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A Microcosm Study on P-Nitrophenol Biodegradation in Soil Slurry by *Alcaligenes faecalis*: Plackett-Burman Design

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Abstract

Contamination of soils with nitroaromatic hydrocarbons, due to their toxic effects, is one of the environmental issues. Therefore, the purpose of this study is to examine para-nitrophenol (PNP) biodegradation in a microcosm consisting of kaolan clayey soil by A. faecalis, the superior bacterium among the four examined bacterial species. Preliminary experiments were performed in slurry phase to investigate biodegradation of PNP by A. faecalis in shaking flasks at initial concentrations of 25 and 50mgkg^{-1} which resulted in 72 and 57% PNP removals after 20 days, respectively. To identify the effective factors on PNP biodegradation, experiments were carried out in shake flasks at various levels of eight factors, i.e. PNP, glucose and yeast concentrations, temperature, soil-water ratio, initial pH, inoculum size as well as PNP concentration in pre-exposure based on Plackett-Burman design method. Results showed 20.9 up to 75.5% PNP removal in soil slurry after 12 days within the design space. Analysis of variance revealed that temperature, inoculum size, yeast extract concentration, pH and soil-water ratio are the most effective factors on PNP biodegradation, respectively.

Keywords: *Alcaligenes faecalis, Bioremediation, Contaminated Soil, P-Nitrophenol, Plackett-Burman*

1- Introduction

Nitroaromatic compounds are one of the environmental concerns. These include chemicals used as herbicides, explosives solvents and industrial chemicals [1]. They are also produced through natural photochemical or biological processes in the environment [2]. P-Nitrophenol (PNP) is a nitroaromatic pollutant used for manufacturing pesticides, dyestuffs, pharmaceuticals (e.g. synthesis of the aspirin and acetaminophen) and as a fungicide to protect leather from fungal mould [3; 4]. It can be released into soil as a result of hydrolysis of organophosphate pesticides: parathion, methyl parathion [5] and herbicides: dinoseb and dinitrocresol [6; 7], can be produced in the atmosphere through photochemical reaction between benzene and nitrogen monoxide and has also been detected in rainwater in Japan [8]. Exposure to PNP may lead to methemoglobin formation, liver and kidney damage, anemia, skin and eye irritation and systemic

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poisoning [9; 10]. Therefore, PNP has been classified as a priority pollutant by the United States Environmental Protection Agency as an EPA class HR-3 compound. So, its rapid removal and detoxification is essential [11]. Traditional methods such as excavation, physicochemical, evaporation, volatilization, hydrolysis, washing, oxidation and photolysis require complicated equipment and high cost for building and maintenance [12; 13]. In addition, in some cases more harmful chemicals are produced, which overall, are far from being optimal [14; 15]. Moreover, these processes are usually time-consuming and hence not feasible when large sites are to be completely remediated [16]. So, the use of microbial potential for the remediation of toxic compounds from soil (bio-remediation) is now accepted [10] as an alternative to conventional methods [17; 18]. Due to the fact that the rate of bioremediation of contaminated soil is often limited by solubility, sorption capacity and slow transfer of organic compounds to aqueous phase [19], agitated slurry phase bioremediation appears to be an alternative to soil bioremediation which facilitates the uptake of contaminants by microorganisms [20]. PNP biodegradation by various bacteria such as *Bacillus, Flavobacterium, Moraxella, Pseudomonas, Sphingomonas, Rhodococcus, Ralstonia and Arthrobacter* has been studied in aqueous phase at different PNP concentrations [13; 21; 22; 23]. However, few studies have been carried out on PNP biodegradation in soil [3; 6; 7; 17; 24] and in slurry [2; 25] phases.

Since the effect of variables such as pH, temperature or nutritional supplementation on PNP biodegradation has not yet been examined in a systematic way; Plackett-Burman design method is utilized in this study to screen the effective factors in a microcosm consisting of clayey soil as thartificial soil contaminated with p-nitrophenol.

2- Materials and methods

2.1- Chemicals and microorganisms

PNP was purchased from Sigma. All other chemicals were of the highest analytical grade available. Four bacterial strains: Pseudomonas aeruginosa (ATCC 1310), Alcaligenes faecalis (ATCC 8750), *Pseudomonas putida* (ATCC 12633) and an indigenous strain of *Pseudomonas*, isolated from oil contaminated soil which was used in a previous study [26], were used to examine PNP bioremediation.

2.2- Media

Nutrient agar was used for preparation of all bacterial subcultures. Luria broth media (0.05% yeast-extract, 1.0% NaCl and 1.0% peptone) supplemented with an appropriate amount of PNP was used for the pre-exposure stage [3] in all experiments. In the aqueous phase and slurry phase experiments, a strongly buffered mineral salt medium (MSM) described previously [13; 24], supplemented with a trace-element solution containing (g/l): 0.611 H_3BO_3, 0.56 $CoCl_2.6H_2O$, 0.56 $ZnSO_4.7H_2O$, 0.385 $MnCl_2$, 0.56 $NiSO_4.6H_2O$, 0.56 $CuSO_4.5H_2O$, 0.28 KI, 0.28 $SnCl_2$ and 0.56 $Al_2(SO_4)_3.6H_2O$ was used. The pH of mineral salt medium was adjusted to 7 with 1 M NaOH. To this solution specified amounts of 4-nitrophenol was added and the medium was autoclaved for 20 min at 121°C.

2.3- Culture pre-exposure to PNP for inoculum preparation

To prepare a PNP adapted inoculum, a pre-exposure stage was carried out in the aqueous phase by transferring a loopful of each strain into a 250mL Erlenmeyer flask containing 50mL Luria broth medium supplemented with 20ppm PNP. The culture was then incubated in an orbital shaker (200rpm, 30°C) for 24h. This was followed by centrifugation of culture at 4,000rpm and 4°C for 20min. The cell pellets were re-suspended in physiological saline (8.5g/l NaCl) to reach an OD_{600} of about 1.2. This cell suspension was then used as inoculum in all aqueous and slurry phase experiments. However, the medium used to prepare PNP adapted inoculum for Placket-Burman experiments contained a specified amount of PNP as given in Table 1, instead of 20ppm PNP mentioned above.

2.4- Soil preparation

For soil microcosm studies, clayey soil supplied from Iran China Clay Industries Corporation containing (%): 0.40 K_2O, 0.30 Na_2O, 0.60 MgO, 1.2 CaO, 0.04 TiO_2, 0.45-0.65 Fe_2O_3, 0.45-0.65 Al_2O_3 and 0.62 SiO_2 was used. Soil samples were artificially contaminated with PNP by acetone [19] up to desirable levels and stored for one month. The contaminated soil was then placed in 100ml Erlenmeyer flasks and sterilized by autoclaving at 121°C before use.

2.5- Experiments

Three sets of experiments were carried out in this work. In aqueous phase experiments, 250ml Erlenmeyer flasks containing 50mL MSM, supplemented with 50mgl^{-1} PNP was inoculated at 5% (v/v) and incubated in an orbital shaker at 200rpm and 30°C. The soil slurry bioremediation experiments were carried out in 100ml Erlenmeyer flasks containing 5gr dry weight of spike-soil and 14ml sterile MSM. Each flask was inoculated by 5% (v/v) cell suspension and incubated at 30°C and 100rpm for 21 days. Slurry samples were examined at 6-day intervals up to 12 days, thereafter at 3-day intervals, and analyzed for PNP concentration. In experiments designed via Plackett-Burman method, which was carried out for 12 days in 100ml Erlenmeyer flasks shaken at 100 rpm, various amounts of medium and inoculum as well as various operational and pre-exposure conditions were used as described in Table 1. Control experiments were carried out in all sets of experiments without inoculation, resulting in only 3-4% non-biological PNP removals.

Table 1. Factor levels for Plackett–Burman experimental design method.

Symbol	Variables	Levels		
		-1	0	+1
A	Glucose Concentration (g/l)	0.5	1	1.5
B	Yeast Extract Concentration (g/l)	0.1	0.55	1
C	PNP Concentration (mg/kg)	30	50	70
D	pH	7	8	9
E	Temperature (°C)	20	27.5	35
F	Soil-water Ratio (%)	20	30	40
G	Inoculum Size (%)	5	7.5	10
H	Pre-exposure Concentration (mg/l)	10	20	30

2.6- Analytical methods

In aqueous phase, bacterial growth was measured by the absorbency at 600nm. For PNP determination, aliquots (3mL) of the culture grown on PNP were harvested at regular time intervals and centrifuged at 15,000rpm for 20min [28, 29]. The pH of the supernatant was adjusted to 12 by NaOH (0.1N) before measuring the absorbance to eliminate the effect of pH [29]. A control medium, without PNP, was used as blank. The spectrophotometric determination of PNP was performed by a spectrophotometer (Metertech, SP80001) at 400nm [13; 29; 30; 31].

For soil slurry experiments, bacterial growth was measured by taking a 1ml sample of the slurry and counting the colony forming unit after appropriate dilution [32]. PNP measurement in slurry phase was performed by drying up the content of each flask at room temperature. This was followed by PNP extraction from 3.0g dry weight of dried soil by 9ml of acidified acetone (acetone with 1.0% 1N HCl) for 16h on a rotary shaker at 200rpm and centrifugation at 4000rpm for 20min. The supernatant was removed and re-extracted with 6ml of acidified acetone for 2h at 200rpm. The extracts were combined and evaporated to dryness [33]. The residue was dissolved in 10ml distillated water and filtered prior to colorimetric analysis as described earlier.

2.7- Plackett–Burman experimental design

In order to probe the central factors affecting PNP biodegradation by *A. faecalis* in slurry phase, Plackett-Burman design method was exploited. This method is a class of saturated orthogonal fractional two-level factorial design with a multiple of 4 (rather than twice the number of factors) number of proposed experiments in which resolution is III and the main effects are aliased with plausible two-factor interactions [34]. In this work, eight variables, i.e. temperature, initial pH, inoculum size, soil-water ratio, initial concentration of PNP, yeast and glucose concentrations as well as pre-exposure of bacterium to PNP were tested, each at two levels: high (+) and low (-) which were selected based on some preliminary experiments (data not shown) as given in Table 1. To check the curvature and repeatability, center points denoted by (0) were also used. Design-Expert software version 7.1.4 was used for statistical analyses.

The twelve experiments based on the Plackett-Burman method as well as the four experiments at the center points (overall 16 runs) and the corresponding PNP removals as the objective function are given in Table 2.

All experiments were conducted in duplicate and the average value of PNP biodegradations was used for statistical analyses. The variables which were significant at 5% level ($P < 0.05$) from the regression analysis were considered to have greater impact on bioremediation of PNP-contaminated soil.

3- Results and discussion

3.1- Selection of superior PNP biodegrading bacterium in aqueous phase

To select the superior PNP biodegrading bacterium among the four different bacteria, experiments were performed at initial PNP concentration of 50mgl^{-1} in aqueous phase. Figure 1 compares the time course of growth

and PNP removal for these four microorganisms.

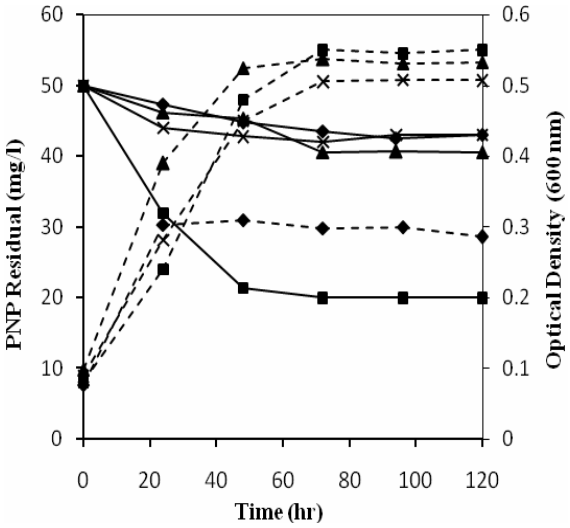

Figure 1. Comparison of growth (---) and PNP biodegradation (—) by four bacterial species at 50mgl^{-1} PNP in aqueous phase. *A. faecalis* (■), *P. putida* (▲), *P. sp.* (◆) and *P. aeruginosa* (×).

As can be seen in Fig. 1, PNP biodegradation occurs alongside bacterial growth, which means that it is growth-associated. Therefore, for all microorganisms, when growth is ceased, no further PNP removal is observed and it remains constant. Although three of these bacteria could grow up to OD_{600} equal to about 0.5, *A. faecalis* showed a maximum PNP removal of 57%, while others showed only about 18% PNP removal. Therefore, *A. faecalis* was selected for further investigation on PNP biodegradation in slurry phase.

3.2- PNP biodegradation by *A. faecalis* in soil slurry

To investigate the effect of PNP content of clayey soil on PNP biodegradation by *A. faecalis* in slurry phase, initial PNP concentrations of 25 and 50mgkg^{-1} were examined. CFU/ml of slurry as a criterion for cell growth and PNP residual at various time intervals are illustrated in Fig. 2. It can be seen that after 20 days PNP removals of 72 and 57% are obtained at initial PNP concentrations of 25 and 50mgkg^{-1}, respectively. Cassedy and co-workers (1999) have reported 22% PNP removal from soil by encapsulated microorganism at an initial PNP of 20mgkg^{-1} [1]. Using an isolated microorganism, Alber and co-workers (2000) have reached 78% PNP removal at an initial PNP of 25mgkg^{-1} [33]. Wang and co-workers (2005) have reported 30% PNP removal at 10mgkg^{-1} by an indigenous microorganism [2].

Figure 2 shows lower biodegradation and growth as well as a long 5-day lag phase at 50 compared to 25mgkg^{-1} PNP due to the inhibitory effect of PNP on the biological activity of *A. faecalis*. Wang and co-workers (2005) have also reported prolonged lag phases when PNP concentration was increased from 10 to 50mgkg^{-1} [2]. It can also be seen that the time reaching the final biodegradation increases at higher initial PNP concentration (see Fig. 2). Hence, a reasonable period of 12-days at 25mgkg^{-1} is selected for comparison purposes in further experiments where soil containing PNP at 30 to 70mgkg^{-1} are used.

3.3- Design and analyses of experiments

Based on the Plackett-Burman design method 16 runs as shown in Table 2 were performed. Runs 11 and 6 resulted in the highest PNP removals of 75.5% and 71.6% at initial PNP concentrations of 30 and 70mg/kg, respectively.

Table 2. Plackett-Burman* with central point design and the corresponding PNP removals in soil slurry phase.

Run	Temperature, °C	pH	Inoculum Size, %	Soil-water Ratio, %	PNP, $mgkg^{-1}$	Yeast Extract, gl^{-1}	Glucose, gl^{-1}	Pre-exposure, $mgkg^{-1}$	PNP Removal, %
1	27.5	8	7.5	30	50	0.55	1	20	69.3
2	35	7	5	20	70	0.1	1.5	30	70.5
3	27.5	8	7.5	30	50	0.55	1	20	65.8
4	20	9	5	40	70	0.1	1.5	30	20.9
5	35	9	5	20	30	1	0.5	30	68.1
6	35	7	10	40	70	0.1	0.5	10	71.6
7	20	9	10	20	70	1	1.5	10	59.0
8	20	7	10	20	70	1	0.5	30	51.0
9	20	7	5	20	30	0.1	0.5	10	39.1
10	20	9	10	40	30	0.1	0.5	30	49.8
11	35	7	10	40	30	1	1.5	30	75.5
12	27.5	8	7.5	30	50	0.55	1	20	68.1
13	20	7	5	40	30	1	1.5	10	49.7
14	35	9	10	20	30	0.1	1.5	10	65.7
15	35	9	5	40	70	1	0.5	10	64.2
16	27.5	8	7.5	30	50	0.55	1	20	67.9

* Three dummy variables are not shown.

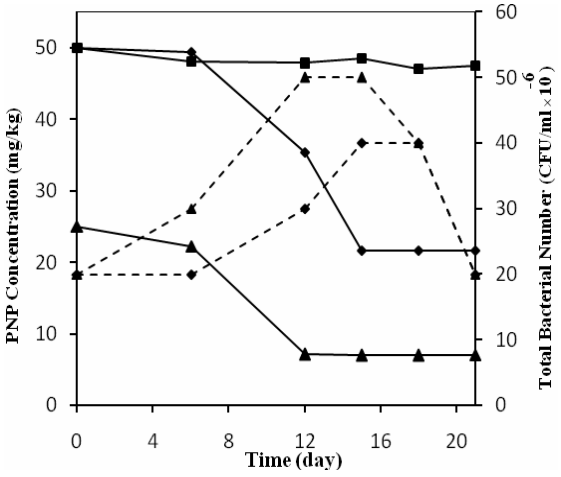

Figure 2. Comparison of growth (---) and PNP residual (—) in soil slurry containing initial PNP of 50 (◆) and $25mgkg^{-1}$ (▲) using *A. faecalis*, control experiment (■).

Regression analyses of the results are given in Table 3. The model F-value of 124.01 implies that the linear model assumed is relatively suitable. There is only a 0.11% chance that a model F-value could occur due to noise. In addition, R^2 (a measure of the goodness of fit of the model) is very much significant at the level of 98%, meaning that the model is unable to explain only 2% of the total variations. The adjusted R^2 value of 0.95 also indicates the significance of the model to describe the experimental observations. Adequate precision, which measures the signal to noise ratio, shows a value of 22.82 (which is quite greater than 4) indicating an adequate signal. These results illustrate that the model can be used to navigate the design space. The F-value of the lack of fit of 3.37 implies that it is not significant relative to the pure error, hence, prediction of PNP removal in soil slurry by the model obtained via Plackett-Burman design method as given in Eq. (1) seems satisfactory.

$$Y= 1.44398 + 1.652223x_1-2.75833x_2+2.11000x_3 – 0.15750\ x_4 – 0.063750\ x_5 + 10.24074\ x_6 + 0.35000\ x_7 – 0.067500\ x_8 \quad (1)$$

Table 3. Regression analyses of Plackett-Burman design in soil slurry bioremediation.

Parameters	Values
R^2	0.9822
Adjusted R^2	0.9586
Predicted R^2	0.8079
Adequate Precision	22.822
Model F-value	124.01
Lack of fit F-value	3.37
Curvature F-value	153.85

Using the four center points resulted in a curvature F-value of 153.85, which shows that there exists a significant curvature (as measured by the difference between the average of the center points and the average of factorial points) in the design space.

Results of analysis of variance (ANOVA) are shown in Table 4. Five factors, i.e. temperature, inoculum size, yeast extract concentration, pH and soil-water ratio are recognized as the most effective factors on PNP biodegradation, respectively, based on the criterion of P-values less than 0.05. In contrast, PNP and glucose concentrations as well as pre-exposure to PNP have insignificant effects. Although the initial PNP concentration is nominated to have an insignificant effect due to its P-value of 0.056, we have already seen its inhibitory effect on bacterial growth and PNP biodegradation (see Fig. 2). It could be reasoned that the selected variables have lessened the effect of this factor when they are involved in significant interactions. Wang and co-workers have also reported that increasing the inoculum size could counteract the negative effect of increasing PNP concentration [2].

Table 4. ANOVA Results of Plackett-Burman design using 8 factors in soil slurry bioremediation.

Source	Sum of square	df*	Mean square	F-value	P-value	Effect grade
Model	2886.17	11	262.38	124.01	0.0011	significant
A – Temperature (x_1)	1842.64	1	1842.64	870.88	< 0.0001	significant
B – pH (x_2)	91.30	1	91.30	43.15	0.0072	significant
C – Inoculum (x_3)	333.91	1	333.91	158.71	0.0011	significant
D – Slurry (x_4)	29.77	1	29.77	14.07	0.0331	significant
E – PNP (x_5)	19.51	1	19.51	9.22	0.0560	-
F – Yeast Extract (x_6)	254.84	1	254.84	120.44	0.0016	significant
G – Glucose (x_7)	0.37	1	0.37	0.17	0.7049	-
H – Pre-exposure (x_8)	5.47	1	5.47	2.58	0.2063	-
Curvature	325.52	1	325.52	153.85	0.0011	significant
Pure Error	6.35	3	2.12			-
Cor Total	3218.04	15				-

*df: Degree of freedom

The P-value of 0.2063 for pre-exposure to PNP for the adaptation of *A. faecalis* confirms the insignificance of PNP concentration as mentioned by Nishino and co-workers [35], though pre-exposure in essence is necessary. With regard to glucose concentration, our result is in accordance with those obtained by Zhang and co-workers (2008) demonstrating the unimportant effect of glucose on PNP degradation [13].

L, J and K in the design space, as dummy variables, are used for finding the error variance. One can see that the effect of dummy factor K is close to zero in the Pareto chart (Fig. 3), but the dummy factor L and J appear as significant on the probability plot of effects. So, an aliased interaction must be the culprit. This shows the role of interaction between the significant factors, which are aliased in this method.

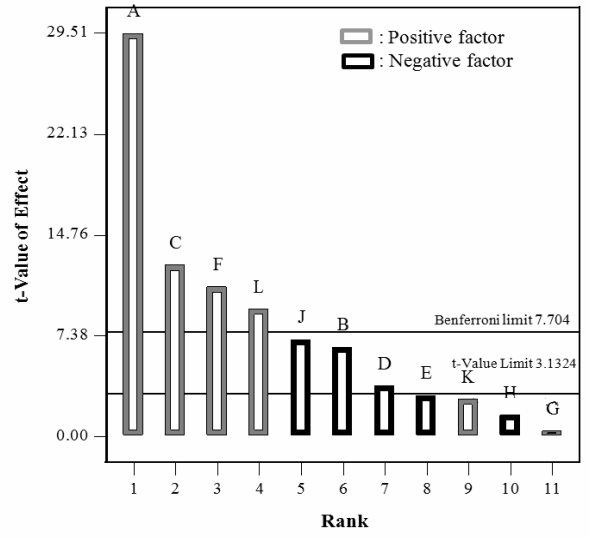

Figure 3. Pareto chart of PNP removal in soil slurry phase. A-H are experimental variables as given in Table 2 and L, J and K are dummy variables.

Figure 4 illustrates the effect of the five important factors on PNP removal. Positive effects are observed for temperature, inoculum size and yeast extract concentration. However in cases of inoculum size and yeast extract concentration, center points have led to higher biodegradations. Labana and co-workers (2005) proved that increasing the inoculum size up to sufficient amounts, increases the chances of successful bioremediation [17]. On the other hand, soil-water ratio and pH showed negative effects. Additionally, the results obtained by center points indicate that a pH of 7-8, as well as a soil-water ratio of 20–30% can improve PNP biodegradation. Zhang and co-workers [13] and Shen and co-workers [36] have also reported that the slightly alkaline pH could enhance nitrophenol biodegradation.

4- Conclusions

Among the selected bacteria, *Alcaligenes faecalis*, found as the superior bacterial species for PNP biodegradation in aqueous phase, led to significant PNP removal in soil slurry. Based on Plackett-Burman design method, it was found that 5 out of the 8 factors investigated, i.e. temperature, inoculum size, yeast extract concentration, pH and soil-water ratio are the most effective factors on PNP biodegradation in slurry phase, respectively. Due to the significant effect of curvature and dummy factors in Plackett-Burman design, in the next step, more levels at the specified range should be examined and/or other experimental design methods capable of presenting higher order models (covering non-linear models) are required.

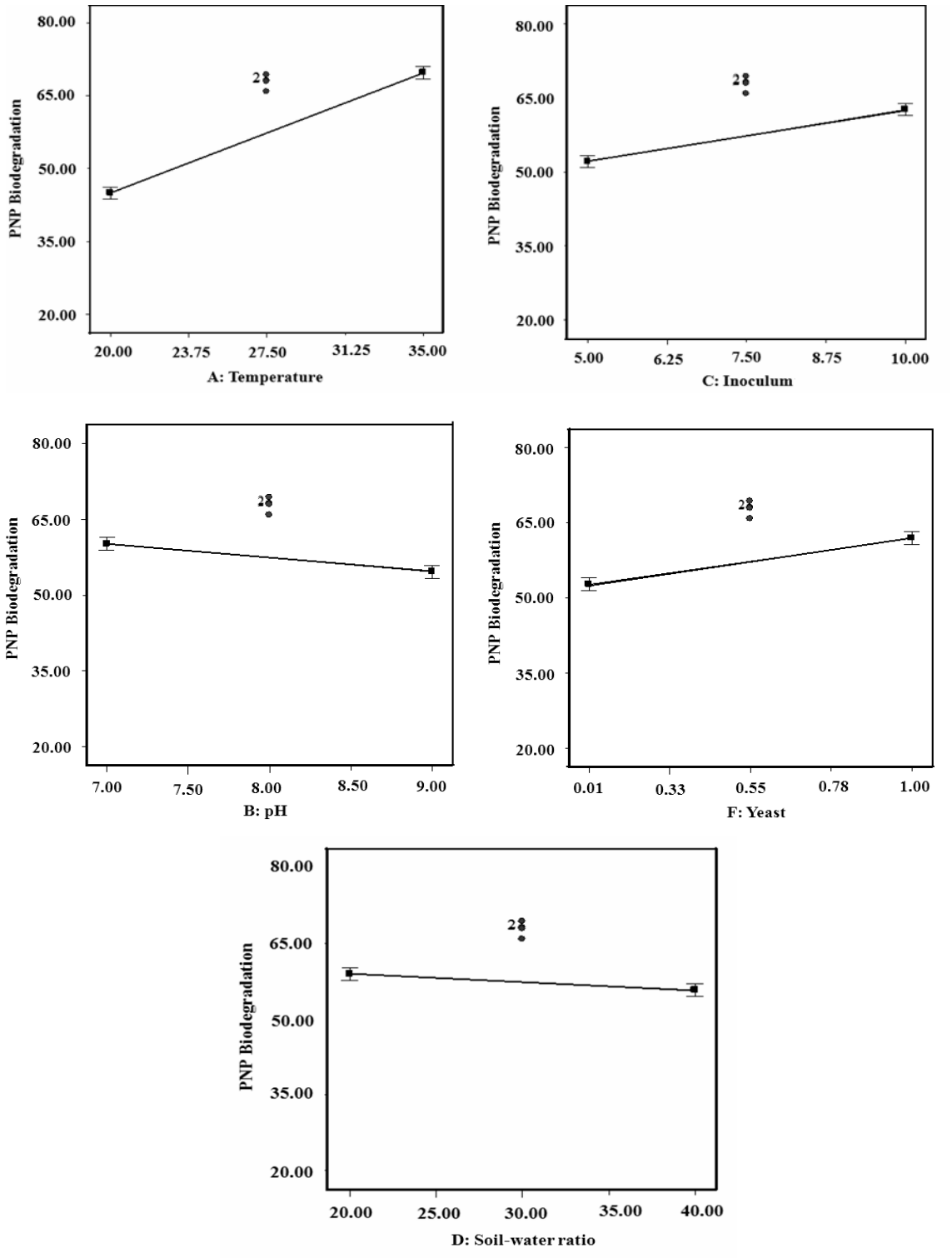

Figure 4. Effects of temperature (A), pH (B), inoculum size (C), soil-water ratio (D) and yeast extract concentration (F) on PNP biodegradation in soil slurry.

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