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Optimization of Physicochemical Parameters for Enhanced *P*-Nitrophenol Biodegradation by an Isolated Microbial Consortium Using Response Surface Methodology

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Abstract: *Para-nitrophenol (PNP), a nitroaromatic compound that is commonly used as a precursor in the manufacturing of pharmaceuticals, insecticides, fertilizers, azo dyes, solvents and to darken leathers. It is highly toxic and affects the soil micro-flora and aquatic life and is mutagenic and carcinogenic. As a result, it is critical to eliminate PNP or reduce it to allowable levels before releasing it into the environment. Among the available physical and chemical treatment methods, the biological methods of removal of PNP are cost effective, efficient and eco-friendly. Recent reports suggestthat microbial consortium is more efficient than pure cultures in degrading xenobiotics. In our study, we have attempted to optimize the physicochemical parameters for PNP degradation by microbial consortium isolated in our laboratory, using a statistical optimization technique called Response Surface Methodology (RSM). Five factors and their different levels were investigated for their impact on PNP biodegradation by the microbial consortium. The optimal pH, inoculum dosage, yeast extract concentration were found to be 10, 0.44 AU (OD600) and 0.2%, respectively, by the Central Composite Design (CCD). Temperature and agitation speed were found to have less significance on the PNP degradation and were kept in a range between 32 ^{0} C and 34 ^{0} C, 120 and 150 rpm. The PNP degradation of 99.36% was obtained within 72 hours when the initial concentration of 1000 mg/L was provided under the optimized conditions. The results suggest that RSM can be used as a valuabletool to optimize the operating parameters leading to enhanced pollutant degradation.*

Keywords: *CCD; Microbial consortium; PNP; RSM*

1. Introduction

Nitroaromatic compounds are used in various industries in the production of drugs, pesticides, herbicides, fungicides, explosives, petrochemicals and precursors for dyes and plasticizers. The accumulation of nitro-aromatic compounds interferes with aquatic life when effluent water mixes with water bodies because of its toxicity. The discharge of nitroaromatic compounds in wastewater and application as pesticides (Parathion, Dinoseb, Fenitrothion) have broadened their environmental impact and called for solutions for the redemption of these toxic compounds. Some are highly mutagenic and toxic.

4- Nitrophenol is mainly used in the manufacturing of drugs, fungicides, insecticides (parathion), herbicides (fluoridofen) and azo dyes. It occurs as a contaminant in effluents of these industries and affects the ground soil and water. Acute inhalation leads to nausea, drowsiness, headaches and cyanosis and irritates eyes on contact [1].

In studies on rat reports, acute inhalation caused methemoglobinemia by delaying the contact with blood forms methemoglobin [1] and led to kidney and liver damage. 4-Nitrophenol or p-Nitrophenol (PNP) is harmful compared to other nitrophenols. Nitrophenols (2-NP, 4-NP, 2,4-DNP) have been classified as priority pollutants by the US Environmental Protection Agency, which recommends maximum contaminant level (MCL) of PNP is 1μg/L for phenols in drinking water [2].

PNP can be readily broken down on surfaces but in deep-down soil and groundwater,it takes a long time. It may accumulate in the food chain. Removal of PNP is necessary because of its toxicity to many living organisms. It can be achieved by physical, chemical and or biological treatment processes. The physical and chemical treatment processes are expensive and produce secondary by products which are sometimes more toxic than the parent compounds. treatment process includes adsorption, ultrasonic irradiation and microwave associated oxidation. Therefore, biodegradation is preferred owing to its minimum expenditure and possibility of complete mineralization [2,3].

The objective is to optimize the physicochemical parameters for the enhanced biodegradation of para nitrophenol (PNP) using Response Surface Methodology (RSM). Central Composite Design is used to determine the influence of the experimental variables and their interaction on the enhanced removal of PNP.

2. Materials and Methods

2.1. Microbial consortium and Culture medium

All chemicals used in this study were of analytical grade and were procured from Sigma- Aldrich. A microbial consortium was isolated from a pesticide-contaminated agricultural field and biochemically characterized in our laboratory and reported in the previous work [4] as *Brevibacterium sp. PNP1*(MH169212), *Pseudomonas sp. PNP2* (MH169213), *Agromyces mediolanus PNP3*(MH169214), *Microbacterium oxydans PNP4*(MH169215). The enrichment media for consortium contains KH_2PO_4-1.2(g/l), K_2HPO_4-4.8 (g/l), $MgSO_4$- 0.25(g/l), $FeSO_4.7H_2O$-0.0025(g/l), $CaCl_2.2H_2O$ 0.03(6g/l) [5] at pH-9. The isolated four membered consortium was grown in a 500 mL Erlenmeyer flask containing 50 mL enrichment media containing yeast extract was supplemented with 1000 mg/L of PNP. Cells were grown for 72 hours then separated and washed with saline solution (0.8% NaCl) by centrifugation at 5000 rpm for 15 min at 4^0 C. These cells were used as inoculum for the degradation tests of the design developed using RSM.

2.2. PNP estimation

PNP concentration in the medium is measured using UV-Spectrometer at 405 nm. The percentage of phenol degradation can be calculated using the equation:

$$\text{PNP degradation (\%)} = (C_i - C_f / C_f) * 100 \quad (1),$$

where C_i and C_f are the initial and final concentrations of PNP respectively.

2.3. Methodology and Design of Experiments

Central composite Design

The significant factors of biodegradation of PNP have been found already using change of one variable at a time (COVT) were pH of the medium, yeast extract concentration (%), agitation (rpm), inoculum dosage (OD_{600}) and temperature (^{0} C). Each factor was assessed at five different levels (-2, -1, 0, 1, 2) to find the interaction between the variables shown in **Table 1**. The biodegradation of phenol was analysed using second-order polynomial equation given:

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ij} X_i X_j + \Sigma \beta_i X_i^2 \quad (2)$$

where Y is the response (percentage PNP degradation); β_0 is constant; β_i, β_{ij}, β_{ii} are linear, quadratic and interactive regression coefficients respectively. X is the significant variable. The experimental design of CCD is developed using Design Expert software (trial version 11) which is also shown in **Table 1.**

TABLE 1: Different levels of the factors

Factors	-2	-1	0	1	2
pH	6.62	8	9	10	11.38
Yeast extract (%)	0.031	0.1	0.15	0.2	0.268
Agitation (rpm)	99.32	120	135	150	170.68
Inoculum dosage (OD_{600})	0.162	0.3	0.4	0.5	0.638
Temperature (^{0} C)	27.24	30	32	34	36.76

2.3.Statistical analysis of the data

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). It includes the Fischer test(F-test), probability factor and R^2 coefficient of determination which measures the goodness of fit to the regression model. The contour plots and surface plots are used to find the responses and interactions between the variables.

The design was developed and the experimental analysis was done using Design Expert software (trial version 11).

TABLE 2: Experimental design and results obtained

S. No.	pH	Temp (^{0} C)	Agitation (rpm)	Inoculum dosage (OD600)	Yeast Extract (%)	Experimental PNP Degradation (%)	Predicted PNP Degradation (%)
1	9	27.24	135	0.4	0.15	14.1	18
2	10	30	120	0.3	0.2	67.92	63.8
3	10	30	120	0.5	0.2	20	22
4	8	30	120	0.5	0.1	82.15	78
5	10	30	120	0.5	0.1	15.02	16.4
6	10	30	120	0.3	0.1	35.7	38.85
7	8	30	120	0.5	0.2	95.1	80
8	8	30	120	0.3	0.2	60	63.46
9	8	30	120	0.3	0.1	75.5	75.7
10	8	30	150	0.3	0.2	99.15	93.6
11	10	30	150	0.5	0.1	39.65	43.75
12	10	30	150	0.5	0.2	40.25	39.62
13	8	30	150	0.3	0.1	21	23
14	10	30	150	0.3	0.1	89.5	89.97
15	8	30	150	0.5	0.1	62.3	61.5
16	8	30	150	0.5	0.2	98.1	93.69
17	10	30	150	0.3	0.2	40.1	41.89
18	9	32	99.3	0.4	0.15	41	44.83
19	9	32	135	0.4	0.031	39.4	40.8
20	9	32	135	0.637	0.15	77	75.48
21	11.38	32	135	0.4	0.15	38.11	40
22	9	32	135	0.4	0.15	49.6	58
23	9	32	135	0.4	0.269	36	42.3
24	9	32	135	0.4	0.15	42.5	45.45
25	9	32	135	0.162	0.15	29.3	26

26	6.62	32	135	0.4	0.15	77.7	74.88
27	9	32	170.67	0.4	0.15	65	65.2
28	10	34	120	0.3	0.1	36.25	38.6
29	8	34	120	0.5	0.1	100	111
30	10	34	120	0.3	0.2	17.76	10.4
31	8	34	120	0.5	0.2	84.38	76.8
32	8	34	120	0.3	0.2	31.17	36
33	10	34	120	0.5	0.2	12	13.38
34	8	34	120	0.3	0.1	83	77.25
35	10	34	120	0.5	0.1	21.7	17
36	8	34	150	0.5	0.2	15	12.08
37	8	34	150	0.3	0.2	100	103
38	10	34	150	0.3	0.1	43	41
39	10	34	150	0.5	0.2	9.93	19.6
40	8	34	150	0.5	0.1	10.57	15
41	10	34	150	0.3	0.2	70	74.1
42	10	34	150	0.5	0.1	64	63.46
43	8	34	150	0.3	0.1	43.06	41.89
44	9	36.75	135	0.4	0.15	15.43	25.87
45	9	32	135	0.4	0.15	49.6	58
46	9	32	135	0.4	0.15	49.6	58
47	9	32	135	0.4	0.15	49.6	58
48	9	32	135	0.4	0.15	42.5	45.45
49	9	32	135	0.4	0.15	42.5	45.45
50	9	32	135	0.4	0.15	42.5	45.45

3. Results and Discussion

3.1. Evaluation of experimental results with Design Expert

The parameters pH, temperature, inoculum dosage, yeast extract concentration and agitation speed were optimized for the enhanced biodegradation of PNP by the microbial consortium using the statistical technique CCD. Based on CCD, 44 runs were performed. The optimization results were analyzed using Design Expert Software (trial version 11). By applying linear regression analysis to the data, the second-order polynomial equation was found to representthe % PNP degradation:

$$Y = 63.1712 + 20.981\ X_1 + 0.460239\ X_2 + 0.869144\ X_3 + 8.25498\ X_4 + 12.3903\ X_5 + 1.69\ X_1X_2 - 0.473125\ X_1X_3 + 8.26313\ X_1X_4 + 0.475\ X_1X_5 + 0.19\ X_2X_3 - 0.68375\ X_2X_4 + 2.67563\ X_2X_5 - 0.244375\ X_3X_4 - 0.0425\ X_3X_5 - 1.7\ X_4X_5 - 0.687338\ X_1^2 - 8.54506\ X_2^2 - 0.961342\ X_3^2 - 1.90975\ X_4^2 - 1.08243\ X_5^2 \quad (3)$$

Where Y (response) is % PNP degradation, X_1, X_2, X_3, X_4 and X_5 are pH, temperature, agitation speed, inoculum dosage and yeast extract concentration respectively. The positiveand negative regression coefficient shows the antagonistic and synergistic effects of eachvariable i.e., A positive sign indicates the direct proportionality of these variables to the response, whereas a negative sign indicates an inverse proportion [3]. The antagonistic effects shown by X_1X_3, X_2X_4, X_3X_4, X_3X_5, X_1^2, X_2^2, X_3^2, X_4^2 and X_5^2. The synergistic effects associated with X_1, X_2, X_3, X_4, X_5, X_1X_2, X_1X_4, X_1X_5, X_2X_3 and X_2X_5. Among these X_1 (pHof the medium) has the highest regression coefficient of 20.981 followed by yeast extract concentration X_5 (12.3903), inoculum dosage X_4 (8.25498), agitation speed X_3 (0.869144) and temperature X_2 (0.460239). X_1, X_5 and X_4 were found to be significant parameters because of their higher impact on PNP degradation compared to X_2 and X_3 regression coefficients.

The predicted values of PNP degradation (%) and experimental values were given in **Table 2**. The results were

analysed by analysis of variance (ANOVA) in **Table 3**. TheF value of the model was 44.94, the lack of fit value was 4.979309 and the value of probability < 0.0001 suggested the model is highly significant. Linear terms X_1, X_4 and X_5 were significant and the quadratic term X_2^2 was significant for PNP degradation. Interactive terms X_1X_4 and X_2X_5 were significant for PNP degradation.

Table 3: ANOVA table

Source	Sum of squares	Degrees of freedom	Mean	F-value	p-value	
Model	34561.44	20	1728.072	44.94293	6.67E-14	Significant
A-pH	19066.81	1	19066.81	495.8811	4.59E-17	Significant
B-Temp.	9.174718	1	9.174718	0.238612	0.629835	
C-Agitation	32.71968	1	32.71968	0.850959	0.365858	
D-Inoculum dosage	2951.6	1	2951.6	76.7639	8.71E-09	Significant
E-Yeast Extract	6649.461	1	6649.461	172.9362	3.48E-12	Significant
AB	91.3952	1	91.3952	2.376966	0.136784	
AC	7.163112	1	7.163112	0.186295	0.670037	
AD	2184.936	1	2184.936	56.82483	1.17E-07	Significant
AE	7.22	1	7.22	0.187775	0.668812	
BC	1.1552	1	1.1552	0.030044	0.863907	
BD	14.96045	1	14.96045	0.389085	0.538919	
BE	229.087	1	229.087	5.957993	0.022764	Significant
CD	1.911012	1	1.911012	0.049701	0.825553	
CE	0.0578	1	0.0578	0.001503	0.969407	
DE	92.48	1	92.48	2.405179	0.134587	
A^2	15.23861	1	15.23861	0.396319	0.5352	
B^2	2355.238	1	2355.238	61.25398	6.24E-08	Significant
C^2	29.80989	1	29.80989	0.775282	0.387696	
D^2	117.6405	1	117.6405	3.059543	0.093599	
E^2	37.79265	1	37.79265	0.982895	0.331801	
Residual	884.3584	23	38.45036			
Lack of Fit	876.3584	22	39.83447	4.979309	0.341567	Not significant
Pure Error	8.00	1	8.00			
Cor Total	35445.8	43				

Std.Dev. 6.20, **R^2** 0.9751, **Adeq. Precision** 23.2976, **Mean** 50.19, **Adjusted R^2** 0.9534, **Predicted R^2** 0.8958, **C.V. %** 12.35.

The R^2 coefficient of determination is used to measure the goodness of fit for the model. Values of R^2 closer to 1 indicate a stronger model and good prediction of the response. The **coefficient of determination** (R^2) was found to be **0.9751**, which indicates that there is a high agreement between predicted and experimental values. Predicted and adjusted R^2 values are **0.9534** and **0.8958** respectively. The optimum levels of each variable and the effects of their interaction on PNP degradationwere studied by response surface plots and their corresponding contour plots. Contour plots area projection of the response surface on a two-dimensional plane whereas the surface plots are projections on a three-dimensional plane. The 3D response surface is a three-dimensional graphic representation used to determine the individual and cumulative effect of the variable and the mutual interaction between the variable and the dependent variable. The response surface analyses the geometric nature of the surface, the maxima and minima of the response and the significance of the coefficients of the canonical equation. The polynomial response surface model obtained may be maximized or minimized to obtain the optimum points.

Whereas, a contour plot is a graphical technique for representing a three-dimensional surface by plotting constant z-slices called contours, on a two-dimensional format. That is, given a value for z, lines are drawn to connect the (x, y) coordinates where the z value occurs.

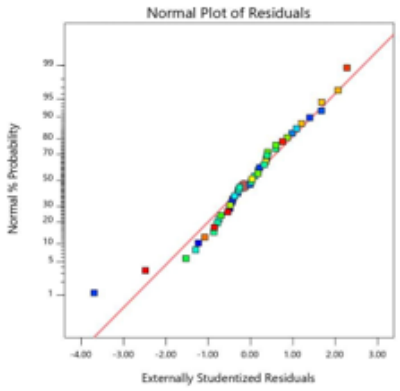

Fig 1: Normal plot

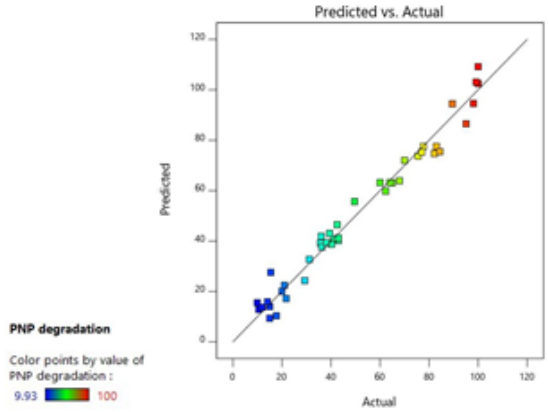

Fig 2: Predicted vs Actual response

The normality of the data was done by means of a **normal probability plot** which is shown in **Fig 1**. It shows the distribution of the data in a straight line. It is used for checking fixed distribution assumptions. The predicted versus actual response is shown in **Fig 2**.

3.1.1. Interaction between the variables

Effects due to the interaction between the variables i.e., parameters are shown in **Fig 3.** The interactive effect between variables A and B was insignificant **Fig 3(a).** The degradation of PNP (%) increased when the pH of the reaction medium increased, whereas temperature change did not affect the degradation of PNP (%). Similarly, the interactive effects between A and C **(Fig 3(b))** & A and E **(Fig 3(c))** were insignificant. Only the pH change showed the difference in the degradation of PNP (%). The interaction between variables A and D showeda significant **(Fig 3(d))** effect on the degradation of PNP. At low pH of 8, the degradation PNP had no effect even when the inoculum dosage was high. Upon increasing the pH to 9 and 10,the degradation of PNP became significant even at lower inoculum dosage levels. The variables B and C **(Fig 3(e))** & B and D **(Fig 3(f))** showed no interactive effects on the degradation of PNP. Variables B and E **(Fig 3(g))** showed a significant interactive effect on the degradation of PNP. At lower temperatures, the degradation of PNP was 60%. Upon mildly increasing the temperature and yeast extract concentration, the degradation of PNP increased to 70%. Yet, at higher temperatures, a fall in the degradation of PNP occurred. the interactive effects between C and D **(Fig 3(h))**, C and D **(Fig 3(i))** & D and E **(Fig 3(j))** were also insignificant in the degradation of PNP. [A - pH; B - Temperature; C - Agitation Speed; D - Inoculum dosage; E- Yeast Extract (Y.E.)]

3.1.2. Validation of the model

Since the temperature and agitation are not significant between the given range, both the variables can be varied in the given range. pH: 10, inoculum dosage: 0.44 AU (OD 600), Y.E.: 0.2%, temperature: 32 ^{0} C (varied 32 ^{0} C -34 ^{0} C), agitation speed: 140 rpm (varied 120 - 150). The percentage degradation of PNP under the optimized condition was 99.36%, which is closer to the predicted PNP degradation (100.3%) for the optimized condition, so the model is validated.

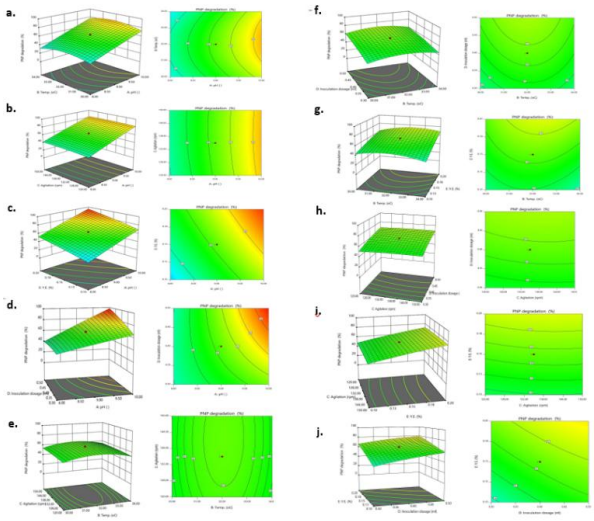

Fig 3: 3D surface plots and corresponding contour plots between the variables **(a)**. pH and temperature (agitation = 135; inoculum dosage = 0.4 OD_{600} AU; Y.E. = 0.15).**(b).** pH and agitation speed (temperature = 32 ^{0} C; inoculum dosage = 0.4 OD_{600} AU; Y.E. = 0.15). **(c)**. pH and yeast extract concentration (temperature = 32 ^{0} C; agitation = 135; inoculum dosage = 0.4 OD_{600} AU). **(d)** pH and inoculum dosage (temperature = 32 ^{0} C; agitation = 135; Y.E. = 0.15) temperature and Agitation speed (pH = 9; inoculum dosage = 0.4 OD_{600} AU; Y.E. = 0.15).**(e)** temperature and inoculum dosage (pH = 9; agitation = 135; Y.E. = 0.15). **(g)** temperatureand yeast extract concentration (pH = 9;agitation = 135; inoculum dosage = 0.4 OD_{600} AU). **(h)** 3D surface plots and corresponding contour plots between the variables agitation speed and inoculum dosage (pH = 9; temperature = 32 ^{0} C; Y.E. = 0.15). **(i)** agitation speed and yeast extract concentration (pH = 9; temperature = 32 ^{0} C; inoculum dosage = 0.4 OD_{600} AU). **(j)** inoculum dosage and yeast extract concentration (pH = 9; temperature = 32 ^{0} C; agitation = 135).

4. Conclusion

The microbial consortium is able to utilize 1000 mg/L of PNP as a co-substrate when supplemented with yeast extract as an energy and carbon source. The optimized conditions for the enhanced PNP biodegradation by microbial consortium were found using Design Expert Software by Response Surface Methodology (CCD) to be pH - 10, inoculum dosage - 0.44 AU (OD_{600}) and yeast extract concentration - 0.2%. Temperature and agitation speed was varied between 32 ^{0} C and 34 ^{0} C and 120 - 150 rpm respectively. The microbial consortium degraded 99.36% of the initial PNP concentration under optimized conditions within 72 hrs. Thus, the isolated consortium holds the potential for the clean-up of PNP laden wastewaters and RSM can be utilized a practical parameter optimization tool.

5. Reference

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