Screening and Optimization of Process Parameters for Perchlorate Biodegradation by *Burkholderia* sp. and *Pseudoxanthomonas* sp. Utilizing Succinate and Phenol as Respective C-source

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***Abstract*: Plackett–Burman screening and Taguchi design was employed for screening 5 parameters for perchlorate degradation from two isolated bacterial strains *Burkholderia* sp. and *Pseudoxanthomonas* sp., utilizing succinate and phenol as Carbon-source (C-source). Four physical parameters: temperature, pH, inoculums age and inoculums volume were selected along with the ration of carbon source and perchlorate concentration. Coefficients and sum of squares ratio in percentage (SS%) of these variables were calculated by subjecting the experimental data to statistical analysis. Temperature, inoculum age and carbon to perchlorate ration showed significant importance in perchlorate degradation with *Burkholderia* sp. Using succinate as C-source and temperature, inoculum age and pH showed significant effect in case of *Pseudoxanthomonas* sp. Using phenol as C-source. Those factors were further analyzed for optimization by Taguchi method to get the optimum culture conditions. Use of this design is scarce in perchlorate degradation and has not been attempted previously.**

***Keywords*: Plackett–Burman screening, Taguchi design, Perchlorate, C-source, Optimization.**

I. Introduction

Perchlorate (ClO_4^-) contamination is a significant concern in surface and ground waters over the years. A major source of perchlorate contamination comes from solid propellants for rocket fuel, missiles, road flares and fireworks (**Atikovic et al 2008, Herman and Frankenberger 1999**). It has recently been added to the drinking water Candidate Contaminant List (CCL) by the United States Environmental Protection Agency (USEPA) (EPA 2005) as it is a threat to indigenous wildlife as well as human health hazards (**Pontius et al 1999, Tan et al 2004**).

The perchlorate ion (ClO_4^-) is nonvolatile, highly soluble, and very stable in the aqueous phase. The use of perchlorate-contaminated water interferes with iodine uptake by human thyroid, which affects several vital body functions. Several studies indicated that low concentrations of perchlorate significantly inhibit iodide uptake in humans and animals; higher doses even results in fatal bone marrow disorders (Achenbach et al 2001). In 1992, the U.S. Environmental Protection Agency reviewed the health effects of perchlorate administered to patients with hyperthyroidism and found that doses of 6 mg kg^{-1} per day or more over a 2-months period resulted in fatal bone marrow changes (**Rikken et al 1996**)

Perchlorate removal from contaminated water can be achieved both by physico-chemical and biological processes. Ion exchange is used as one of the effective methods for its removal, but it is an incomplete process because it is non-selective and only separates these anions from the contaminated sources (**Urbansky 2000**). Therefore, due to the high affinity of perchlorate for these resins, very high salt concentrations (7–12%) are needed to regenerate the whole column (**Batista and Liu 2001**). Under such condition, bioremediation offers a benign and efficient technological solution, as these pollutants can be eliminated in an environment-friendly manner. (**Bardiya and Bae 2005**)

It has already been recognized that microbial reduction of chlorine oxyanions under anaerobic conditions is possible (**Coates et al 1999**). Perchlorate is used as a terminal electron acceptor by pure and mixed cultures (**Korenkov et al 1976**). Therefore, biological reduction is a promising treatment approach as perchlorate can be reduced to chloride and water by dissimilatory (per)chlorate-reducing bacteria (PRB), i.e., bacteria that reduce perchlorate and chlorate (ClO_3^-) as electron acceptors that provide energy for growth (**Logan et al 2001**). **Hackenthal et al.** proposed the perchlorate reduction pathway as follows (**Hakenthal 1964**),

$$ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$$

The reduction of perchlorate (ClO_4^-) or chlorate (ClO_3^-) to chloride (Cl^-) by bacteria has been confirmed by other researchers (**Coates et al 1999, Logan 1998, Malmqvist et al. 1991**) and none of the intermediates has been reported to be accumulated in solution (**Attaway and Smith 1993, Giblin and Frankenberger 2001, Kengen et al. 1999**). Chlorite (ClO_2^-) disproportionation to chloride and oxygen is a non-energy yielding step catalyzed by the enzyme chlorite dismutase (**Coates et al 1999 and Malmqvist 1991**). All PRB are capable of dissimilatory reduction of chlorate, whereas many of these can also reduce nitrate.

II. MATERIAL AND METHODS

Chemicals and reagents

Chemicals and reagents used in the study was of analytical grade, inorganic salts used in preparing microbial growth media were of reagent grade. Sodium perchlorate ($NaClO_4$. H_2O), procured from Merck, India was used as the source of ClO_4^- in all the experiments. All the other chemicals used in this study were purchased from Merck, India.

Analytical methods

All the anions viz. perchlorate, nitrate, chlorate, sulfate, phosphate and the organic acids such as acetate, formate, citrate, oxalate and succinate were measured using a Metrohm 792 Basic Ion Chromatograph (Metrohm AG, Herisau, Switzerland) equipped with a Dual 3 column (250 mm × 4 mm), a RP guard column, and a conductivity detector. Samples taken during the experiments and centrifuged at 8000 rpm for 10 min and were filtered through a C-18 reverse-phase cartridge and then through 0.45µm filter for analysis. NaOH (5 mM) served as the eluent and sulfuric acid (2.0 mM) as the regenerant in the chromatogram analysis. Scanning electron micrograph (SEM) images of the mixed microbial culture (glued to an aluminum stub and gold sputtered) were obtained by means of a LEO-1430 VP electron microscope.

Microorganism and culture conditions

An indigenous mixed microbial culture, potent to degrade perchlorate, was collected and enriched from a sewage treatment plant located in Guwahati, India. Two different strains *Burkholderia* sp (**Ghosh et al 2011**) and *Pseudoxanthomonas* sp were isolated from the mixed sludge consortium and acclimated for perchlorate degradation by using succinate and phenol as respective C-source. The mixed culture was grown in the medium with low initial concentration of chloride given by **Kim and Logan (2001**) having the following composition per liter of deionized water (Milli-Q System, Millipore Corp., New Bedford, MA, USA): 1555 mg K_2HPO_4, 850mg NaH_2PO_4, 500mg $NH_4H_2PO_4$, 100mg $MgSO_4.7H_2O$, 5mg $FeSO_4.7H_2O$, 0.2 mg $Na_2MoO_4.2H_2O$, 10 mg EDTA and trace minerals contained: 2mg $ZnSO_4.7H_2O$, 1mg $MnCl_2.2H_2O$, 1mg $CaCl_2$ $.2H_2O$, 0.2 mg $CuSO_4.5H_2O$, 0.4mg $CoCl_2.6H_2O$. Initial pH of the media was adjusted by adding required amount of 0.1 M HCl or 0.1 M NaOH solution.

Experimantal Design

Plackett-Burman design

Screening and optimization of media constituents can be done by either varying one variable at a time or non-conventionally using statistical methods. Conventional screening and optimization techniques involve varying factors and their levels by maintaining the other factors at an unspecified constant level, and by doing so, the combined effect of the factors is generally neglected; moreover, the technique is time-consuming and requires a sufficiently large number of experimental runs. These limitations of a classical method can be eliminated by screening and optimizing all the affecting factors collectively by employing statistical experimental design and empirical model building using regression analysis. To reduce the number of factors to be used in an optimization study, screening of factors is normally performed by employing another statistical design such as Plackett– Burman. Plackett-Burman design, an efficient way to identify the important factors among a large number of variables (**Stanbury et al., 1986**), was used in the present study to screen the important variables that significantly influenced perchlorate degradation. In this study, a 16-run Plackett-Burman design was applied to evaluate five factors. Each variable was examined at two levels: –1 for the low level and +1 for the high level. Table 1 illustrates the variables and their corresponding levels used in the experimental design. The values of two levels were set according to our previous preliminary experimental results. The Plackett-Burman design and the response value of phenol degradation are shown in Table 2. The effect of individual variable on perchlorate degradation was calculated by the following Eq. 1:

$$E(X_i) = 2\left(\sum M_i^{+} - M_i^{+}\right)/N$$

where, $E(X_i)$ is the effect of the tested variable (X_i) and M_i^{+} and M_i^{+} are responses (phenol degradation) of trials at which the variable is at its high or low levels respectively

N is the total number of trials. Experimental error was estimated by calculating the variance between the two dummy variables using following Eq. 2:

$$V\text{eff} = (E_d)^2/n$$

where, V_{eff} is the variance of the effect, E_d is the effect for the dummy variable and n is the number of dummy variables used in the experiment.

The standard error (SE) of the effect was the square root of V_{eff} and the significance (p-value) of the effect of each variable on phenol degradation was measured by Student's t-test according to the Eq. 3: $t(X_i) = E(X_i)/\text{SE}$

where, $E(X_i)$ is the effect of variable X_i.

Table 1: Plackett-Burman design using succinate as sole C-source

Serial No	*Temperature (^0C)*	*pH*	*Inoculum volume (mg/l)*	*Inoculum age (days)*	*Carbon: perchlorate ratio*
1	24	6.5	3.5	2	1
2	32	7.5	6.5	2	2
3	24	7.5	3.5	2	1
4	32	7.5	3.5	4	1
5	28	7.0	5.0	3	1.5
6	32	7.5	3.5	4	2
7	24	7.5	6.5	2	2
8	24	7.5	6.5	4	1
9	28	7.0	5.0	3	1.5
10	32	6.5	6.5	2	1
11	28	7.0	5.0	3	1.5
12	28	7.0	5.0	3	1.5
13	32	6.5	6.5	4	1
14	24	6.5	6.5	4	2
15	24	3.5	3.5	4	2
16	32	3.5	3.5	2	2

Serial No	*Temperature (^0C)*	*Inoculum age (days)*	*Inoculum volume (mg/l)*	*pH*	*Carbon: perchlorate ratio*
1	32	2	3.5	6.5	2
2	24	4	3.5	6.5	1
3	28	3	5.0	7.0	1.5
4	32	2	6.5	6.5	1
5	32	4	6.5	6.5	2
6	28	3	5.0	7.0	1.5
7	32	4	3.5	7.5	1
8	24	2	3.5	6.5	1
9	28	3	5.0	7.0	1.5
10	24	2	6.5	7.5	2
11	32	2	6.5	7.5	1
12	24	4	6.5	6.5	2
13	24	4	3.5	7.5	2
14	24	2	3.5	7.5	2
15	24	4	6.5	7.5	1
16	28	3	5.0	7	1.5

Table 2: Plackett-Burman design using phenol as sole C-source

Taguchi design

The Taguchi method, one of the optimization, has good reappearance of experiments concerned only with the main effects of design parameters. In principle, the Taguchi's design of experiments is used to get information such as main effects and interaction effects of design parameters from minimum number of experiments. The objectives of Taguchi method for parameter design were to find out the best combination of design parameters and reduce the variation for quality. Based on our previous work the main operational parameters and their levels were selected and showed in Table 3. The orthogonal array of L16 type was used, and is represented in Table 4. L and 16 mean Latin square and the replication number of the experiment, respectively. Five–four level factors can be positioned in an L16 orthogonal array table. The number in table indicates the levels of a factor (**Kim et al, 2004**).

Serial no.	Inoculum age (days)	Temperature (ºC)	Carbon: Perchlorate ratio
1	1	24	0.5
2	1	24	0.5
3	1	24	0.5
4	1	28	1.0
5	1	28	1.0
6	1	28	1.0
7	1	32	2.0
8	1	32	2.0
9	1	32	2.0
10	3	24	1.0
11	3	24	1.0
12	3	24	1.0
13	3	28	2.0
14	3	28	2.0
15	3	28	2.0
16	3	32	0.5
17	3	32	0.5
18	3	32	0.5
19	5	24	2.0
20	5	24	2.0
21	5	24	2.0
22	5	28	0.5
23	5	28	0.5
24	5	28	0.5
25	5	32	1.0
26	5	32	1.0
27	5	32	1.0

Table 3: Design for Taguchi method using succinate as a sole carbon-source

Table 4: Design for Taguchi method using phenol as sole carbon source

Serial no.	Inoculum age (days)	Temperature (ºC)	pH
1	2	21	5
2	2	21	5
3	2	21	5
4	2	28	7
5	2	28	7
6	2	28	7
7	2	37	9
8	2	37	9
9	2	37	9
10	3	21	5
11	3	21	5
12	3	21	5
13	3	28	7
14	3	28	7
15	3	28	7
16	3	37	9
17	3	37	9
18	3	37	9
19	4	21	5
20	4	21	5
21	4	21	5
22	4	28	7
23	4	28	7
24	4	28	7
25	4	37	9
26	4	37	9
27	4	37	9

III. RESULT AND DISCUSSION

Temperature, inoculum age, Carbon to perchlorate ratio were found to be more significant according to the results obtained by Plackett-Burman method of screening using succinate as a sole C-source for degradation of perchlorate when phenol was the sole C-source temperature, pH and inoculum age were found to be significant among all the five factors.

Table 5: Estimated effects and co-efficient using succinate

Terms	*Effect*	*Co-efficient*	*SE Co-efficient*	*T*	*P*
Constant		27.533	1.863	14.78	0.000
Temperature	-16.000	-8.000	1.863	-4.29	0.002
pH	-5.667	-2.833	1.863	-1.52	0.163
Inoculum vol	10.200	5.100	1.863	2.74	0.023
Inoculum age	-7.267	-3.633	1.863	-1.95	0.083
Carbon	14.267	7.133	1.863	3.83	0.004
Ct Pt		-3.933	3.727	-1.06	0.319

S=6.45520 PRESS=1137.8

R-Sq=84.16% R-Sq(adj)=73.59%

Table 6: Analysis of variance (ANOVA) using succinate

Source	*DF*	*Seq SS*	*Adj SS*	*Adj MS*	*F*	*P*
Main effects	5	1945.48	1945.48	389.10	9.34	0.002
Curvature	1	46.41	46.41	46.41	1.11	0.319
Residual error	9	375.03	375.03	41.67		
Lack of fit	6	219.91	219.91	36.65	0.71	0.671
Pure error	3	155.12	155.12	57.71		
Total	15	2366.92				

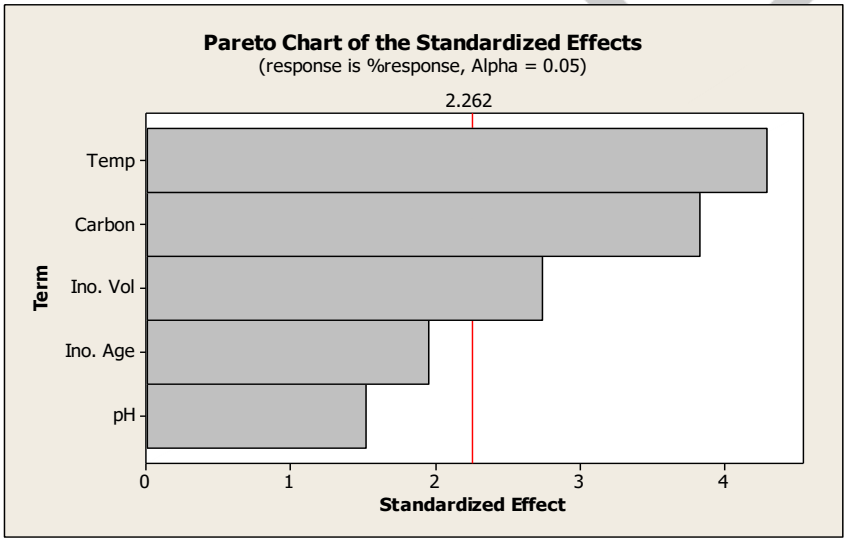

Fig.1: Pareto chart showing the standardized effects of the parameters using succinate as C-source

Table 7: Estimate effects and co-efficient using phenol

Terms	*Effects*	*Co-efficient*	*SE Co-efficient*	*T*	*P*
Constants		92.024	0 .2790	321.89	0.000
Temperature	-2.228	-1.114	0.2790	-3.99	0.003
pH	1.382	0.691	0.2790	2.48	0.035
Inoculum vol	-0.052	-0.026	0.2790	-0.09	0.928
Inoculum age	-1.532	-0.816	0.2790	-2.92	0.017
Carbon	0.465	0.233	0.2790	0.83	0.426
Ct Pt		3.976	0.5581	7.12	0.000

S= 0.966574 PRESS=107.982

R-Sq=90.12% R-Sq(adj)=83.53%

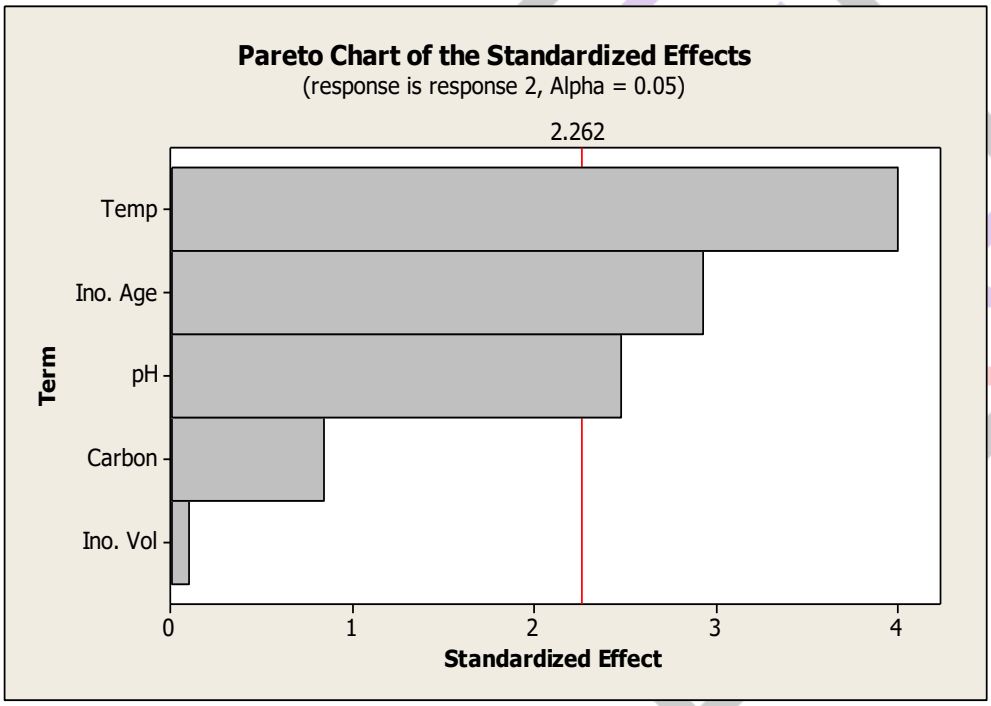

Fig. 2: Pareto chart showing the standardized effects of the parameters using phenol as C-source

A Taguchi method was used to identify the optimal conditions and to select the parameters having the most principle influence on the dye removal. The structure of Taguchi's L 27 design has been shown in Table 3 and 4. In the Taguchi method, the terms 'signal' and 'noise' represent the desirable and undesirable values for the output characteristic, respectively. Taguchi method uses the S/N ratio to measure the quality characteristic deviating from the desired value. The S/N ratios are different according to the type of characteristic. In the case that bigger characteristics are better, the S/N ratio is defined as Eq. 4

$$\frac{S}{N} = \frac{-10 \log (1/Y_2^1 + 1/Y_2^2 + 1/Y_2^3 + \cdots\cdots\cdots + 1/Y_2^n)}{n}$$

where Y_i is the characteristic property, n is the replication number of the experiment. The unit of S/N ratio is decibel (dB), which is frequently used in communication engineering. Table. 9 and 11 shows the S/N ratio for % degradation calculated using Eq. 4. Fig. 3 and 4 shows the S/N response graph for % degradation of perchlorate.

The optimum conditions for perchlorate degradation using succinate as well as phenol were listed in the table titled factor level for prediction.

Table 8: Analysis of variance (ANOVA) using succinate

Source	*DF*	*Seq SS*	*Adj SS*	*Adj MS*	*F*	*P*
Temperature	2	11277.9	11277.9	5638.9	238.35	0.000
Inoculum age	2	9423.8	9423.8	4711.9	199.6	0.000
c/p ratio	2	277.3	277.3	138.6	5.86	0.010
Error	20	473.2	473.2	23.7		
Total	26	21452.1				

S= 4.86402 R-Sq= 97.79% R-Sq(adj)=97.13%

Table 9: Response table for means

Level	*Inoculum age*	*Temperature*	*pH*
1	24.47	25.91	47.10
2	74.10	44.82	47.21
3	43.62	71.46	47.88
Delta	49.63	45.54	0.78
Rank	1	2	3

Predicted values

S/N Ratio	Mean	StDev	Ln(St Dev)
43.0990	98.6407	1.45098	-0.307821

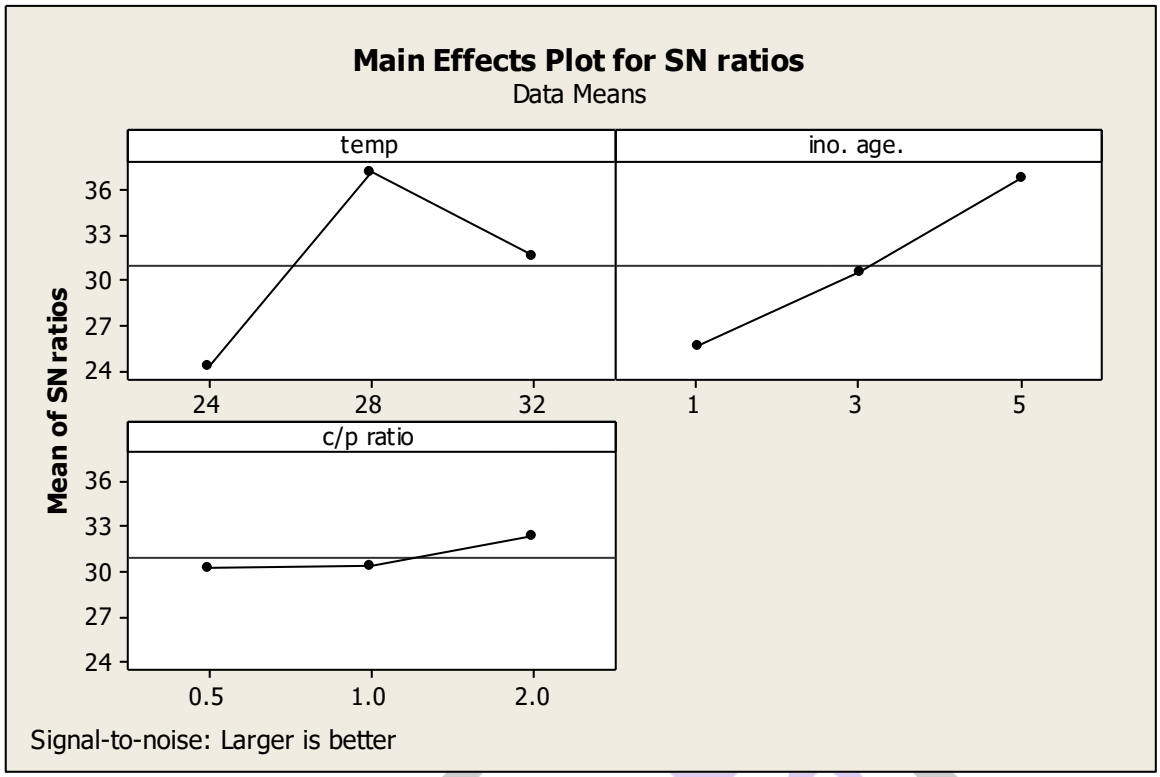

Fig.3: Effect of each parameter on perchlorate degradation using succinate

Table 10: Analysis of variance (ANOVA) using phenol

source	DF	SS	Adj SS	Adj MS	F	P
Inoculum age	2	4711.9	4711.9	4711.9	199.16	0.000
Temperature	2	27.3	27.3	27.3	5.86	0.048
pH	2	5417.6	5417.6	5417.6	238.35	0.000
Error	20	451.3	451.3	23.7		
Total	26	21452.1				

S = 5.6758 R-Sq = 96.19% R-Sq(adj) = 96.23%

Table 11: Response table for means

Level	*Temperature*	*Inoculum age*	*C/P ratio*
1	24.26	25.56	30.21
2	37.10	30.58	30.42
3	31.58	36.79	32.31
Delta	12.84	11.23	2.10
Rank	1	2	3

Predicted values

S/N ratio	Mean	St Dev	Ln(St Dev)
44.2462	99.4963	1.29013	-0.302599

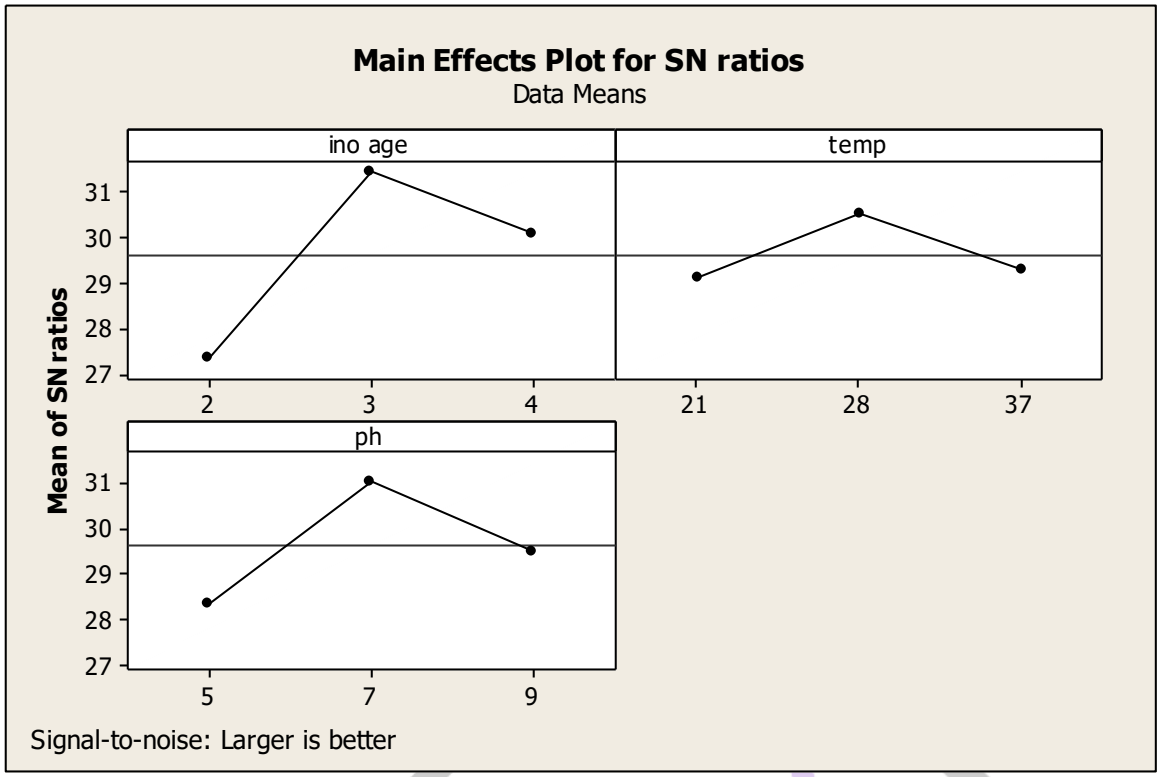

Fig. 4: Effect of each parameter on perchlorate degradation using phenol.

IV.CONCLUSION

In order to conduct an analysis of the relative importance of each factor more systematically, an analysis of variance (ANOVA) was applied to the data. The main objective of ANOVA is to extract from the results how much variations each factor causes relative to the total variation observed in the result. The carbon to perchlorate ratio had the largest variance in case of succinate and temperature in case of phenol. Result shows 28^{o}C is the optimum temperature for both cases i.e., perchlorate degradation in presence of succinate and as well as phenol which is also reported to be the optimum culture condition for known bacterial species responsible for perchlorate and chlorate respiration growing in anaerobic condition. The inoculum age was found to be optimum as 3 days in case of degradation using succinate and 5 in case of degradation using phenol which falls within the early log phase of both of the isolated strains as observed in our previous study and this observation is also similar to the previously reported studies where it has been reported that perchlorate usually gets uptake by the PRB (perchlorate degrading bacteria) in early and mid-log phase. The pH was found to be a significant variable in case of degradation using phenol but not in case of degradation in case of succinate which indicates some effect on the organism due to the presence of phenol as toxic substance which was used as C-source.

V.ACKNOWLEDGEMENT

This work was done under valuable guidance of Dr. (Prof.) K. Pakshirajan of the Department of Bioscience and Bioengineering and Dr. (Prof.) P.K Ghosh of the Department of Civil Engineering, Indian Institute of Technology, Guwahati, India.

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