

Zabermawi, N. M. O., Alyhaiby, A. H. and El-Bestawy, E. A. (2022). Microbiological analysis and bioremediation bioassay for characterization of industrial effluent. *Sci. Rep.* **12**: 18889. *https://doi.org/10.1038/s41598-022-23480-7.*