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Growth Parameter Optimization of copper-tolerant Bacterium, *Stenotrophomonas acidaminiphila* MYS2 through Response Surface Methodology

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Abstract—*This study was conducted to establish the optimized growth parameters of Stenotrophomonas acidaminiphila MYS2, rhizosphere bacterium isolated from brass industry effluent contaminated soil. Bacterium was enriched in increasing concentration of copper sulphate from 50-600mg/L. Optimization of growth parameters was carried out with response surface methodology. Significance of the quadratic model is revealed by high R-Squared (0.9610) low P (<0.0001) and F value (44.85). Results revealed optimum pH-6.5, temperature-40°C and copper concentration of 250 mg/L. Results showed that maximum bacterial growth of 12.95µg/mg was obtained at optimized conditions. At optimized growth conditions bacteria grow well and could remove copper from the contaminated media efficiently.*

Keywords*: Stenotrophomonas acidaminiphila MYS2, Copper-tolerant, growth optimization, response surface methodology.*

1. INTRODUCTION

Copper is an essential micronutrient for prokaryotic and eukaryotic cells as it is involved in vital metabolic processes. It is used as co-factor in many metallo-enzymes and hence plays an important role in enzymes active sites. However, above optimum levels it becomes toxic [1, 2]. Further the toxicity of the metal increases due to its ability to generate free radicals. In elevated copper concentration, copper (II) ions get accumulated in essential organs including brain, myocardium, liver, and pancreas [3]. This will escort to nausea, headache, vomiting, diarrhea, trouble in respiration, breakdown of kidney and liver, and lastly death [4]. Mining, agriculture, coal fired thermal power plants, metal producing industries such as brass industries, sewage treatment processes, waste incinerators are the main contributors of copper contamination.

Bacteria acquired different mechanisms to combat the heavy metal stress. Different mechanisms involved include intracellular and extracellular sequestration, efflux, reduced permeability and extracellular detoxification. Exploitation of these bacterial mechanisms for remediation of heavy metals has drawn much consideration among researchers. Many bacteria use bioaccumulation strategy to remove metals. Chowdhury *et al*. [5] reported about the possible use of metal accumulating bacteria to remove metals from the contaminated industrial effluents. Heavy metal tolerant bacteria could be used for remediation of contaminated sites either alone or with metal accumulating plants.

This study aims with designing a growth model using bacteria against copper contamination. For this, copper-tolerant bacterium was isolated from contaminated site and optimization of pH, temperature and copper concentration on bacterial growth was demonstrated to use it for bioremediation purpose. Response surface methodology is mostly used technique for optimization studies in recent years. Design-Expert (trial version 9.0.6.2) was used for the concerned study.

2. MATERIALS AND METHODOLOGY

2.1. Isolation of the bacterium

Bacterium was isolated from rhizosphere soil of *Cynodon dactylon*, growing in brass effluent contaminated soil. Soil suspension was prepared by serial dilution method. 100µL suspension was spread on nutrient agar medium and incubated at 30°C for 7 days. The nutrient media was supplemented with cycloheximide to hamper the fungal development. Colonies were enriched in copper sulphate ($CuSO_4.5H_2O$) in increasing concentration from 50 to 600 mg/L.

2.2. Characterization of the bacterium

Isolated rhizosphere bacterium was gram-negative bacilli. 16S ribosomal gene sequencing revealed 95% similarity with *Stenotrophomonas acidaminiphila*. The sequence was submitted to GenBank under accession number-KJ664228.

2.3. Stock solution of copper

1 g/L stock solution was prepared by dissolving 3.931 g $CuSO_4$ in one litre distilled water. This stock solution was further used for preparation of different concentration of copper solutions.

2.4. Bacterial growth optimization studies

2.4.1. Response surface methodology (RSM)

Design-expert (Trial version 9.0.6.2, Stat-Ease) was used for the optimization of growth parameters. Box-Behnken design with quadratic model was selected. Total 17 experimental runs were carried out to achieve the optimum pH, temperature and copper concentration for maximum bacterial growth.

2.4.2. Optimization of growth parameters by RSM

For growth study three parameters namely pH, temperature and copper concentration in the range of 5-8, 25°C- 40°C and 150 to 600 mg/L, respectively were chosen. The response was assessed as bacterial growth. Nutrient broth supplemented with different concentration of copper sulphate was used. Medium pH was adjusted with 0.1 M NaOH and 0.1 M HCl and autoclaved. Then the broth was inoculated with the bacterial culture and incubated at 120 rpm for 24 hours. Bacterial culture were sampled and centrifuged at 10,000 rpm for 5 minutes. The pellet so obtained was further studied for bacterial protein by Bradford assay [6]. The bacterial growth was assessed through protein.

2.5. Statistical analysis

The experiments were carried out in triplicates and statistical calculations were done with Microsoft Office Excel 2007. RSM was studied with Design-Expert (Trial version 9.0.6.2).

3. RESULTS AND DISCUSSION

Isolated rhizosphere bacterium showed very high copper tolerance of 600 mg/L. It tolerated copper (II) concentration up to 600 mg/L.

3.1. Response surface study

Copper-tolerant *Stenotrophomas acidaminiphila* MYS2 growth was optimized by employing Box-Behnken design with quadratic model in response surface methodology. Three parameters that influence bacterial growth were pH (5-8), temperature (25-40ºC) and copper concentration (150-1000 mg/L). Experimental and predicted bacterial growth values are listed in Table 1 and Fig. 1. Maximum bacterial growth was observed in run 10 showing 12.95 µg/mg. The predicted growth 12.47 µg/mg was lesser than that obtained during experimental studies. This implies the significance of the model. The design and results obtained were utilized to produce contour and response surface plots for evaluating the effect of different independent variables on response. Analysis of variance (ANOVA) for quadratic model is listed in Table 2, indicating model's high significance ($p<0.0001$). Further model significance is confirmed by F value of 44.85. A, C, BC, A^2, C^2 are the significant terms for this model. Lack of Fit value of 2.99 implies insignificant lack of Fit, which is good. The R-Squared obtained in this study is 0.9830, which is close to 1. The predicted R-Squared (0.8032) and Adjusted R-Squared (0.9610) are in reasonable agreement with each other and it revealed very high relationship between experimental and predicted response. The relationship between response (bacterial growth) and variables is demonstrated by second order polynomial equation (1):

$$\text{Bacterial growth } (\mu g/mg) = +5.70 + 2.70A - 0.012B - 3.66C + 0.14AB + 0.46AC - 1.02BC - 0.83A^2 + 0.75B^2 + 1.35C^2 \quad \text{.................................} (1)$$

Table 1: Experimental and predicted bacterial growth values obtained by response surface methodology

Run No.	A:(pH)	B:Temp. (°C)	C:Cu conc. (mg/L)	Bacterium growth (µg/mg) Experimental	Predicted
1	5	40	625	2.14	2.75
2	6.5	25	250	10.48	10.44
3	6.5	32.5	625	6.3	5.69
4	8	40	625	8.62	8.44
5	6.5	32.5	625	5.68	5.69
6	6.5	32.5	625	6.0	5.69
7	6.5	32.5	625	5.0	5.69
8	8	32.5	250	11.46	12.12
9	6.5	40	1000	3.06	3.1
10	6.5	40	250	12.95	12.47
11	6.5	32.5	625	5.5	5.7
12	8	25	625	8.8	8.18
13	5	25	625	2.9	3.07

14	6.5	25	1000	4.69	5.17
15	5	32.5	250	7.77	7.64
16	5	32.5	1000	0.006	-0.59
17	8	32.5	1000	5.59	5.72

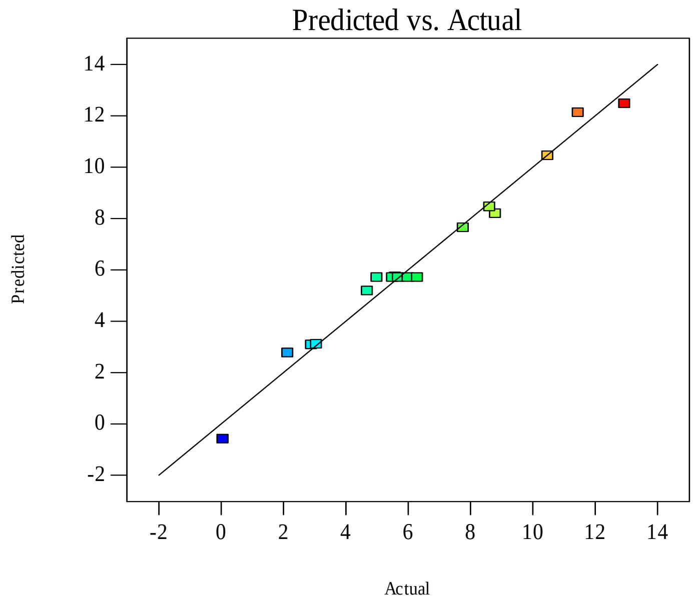


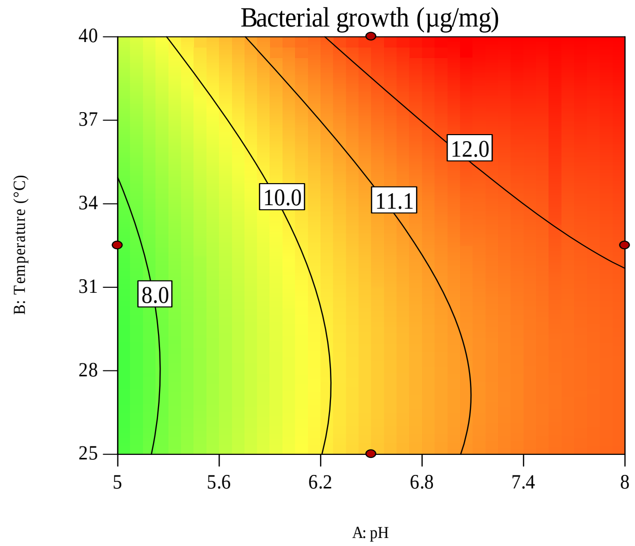
Fig. 1: Plot showing actual and predicted bacterial growth (µg/mg)

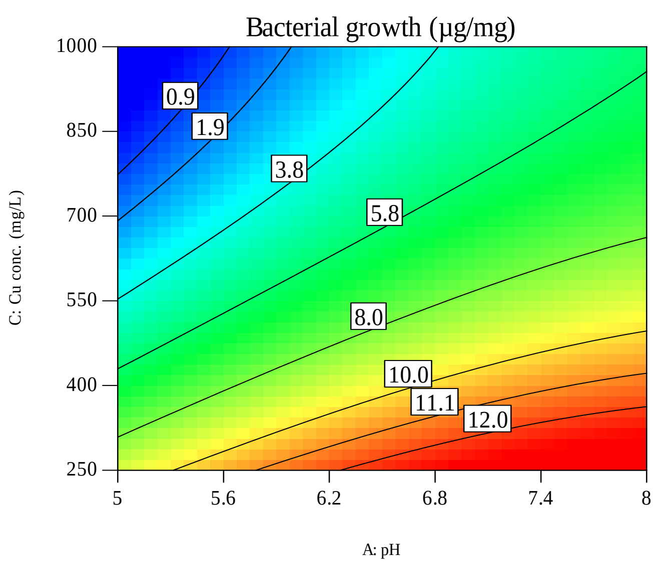
Table 2: Analysis of variance for quadratic model selected for growth optimization

Source	Sum of squares	df	Mean square	F value	P Value
Model	183.14	9	20.35	44.85	<0.0001
A-pH	58.32	1	58.32	128.55	<0.0001
B-Temp.	1.25E-003	1	1.25	2.75	0.9596
C-Conc.	107.02	1	107.02	235..8	<0.0001
AB	0.084	1	0.084	0.19	0.6797
AC	0.85	1	0.85	1.87	0.2142
BC	4.20	1	4.20	9.26	0.0188
A^2	2.89	1	2.89	6.36	0.0397
B^2	2.35	1	2.35	5.18	0.0570
C^2	7.70	1	7.70	16.96	0.0045
Residual	3.18	7	0.45		
Lack of fit	2.20	3	0.73	2.99	0.1591
Pure error	0.98	4	0.25		
Cor Total	186.31	16			

3.2. Interaction among parameters

Interactive effect of two variables on response can be understood by contour plots and response surface graphs (Fig. 2 and 3). These graphs elucidate the effect of three important variables; pH, temperature and copper concentration on the bacterial growth. Fig. 2 (a) and 3 (a) shows the effect of pH and temperature of the medium on bacterial growth. The bacterial growth increased with the increase in temperature and pH. An elevated bacterial growth was observed above pH 6.2. Likewise, Hussain *et al.* [7] and Joshi and Modi [8] report optimum growth pH of 6.0 for copper tolerant *Pseudomonas sp* No. 16 and PGCU 5 .Effect of pH and copper concentration on bacterial growth are shown in Fig. 2 (b) and 3 (b). Bacterial growth decreased with increase in copper concentration because at higher copper concentration pH of the medium decreases. Fig. 2 (c) and 3(c) demonstrate the effect of copper concentration and temperature on bacterial growth and it entails that maximum bacterial growth could be achieved at a temperature range of 34°C to 40°C for 250 mg/L of copper concentration. Rohini and Jayalakshmi, [9] reported optimal growth temperature for *Bacillus cereus* as 40°C with maximum copper accumulation.





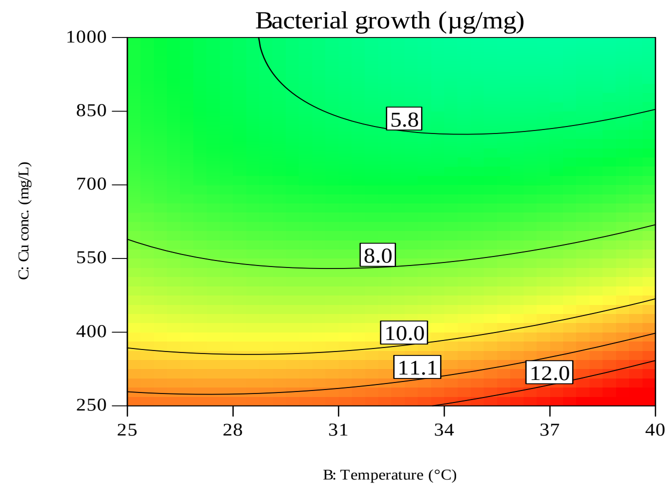
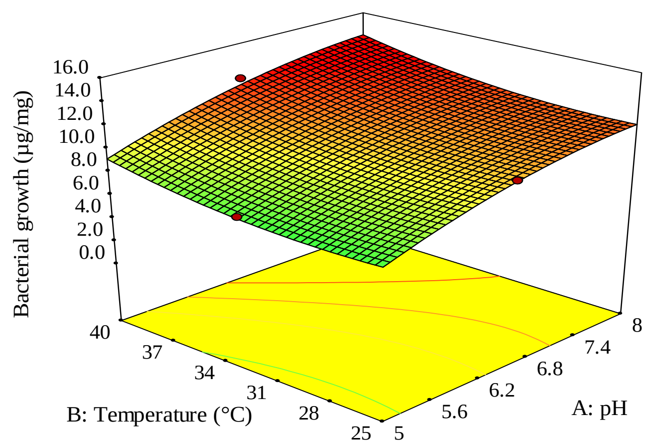
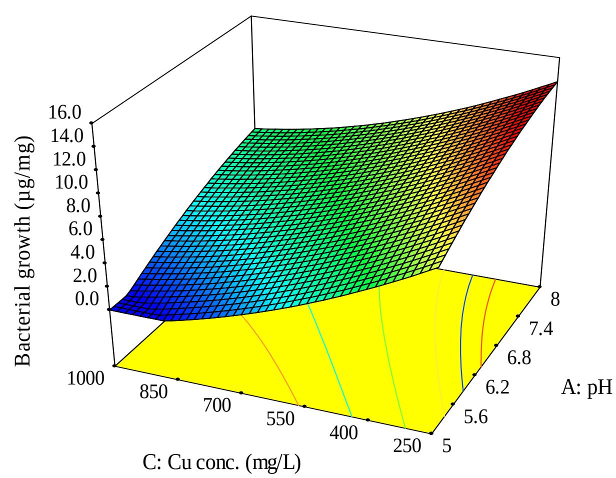


Fig. 2: Two-dimensional contour graphs showing interaction between: (a) pH and temperature, (b) pH and copper concentration and (c) temperature and copper concentration





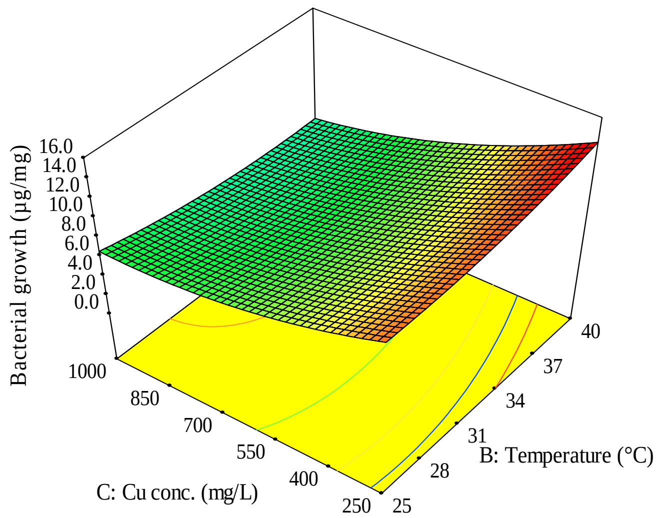


Fig. 3: Three-dimensional response surface graphs showing interaction between: (a) pH and temperature, (b) pH and copper concentration and (c) temperature and copper concentration

The optimum conditions were at pH-6.5, temperature- 40°C and copper concentration of 250 mg/L for maximum bacterial growth of 12.97 µg/mg experimentally and 12.45µg/mg predictably.

4. CONCLUSIONS

The gram-negative bacilli *Stenotrophomonas acidaminiphila* MYS2 showed high copper-tolerance. Optimum bacterial growth conditions were obtained at 6.5 pH, 40°C and 250 mg/L of copper concentration. The bacterium showed maximum growth of 12.95 µg/mg. This study concludes that at optimized growth conditions, bacterium showed good growth at 250 mg/L copper concentration and could be used in copper bioremediation.

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