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RESEARCH PAPER

Copper(II) Bioremoval by a Rhizosphere Bacterium, *Stenotrophomonas acidaminiphila* MYS1-Process Optimization by RSM Using Box–Behnken Design

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Abstract A copper-tolerant bacterium strain MYS1 was isolated from Brass effluent-contaminated *Cynodon dactylon* rhizosphere and identified as *Stenotrophomonas acidaminipila* by 16S rRNA analysis. Bacterium culture was enriched in copper(II) supplemented nutrient broth. Bacterial potential for copper(II) bioremoval was investigated under optimized parameters. Three parameters—pH, temperature and copper(II) concentration—were optimized through response surface methodology (RSM). Box–Behnken design (BBD) with quadratic model was selected. Seventeen experimental runs were carried out to get the desired response. Model's significance was confirmed by high R^2 value (0.9941), low P value (<0.0001) and F value (131.32). Effect of different parameters on bioremoval of copper(II) was determined by response contour and surface graphs. Results showed that optimum values for copper(II) removal were obtained at pH (5.0), temperature (32.5 °C) and copper(II) concentration (250 mg/L). Under these optimized conditions, maximum bacterium growth (2.87 µg/mg) and copper(II) bioremoval (94.1%) were demonstrated after 120 and 168 h of incubation, respectively. High percentage of copper(II) removal at such a higher concentration confirmed the feasibility of bacterium *Stenotrophomonas acidaminiphila* MYS1 in copper bioremediation and industrial effluent treatment.

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Introduction

Increased industrialization has led to the contamination of the environment. Soil and water contamination due to industrial effluent discharge is of greater concern. Most of the industrial wastewater contains a number of hazardous organic and inorganic substances, depending on the type of industry. Among inorganic pollutants, copper is also one of the lethal substances above a certain concentration. Copper is present in electroplating and electrolysis, alloy, fertilizer and metal cleaning industrial effluents in alarming concentration (Özer et al. 2004). Copper is the main trace element required for metabolic processes in microbes, plants and animals including humans. But in higher concentration, it affects negatively and becomes toxic to the organisms. Higher copper concentration in water bodies is reported to damage marine life (Karthikeyan et al. 2007; Berehanu et al. 2015). Excessive copper ion accumulation has been observed to damage brain, liver and kidney, Schizophrenia and even led to death in humans (Tapiero et al. 2003).

Different treatment technologies exist for Cu(II) removal from the wastewater, including oxidation, ion exchange, precipitation, evaporation, electroplating and membrane filtration. However, these technologies have negative effects on the environment and require complicated technical setup and high operating cost. Therefore, there is necessity of a novel eco-friendly and cost-effective tool which can remove copper ions from the aqueous solution.

Bioremediation is the well-liked area of the environmental microbiology due to its economical and eco-friendly nature. Its ability to degrade and remove lethal organic and inorganic contaminants with less complexity using naturally available bacteria, fungi and algae makes the technology more attractive among others (Congeevaram et al. 2007; Andreazza et al. 2011; Bestawy et al. 2013). However, toxicity and certain environmental conditions such as temperature and pH of the media affect the sensitivity of the process. Microorganisms have evolved a number of mechanisms to overcome the metal stress. Utilization of these different microbial mechanisms for bioremediation of heavy metals has drained much contemplation among the researchers. Many microbes use biosorption and bioaccumulation strategy to remove metals. Chowdhury et al. (2008) reported about the possible use of metal accumulating bacteria to remove metals from the contaminated industrial effluents. Therefore, these metal-tolerant bacteria can be utilized for the bioremediation of contaminated environment either alone or with synergistic association of plants in phytoremediation.

Generally, conventional methods for optimization of different parameters are time consuming, lengthy and laborious. These methods separately require batch studies for optimization of different parameters such as pH, temperature, time, and metal concentration. These drawbacks of conventional optimization methods are overcome by the use of response surface methodology (RSM). It is a statistical tool which is used to optimize all the parameters of interest collectively and for evaluating their interactions. Application of these statistical tools for optimization study reduces experimental time, capital cost and increases the accuracy percentage. Nowadays, this practice is generally used for optimization of heavy metal removal (Venil et al. 2011; Ghosh and Saha 2013). Therefore, response surface methodology of Design-Expert (version 9.0.6.2) software was used to obtain optimized parameters.

In this study, an effort is made for optimization of copper(II) removal parameters and bioremoval capacity of a rhizosphere bacterium, *Stenotrophomonas acidaminiphila* strain MYS1. Bacterium was isolated from Brass effluent-contaminated rhizosphere soil of *Cynodon dactylon* grown in Karula Nala, Moradabad, India. It is a slow growing Gram-negative bacillus.

Materials and Methods

Isolation and Cultivation of the Bacterium

Healthy *Cynodon dactylon* plants were collected from Brass effluent polluted soil. Bacterium was isolated from *Cynodon dactylon* rhizosphere soil by serial dilution method on nutrient agar medium. Petri plates were kept for incubation at 30 °C for 168 h. Cycloheximide was supplemented to the medium to control the fungal growth. Pure colonies were enriched in copper(II) sulfate in 50–600 mg/L range. Bacterial cells showed good growth up to 600 mg/L of copper(II) concentration, after which the growth ceased. Enrichment was stopped at 600 mg/L of copper.

Characterization of the Bacterium

The isolated rhizosphere bacterium was a Gram-negative bacillus. 16S ribosomal gene sequencing revealed 99% similarity with *Stenotrophomonas acidaminiphila* MYS1 and deposited to GenBank under accession number-KJ664227.

Copper Stock Solution Preparation

Copper stock solution was prepared from copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) by dissolving 3.931 g $CuSO_4$ in one liter distilled water. This stock solution was used for preparation of different copper(II) concentration solutions.

Copper(II) Bioremoval Study

Copper(II) bioremoval experiments were carried out in nutrient medium with different range of copper(II) concentration, pH and temperature. pH of the medium was accustomed with 0.1 M NaOH and 0.1 M HCl solution. 1 mL bacterial culture was inoculated to 50 mL of nutrient broth containing copper(II) sulfate in Erlenmeyer flask. Flasks were put for incubation in incubator shaker at 120 rpm for 9 days. 1 mL of suspension was taken on every alternate day and centrifuged at 10,000 rpm for 5 min. Supernatant was acid digested and copper(II) concentration was determined by atomic absorption spectrophotometer (AAS).

Response surface methodology

Design-expert 9 (Trial version, Stat-Ease) is a statistical software, especially devoted to execute design of experiments. It offers comparative tests, characterization, screening, optimization and tough parameter designs. Around 50 variables can be used in design expert. The impact of the variables on the response is analyzed statistically through ANOVA and represented graphically through 3D curves. In the present study, Box–Behnken design (BBD) of quadratic model was used to optimize three variables—pH, temperature and copper(II)

concentration. Total seventeen runs were carried out to obtain optimized levels of the three selected key variables for maximum copper(II) bioremoval.

Optimization of variables

For copper(II) bioremoval study, three key variables selected were: pH ranged from 5 to 8, temperature ranged from 25 to 40 °C and Cu(II) concentration ranged from 250 to 1000 mg/L. Cu(II) bioremoval response was studied by the statistical experiments planned by RSM. Preliminary experiments were carried out for selection of the ranges of different variables. Medium pH was adjusted with 0.1 M HCl/NaOH. 50 mL copper(II) supplemented broth was autoclaved, inoculated with bacterium culture and incubated in incubator shaker at 120 rpm for 24 h. 1 mL bacterial suspension was centrifuged at 10,000 rpm for 5 min. Supernatant copper(II) concentration was determined by atomic absorption spectroscopy (AAS).

The correlation between different variables and response was demonstrated by second-order polynomial Eq. (1)

$$Y = \alpha_0 + \alpha_1 A + \alpha_2 B + \alpha_3 C + \alpha_{12} AB + \alpha_{13} AC + \alpha_{23} BC + \alpha_{11} A^2 + \alpha_{22} B^2 + \alpha_{33} C^2 \quad (1)$$

where Y designates copper(II) removal, α_0 is for the fixed response, α_1, α_2, and α_3 are the linear coefficients, α_{12}, α_{13}, and α_{23} are interaction terms and α_{11}, α_{22}, and α_{33} are quadratic coefficients.

Bacterial growth and copper(II) bioremoval study at optimized variables

A growth curve for *Stenotrophomonas acidaminiphila* MYS1 at optimized conditions was determined in terms of cell biomass produced. 50 mL of nutrient broth was inoculated with 1 mL of bacterial inoculum and incubated at optimized pH 5, temperature 32.5 °C and copper concentration of 250 mg/L for 9 days. Sampling was done on every alternate day and analyzed for growth and copper(II) bioremoval efficiency. Bacterial cell protein content was determined by Bradford (1976) assay. 1 mL bacterial suspension was centrifuged at 10,000 rpm for 5 min. Pellet was used for bacterial growth study and supernatant was analyzed for copper(II) concentration. Percent Cu(II) bioremoval by bacterium was calculated by the following formula:

$$\%\ \text{Cu (II) bioremoval} = \frac{\text{Cu}_{\text{initial}} - \text{Cu}_{\text{final}} \times 100}{\text{Cu}_{\text{initial}}}$$

where $\text{Cu}_{\text{initial}}$ is the Cu(II) concentration in the medium before incubation and Cu_{final} is the copper(II) concentration left in the medium after incubation.

Statistical Analysis

Experimental work was done in triplicate. Mean and standard deviation calculation were done with Microsoft Office Excel 2007 and optimization study was carried out with Design-Expert 9 software.

Results and Discussion

Copper-tolerant rhizosphere bacterium, *Stenotrophomonas acidaminiphila* MYS1, showed copper tolerance of 600 mg/L in nutrient broth. It showed 99% 16S rRNA gene sequence similarity with *Stenotrophomonas acidaminiphila*.

Response Surface Experiments for Maximum Copper(II) Removal

Response surface methods were used to determine the effect of different variables such as pH 5.0–8.0, temperature 25–40 °C and Cu(II) concentration 250–1000 mg/L on copper(II) bioremoval percentage. Quadratic model of three factorial Box–Behnken design (BBD) was selected. Total seventeen runs were conducted to get the best levels of variables. Three parameters (pH, temperature and copper concentration) were taken as independent variables and copper(II) removal percentage was taken as response of the study. The experimental and predicted (%) values for Cu(II) bioremoval are listed in Table 1. Maximum Cu(II) bioremoval percentage was acquired in run 14, i.e., 88% experimentally and 88.2% predictably, which entail about the significance of the model. Table 2 lists the results of analysis of variance (ANOVA) obtained for quadratic model from Box–Behnken design. P value (<0.0001) obtained revealed the regression model's significance. F value (131.32) obtained further implies that the model is highly significant for the bioremoval of copper(II). There is only a 0.01% chance that large F value could occur due to noise. Values of "Prob > F" less than 0.0500 indicate that model terms are significant. In this case, B, C, BC, B^2, C^2 are the significant model terms. Lack of fit F value—1.09 implies non-significant lack of fit. The goodness of the model was restricted by R^2 (coefficient of determination). High R^2 value (0.9941) obtained is very close to 1.0, and advocates great correlation between the predicted and actual values. The value of Predicted R^2 (0.9525) and Adjusted R^2 (0.9865) are in reasonable agreement. The regression model selected provides brilliant explanation of relationship between the variables and response. Plot shown in Fig. 1 was used for assessing the competence of the model. The relationship between the selected variables

Table 1 Predicted and observed values for Cu(II) bioremoval efficiency obtained from quadratic model

Run no.	*A*: (pH)	*B*: Temperature (°C)	*C*: Cu conc. (mg/L)	Removal efficiency (%)	
				Experimental	Predicted
1	6.5	40	250	84.80	84.69
2	6.5	40	1000	83.80	84.84
3	6.5	32.5	625	85.12	84.69
4	6.5	25	250	66.00	64.96
5	8	32.5	250	87.60	88.54
6	6.5	32.5	625	85.20	84.69
7	5	32.5	1000	85.00	84.69
8	8	40	625	83.40	82.57
9	5	40	625	83.50	83.40
10	6.5	32.5	625	85.44	84.69
11	6.5	32.5	625	85.28	84.69
12	8	25	625	59.20	59.30
13	5	25	625	57.40	58.23
14	5	32.5	250	88.00	88.21
15	6.5	25	1000	56.00	56.11
16	6.5	32.5	625	82.40	84.69
17	8	32.5	1000	84.20	83.99

Table 2 Analysis of variance (ANOVA) for quadratic model of Box–Behnken design

Source	Sum of squares	*df*	Mean square	*F* value	*P* value	Prob > *F*
Model	2027.02	9	225.25	131.32	<0.0001	Significant
A—pH	0.031	1	0.031	0.018	0.8964	
B—Temperature	1173.70	1	1173.70	684.25	<0.0001	
C—Concen.	37.84	1	37.84	22.06	0.0022	
AB	0.90	1	0.90	0.53	0.4918	
AC	0.040	1	0.040	0.023	0.8829	
BC	20.25		0.25	11.81	0.0109	
A^2	0.073	1	0.073	0.042	0.8426	
B^2	788.14	1	788.14	459.47	<0.0001	
C^2	11.37	1	11.37	6.63	0.0367	
Residual	12.01	7	1.72			
Lack of fit	5.41	3	1.80	1.09	0.4486	Not significant
Pure error	6.60	4	1.65			
Cor total	2039.23	16				

$R^2 = 0.9941$, Adj $R^2 = 0.9865$, Pred $R^2 = 0.9525$

and response (Cu removal) for quadratic model was demonstrated by second-order polynomial equation.

$$\text{Cu (II) removal} = +84.69 + 0.062A + 12.11B - 2.17C - 0.48AB - 0.100AC + 2.25BC - 0.13A^2 - 13.68B^2 + 1.64C^2$$

where *A* denotes pH, *B* denotes temperature and *C* denotes copper(II) concentration.

Interactive Effects of Variables

To demonstrate the effect of each independent variable, two-dimensional (2D) contour and three-dimensional (3D) surface graphs were used. These graphs are helpful to investigate the interactive effect of two variables on copper(II) removal percent in the experimental ranges as given in Figs. 2 and 3. The observations obtained are discussed below.

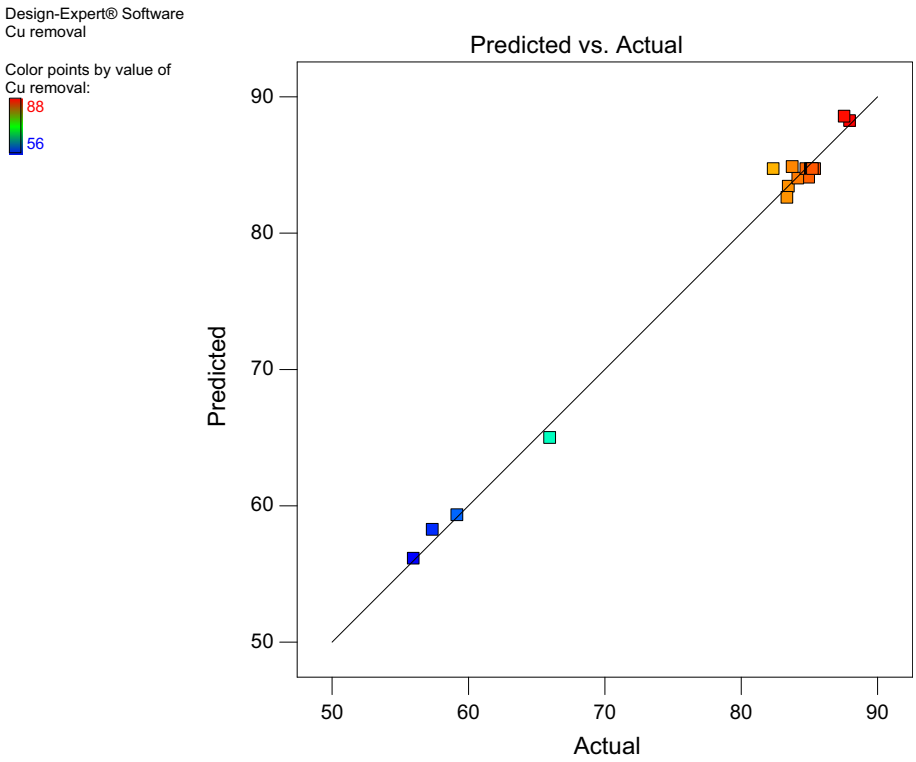

Fig. 1 Plot presenting the distribution of predicted vs. actual values of % Cu(II) removal

Effect of Initial pH and Temperature

Three independent variables, pH (5.0–8.0) temperature (25–40 °C) and copper(II) concentration (250–1000 mg/L), were selected to obtain maximum copper(II) removal percent by *Stenotrophomonas acidaminiphila* MYS1. Contour and surface graphs shown in Figs. 2a and 3a) depict the interactive effect of pH and temperature on copper(II) removal (%). As indicated in the figures, copper(II) removal percent was decreases with increase in the pH of the medium. Maximum copper removal percent was achieved at pH 5. Similar results regarding optimum pH of copper removal by *Pseudomonas putida* CZ1 and *Bacillus subtilis* are reported by Chen et al. (2005) and Fang et al. (2014), respectively. Medium pH affects the solubility of metal and ionization of chemical groups present on the bacterial cell wall of (Galun et al.1987). Therefore, optimum pH is required for metal bioremediation. At pH 5, copper ions are biologically active and are available to bind with functional groups present on the bacterial surface and are metabolically up taken by bacterial cells. On the other hand, at higher pH solubility of copper ions decreases and precipitation occurs due to which bacterial cells are not able to bind with copper ions (Goksungur et al. 2003). While in case of temperature, copper(II) removal (%) showed a sharp increase from 31 to 40 °C. Maximum copper(II) removal (%) was observed at temperature 32.5 °C.

Effect of pH and Cu(II) Concentration

Interactive effect of initial pH and copper(II) concentration on copper removal percent was shown by contour (Fig. 2b) and three-dimensional surface graphs (Fig. 3b). As copper concentration of the medium increases, the removal (%) decreases. The highest copper removal (%) was observed at 250 mg/L of copper(II) concentration, after which no significant increase in removal percent was observed. The possible reason might be the saturation of binding sites present on the surface of bacteria. As copper concentration in the medium increases, binding sites present on bacterial cell surface become saturated with copper ions and are not available for further binding of copper ions, leading to no further increase in copper removal percent after 250 mg/L of copper concentration. Optimum copper concentration obtained in the present study is much higher than that reported by Ghosh and Saha (2013).

Effect of Cu(II) concentration and temperature

As seen in Figs. 2c and 3c, it is clearly evident from the graphs that both the variables strongly influenced the copper(II) removal response. Maximum copper removal was obtained at 250 mg/L copper concentration and temperature 32.5 °C. Temperature of the medium is an important factor affecting copper removal as it affects

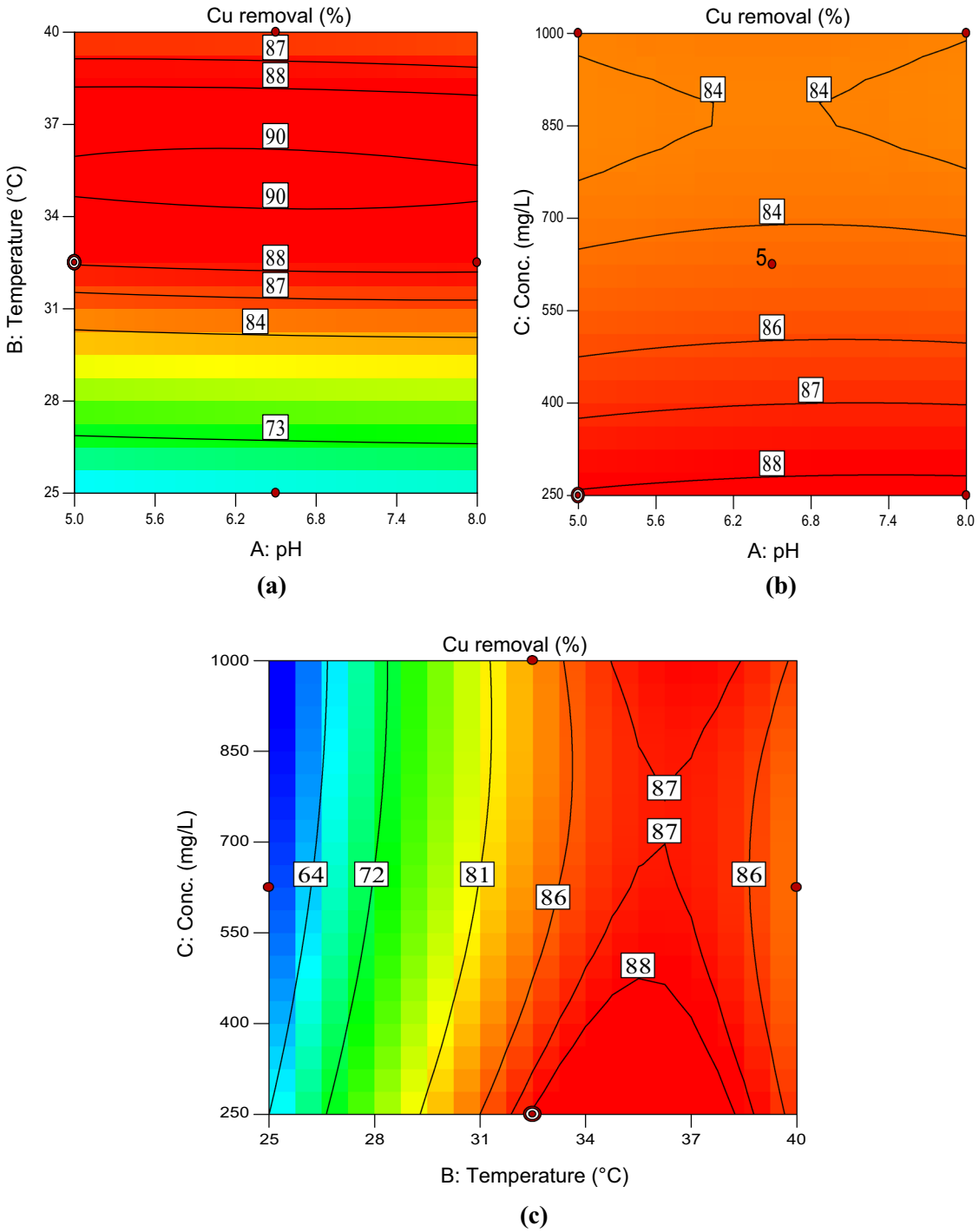

Fig. 2 Two-dimensional (2D) contour graph depicting effect of **a** pH and temperature, **b** Cu(II) concentration and pH and **c** Cu(II) concentration and temperature on copper(II) bioremoval percent

stability, configuration of bacterial cell wall and ionization state of chemical groups present on the surface of bacteria (Congeevaram et al. 2007). Therefore, to achieve maximum copper removal optimum temperature of the copper removal medium is required.

Bacterial growth and Copper(II) Bioremoval Study at Optimized Variables

Stenotrophomonas acidaminiphila MYS1 was studied for growth and copper(II) bioremoval at optimized levels of

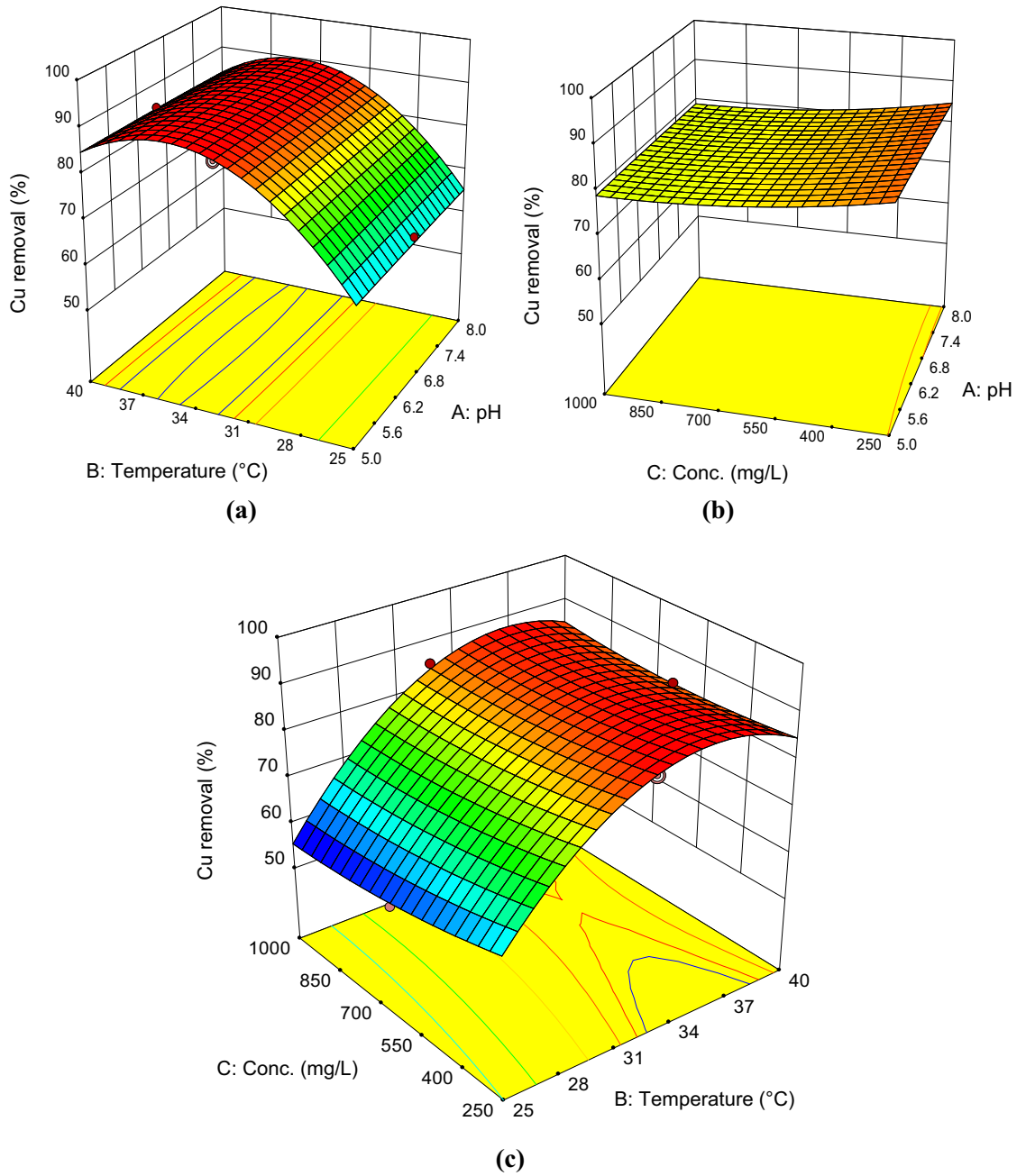

Fig. 3 Three-dimensional (3D) surface graphs depicting effect of **a** pH and temperature, **b** Cu(II) concentration and pH and **c** Cu(II) concentration and temperature on copper(II) bioremoval percent

temperature (32.5 °C), pH (5.0), and copper(II) concentration (250 mg/L) at pre-determined time period. Bacterium growth and copper(II) removal (%) are depicted in Fig. 4. Bacterial growth followed a sigmiodal curve, lag phase extended up to 24 h of incubation and exponential phase was observed from 24 to 72 h of incubation. Maximum growth was achieved at 120 h of incubation after which growth did not increase as a function of incubation time. Similarly, copper removal percent increases with increase in bacterial growth because more number of bacterial cells or metal binding sites are available for copper removal. However, bacterial cells remained metabolically active in stationary phase and continuously removed the copper ions from the medium. Therefore, maximum copper removal percent (94.1) was achieved in stationary phase at 168 h of incubation time. The copper(II) percent removal by the bacterium is much higher than those reported in earlier studies. Baltazar et al. (2014) reported 30% of 160 mg/L and 50% of 320 mg/L copper bioremoval by *Pseudomonas aeruginosa* and *Enterobacter*

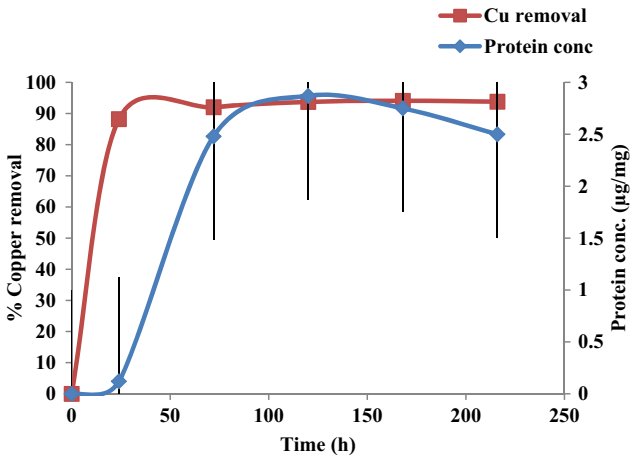

Fig. 4 Graph presenting growth curve and Cu(II) removal % by *Stenotrophomonas acidaminiphila* MYS1 at optimized conditions

cloacae. Similarly, Gosh and Saha (2013) reported 90% of 50 mg/L copper removal by *Stenotrophomonas maltophilia* PD2.

Conclusions

This study reveals that the copper-tolerant rhizosphere bacteria showed considerably a very high tolerance and removal percent of copper(II) sulfate in nutrient broth. The bacterium was Gram-negative bacilli and identified as *Stenotrophomonas acidaminiphila* MYS1. RSM was used for the optimization of copper(II) removal variables. An experimental association between the desired response and independent parameters was obtained from the second-order polynomial equation. ANOVA showed a high R^2 value (0.9941), thus ensuring an agreeable tuning of the model and experimental data. Optimum values obtained were pH 5.0, temperature 32.5 °C and copper(II) concentration 250 mg/L. At the optimized conditions, the bacterium showed maximum growth and Cu(II) removal (235.3 mg/L) after 120 and 168 h of incubation. Higher copper(II) bioremoval at such a higher concentration confirmed the feasibility of *Stenotrophomonas acidaminiphila* MYS1 in bioremediation of copper.

Compliance with ethical standards

The present article does not include any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no challenging interests.

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