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Optimization of *Pseudomonas aeruginosa* for Chlorpyrifos Degradation using Response Surface Methodology

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Abstract

Chlorpyrifos is a hazardous organophosphate pesticide used worldwide thus its biodegradation is need of the time. In the present study 13 bacterial strains were isolated from collected soil samples of different agro climatic regions and out of them one bacterial strain was screened out as potent degrader of Chlorpyrifos on the basis of percentage degradation using high pressure liquid chromatography (HPLC). Present HPLC results indicated this potent degrader identified as *Pseudomonas sp*. (Sanger's sequencing) degrading up to 96% of pesticide at end of 10^{th} day of incubation. For effective results, optimisation of physical parameters using response surface methodology used to enhance the ability of the strain to degrade the pesticide in minimum period of incubation. Gas chromatography (GC) equipped with electron captured detector used to optimize the physical parameters X1 (pH 6-8), X2 (temperature 20-40 °C) and X3 (1-3 ml, 3×10^8 cells per ml) was performed using Design expert software of response surface methodology (Box behnken design). Model was found to be fit with R^2 (0.91), non-significant lack of fit and P <0.05 of quadratic terms. These result indicated that strains (*Pseudomonas sp*.) degrade up to 64% chlorpyrifos within three days of incubation period with in optimized conditions and found its minimum residues after ten days of incubation can be used to eliminate the chlorpyrifos contaminated environment.

Keyword: *Pseudomonas sp*; HPLC; RSM; Chlorpyrifos; Organophosphate

Introduction

India is primarily an agriculture based country with more 60-70% of its population dependent on agriculture. Indian population is increasing with a very fast rate, annual food grain production must be increased or minimize the crop loss so it should be meet the requirement of food and cloth of increasing population [1,2]. Use of fertilizers hybrid seeds and pesticides become common practices. About 30% of agricultural product is lost due to pests to prevent this loss [3] extensive use of pesticides is inevitable [4,5]. Due to over apprehensive use of pesticide has led to causing short and long term health hazards. The extensive use of these pesticides over the years and their interaction with the biological system resulted problems in the environment [6]. Currently among the various groups of pesticides that are being used all over the world, organophosphorus group forms a most widely used group accounting for more than 36% of the total world market. Some of the main agricultural pesticides are Qinalphos, Monocrotophos, Chlorpyriphos, Malathion and Parathion. Out of them chlorpyrifos used extensively and less studied.

Chlorpyrifos $C_9H_{11}C_{l3}NO_3PS$ used for insect control in cereal cotton fruit nut and vegetable crops [7] Chlorpyrifos has a high soil sorption co-efficient [8]. 3, 5, 6-trichloro-2-pyridinol (TCP) is the hydrolysis product of chlorpyrifos and transported to long distances [9] also found resistant to enhanced degradation and exhibit antimicrobial activity [10]. When these residues entered in the food chain directly or indirectly can affect ecosystems and human health. Acetylcholine is a vital component of the nervous system in insects and mammals. Organophosphate compounds inhibit the activity of acetylcholinesterases enzyme and results overstimulation of acetylcholine receptors in synapses of autonomic and central nervous systems. Organophosphate poisoning is a world-wide serious health issue with 200000 deaths annually [11-13].

To overcome these problems we have to look for the best method out of chemical landfills incineration method. The most reliable method for destruction of these compounds has met serious public opposition because of the potentially toxic emission and is economically restrictive [14]. Ability of microorganism to remove pollutants from containment sites is an effective strategy minimally hazardous and environment friendly is the process known as bioremediation [15,16].

Response surface methodology (Design expert 10.0.6 software) is a statistical tool used to establish the optimization of different parameters for maximum output of response [2,17,18]. In this study Box-Behnken design is quadratic model which requires a few

number of experiments to optimize the different factors for maximum response [18-20]. With the understanding of bioremediation of chlorpyrifos the challenge is now to build the cost effective way to deliver optimization of physical parameters for enhanced degradation of target pesticide.

Materials and Methods

Chemical and Media

Minimal salt media (100 ml) in Erlenmeyer flask contained KH_2PO_4 (1.5gL^{-1}) K_2HPO_4 (0.5gL^{-1}) $(NH4)_2SO_4$ (1.0gL^{-1}) $MgSO_4$ (0.2gL^{-1}) FeSO4 (0.02 gL^{-1}) and NaCl 0.5gL^{-1} with pH 7.2 amended with chlorpyrifos analytical grade (Sigma Fluka) as a carbon source at a concentration of 25mgL^{-1} agar and other media are nutrient agar and nutrient broth.

Enrichment and Isolation of Chlorpyrifos Degrading Strain

Soil sample was serially diluted (Mac Fedin 1985) with distilled water dissolved in 100ml of sterile nutrient broth with 25mg/l chlorpyrifos in 250 ml conical flask incubated at 37 °C at 100rpm for first enrichment of one week. 10 ml of first enrichment transferred to fresh 100ml of minimum salt media with increased concentration of chlorpyrifos (50mg/l) kept for one week under same condition were checked on pesticide amended MSM agar plates to obtain pure isolated colonies. Plates showed growth with different morphological colonies of bacterial strain. 13 bacterial strains were isolated which can use chlorpyrifos as a carbon source able to degrade chlorpyrifos. These bacterial strains were checked on media containing high concentration of chlorpyrifos/litre kept at 37 °C for 24hrs so out of them potent degraders were preserved in glycerol stock and kept in – 20 °C.

Biochemical Characterization

Biochemical test such as starch hydrolysis, casein, catalase and urease test were performed with isolated strain manually. While some other tests also performed for selected strain such as gelatin, esculin substrate utilization of lysine, ornithine, methyl red, malonate, catalase and urease test, starch, mcconkye, ONPG, citrate, indole, voges proskauer's, nitrate reduction, glucose, cellobiose, trehalose, saccharose, raffinose xylose and casein on ready to use kit from Sigma Aldrich.

Analytical Instrumentation and Monitoring of Percentage of Degradation

All 13 strains were screened and best degraders was excluded out by analysis of pesticide residue performed on WATERS HPLC equipped with waters column C_{18} symmetry 300 Tm (4.6 mm×250 mm) 2487 detector and pump 600. Analyte was detected at 290 nm after 4.5 min (Retention time). The isocratic solvent system as (88 ACN: 12 H_2O) was used with a flow rate 1ml/min it was observed that retention time was temperature dependent. Different concentrations of analytical grade standard stock solutions 1 to 100 µg/ml of chlorpyrifos Sigma (Fluka) were prepared in acetonitrile (HPLC grade). Preserved glycerol stock revived by incubating in nutrient broth overnight in standard conditions and sub cultured twice and used the dilution plate count technique for inoculating in media. 25mg/l chlorpyrifos supplemented in 100ml MSM inoculated with inoculum size of 1ml (3×10^8 CFU/ml) in standard condition for 10 days (37 °C temperature and 100 rpm) and without inoculated flask kept as a control under identical conditions. Chlorpyrifos residues were detected at different intervals by taking 5 ml of samples and centrifuged at 10000 rpm. Clear supernatant was collected and passed through 0.45 µm nylon syringe filter and subjected to HPLC analysis.

Molecular Identification

Out of total bacterial strains, best degrading strain with maximum degrading capacity was selected for molecular identification (Sanger's method) from Macrogen Korea.

Gas Liquid Chromatography (Instrumentation) and Sample Extraction

A Shimadzu gas chromatography model GC 2010 plus equipped with autosampler AOC-20i mounted on a split/splitless injector port connected to Ni electron capture detector through Equity 5 capillary column (30m ×0.25µm I.D). Initiated temperature of oven was 60 °C kept for 0.5 min then at a rate of 20 °C/min to 204 °C followed by rate of 2 °C/min to 208 °C similarly by rate of 0.5 °C/min to 210 °C and finally by 20 °C/min to 300 °C (hold for 3min) with total run of 23.3 min. In present study standard of chlorpyrifos (Sigma Aldrich) were used for maximum degradation by identified strains and optimization of physical parameters (pH, temperature and inoculum size) of potent degrader using RSM software.

Chlorpyrifos 25mg/l emended in 100 ml of MSM inoculated with specific strain. 10 ml of samples is withdrawn after incubation period of 4 days add equal of n-hexane followed by 10 min shaking and finally separated and collected the organic phase. Repeated the same process for three times total organic solvent evaporated and remaining residues made a total volume of 10ml of methanol. 4 ml of extracted sample adjusted to 10, 50 and 100ml of total volume which will be consider as dilution factor (Dilution is necessary because the limitation of GC column high concentration of analyte can block or damage the column) and finally syringe filter (0.45mm) and finally aliquot transferred to Gas Chromatography analysis.

RSM Design of Physical Parameters for Optimization

Design expert 10.0.6 software was used for response surface methodology to optimize the three independent variables (Temperature pH and inoculum size) and their consequence on degradation level using bacterial strain. Box Behnken Design (BBD) model with 17 experiments (Table 1) indicate the estimation of percentage of degradation of chlorpyrifos termed as response (R). Independent variables X1 (pH 6-8) X2 (temperature 20-40 degree Celsius) and X3 (1-3 ml per ml contained 3×10^8) required three factorials level (-1, 0 and +1). Analysis of variance (ANOVA) plot of residuals will describe the success of designed model and 3D colored contour plots of fitted surfaces indicate the effect of variables in combined and individual form [21].

Run no.	Factor 1 pH	Factor 2 Temp	Factor 3 Inoculum size	Response % Degradation 3rd day incubation
1	7	30	2	53.6
2	6	40	2	42.8
3	7	40	1	41.6
4	6	30	3	53.6
5	7	30	2	56
6	7	20	3	55.9
7	7	30	2	54.4
8	7	40	3	49.6
9	7	30	2	49.6
10	6	30	1	48.8
11	6	20	2	64
12	8	40	2	60
13	7	30	2	54.6
14	7	20	1	27.2
15	8	30	1	32
16	8	20	2	32.8
17	8	30	3	48.8

Table 1: Combination of Different Experimental Sets with their Response

In this study 100ml minimum salt media amended with 25 mg/l chlorpyrifos Sigma Aldrich were dependent variables and 100rpm incubated for 3 days period were independent variables. This design is able to build a quadratic response achieved by second order polynomial equation.

Result and Discussion

Isolation of Chlorpyrifos Degrading Bacterial Strain by Enrichment Process

Thirteen morphologically different potential bacterial strains were isolated from agriculture soil and water samples. All strain found to be catalase producing indicating their aerobic nature while SA5 (FIT3) found positive for gelatin, esculin substrate utilization of lysine, Ornithine, methyl red, malonate, catalase and urease test and negative for starch, mcconkye, ONPG, citrate, indole, voges proskauer's, nitrate reduction, glucose, Cellobiose, trehalose, saccharose, raffinose xylose and casein. This strain found to be susceptible for streptomycin, rifampicin, and kanamycin while resistant to Chloramphenicol. All individual strains were analyzed by HPLC for their chlorpyrifos degradation ability in minimal salt medium amended with (25mg/l) chlorpyrifos. After completion of incubation period of ten days samples were subjected to find the best degrader.

HPLC Spectra (Figure 1) showed a peak over 4.5 min at 290 nm. Its absorbance increases with increase in concentration so directly a relation in between concentration and height of peak was set up. Standard graph reveals a linear function in between concentration and height. Here y = 1390x + 3247 here y is height of the peak and c is the concentration of chlorpyrifos remaining in the sample. By this easy calculation concentration of chlorpyrifos residues in each sample were derived and showed the result for percentage degradation of chlorpyrifos. Degradation of 13 bacterial strains found to be from 69.14 to 96.07 percent (Table 2). Out of them a single strain with maximum degradation up to 96.07. Out of thirteen strains, SA5HB-SH-17-FIT3 found to be best degrader of chlorpyrifos pesticide. Result of 16s rDNA analysis determined the strain as *Pseudomonas aeruginosa* resembles 99% to sequence of KT966462 found to degrade more than 96% of chlorpyrifos. Chlorpyrifos degradation can be possible due to presence of phosphotriesterase enzyme [22]. It degrades almost 96% of initial chlorpyrifos concentration within 10 days of incubation period under standard conditions. Due to lack of free carbon source growth pattern was different; a clump formation of bacterial biomass was seen. This strain was submitted to GenBank with accession number of KY07250. A number of bacterial strains reported as chlorpyrifos degrading including *Enterobacter sp. B. subtilis B. pumilus and Flavobacterium sp* [15,23,24].

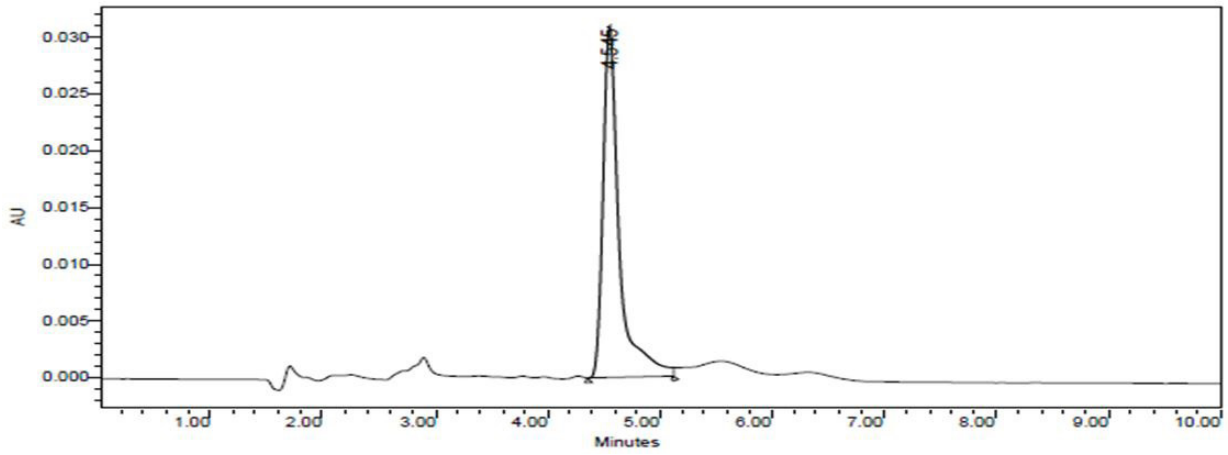


Figure 1: Remarkable Peak Of Analyte (Chlorpyrifos) at 4.5 Min

Strains	Run number	RT	Area	Height	Percentage %degradation 10th day
SA4(3)A11 HB-MN-25	176	4.346	30727	3146	89.87
SA5(5)Green HB-SH-17 FIT3	**252**	**4.63**	**16491**	**1218**	**96.07**
SA(9)New4 HB-BH-4	240	4.065	57520	6017	80.61
SA(10)Gr11 HB-KD-23	224	4.028	82867	8786	71.27
SA(11)Orange HB-SH-24	209	4.356	21001	2709	91.27
SA(12)Vons HB-SR-24	177	4.461	47493	5218	83.26
SA(14)B7 HB-DL-7	235	4.178	52158	5925	80.92
SA(15)Fibri HB-KU-3	239	3.845	48061	5582	82.02
SA(16)Fibritic HB-HS-19	226	4.035	72140	7420	76.10
SA(17)Pill HB-EL-29	185	4.38	41458	4770	84.63
SA(18)Ranbo HB-FT-15	223	4.021	96838	10354	66.6
SA(19)X HB-BU-17	183	4.451	68724	7621	75.45
SA(20)M20 HB-HS-20	225	4.050	104738	10512	69.14

Table 2: Pesticide Degradation (HPLC) Result by Thirteen Different Bacterial Strains

Gas Chromatomatography and Samples Analysis

Five different concentrations of analytical grade sigma fluka analyzed on GC (Detector ECD) to check the response. Analyte peak found very clear and its retention time fixed around 16.2 minute in (Figure 2). Standard calibration curve of the analyzed chlorpyrifos pesticide represented a good reggresion in the range of explored concentrations from 50ppb to 200ppb (ng/ml). It was revealed that all concentration of organophosphate pesticide under study were collinear and thus calibration curves were further employed for the detection of analytes under study. Chlorpyrifos standard curve increased proportionaly with corresponding increase in concentration and the coefficient of determination (r^2) were found to be more than 0.9 which indicates good linearity. Some interesting fact about degradation of chlorpyrifos indicated in (Figure 3) revealed the presence of secondary metabolite TCP in sample analysis with retention time 9.32 min. In chlorpyrifos degradation pathway 3, 5, 6-trichloro-2-pyridinol formed which indicate the degradation of chlorpyrifos [25]. Observation regarding various parameters discussed so far revealed that instrumental response with respect to area retention time remained within expected limits. Thus the instrument used was found to fit for the purpose. GC 2010 model shimadzu equipped used to optimize the physical parameters. X1 (pH 6-8) X2 (temperature 20-40 °C) and X3 (1-3 ml per ml contained 3×10^8) physical parameters should be given first priority [26] using Design expert software of Box behnken design.

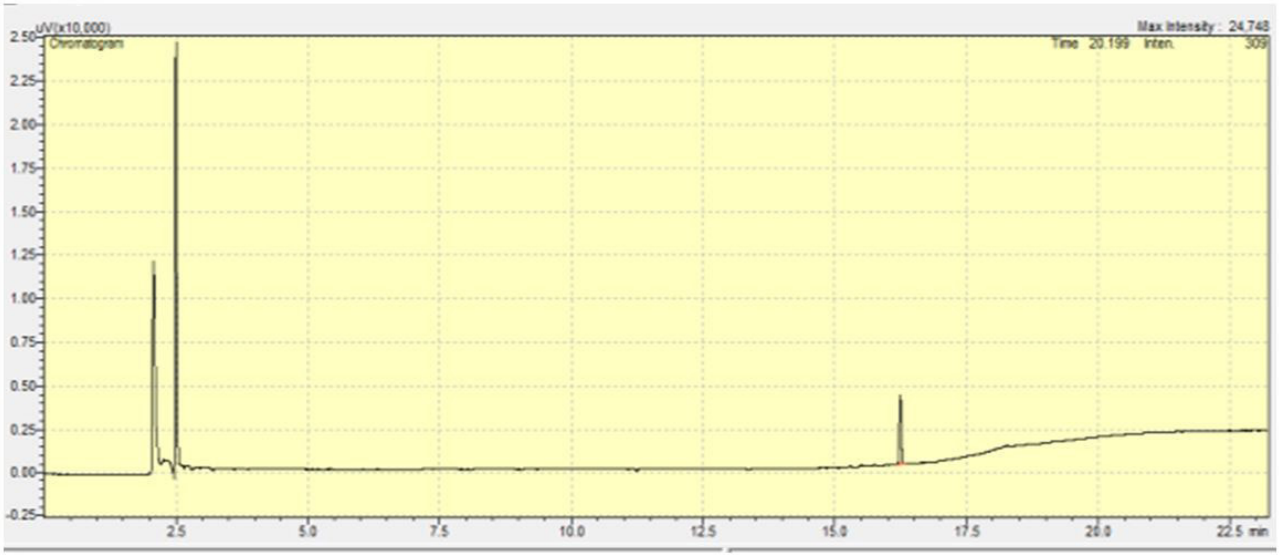


Figure 2: Gas Chromatography Chromatogram of (150ppb) Chlorpyrifos Standard

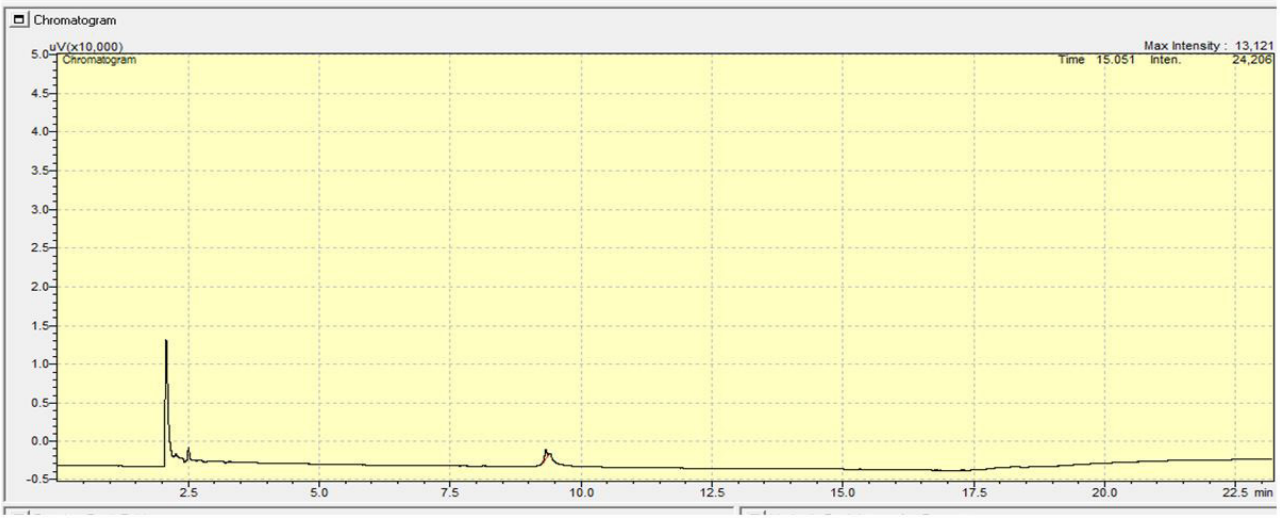


Figure 3: Gas Chromatography Chromatogram of Tcp Secondary Metabolite

Response Surface Methodology

BBD is a quadratic rotatable model; its function gives estimation of percentage of degradation of chlorpyrifos (Y), 3D colored plots of fitted surfaces, test of lack of fit ANOVA and plot of residuals. Effective degradation could be achieved by providing most suitable environmental conditions to the degrading strain. Statistical tool BBD model of response surface methodology (Meilgaard *et al.*, 2003) used to find the most suitable physical parameter for decontamination of natural resources from such chemical entities in cost effective manner. Response surface methodology BBD model (Meilgaard *et al.*, 2011) [27] gives a full description of ANOVA estimated the fitness of designed model. F-value 20.90 and P- value 0.0003 implies the model is significant (Table 2). There is only a 0.03% chance that an f- value this large could occur due to noise. P- Values less than 0.05 indicate model terms are significant. The lack of fit F-value of 2.07 (Table 3) implies that it is not significant relatives to the pure error. There is a 24.72% chance that a lack of fit F-value this large could occur due to noise. Non-significant lack of fit is good that model is significant. Maximum degradation achieved under the condition of (pH 6) (temperature 20 ^{o}C) and (inoculum size of 2 ml per ml contained 3× 10^{8}) around 64% within 72 hours of incubation period. Pino *et al.*, 2011 described adaptation and acclimatization with environmental conditions explains its effective degradation [28].

ANOVA for response surface quadratic model Analysis of variance table (Partial sum of squares –Type *III*)						
Source	**Sum of squares**	**DF**	**Mean Square**	**F value**	**P-value**	**Remarks**
Model	1602.98	9	178.11	20.90	0.0003	Significant
A- pH	158.42	1	158.42	18.59	0.0035	
B-Temp	24.85	1	24.85	2.92	0.1315	

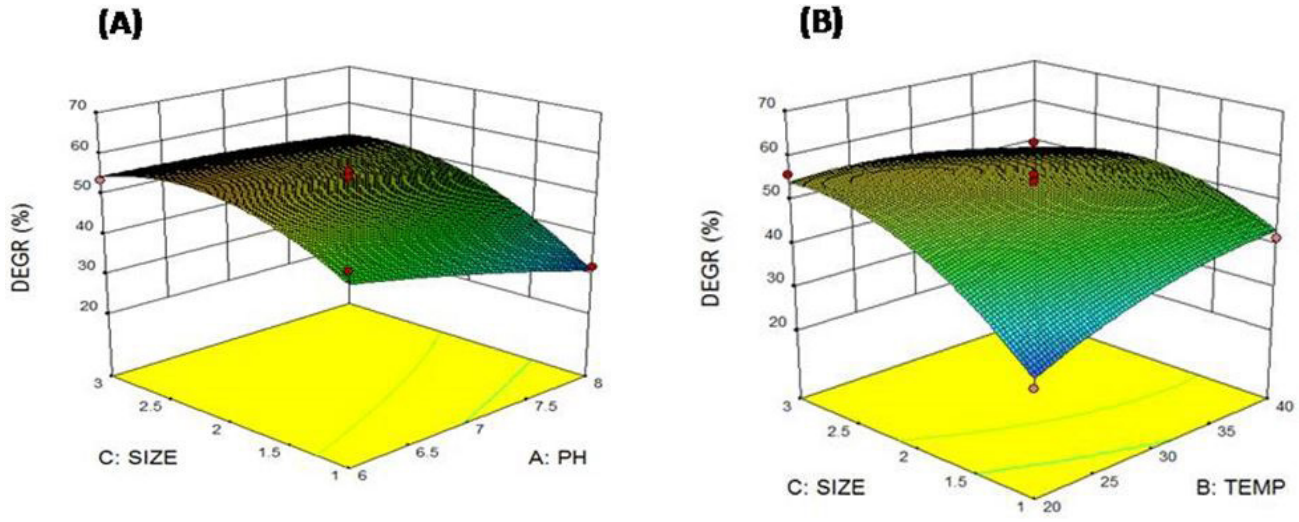
ANOVA for response surface quadratic model Analysis of variance table (Partial sum of squares –Type *III*)						
Source	**Sum of squares**	**DF**	**Mean Square**	**F value**	**P-value**	**Remarks**
C-Size	424.86	1	424.86	49.85	0.0002	
AB	585.64	1	585.64	68.71	0.0001	
AC	36.00	1	36.00	4.22	0.0789	
BC	107.12	1	107.12	12.57	0.0094	
A^2	2.42	1	2.42	0.28	0.6109	
B^2	37.45	1	37.45	4.39	0.0743	
C^2	211.21	1	211.21	24.78	0.0016	
Residual	59.66	7	8.52			
Lack of fit	36.27	3	12.09	2.07	0.2472	Not significant
Pure error	23.39	4	5.85			
Cor total	1662.64	16				

Table 3: ANOVA Result Data Sheet of Designed Model

Determination of coefficient exhibited the harmony b/w experimental result and predicted model. The predicted R- Squared of 0.6290 is not close to the adjusted R-squared of 0.91 (Table 4) as one might normally expect that the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model and the data things to consider are model reduction response transformation outliers and etc. All empirical models should be tested by doing confirmation runs. Adequate precision measures the signals to noise ratio. A ratio greater than 4 is desirables. Our ratio of 15.78 indicates an adequate signal. Value of coefficient of variance (1.33%) should not be more than 10% (Table 4) exactly achieved by the model which denoted the degree of precision [2,20]. This model can be used to navigate the design space. Normal probability plot of the studentized residuals was performed to check for normality of residuals and found to be normal fit to model and homogeneous various assumptions. By keeping a third variable at center point and effect of other variables on response can be seen (Figure 3). Response curve surfaces plotted to explain to determine the optimum level of each factor to reach a maximum response. Regression (R^2) 0.94 and adjusted R^2 (0.91) indicate the high degree of correlation in experimental and theoretical assumption. Highly precised coefficient of determination (R^2 0.96) of BBD model found in previous study [29]. Coloured graph Figure 4A represented two independent variables (Inoculum size and pH) against R (percentage of degradation), Figure 4B temperature and Inoculum size against percentage of degradation, Figure 4C pH versus temperature while third variable kept as control.

Std. Dev	2.92	R-Squared	0.9641
Mean	48.55	Adj R-Squared	0.9180
C.V %	6.01	Pred R-Squared	0.6290
PRESS	616.91	BIC	97.92

Table 4: Regression Analysis Result



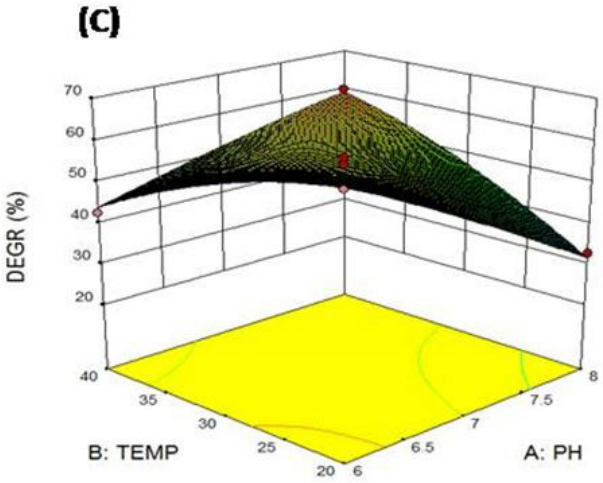


Figure 4: (A) Coloured Graph Represent Two Independent Variables (Inoculum Size And Ph) Against Percentage of Degradation; **(B)** Inoculum Size and Temperature Against Percentage of Degradation **(C)** Temperature Versus Ph While Third Variable Kept as Control

Equation in terms of actual factors

Degradation%(Response)=+237.68–36.145×pH-5.46925×Temp+30.1425×Size+1.21×pH×Temp+3.0×pH×Size-0.5175×Temp×Size-0.7575 pH^2-0.029825×$Temp^2$-7.0825×$Size^2$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. Here AC, AB, BC and C^2 (Table 3) are significant model terms. Value greater than 0.1000 indicate the model terms are not significant. Here the levels should be specified in the original units for each factor. The equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space [30,31].

Conclusion

This study was conducted to isolate a chlorpyrifos degrading bacterial strain and optimize its physical parameters for affective degradation. *Pseudomonas aeruginosa* is the highly degrader of chlorpyrifos pesticide result shows its 96% degradation in ten days of incubation period within optimize conditions. Awareness regarding environment and development of safe methods to keep it pollution free seems to be most concern area these days. Biodegradation is the safest method to eliminate these toxic compounds such as pesticides without leaving any side effect. This can be a great help in future prospect of pollution free environment.

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