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# BOX-BEHNKEN EXPERIMENTAL DESIGN MEDIATED OPTIMIZATION OF AQUEOUS METHYLPARATHION BIODEGRADATION BY *Pseudomonas aeruginosa* mpd STRAIN

*Krishnaswamy Usharani* * [1,2,3], *Perumalsamy Lakshmanaperumalsamy* [2]

***Address(es):***
[1]Division of Environmental Microbiology.
[2]Division of Environmental Engineering and Technology, Department of Environmental Sciences, Bharathiar University, TN, India.
[3]Department of Environmental Science, Central University of Kerala, KL , India.

*Corresponding author: usharanik05@yahoo.com

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**ABSTRACT**

Biotreatment of methylparathion was studied in aqueous mineral salts medium containing bacterial culture to demonstrate the potential of the novel strain of *Pseudomonas aeruginosa* mpd. A statistical Box–Behnken Design (BBD) of experiments was performed to evaluate the effects of individual operating variables and their interactions on the methylparathion removal with initial concentration of 1000 mg $l^{-1}$ as fixed input parameter. The temperature ($X_1$), pH ($X_2$), reaction time ($X_3$) and agitation ($X_4$) were used as design factors. The result was shown that experimental data fitted with the polynomial model. Analysis of variance showed a high coefficient of determination value 0.9. The optimum biodegradation of MP in terms MP removal ($Y_1$), COD removal ($Y_2$) and TOC removal ($Y_3$) were found to be 95.2 %, 82 % and 61.2 % respectively. The maximum growth ($Y_4$) was 2.18 optical density (OD). The optimum biodegradation correspond to the factors combination of middle level of $X_1$ (33 °C), $X_2$ (7.0), $X_4$ (150 rpm) and the highest level of $X_3$ (96h). MP removal and its residues were detected using spectral analysis. The study demonstrates the optimum MP biodegradation potential of this strain could use MP as the sole Carbon/Phosphate source. BBD confirmed to be dependable in developing the model, optimizing factors and analyzing interaction effects. Data from this study will be helpful in the design of small-scale field experiments and subsequently an in situ methylparathion biotreatment system for field application.

**Keywords:** Wastewater Biotreatment. Design Optimization. *Pseudomonas aeruginosa*. Biodegradation. *O,O*-dimethyl -*O*-4-nitrophenylphosphorothioate

## INTRODUCTION

Continuous and excessive use of organophosphorus (OP) compounds has led to the contamination of several ecosystems in different parts of the world (**Cisar and Snyder, 2000; Tse *et al.*, 2004**). Thiophosphoric acid esters, such as parathion, methylparathion (MP) and tetrachlorvinphos, are hazardous pollutants and their accumulation in the environment is a recognized ecological threat (**Kaloyanova and Tarkowski, 1981**). Methods for their enhanced degradation are an urgent task of contemporary chemical technology and biotechnology. Its widespread use has caused environmental concern due to its frequent leakage into surface and ground waters. The drinking water directive (Council directive 98/ 83/ EC) sets an allowed contaminant level of 0.1 mg/L for a single pesticide and 0.5 mg/L for the total sum of pesticides. Industries manufacturing pesticides release wastewater in water bodies or land. Although industries treat their wastewater by activated sludge process, no attention is paid to remove the specific pesticides or their metabolites which exert toxicity at very low concentrations. Therefore, there is a need for economically dependable methods for organophosphorus (OPs) detoxification from the environment. To date, bacterial transformations have been the main focus in research on organophosphate pesticide degradation. *Pseudomonas aeruginosa*, *Clavibacter michiganense* (**Subhas and Dileep, 2003**), *Arthrobacter atrocyaneus*, *Bacillus megaterium* and *Pseudomonas mendocina* (**Bhadbhade *et al.*, 2002**), *Agrobacterium radiobacter* (**Horne *et al.*, 2002**), and other *Pseudomonas* species (**Ramanathan and Lalithakumari, 1999**) have been reported to degrade OP in solutions and soils. Use of specific microorganism adapted to the pesticides, in treatment of industrial effluents is not in practice (**Kanekar *et al.*, 2004**). Therefore, research should be concentrated to develop economical but effective microbial processes for the treatment of industrial effluents containing pesticides and take them to field. The aim of this research was to optimize the process variables for the biodegradation potential of the *Pseudomonas aeruginosa* mpd novel strain using response surface methodology (RSM).

## MATERIAL AND METHODS

### Bacterial culture conditions

A potential bacterial strain (*Pseudomonas aeruginosa* mpd) was isolated from pesticide exposed agricultural soil. The initial enrichment cultures were established in a synthetic wastewater containing mineral salts medium amended with the methylparathion (Devithion™ 50% EC) as the sole source of carbon and energy. The concentration of methylparathion used was 0.1%, pH was adjusted using 1N NaOH and 1N HCl (ELICO - L1127, India). The methylparathion contaminated synthetic wastewater was neutral pH and the mean value of methylparathion (MP) content was 1000 mg/L, chemical oxygen demand (COD) was 41950 mg/L and total organic carbon (TOC) was 10459 mg/L. The synthetic wastewater containing higher concentration of methylparathion with maximum level of 1000 mg/L was used in the present study. Stock solution of pure methylparathion (98.5%) was prepared by dissolving 1g in 100 mL methanol, made up to 1000 mL of distilled water and was used as a reference for instrumental analysis. It was reported that the pesticide pollution due to wastewater released from formulating or manufacturing pesticide industry were up to 1000 $mgL^{-1}$ (**Chiron *et al.*, 1997**). Therefore in this research, synthetic wastewater containing methylparathion with maximum concentration of 1000 $mgL^{-1}$ was used.

Organisms were subsequently grown on nutrient agar medium plates to obtain single colonies. A pure culture of methylparathion-degrading *Pseudomonas aeruginosa* was isolated by series of replating on MSM with methylparathion agar plates. Minimum inhibitory concentration (MIC) test with plate screening method was carried out to screen methylparathion resistant bacteria using methylparathion MSM with methylparathion agar plates. Based on the MIC test, the five potential bacterial cultures isolated were identified based on their morphological characters and biochemical tests as given in Bergey's Manual of Determinative Bacteriology (**Holt *et al.*, 2000**). For degradation studies, *Pseudomonas aeruginosa* was inoculated into sterile shake-bottles containing 250

mL of MSM, 0.1% (w/v) methylparathion and incubated under aerobic conditions on a shaker (150 rpm) for 96 h. The other parameters, i.e., pH value, culture temperature, time and agitation, were part of the experimental design. All experiments were performed in triplicate, and the results are expressed as an average of three replicates.

**Optimization of methylparathion degrading condition by *Pseudomonas aeruginosa* mpd**

In order to study the effect of variables on the degradation of methylparathion by biotreatment using potential bacterial strain, the process variables include pH, temperature time and agitation were optimized. Experimental design was set using the variables such as pH, temperature, time and agitation. The synthetic wastewater which consists of mineral salts medium amended with the methylparathion was set at various temperature (25-40°C), pH (5-9), time (24-168 h) and agitation (120-180 rpm) for analysis. The concentration of methylparathion used was 1000 $mgL^{-1}$. The pH was adjusted using 1N NaOH and 1N HCl with the help of pH meter (ELICO - L1127, India). During this process, estimation of various parameters such as residual methylparathion, COD removal, TOC removal and pH were analysed to measure the degradability of methylparathion. Response surface methodology (RSM) based on the Box-Behnken design of experiment was used to optimize these parameters and their interaction which significantly influenced methylparathion biodegradation.

**Box–Behnken Experimental Design (BBD) of methylparathion bioremoval using RSM**

A standard RSM design called Box-Behnken's Design (BBD) for biotreatment process was adopted to study the influence of variables for the removal of aqueous methylparathion. The method can reduce the number of experimental trials needed to evaluate multiple parameters and their interactions and for finding the most suitable condition and prediction of response (**Box and Behnken, 1960; Myers and Montgomery, 2002**). Among all the RSM designs, BBD requires fewer runs than the others, e.g., 29 runs for a 4-factor experimental design. By careful design and analysis of experiments, Box-Behnken design allows calculations of the response function at intermediate levels which were not experimentally studied and shows the direction if one wishes to change the input levels to determine the effects on the response (**Hamed and Sakr, 2001, Martínez-Toledo and Rodríguez-Vázquez, 2011**).

**Table 1** The levels of variables in Box-Behnken statistical experiment design

| Variable | Name | Coded level | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| $X_1$:A | Temperature (°C) | 25 | 32.5 | 40 |
| $X_2$:B | pH | 5 | 7 | 9 |
| $X_3$:C | Time (h) | 24 | 96 | 168 |
| $X_4$:D | Agitation (rpm) | 120 | 150 | 180 |

The relation between the code values and none code values were:
$X_1 = (A- 32.5)/7.5$, $X_2 = (B - 7)/2$, $X_3 = (C - 96)/72$, $X_4 = (D - 150)/30$.

**Table 2** Experimental design with coded and actual values

| Run | Temp (°C) | pH | Time (h) | Agitation (rpm) | Temp (°C) | pH | Time (h) | Agitation (rpm) |
|---|---|---|---|---|---|---|---|---|
| | Coded Values | | | | Actual Values | | | |
| 1 | 1 | 0 | 0 | -1 | 40 | 7 | 96 | 120 |
| 2 | 0 | 1 | 1 | 0 | 32.5 | 9 | 168 | 150 |
| 3 | 0 | 0 | -1 | -1 | 32.5 | 7 | 24 | 120 |
| 4 | -1 | 0 | 0 | -1 | 25 | 7 | 96 | 120 |
| 5 | 1 | 0 | 1 | 0 | 40 | 7 | 168 | 150 |
| 6 | 1 | 1 | 0 | 0 | 40 | 9 | 96 | 150 |
| 7 | -1 | 0 | 1 | 0 | 25 | 7 | 168 | 150 |
| 8 | 0 | 0 | 1 | 1 | 32.5 | 7 | 168 | 180 |
| 9 | -1 | 0 | -1 | 0 | 25 | 7 | 24 | 150 |
| 10 | 1 | 0 | -1 | 0 | 40 | 7 | 24 | 150 |
| 11 | 0 | -1 | 0 | -1 | 32.5 | 5 | 96 | 120 |
| 12 | 0 | 0 | 1 | -1 | 32.5 | 7 | 168 | 120 |
| 13 | 0 | 0 | 0 | 0 | 32.5 | 7 | 96 | 150 |
| 14 | -1 | 0 | 0 | 1 | 25 | 7 | 96 | 180 |
| 15 | 0 | 1 | -1 | 0 | 32.5 | 9 | 24 | 150 |
| 16 | 0 | 0 | 0 | 0 | 32.5 | 7 | 96 | 150 |
| 17 | 0 | 1 | 0 | 1 | 32.5 | 9 | 96 | 180 |
| 18 | 0 | -1 | -1 | 0 | 32.5 | 5 | 24 | 150 |
| 19 | 0 | 1 | 0 | -1 | 32.5 | 9 | 96 | 120 |
| 20 | 0 | -1 | 1 | 0 | 32.5 | 5 | 168 | 150 |
| 21 | 1 | 0 | 0 | 1 | 40 | 7 | 96 | 180 |
| 22 | 0 | -1 | 0 | 1 | 32.5 | 5 | 96 | 180 |
| 23 | 0 | 0 | 0 | 0 | 32.5 | 7 | 96 | 150 |
| 24 | 0 | 0 | 0 | 0 | 32.5 | 7 | 96 | 150 |
| 25 | -1 | 1 | 0 | 0 | 25 | 9 | 96 | 150 |
| 26 | -1 | -1 | 0 | 0 | 25 | 5 | 96 | 150 |
| 27 | 0 | 0 | 0 | 0 | 32.5 | 7 | 96 | 150 |
| 28 | 1 | -1 | 0 | 0 | 40 | 5 | 96 | 150 |
| 29 | 0 | 0 | -1 | 1 | 32.5 | 7 | 24 | 180 |

Response surface methodology (RSM) based on the BBD of experiment was used to optimize the variables and their interaction which significantly influenced methylparathion biodegradation by the individual strains of *Pseudomonas aeruginosa* mpd. A four-factor, three-level Box-Behnken design was used in the biotreatment process. The Box-Behnken design is an independent, rotatable quadratic design with no embedded factorial or fractional factorial points where the variable combinations are at the mid-points of the edges of the variable space and at the center. Among all statistical experiment designs, Box-Behnken design requires fewer runs than the others, e.g., 29 runs for a 4-factor experimental design. The low, middle and high levels of each variable were designated as −1, 0, and +1 respectively, as given in Table 1. For this biotreatment process, the variables and their values in brackets were three levels include temperature (25-40°C), pH (5-9), time (24-168 h) and agitation (120-180 rpm), at constant methylparathion concentration 1000 $mgL^{-1}$(0.1%). This also enabled the identification of significant effects of interactions for the batch studies. This also enabled the identification of significant effects of interactions for the batch studies. In system involving four significant independent variables $X_1$, $X_2$, $X_3$, and $X_4$, the mathematical relationship of the response of these variables can be

approximated by quadratic (second degree) polynomial equation (**Box and Behnken, 1960**). A total of 29 experiments were carried out. The design consists of three replicated center points, and a set of six points lying at the midpoints of each edge of the multidimensional cube (Table 2). Response functions, describing variations of dependent factors (*Y*) (methylparathion removal, COD removal, TOC removal, bacterial growth for bacterial with the independent variables ($X_i$) (temperature, pH, time and agitation) can be written as follows (Eq.1):

$$Y = b_0 + \sum b_i x_i + \sum_{\dots} b_{ij} x_i x_j + \sum b_{ii} x^2_{ii} \quad \dots (1)$$

Linear Interaction Square

Where, Y is the predicted response in percentage of methylparathion removal, COD removal, TOC removal and bacterial growth in terms of optical density, $b_o$ is the offset term and $b_i$ is the linear effect while $b_{ii}$ and $b_{ij}$ are the square and the interaction effects, respectively. Experimental data points used in Box-Behnken statistical experiment design are presented in Table 1. The response function coefficients were determined by regression using the experimental data and the Stat-Ease Design Expert 8.0.4 program.

The response functions for percentage of methylparathion removal, COD removal, TOC removal and bacterial growth in terms of optical density were approximated by the standard quadratic polynomial equation as presented in Eq. 2.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad \dots (2)$$

Where *Y* is the predicted response, i.e. the methylparathion removal; $X_1$, $X_2$, $X_3$ and $X_4$ are the coded levels of the independent factors: temperature, pH, time and agitation. The regression coefficients are: $b_0$ – the intercept term; $b_1$, $b_2$, $b_3$ and $b_4$ – the linear coefficients; $b_{12}$, $b_{13}$, $b_{14}$, $b_{23}$ $b_{24}$ $b_{34}$ – the interaction coefficients and $b_{11}$, $b_{22}$, $b_{33}$, $b_{44}$ – the quadratic coefficients. The model evaluates the effect of each independent factor on the response.

The normal practice is to test within the feasible range, so that the variation in the process does not mask the factor effect. A total of 29 trials were necessary to estimate the coefficients of the model using multiple linear regressions. Hence, about 29 treatments were conducted in the present study and analysis the variance. The data obtained from 29 experiments, were used to find out the optimum point of the process parameters using Box-Behnken Design in Response surface methodology. All the data were treated with the aid of Design Expert by Stat Ease Inc, Minneapolis (Design Expert. 8.0.4). For this bacterial biotreatment process, the methylparathion removal conditions are presented in Table 2, according to the experimental design (Table 1).

**Preparation of Sample for Residual Analysis**

The biotreated samples were centrifuged at 10,000 rpm for 15 min using high-speed refrigerator centrifuge (CR22GII- Hitachi, Japan). The centrifuged samples were filtered through 0.2 µm sterile syringe nylon filters and then used for analysis of residual methylparathion and intermediate products using HPLC.

**Methylparathion determination**

The methylparathion removal efficiency of the bacterial biotreatment process was analyzed in terms of COD, TOC and residual methylparathion concentration of the wastewater before and after the treatment process. The samples were withdrawn at different time intervals after biotreatment from 0 to 168 h were analyzed for COD, TOC as per standard procedure laid down in APHA (1998). All experiments were performed in triplicates. The pH of the treated wastewater was adjusted and monitored using pH meter (ELICO - L1127, India). The residual methylparathion was analysed using UV-Vis spectrophotometer (Shimadzu - UV- 3600, Japan), HPLC (Shimadzu, SPD-20A, Japan) and GC-MS (Perkin Elmer-Clarus 600, Germany).

**Estimation of Growth**

Growth in terms of optical density (Bacteria) was estimated. The increase in growth of bacteria for every 24 h was monitored by measuring optical density (OD) at 600 nm on a UV-Visible Spectrophotometer (Shimadzu - UV- 3600, Japan).

**Spectral Analysis**

Degradation of methyl parathion and subsequent formation and eventual disappearance of intermediate products in the reaction mixture as a function of pH and time was monitored using UV-Vis spectroscopy. The filtered samples were scanned using UV-Vis-NIR Spectrophotometer (Shimadzu - UV- 3600, Japan), at 277nm. The centrifuged and filtered samples were analyzed for residual methylparathion using HPLC, (Shimadzu, Japan) on a reverse phase C18 column [(250 x 4.60 mm) (Desc. Luna 5µ C18 (20)-100A Phenomenex)], at a flow rate of 1.5 mL $min^{-1}$. Mobile phases consisted of solution A (HPLC grade water) and solution B (HPLC grade methanol) in the ratio of 1:4 respectively. The isocratic gradient mode with pressure limit of 20 MPa and the total run time for 20 min. The sample was injected at a rate of 20 µL and was detected at 277nm using UV detector (SPD-20A, Japan). Under the conditions described above, the retention time (RT) of methylparathion standard was 3.3 min.

**RESULTS AND DISCUSSION**

**Box-Behnken Experimental design and statistical analysis of methylparathion biotreatment**

Box-behnken statistical experimental design was used to investigate the effects of the three independent variables on the response function and to determine the optimal conditions for maximizing the removal of methylparathion, COD, TOC and growth of bacteria. The optimization procedure involves studying the response of the statistically designed combinations, estimating the coefficients by fitting the experimental data to the response functions, predicting the response of the fitted model and checking the adequacy of the model. The independent variables were the temperature ($X_1$), pH ($X_2$), time ($X_3$) and agitation ($X_4$). The low, center (middle) and high levels of each variable are designated as -1, 0, and +1, respectively as shown in Table 1. The response functions are the methylparathion removal ($Y_1$), COD removal ($Y_2$), TOC removal ($Y_3$) and growth of bacteria ($Y_4$). The experimental values and predicted values are presented in Table 2. The center point (0, 0, 0, 0, 0) was repeated five times and the same results were obtained indicating the reproducibility of the data. Observed and predicted removal (%) for methylparathion, COD, TOC and bacterial growth (OD) are compared in Table 3. Yuan et al., (2006) reported optimization of a medium for enhancing nicotine biodegradation by Ochrobactrum intermedium DN2 by using RSM. Furthermore **Usharani *et al.*, (2013)** reported optimization of Phosphate removal by bacterial consortium in batch scale process using response surface methodology. Results obtained during the present study showed the importance of using RSM based on the BBD of experiment for the optimization of aqueous methylparathion biotreatment and degradation by potential microbial strains.

**Analysis of Variance**

The data obtained from the experiments were used for the analysis of variance. Table 4 and 5, shows the ANOVA results of the model of response surface showing the removal of methylparathion, COD and TOC by *Pseudomonas aeruginosa* mpd and its growth in terms of optical density as a function of temperature, pH, time and agitation. The model F-value obtained (6903.04, 3961.11, 4683.83 and 4.55) from each source implied the respective model was significant for the removal of methylparathion, COD, TOC and bacterial growth in terms of optical density. The 'P' value lower than 0.01% (or 0.0001) indicates that the respective model is considered to be statistically significant (**Montgomery 1991, 2004**). In Table 5, the "lack of fit F- value" of 1.45 for methylparathion removal, 0.36 for COD removal, 2.90 for TOC removal and 1.5 for growth in OD implies that the lack of fit phenomenon is not important relatively to pure error, indicating the suggested model is well fitted to the observed methylparathion removal, COD removal, TOC removal and growth of *Pseudomonas* sp in OD. Figure 1, show their actual and predicted plot for (a) methylparathion removal, (b) COD removal, (c) TOC removal and (d) growth. The actual values are the measured response data for particular run and the predicted values are the results generated using the approximating functions. It was found that the removal of methylparathion, COD, TOC and bacterial growth (OD) which measured the signal to noise ratio was greater than 4, reaching the ratio of 135.92, 69.66, 43.75 and 30.60, respectively indicates an adequate signal. This indicates the model is adequate to be used to navigate the design space.

**The Regression Model Coefficients**

The application of RSM offers an empirical relationship between the response function and the independent variables. The mathematical relationship between the response function (Y) and the independent variables (X) can be approximated by a quadratic polynomial equation as given in Eq. 2. By applying multiple regression analysis of the experimental data, the experimental results were fitted with a second-order polynomial equation. Thus, mathematical regression models for methylparathion removal using the coded factors are given in Eqs. (1) – (4).

$$Y_1 = 95.2 + 4.67X_1 + 2.75X_2 + 5.75X_3 - 2.67X_4 - 20.81X_1^2 - 18.18X_2^2 - 19.18X_3^2 - 14.31X_4^2 - 0.50X_1X_2 + 4.25X_1X_3 - 7.75X_1X_4 - 4.50X_2X_3 + 2.25X_2X_4 - 3.0X_3X_4 \quad \dots (1)$$

$$Y_2 = 82.0 - 0.757X_1 + 2.5X_2 + 1.33X_3 + 2.58X_4 - 13.25X_1^2 - 11.87X_2^2 - 15.62X_3^2 - 17.50X_4^2 - 1.50X_1X_2 + 1.0X_1X_3 - 4.25X_1X_4 + 1.0X_2X_3 + 1.0\ X_2X_4 + 2.50X_3X_4 \quad \ldots (2)$$

$$Y_3 = 61.2 - 1.257X_1 + 2.58X_2 + 0.92X_3 + 1.75X_4 - 15.98X_1^2 - 12.73X_2^2 - 15.47X_3^2 - 19.22X_4^2 - 0.25X_1X_2 + 2.25X_1X_3 - 4.75X_1X_4 + 4.25X_2X_3 + 2.25\ X_2X_4 + 1.25X_3X_4 \quad \ldots (3)$$

$$Y_4 = 2.18 - 0.075X_1 + 0.13X_2 + 0.13X_3 + 0.12X_4 - 0.38X_1^2 - 0.44X_2^2 - 0.38X_3^2 - 0.56X_4^2 - 0.025X_1X_2 - 0.025X_1X_3 - 0.17X_1X_4 + 0.25X_2X_3 + 0.13\ X_2X_4 + 0.15X_3X_4 \quad \ldots (4)$$

Where $Y_1$ (% methylparathion removal), $Y_2$ (% COD removal), $Y_3$ (% TOC removal) and $Y_4$ (bacterial growth in OD) is the predicted responses where as the $X_1$ (temperature), $X_2$ (initial pH), $X_3$ (time) and $X_4$ (agitation) are the coded variables.

**Table 3** The observed (experimental) values and model response (predicted) values obtained from combination of process variables

| Run | $Y_1$ | | $Y_2$ | | $Y_3$ | | $Y_4$ | |
|---|---|---|---|---|---|---|---|---|
| Run | MP Removal (%) | | COD Removal (%) | | TOC Removal (%) | | Growth-$MP^+$ (OD) | |
| | EV | PV | EV | PV | EV | PV | EV | PV |
| **1** | 75 | 75.17 | 52 | 52.17 | 28 | 27.75 | 1.2 | 2.352 |
| **2** | 62 | 61.84 | 59 | 59.34 | 41 | 40.75 | 1.8 | 1.871 |
| **3** | 55 | 55.63 | 48 | 47.47 | 24 | 25.09 | 1.2 | 2.277 |
| **4** | 51 | 50.35 | 45 | 45.17 | 21 | 20.75 | 1 | 2.152 |
| **5** | 70 | 69.88 | 55 | 54.71 | 32 | 31.67 | 1.5 | 1.451 |
| **6** | 63 | 63.13 | 57 | 57.13 | 33 | 33.57 | 1.4 | 1.396 |
| **7** | 52 | 52.06 | 54 | 54.21 | 30 | 29.67 | 1.7 | 1.651 |
| **8** | 62 | 61.81 | 55 | 55.29 | 32 | 30.43 | 1.6 | 2.759 |
| **9** | 49 | 49.06 | 53 | 53.55 | 32 | 32.33 | 1.3 | 1.351 |
| **10** | 50 | 49.88 | 50 | 50.05 | 25 | 25.33 | 1.2 | 1.251 |
| **11** | 65 | 60.38 | 48 | 48.55 | 28 | 27.17 | 1 | 2.181 |
| **12** | 73 | 73.13 | 45 | 45.13 | 23 | 24.43 | 1.1 | 2.227 |
| **13** | 95 | 95.2 | 81 | 82 | 60 | 61.2 | 2.2 | 2.18 |
| **14** | 61 | 60.53 | 59 | 58.83 | 33 | 33.75 | 1.6 | 2.734 |
| **15** | 60 | 59.34 | 54 | 54.68 | 31 | 30.41 | 1.1 | 1.121 |
| **16** | 95 | 95.2 | 82 | 82 | 61 | 61.2 | 2.1 | 2.18 |
| **17** | 65 | 60.56 | 59 | 58.71 | 35 | 35.83 | 1.6 | 2.679 |
| **18** | 45 | 44.84 | 52 | 51.68 | 33 | 33.75 | 1.4 | 1.355 |
| **19** | 66 | 70.38 | 52 | 51.55 | 29 | 27.83 | 1.1 | 2.197 |
| **20** | 65 | 65.34 | 53 | 52.34 | 26 | 27.09 | 1.1 | 1.105 |
| **21** | 54 | 54.35 | 49 | 48.83 | 21 | 21.75 | 1.1 | 2.234 |
| **22** | 55 | 59.56 | 51 | 51.71 | 25 | 26.17 | 1 | 2.163 |
| **23** | 95 | 95.2 | 83 | 82 | 62 | 61.2 | 2.2 | 2.18 |
| **24** | 96 | 95.2 | 83 | 82 | 62 | 61.2 | 2.2 | 2.18 |
| **25** | 54 | 54.81 | 62 | 61.63 | 36 | 36.57 | 1.6 | 1.596 |
| **26** | 48 | 48.31 | 54 | 53.63 | 32 | 30.91 | 1.3 | 1.28 |
| **27** | 95 | 95.2 | 81 | 82 | 61 | 61.2 | 2.2 | 2.18 |
| **28** | 59 | 58.63 | 55 | 55.13 | 30 | 28.91 | 1.2 | 1.18 |
| **29** | 56 | 56.31 | 48 | 47.63 | 28 | 26.09 | 1.1 | 2.209 |

Experimental values - EV; Predicted values - PV

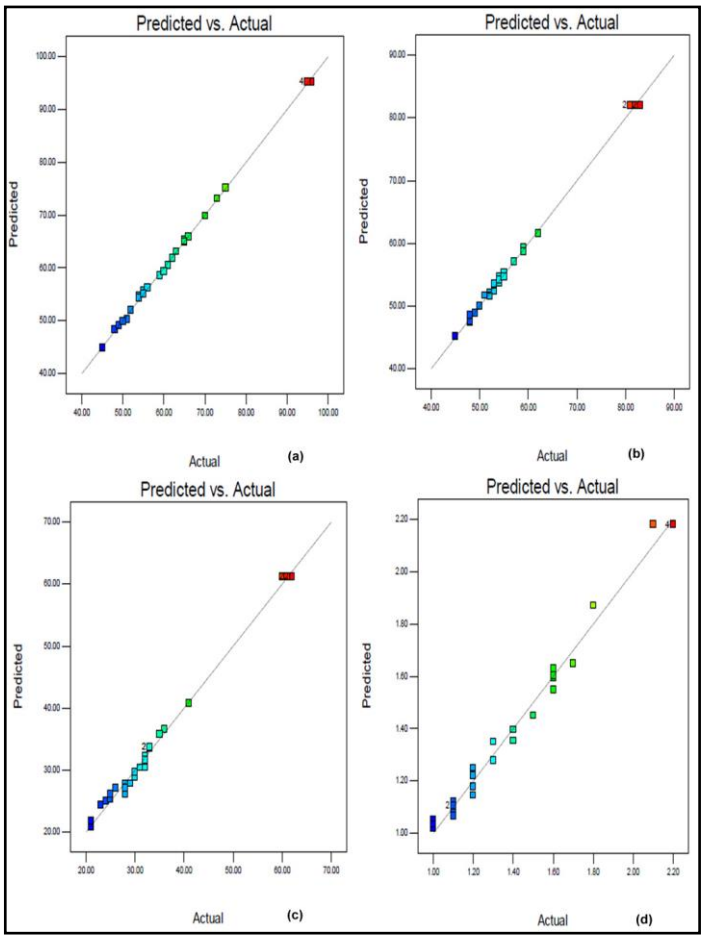


**Figure 1** The actual and predicted plot for (a) Methylparathion removal (%), (b) COD removal (%), (c) TOC removal (%) and (d) growth (OD)

**Analysis of optimized process variables by response surface plots**

The optimum values of the selected variables were obtained by solving their regression equation and analyzing response surface contour plots. Response Surface plots as a function of four factor at a time maintaining all other factors at a fixed level (zero for instance) are more helpful in understanding both the main and interaction effects of the four factors. The plots can be easily obtained by calculating the data from the model. The values were taken by one factor, where the second varies with constant of a given Y-values. The yield values of the different concentrations and range of the variable can also be predicted from respective response surface plots. The coordinates of the central point within the highest optimum concentration of the respective components. Figures 2 to 5 show their response surface obtained as a function of temperature, pH, time and agitation against methylparathion removal, COD removal, TOC removal and growth of bacteria in terms of optical density.

**Optimum values and validation of the model**

The methylparathion removal by *Pseudomonas aeruginosa* mpd was predominantly influenced by the combined effects of the environmental factors include temperature, pH, incubation period (time) and agitation. The point prediction from the analysis of variables for the response surface model showed the maximum methylparathion removal (95.2 %), COD removal (82 %), TOC removal (61.2 %) and growth (2.18 OD) by *Pseudomonas aeruginosa* (mpd) in synthetic wastewater containing 1000 $mgL^{-1}$ of methylparathion at optimum conditions of pH (7), temperature (32.5 $^{o}$C) and agitation at 150 rpm for 96 h of incubation period. As can be seen, there is not much difference between the experimental values and model response values obtained. This confirmed that RSM could be effectively used to predict the removal performance of methylparathion from wastewater by potential bacterial strain (*Pseudomonas aeruginosa* mpd).

The maximum experimental response for methylparathion removal was 95 % whereas the predicted value was 95.2 % indicating a strong agreement between them. The optimum values of the tested variables are at pH 7, 32.5$^{o}$C temperature and agitation at 150 rpm for 96 h of incubation time as shown in perturbation graph (Figure 6.). The model was also validated by conducting the experiments under the optimized conditions, which resulted in the methylparathion removal of 96 % (Predicted response 95.2 %), thus proving the validity of the model.

The temperature is the most suitable variable for the growth of the isolates as well as the methylparathion (MP) removal which was found to be growth related processes. Temperature is another abiotic factor that influences the rate and extent of bioremediation since it affects microbial activity with rates of metabolic reactions generally increasing with increasing temperature (**Baker, 1994 and Hong *et al.*, 2007**). Shake culture or aerated culture conditions are better for the growth and removal of methylparathion. The rise in temperature of the synthetic wastewater medium may accelerate the chemical reactions, reduces solubility of gases, amplifies taste and odour and elevates metabolic activity of organisms. This in turns reduce the organic loads in terms of COD and TOC in the wastewater. The decrease in COD and TOC may increase the biodegradation of methylparathion. This may be the cause for the increase in the biodegradability of methylparathion from the medium. So, the organic loads in terms of these parameters may increase the removal of methylparathion from aqueous solution. It was noted that the removal of organic load in terms of COD was proportional to the disappearance of cypermethrin (**Jilani and Khan, 2006**). Similar correlations were also observed by **Berchtold *et al.*, (1995).**

The optimum growth of the strain was found to be pH 7. The strain mpd can degrade methylparathion from pH7; this could perhaps be due to increased bioavailability of methylparathion and optimal biotic activity of cells in this pH. The pH from 5 to 8 suggested that dissipation of methylparathion was mediated by the cometabolic activities of the bacteria and also the rate of degradation of methylparathion was low in acidic but increased considerably with an increase in pH. **Brajesh *et al.*, (2004)** also reported similarly that the pH from 4.7 to 8.4 for chlorpyrifos by *Enterobacter* strain (B-14). The optimum conditions were more favorable for the growth of the bacteria. It may be either metabolize or co-metabolize the methylarathion in the medium as a nutrient or energy source for their growth and metabolism. The degradation of methylparathion supported cell growth, indicating that isolated strain could utilize methylparathion as a phosphorus source. The pH condition would be significance while emergent an effective remediation strategy.

The optimum time for the incubation period was enhanced the growth of the bacteria and increase its metabolic activity. The log phase of the bacteria was extended and the secondary metabolites which include the release of the respective enzymes responsible for the hydrolysis of methylparathion degradation, or oxidation and reduction process may occur. This in turns may results in the higher reactivity of the pollutant and increases the degradation process. The optimum agitation observed was more encouraged for the growth of the bacteria by utilizing the nutrients from the uniformly distributed and suspended nutrients in the medium which may helps in oxidation process. It may be either metabolize or co-metabolize the methylarathion in the medium. Shake culture or aerated culture conditions are better for the growth and removal of methylparathion. Methylparathion removal under aerobic conditions suggesting that a constitutively expressed enzyme could be involved in the degradation. Repeated application of pesticides results in the enhanced ability of microbial population to degrade the pesticide. The study also suggests that methylparathion degrading bacterial culture should preferably be used for the management of methylparathion containing wastewater.

**Table 4** ANOVA table for $Y_1$ (methylparathion removal in %), $Y_2$ (COD removal in %), $Y_3$ (TOC removal in %) and $Y_4$ (growth in optical density) responses

| Source | DF | $Y_1$ | | | | $Y_2$ | | | | $Y_3$ | | | | $Y_4$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SS | MS | F | P | SS | MS | F | P | SS | MS | F | P | SS | MS | F | P |
| **Model** | 14 | 6903 | 493 | 1857 | < 0.0001 | 3961 | 282 | 522 | < 0.0001 | 4683 | 334 | 202 | < 0.0001 | 4.55 | 0.32 | 117 | < 0.0001 |
| **A-$X_1$- Temp ($^oC$)** | 1 | 261 | 261 | 984 | < 0.0001 | 6.7 | 6.7 | 12.4 | 0.0033 | 18.7 | 18.7 | 11.3 | 0.0046 | 0.06 | 0.06 | 24.3 | 0.0002 |
| **B-$X_2$- pH** | 1 | 90.7 | 90.7 | 341 | < 0.0001 | 75 | 75 | 138 | < 0.0001 | 80 | 80 | 48.4 | < 0.0001 | 0.21 | 0.21 | 76.9 | < 0.0001 |
| **C-$X_3$- Time (h)** | 1 | 396 | 396 | 1494 | < 0.0001 | 21.3 | 21.3 | 39.3 | < 0.0001 | 10 | 10 | 6.1 | 0.0270 | 0.18 | 0.18 | 67.5 | < 0.0001 |
| **D- $X_4$- Agitation (rpm)** | 1 | 85.3 | 85.3 | 321 | < 0.0001 | 80 | 80 | 147 | < 0.0001 | 36.7 | 36.7 | 22.2 | 0.0003 | 0.16 | 0.16 | 58.8 | < 0.0001 |
| **$X_1$ $X_2$** | 1 | 1 | 1 | 3.76 | 0.0727 | 9 | 9 | 16.6 | 0.0011 | 0.25 | 0.25 | 0.15 | 0.7032 | 0.00 | 0.00 | 0.90 | 0.3585 |
| **$X_1$ $X_3$** | 1 | 72.2 | 72.2 | 272 | < 0.0001 | 4 | 4 | 7.38 | 0.0167 | 20.2 | 20.2 | 12.2 | 0.0035 | 0.00 | 0.00 | 0.90 | 0.3585 |
| **$X_1$ $X_4$** | 1 | 240 | 240 | 904 | < 0.0001 | 72.2 | 72.2 | 133 | < 0.0001 | 90.2 | 90.2 | 54.6 | < 0.0001 | 0.12 | 0.12 | 44.1 | < 0.0001 |
| **$X_2$ $X_3$** | 1 | 81 | 81 | 305 | < 0.0001 | 4 | 4 | 7.38 | 0.0167 | 72.2 | 72.2 | 43.7 | < 0.0001 | 0.25 | 0.25 | 90.1 | < 0.0001 |
| **$X_2$ $X_4$** | 1 | 20.2 | 20.2 | 76.2 | < 0.0001 | 4 | 4 | 7.38 | 0.0167 | 20.2 | 20.2 | 12.2 | 0.0035 | 0.06 | 0.06 | 22.5 | 0.0003 |
| **$X_3$ $X_4$** | 1 | 36 | 36 | 135 | < 0.0001 | 25 | 25 | 46.1 | < 0.0001 | 6.25 | 6.25 | 3.7 | 0.0722 | 0.09 | 0.09 | 32.4 | < 0.0001 |
| **$X_1^2$** | 1 | 2808 | 2808 | 10579 | < 0.0001 | 1138 | 1138 | 2102 | < 0.0001 | 1655 | 1655 | 1001 | < 0.0001 | 0.92 | 0.92 | 333 | < 0.0001 |
| **$X_2^2$** | 1 | 2144 | 2144 | 8078 | < 0.0001 | 914 | 914 | 1688 | < 0.0001 | 1050 | 1050 | 635 | < 0.0001 | 1.25 | 1.25 | 452 | < 0.0001 |
| **$X_3^2$** | 1 | 2387 | 2387 | 8991 | < 0.0001 | 1583 | 1583 | 2923 | < 0.0001 | 1553 | 1553 | 940 | < 0.0001 | 0.92 | 0.92 | 333 | < 0.0001 |
| **$X_4^2$** | 1 | 1327 | 1327 | 5002 | < 0.0001 | 1986 | 1986 | 3667 | < 0.0001 | 2397 | 2397 | 1450 | < 0.0001 | 2.07 | 2.07 | 746 | < 0.0001 |
| **Residual** | 14 | 3.71 | 0.26 | | | 7.58 | 0.54 | | | 23.1 | 1.67 | | | 0.03 | 0.00 | | |
| **Lack of Fit** | 10 | 2.91 | 0.29 | 1.458 | 0.3821 | 3.58 | 0.36 | 0.36 | 0.9143 | 20.3 | 2.0 | 2.9 | 0.157 | 0.03 | 0.00 | 1.54 | 0.3594 |
| **Pure Error** | 4 | 0.8 | 0.2 | | | 4 | 1 | | | 2.8 | 0.7 | | | 0.00 | 0.00 | | |
| **Cor Total** | 28 | 6906 | | | | 3968 | | | | 4706 | | | | 4.59 | | | |

**Table 5** Analysis of variance (A NOVA) results of the model of methylparathion removal, COD and TOC removal by *Pseudomonas aeruginosa* mpd

| Source | Sum of squares | Degree of freedom | Mean Square | F-value | Prob > F | Remarks |
|---|---|---|---|---|---|---|
| [a]**MP-Removal (%)** | | | | | | |
| Model | 6903 | 14 | 493.07 | 1857.3 | <0.0001 | **Significant** |
| Residual | 3.716 | 14 | 0.2654 | | | |
| Lack of fit | 2.916 | 10 | 0.2916 | 1.4583 | 0.3821 | Not- Significant |
| Pure error | 0.8 | 4 | 0.2 | | | |
| Cor Total | 6906 | 28 | | | | |
| [b]**COD Removal (%)** | | | | | | |
| Model | 3961 | 14 | 282.94 | 522.3 | <0.0001 | **Significant** |
| Residual | 7.58 | 14 | 0.54 | | | |
| Lack of fit | 3.58 | 10 | 0.36 | 0.36 | 0.9143 | Not- Significant |
| Pure error | 4.00 | 4 | 1.00 | | | |
| Cor Total | 3968 | 28 | | | | |
| [c]**TOC Removal (%)** | | | | | | |
| Model | 4683 | 14 | 334.55 | 202.47 | <0.0001 | **Significant** |
| Residual | 23.13 | 14 | 1.6523 | | | |
| Lack of fit | 20.33 | 10 | 2.0333 | 2.9047 | 0.1579 | Not- Significant |
| Pure error | 2.8 | 4 | 0.7 | | | |
| Cor Total | 4706 | 28 | | | | |
| [d]**G-MP$^+$ (OD)** | | | | | | |
| Model | 4.553 | 14 | 0.3252 | 117.25 | <0.0001 | **Significant** |
| Residual | 0.038 | 14 | 0.0027 | | | |
| Lack of fit | 0.030 | 10 | 0.0030 | 1.5416 | 0.3594 | Not- Significant |
| Pure error | 0.008 | 4 | 0.002 | | | |
| Cor Total | 4.592 | 28 | | | | |

| R-squared | Adj R-squared | Pred R-squared | Adequate precision |
|---|---|---|---|
| [a]$R^2$ = 0.9994; | $R^2_{adj}$= 0.9989; | $R^2_{pred}$= 0.9973 | Adeq precision = 135.92 |
| [b]$R^2$ = 0.9981; | $R^2_{adj}$ = 0.9962; | $R^2_{pred}$= 0.9932 | Adeq precision = 69.66 |
| [c]$R^2$ = 0.9950; | $R^2_{adj}$ = 0.9901; | $R^2_{pred}$= 0.9741 | Adeq precision = 43.75 |
| [d]$R^2$ = 0.9915; | $R^2_{adj}$ = 0.9830; | $R^2_{pred}$= 0.9586 | Adeq precision = 30.60 |

***MP****- Methylparathion,* ***COD****- Chemical oxygen demand,* ***TOC****- Total organic carbon,*
***G-MP$^+$*** *- Growth in presence of methylparathion,* ***OD****- Optical density*

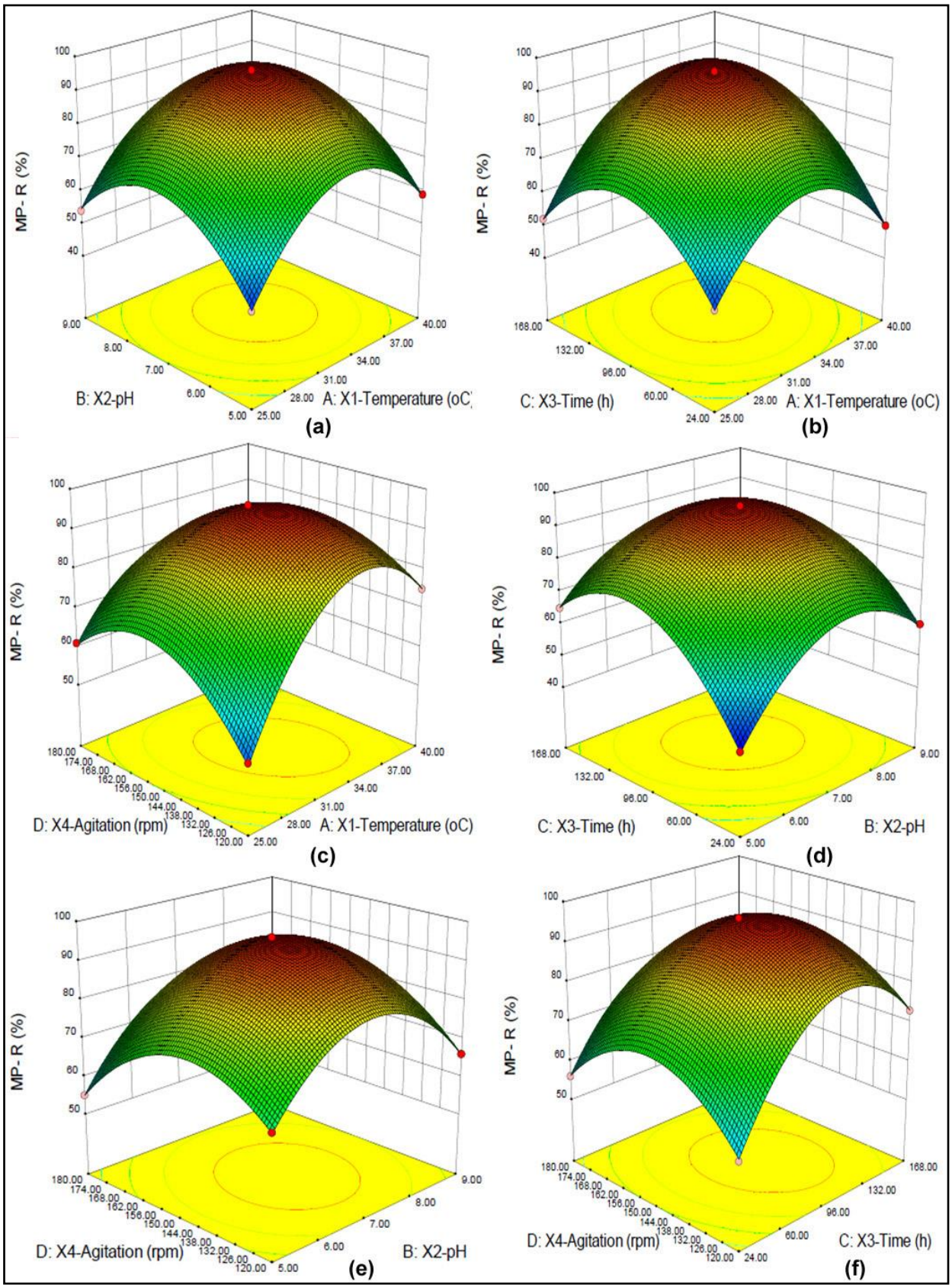


**Figure 2a** Response surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of Methylparathion by Pseudomonas aeruginosa mpd

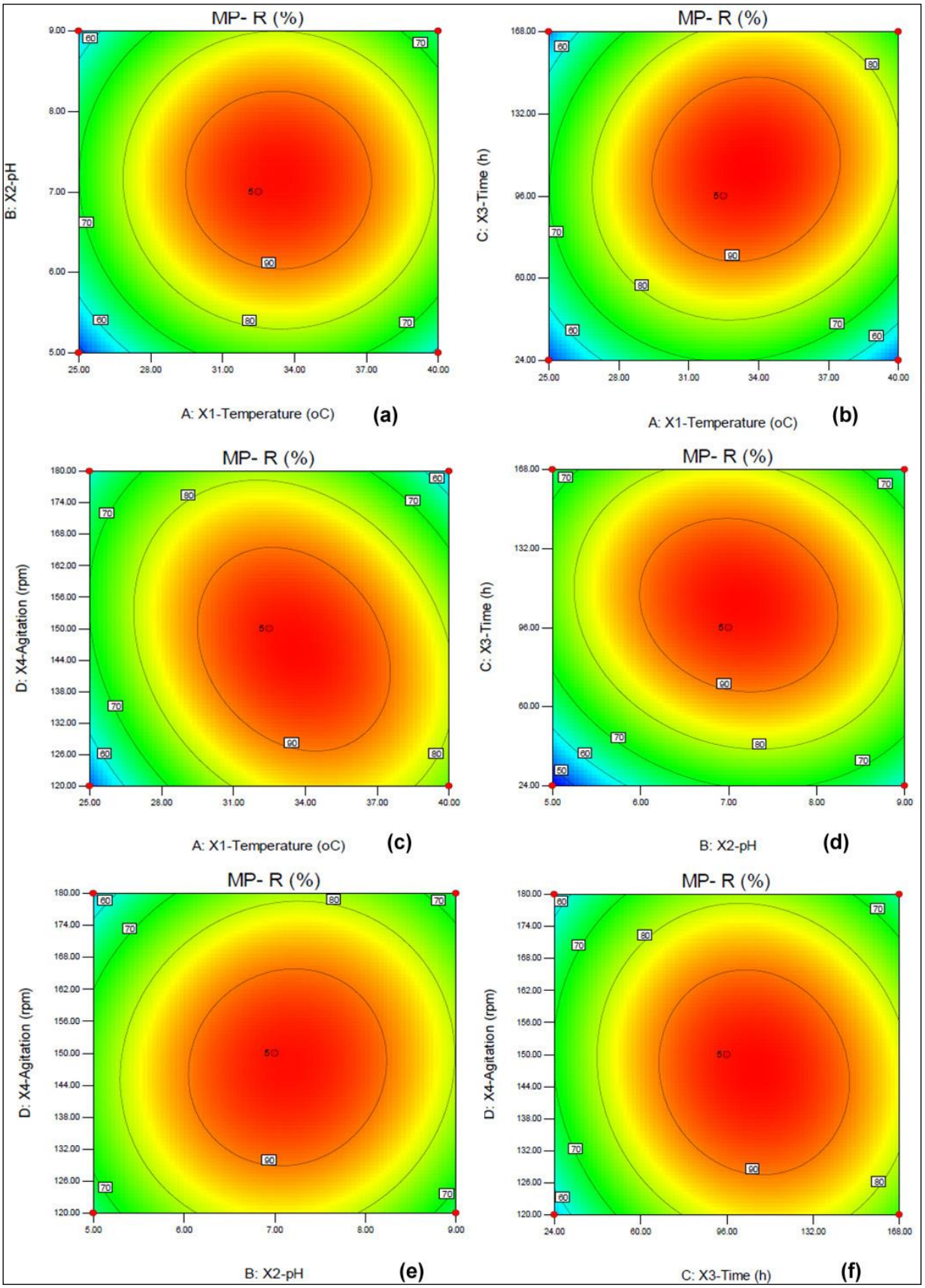


**Figure 2b** Contour surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of Methylparathion by *Pseudomonas aeruginosa* mpd

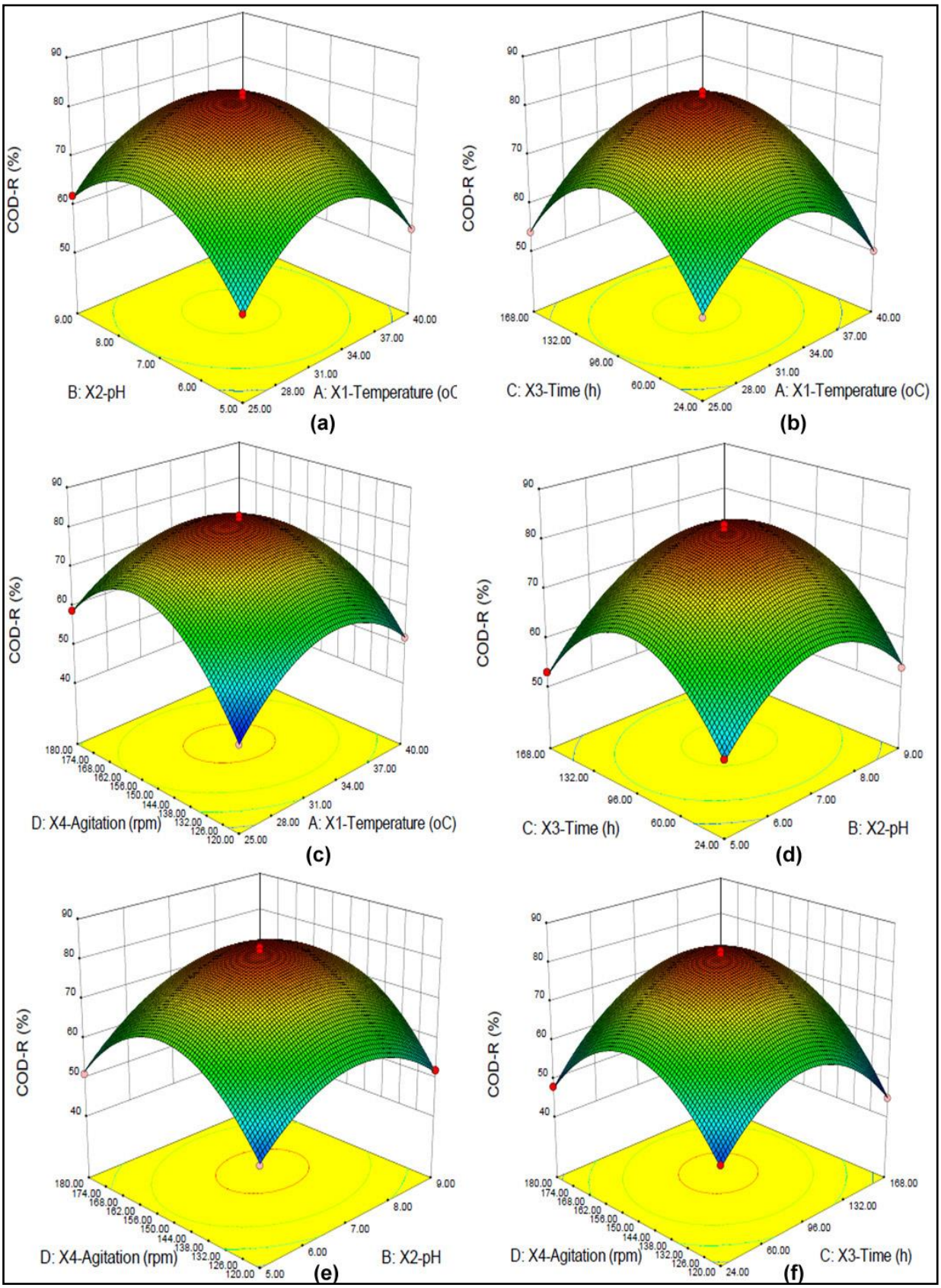


**Figure 3** Response surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of COD by *Pseudomonas aeruginosa*mpd

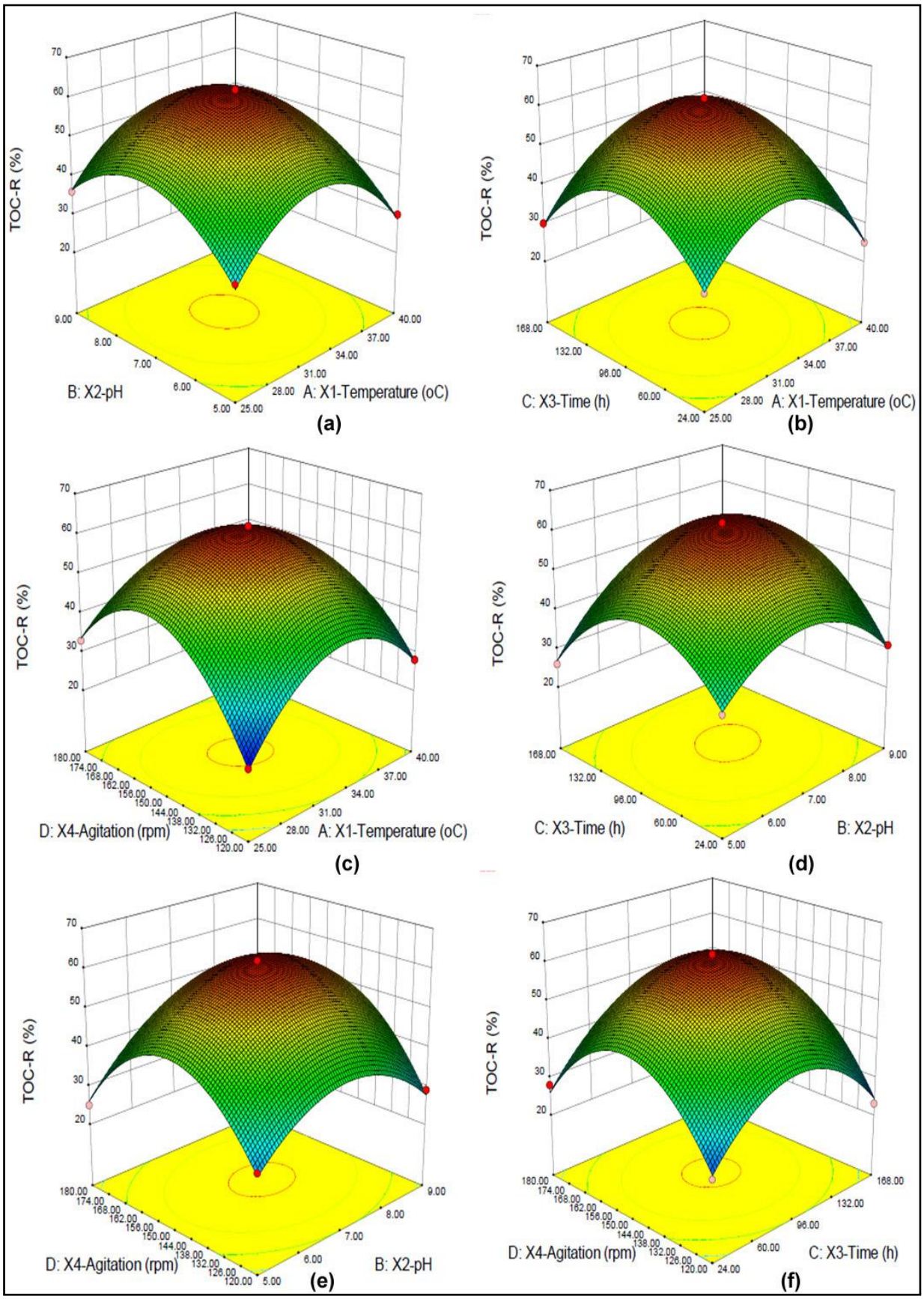


**Figure 4** Response surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of TOC by *Pseudomonas aeruginosa* mpd

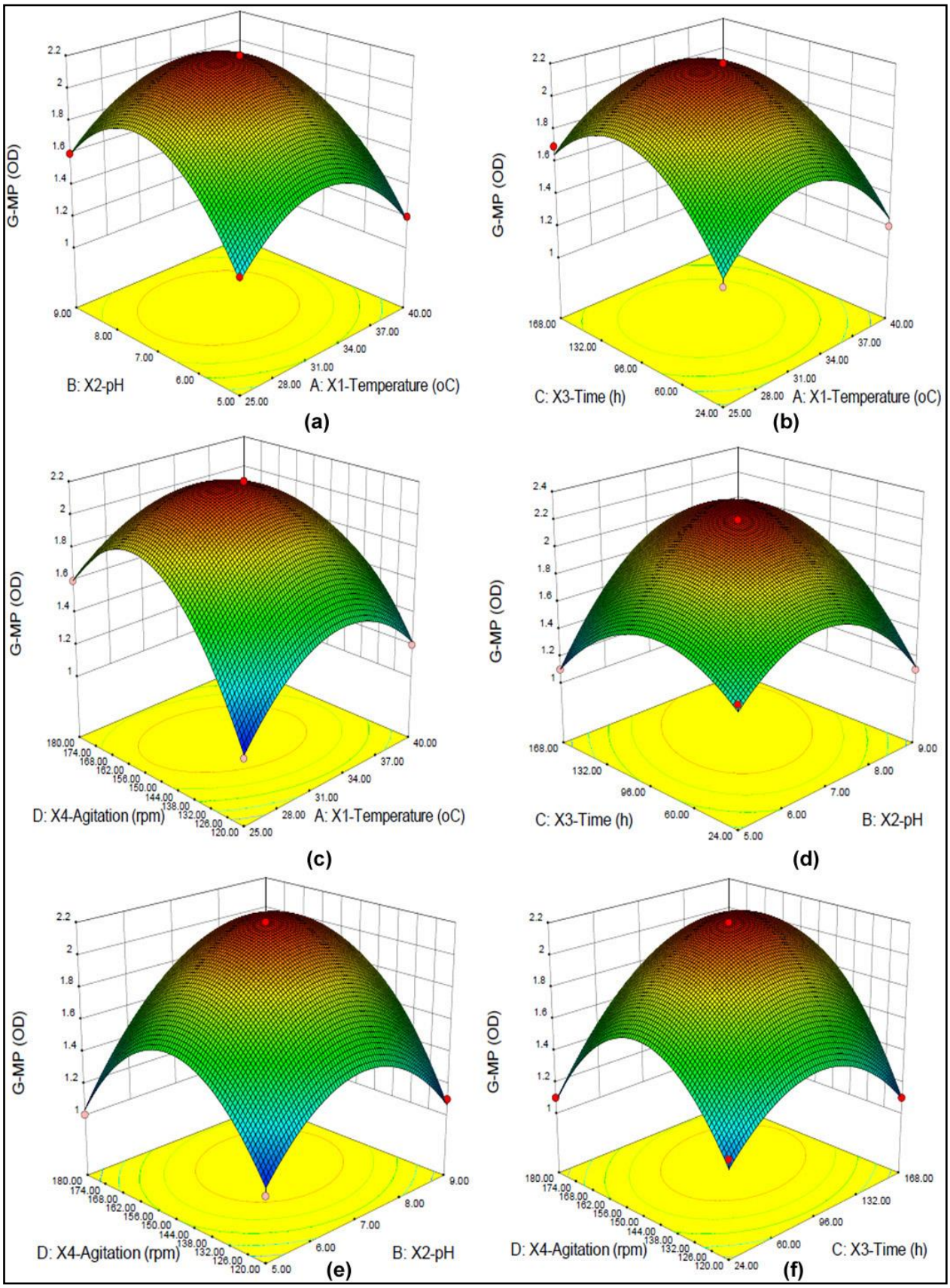


**Figure 5** Response surface plot of the combined effects of pH, temperature, time and agitation on the growth of *Pseudomonas aeruginosa* mpd

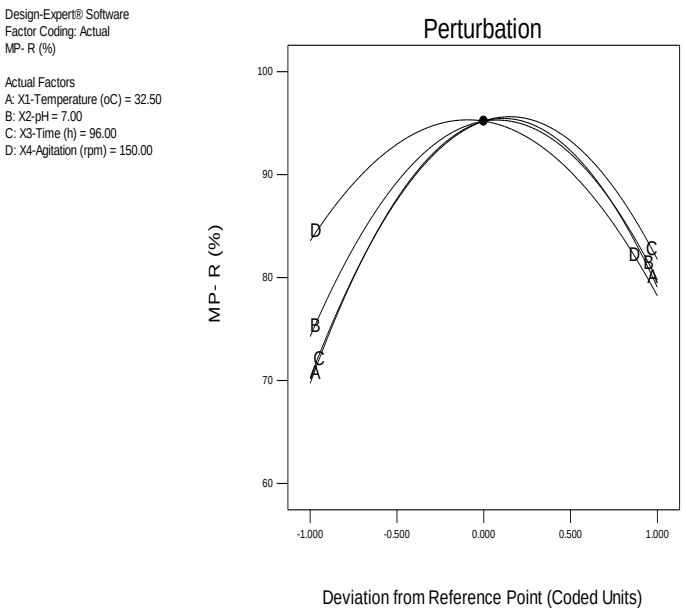


**Figure 6** Perturbation graph showing the optimum values of the tested variables

However, in the presence of other carbon sources (such as glucose), initially it delayed to degrade methylparathion but with the passage of time it degraded to 95 % within 96 h, indicating that when glucose was depleted, it started to utilize methylparathion as a source of carbon. Similar results were reported by **Brajesh *et al.*, (2004)**. Glucose was chosen because it is a primarily substratum and the main carbon source for the bacteria. Glucose addition is important to improve the efficacy of bioremediation of persistent compounds like pesticides (**Sampaio, 2005; Singh, 2006; Yang *et al.*, 2009; Yugui et al., 2008**). **Qiu *et al.*, (2006)** reported that the additional nutrients such as glucose and organic nitrogen greatly enhanced the growth of *Ochrobactrum* sp B2. Singh (2006), reports that the addition of glucose produces substances of high reactivity, which react more easily with the pollutant. Previous reports concerning isolation of organophosphorus degrading microorganisms suggest that the bacteria mainly degrade the compounds cometabolically (**Horne *et al.*, 2002; Zhongli *et al.*, 2001**). Some reports showed that the isolated bacterium can utilize organophosphates as a source of carbon or phosphorus (**Subhas and Dileep, 2003**) from the hydrolysis products (**Serdar and Gibson, 1985**). In natural environments, the competition for carbon sources is immense and the utilization of pesticide as an energy source by this bacterium provides it with a substantial competitive advantage over other microorganisms (**Malghani *et al.*, 2009**).

**UV-Vis Spectral Analysis**

In order to investigate the formation and eventual disappearance of intermediate compounds in the reaction mixture, the biotreated synthetic wastewater containing methylparathion was monitored using UV-Vis spectroscopy as a function of time. The UV-Vis spectroscopy scanning profile shows a peak formation with lambda max (λmax) at 277 nm as shown in Figure 7. The extended biotreatment after 96 h shows the same band decrease its intensity and eventually disappeared. The absorbance value was found to be reduced at maximum time of 96 h at optimized process variables. The wavelength at 277nm shows a displacement to higher wavelengths and formation of band at 400 nm that can be attributed to the p-nitrophenol absorption bands. **Zhongli *et al* (2001)** reported that the maximum absorption peak of methylparathion was recorded at 273nm by *Plesiomonas* strain (M6). **Wu and Linden (2008)** reported that the parathion produces a maximum absorbance (λmax) at 275nm. Further, the biotreated samples were analysed by HPLC for the confirmation of the residual MP and intermediates formation.

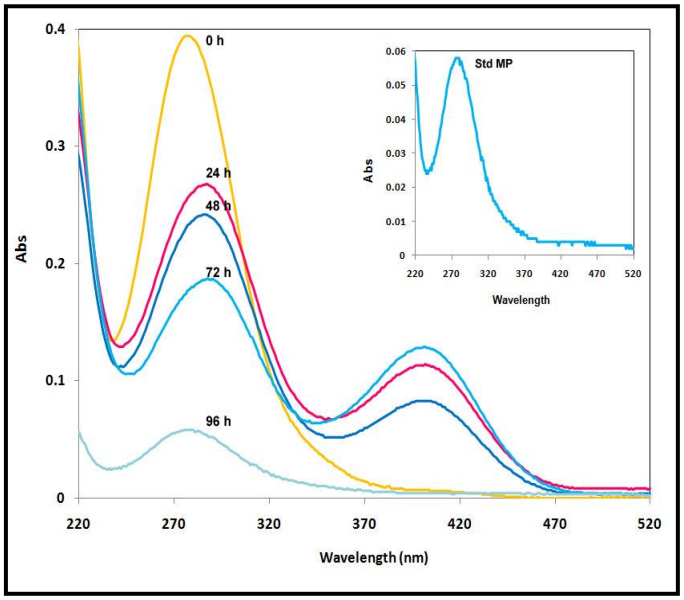


**Figure 7** UV –Vis NIR Spectroscopic scanning profile of MP degradation by *Pseudomonas aeruginosa* mpd at different treatment time (h)

**HPLC Analysis**

The biotreated samples were analysed by HPLC for the confirmation of the residual methylparathion and their byproducts or intermediatediates formation. The retention time for methylparathion was found to be 3.3min which was confirmed by the spectra as shown in Figure 8. The percentage degradation of methylparathion by *Pseudomonas aeruginosa* mpd was found to be 95 %. Treated samples showed that the peak reduction at 3.3 retention time (RT), hence it proves the degradation of methylparathion by biotreatment (*Pseudomonas aeruginosa* mpd) process. The peak at retention time of 4.0, 4.4 and 10 min in treated sample were observed as the intermediate products of methylparathion degradation during the biotreatment process. Moreover, methylparathion was rapidly oxidized into other organic compounds.

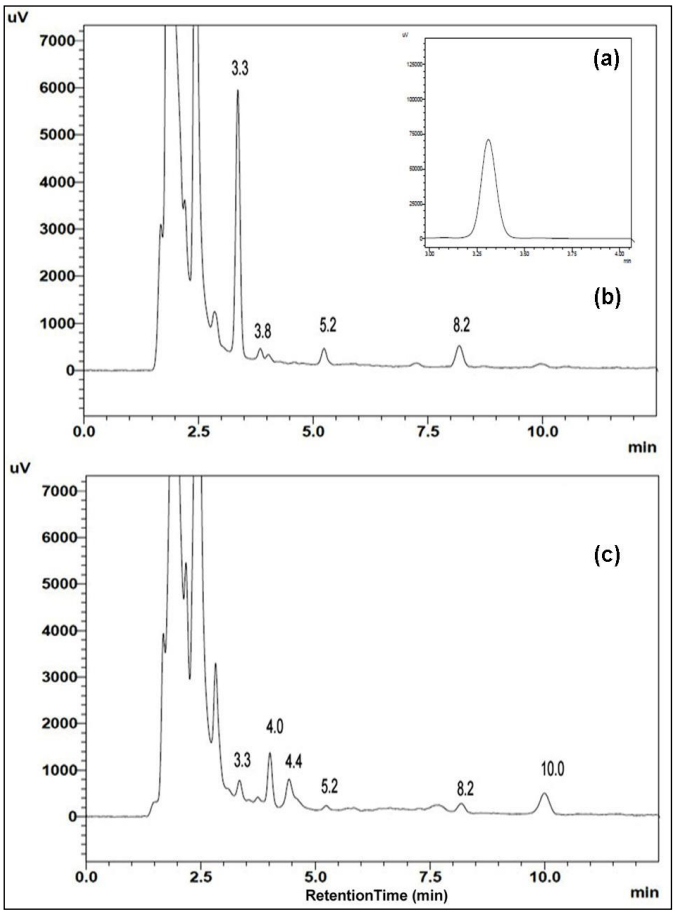


**Figure 8** HPLC profiles of methylparathion biodegradation by *Pseudomonas aeruginosa* mpd after 72h (a) Standard, (b) Control and (b) Treated after 72 h

## CONCLUSION

RSM was used in this study to establish the optimum variables initial pH, time, culture temperature and agitation for methylparathion biodegradation. It was concluded that the optimal conditions for methylparathion removal are pH 7 and 32.5 $^{o}$C temperature and agitation at 150 rpm for 96 h of incubation period. The predicted extent of methylparathion biodegradation by this strain of *Pseudomonas aeruginosa* under these optimum conditions was 95.2 %, and the experimental results were in close agreement with this prediction. The point prediction from the analysis of variable for response surface model for methylparathion removal (95.2 %), COD removal (82 %), (c) TOC removal (61.2 %) and (d) growth (2.18 OD) by *Pseudomonas aeruginosa* (mpd) from waste water with 1000 mg/L of medium at optimum conditions of pH, temperature and agitation for 96 h of incubation period. The predicted optimal and experimental measured methylparathion removal efficiencies agreed well with high coefficients of determination ($R^2$ = 0.9994, $R^2_{adj}$ = 0.9989), and the COD removal ($R^2$ = 0.9981 $R^2_{adj}$ = 0.9962) and TOC removal ($R^2$ = 0.9950, $R^2_{adj}$ = 0.9901) are also agreed well. Moreover the growth of the strain in terms of its OD were also agreed well ($R^2$ = 0.9915, $R^2_{adj}$ = 0.9830). Hence this study was an attempt for methylparathion removal using *Pseudomonas aeruginosa* strain with RSM model, has helped to recognize the important operating variables and optimum levels with least effort and time. The isolate of the present study was found to have potential in methylparathion removal at optimized condition and suggested for biotreatment of methylparathion wastewater. This study will form the basis for the further utilization of the bacterial strain, grown on suitable substrates, in biofiltration systems for the treatment of wastewaters.

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