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Molecular Characterization of Copper Resistance Bacterial Strains and its Optimization Using Statistical Methods

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Abstract

Heavy metal contamination is one of the key environmental complications. Due to some disadvantages of conventional methods, the use of active organisms is becoming more popular technique to remove it. In the present study, primarily 35 bacterial strains were discovered in metal containing media. After being identified resistance power to different copper concentrations (100–1000 mg/l), JRHM33 had the highest level of resistance up to 1000 mg/l of copper. Using the 16S rRNA sequencing, bacterial strain JRHM33 was discovered and revealed 99% similarity to *pseudomonas aeruginosa*. Sequencing and bioinformatics study using conserved domain analysis supported the *laccase* gene is present in JRHM33 and has classification as a member of the multicopper oxidase superfamily, which has reduction capacity of metal ions. Analysis of phenotype microarray (PM) technology provides an insight into the metabolic profiling of microbial cell into *Pseudomonas aeruginosa* JRHM33. Furthermore, Using the central composite design of response surface methodology (CCD-RSM), the successive optimization of the process parameters was attempted for the maximum reduction of the copper. Maximum 68.71% Cu reduction was achieved at 6.71 pH, 90.61 min of incubation time, 5 ml of inoculum size, and 113 rpm of agitation. The generated model has R^2 value of 0.9834, indicating that the ANOVA gave it a very significant result. The findings of the validation experiment showed a remarkable similarity between the projected and experimental results. It is determined that bacterial strains isolated from metal-contaminated effluent employ their natural capacity to change toxic heavy metals into less harmful or nontoxic forms.

Keywords: Copper (Cu), *Pseudomonas aeruginosa*, Optimization, Heavy Metal Tolerance, PBD, CCD-RSM, Laccase

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INTRODUCTION

Numerous kinds of wastewater have been produced as a result of the population boom, urbanization, and industrialization.[1] Because wastewater contains a wide range of organic and inorganic contaminants, it can contaminate soil, groundwater, and surfaces.[2] One of the foremost difficulties globally is the pollution of heavy metals.[3,4] Metal ion concentrations in some industrial wastes are typically far higher than allowable limits. One such business that produces a lot of waste water with a high concentration of metals is electroplating. Cd, lead, mercury, Cr, Ni, Cu, Zn, Co, and so on are some of these metals. Because heavy metals are harmful even at extremely low quantities, they can harm the environment and subsequently cause a variety of disorders in humans.[5] Food, air, and water can all allow them to enter the human body, where they can eventually bioaccumulate.[6] Heavy metals in wastewater tend to accumulate because they are not soluble in it and cannot be broken down. Since many companies produce a great deal of hazardous pollution issues for the environment, removing dangerous heavy metals from wastewater is regarded as among the most essential topics of water treatment.[7]

Dissimilar organic pollutants, there is no complete chemical or biological breakdown of heavy metals. They can therefore only be changed into less dangerous or absorbed forms. One novel technique for treating contaminated wastewater is bioremediation. In bioremediation procedures, tolerance to contaminants, particularly heavy metals, and microbial resistance are crucial. Numerous studies have revealed that certain bacteria are capable of withstand heavy metals by either removing them from the environment or converting them into forms that are entirely safe or less hazardous, which they then use in their metabolic processes to continue growing.[8] The study's objective was to eliminate copper ions from a water-based mixture using microorganisms. We conducted a central composite design examination to identify important factors that influence the elimination of heavy metal from aqueous solution by bacterial isolates and comprehend their impact on the process.[9] We also isolated and identified heavy metal tolerant bacteria, determined the tolerance power of heavy metal tolerating microbes, and selected highly potential bacterial strains for bioremediation of heavy metal. A CCD, which provides a scientific model that illustrates the impact of every variable, was utilized to investigate the impacts of several variables on heavy metal removal, including pH, incubation period, inoculum size, agitation, and primary content of Cu^{+2}.[10]

The variables participate in a testing are changing concurrently in the statistical design. The two most significant benefits are that the interaction of two or more variables may also be determined, in addition to evaluating the impacts of distinct parameters and how significant they are in a certain process.[11] Because of this, it uses low-tech, low-cost treatments that are widely accepted by the public and may frequently be completed on-site.[12]

MATERIALS AND METHODS

Sample collection

Water samples and sludge were taken from wastewater discharge locations of three distinct electroplating industries that were contaminated with heavy metals. The samples were taken from the same electroplating industry, Makarpura GIDC, located in Baroda, Gujarat, India. The waste product of this sector is copper (Cu), which is used to metal plate automobile gasoline pipes. Following standard procedures, wastewater and sludge samples were collected and aseptically transferred to the lab in screw-cap sampling vials at 4°C.[13]

Isolation of bacteria

Using a nutrient agar medium (HiMedia), the heavy metal resistance bacterial strains were isolated by a serial dilution procedure. In 10 milliliter Erlenmeyer flasks with distilled water, one gram of sludge was added, and the mixture was stirred for 20 minutes at 200 rpm. Each suspension was diluted in steps of 10^{-1} to 10^{-6}. To separate the organisms from diluted samples, the Spread plate technique was used. Using a glass spreader, 0.1 ml was pipetted onto nutrient agar plates, which were then incubated for 24 hours at 37°C. The nutrient broth (100 ml) was directly inoculated with 10 ml of the polluted water from the wastewater

sample, and it was then incubated for 24 hours at 150 rpm and 37°C in an incubator shaker (REMI). Utilizing a glass spreader, 0.1 ml of nutrient broth containing the contaminated water sample was applied to the nutrient agar plates and incubated for 24 hours at 37°C in an incubator.[14] The strain with the resistance to a copper heavy metal was exposed to further examination.

Primary screening for heavy metal-resistant bacteria

To do a preliminary screening for bacterial resistance to heavy metals, each bacterial isolate was distributed independently onto nutrient agar plates with a medium containing 100 parts per million of $CuSo_4$. After preparing copper-adjusted nutritional agar medium in distilled water, bringing the pH to 7.5, and incubating the plates at 37°C for 24 hours, bacterial colony development was observed.

Secondary screening for selection of most efficient heavy metal resistant bacteria

To find the most effectual heavy metal resistance bacterial, secondary screening is done with two strategies; one is all the bacterial isolates were screened for a potential heavy metal tolerance test and the second is to check bacterial growth in the presence of copper heavy metal

Determination of MIC or Heavy metal tolerance test

In these studies on heavy metal removal, the bacterial strains used must be resistant to a specific concentration of heavy metal. The minimum inhibitory concentration test was used to determine whether an identified strain of bacteria was resistant to the heavy metal copper (Cu). Congeevaram *et al.,*[10] where the varying concentrations of metals ranging from 100 ppm to 1000 ppm containing nutrient agar plates were prepared. Stock solutions of metals ($CuSO_4$) were ready in deionized water, decontaminated by filtration, and added to a Nutrient agar medium. All the bacterial strains of 0.1 ml were streaked on above mentioned different concentrations of metal-containing plates, separately. Then all the plates were placed in an incubator and incubated for 48 hours at 37°C. Microbial development on a nutrient agar medium free of heavy metal contaminants served as a positive control.[13]

Growth in the existence of Cu metal ion

Bacterial growth in the existence of Cu was tested rendering to Deng and Wang[15] with changes. Copper was added to a Nutrient broth medium after it had been inoculated with a culture that had been cultured overnight in the medium at 37°C. The following single ingredient concentrations were employed for each mixture: 0 ppm (control), 25 ppm, 100 ppm, 500 ppm, and 1000 ppm. The cultures were incubated at 37°C for 48 hours, and the growth was quantified as an increase in optical density at 600 nm (OD600) using a spectrophotometer (Shimadzu UV-1800).

Characterization of selected isolate

Morphological and biochemical characterization

The form, elevation, edge, surface, and size of the most productive isolates were used to determine the colony morphology, whereas Gram staining was used for microscopic identification. In order to determine the integrity of isolate JRHM33, different biochemical tests were performed, including an oxidase test, a catalase test, an indole production test, a Voges Proskauer test, a Urease test, and a citrate test.[16]

Metabolic profiling of selected bacterial isolates

Utilization trends for Biolog carbon substrates inoculations were made in duplicate using Biolog GP2 MicroPlates (Biolog, Inc., Hayward, CA, USA) and incubated at 30-35°C for 24-36 h. After 12, 24, 36, and 48 hours, the optical density in each well at 590 nm that results from tetrazolium violet reduction was measured using a Biolog Microplate Reader and MicroLog software (Release version 4.0).[17] When a microplate well produced an O.D. 0.59 greater than 0.4 on at least two reading points, it was considered positive.

Molecular identification and phylogenetic analysis of selected bacterial isolates

Using universal forward and reverse primers (27F - 52 AGAGTTTGATCMTGGCTCAG 32 and 1492R - 52 TACGGYTACCTTGTTACGACTT 32) in PCR (Polymerase Chain Reaction) the molecular identification of JRHM33 was accomplished.[18] A

similarity search was done using the online BLAST tool of NCBI for the identification of isolates. Sequenced data has been submitted to GenBank for accession numbering. Additionally, NCBI was used to construct a neighbor-joining tree and conduct an evolutionary analysis.

Molecular characterization for laccase enzyme

Several possible laccase gene sequences were found on NCBI by using bioinformatics. With the use of In silico rapid PCR software, the gene-specific primer was created.[17] It was also used to identify the laccase gene found in the bacterial DNA using PCR. The presence of a conserved domain for laccase enzyme was identified using NCBI conserved domain search.

Important variables were screen out for Cu removal using Placket- Burman design

Plackett and Burman[19] design was used to eliminate factors that could influence the maximum removal of heavy metal ions using JRHM33 isolates. Important process variables were chosen in accordance with the literature review, and then, for each assay, the copper removal was calculated in terms of percentage (%). Twelve runs of an experimental design with six particular variables and five dummy variables, each with two levels, were used to select significant variables as depicted in Table 1. Variables with a 95% confidence level or higher were regarded as a relevant factor for the greatest Cu removal by JRHM33, and they were further selected for RSM (CCD) level optimization using Design-Expert (version 13.0.1.0) software.

Table 1. Placket-Burman experimental design was used to remove Cu utilizing independent two-level variables

Factor	Name	Unit	-1	1
1	pH		4	8
2	Incubation Time	Hours	24	120
3	Initial concentration	PPM	10	100
4	Temperature	°C	25	45
5	Inoculum size	ml	01	05
6	Agitation	RPM	0	150
7	G- Dummy: 1			
8	H- Dummy: 2			
9	I- Dummy: 3			
10	J- Dummy: 4			
11	K- Dummy:5			

Optimization of particular variables for maximizing Copper (Cu) removal by JRHM33 isolates using central composite design of response surface methodology (CCD-RSM)

Using the CCD-RSM design, shake flask trials were conducted, and four important variables were investigated to optimize Cu removal. Thirty run experiment design was conducted with four factors at five levels (Table 2). Wherein, highest (+2), high (+1), medium (0), low (-1), and lowest (-2) were the coded levels. The middle point is made again six times to consider only errors and the lack of fit of the offered model. The following second-order polynomial equation explains the model's behavior:

$$Y= \beta_0+\sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$

where Y is the expected response (percentage of Cu removed), The linear, quadratic, and interaction coefficients are bi, bii, and bij, respectively, while the model constant is denoted by β_0. The system's independent coded variables are shown by Xi and Xj.[20] There was a regression study using the Design-Expert program. The model with the highest F-value, with a p-value less than 0.05 and an insignificant Lack of Fit test, was chosen. Furthermore, a suggested model was evaluated using different fit data metrics such as F-value, R^2-coefficient of determination, SNR-value of precision, and the disparity between adjusted and predicted R^2-values.[21] The appropriateness of the proposed model was also evaluated using different diagnostics tools. Three-factor interaction study employed using surface graphs in three dimensions and counterplots. Finally, optimization was attempted for the maximum heavy metals elimination by optimizing the level of different variables. In the end, authentication trials were conducted to authorize the model's optimum solution.[22]

RESULTS AND DISCUSSION

Isolation and screening bacteria resistance to heavy metal

From industrial sludge, 40 distinct bacterial strains were identified. Using the primary

screening procedure, 35 Cu-resistant bacterial strains were eliminated out of 40 isolates.[23] These 35 isolates were chosen based on their resistance to 100 ppm of Cu and their capacity to withstanding heavy metals, which was assessed by raising the concentration of copper in media with a range of 100 ppm to 1000 ppm.

Additionally, most heavy metal-tolerant bacteria were chosen from the selected 35 isolates using a secondary screening method. In the first phase, heavy metal tolerance was studied by incubating selected isolates in a nutrient agar plate containing copper heavy metal. 23 isolates were tolerated up to 200-300 ppm of Cu, 10 isolates were tolerated up to 600-700 ppm of Cu while 2 isolates JRHM27 and JRHM33 showed growth with 1000 ppm of Cu and JRHM33 also grew on 10,000 ppm Cu (Figure 1a).

The most resilient bacteria were chosen in the second stage by culturing the chosen bacteria in nutrient broth that contained copper heavy metal. Bacterial growth in the presence of Cu in the nutrient broth after 48 hours, JRHM33 gives the highest growth compared to other isolates. So based on growth and tolerance capacity, JRHM33 was selected for further study. Numerous studies (Abou-Shanab, Van Berkum *et al.*[24], Alam and Malik[25]) have documented metal resistance in bacteria that have been isolated from soil pollution and wastewater that contains various heavy metals. Batool *et al.*[26] found that two bacterial isolates were resistant up to 1600 ppm of Cr and Cd.

Morphological and biochemical characterization

Considering the ability to tolerate heavy metals and the current growth in Cu heavy metal, most potential heavy metal removal isolate JRHM33 is used for further study and is found gram-negative, short rod bacteria and give bluish green pigmentation on nutrient agar (Figure 1 a, b and c) and were characterized according to their physical, biochemical, and cultural traits (Table 3).

Table 2. Level of the CCD's experimental variables, both coded and uncoded

Level	Uncoded level	Coded level			
		Inoculum size (Baumler, Ma et al.)	pH Time	Incubation (Hours)	Agitation (RPM)
alpha	-2	0	2	0	0
Low	-1	1	4	24	1
Mid	0	3	6	72	75
High	+1	5	8	120	150
Alpha	+2	7	10	168	225

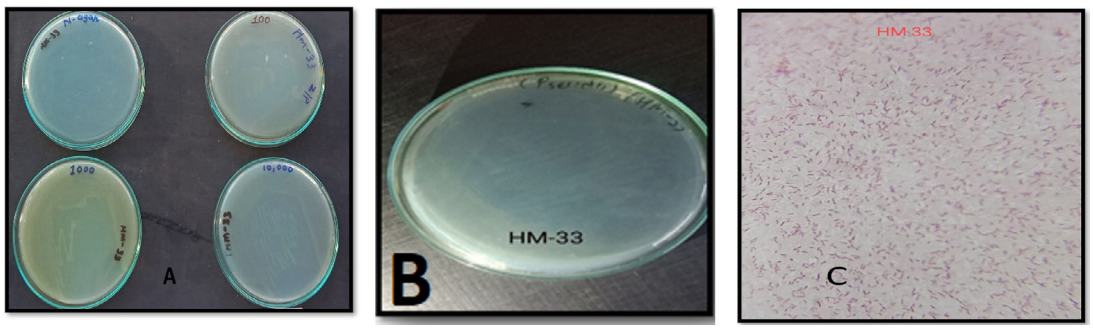

Figure 1. (A) JRHM33 on nutrient agar plate without heavy metal (Cu) and with 100 ppm, 500 ppm & 1000 ppm of heavy metal (Cu) (B) heavy metal Growth of *Pseudomonas aeruginosa* JRHM33 on Cu-containing nutrient agar and (C) Gram staining of JRHM33

Molecular identification and analysis of potential isolates

Molecular identification was done by 16S rRNA sequence identification by HiMedia laboratory, Bombay. The highly similar sequence of the deduced sequence was aligned using BLAST search. A sequence was closely related to the sequence of previously reported bacterial strains, with an average identity of 98%, based on the 16S rRNA sequence analysis by NCBI, the isolates

Table 3. Morphological and biochemical characterization of *Pseudomonas aeruginosa* JRHM33 isolates

Morphological characteristics	Test	Result
	Colony on Nutrient agar	Smooth, Translucent, low convex, and irregular edge
	Gram's reaction	Gram's Negative
	Motility	Motile
	Cell size & shaped	Small, rod
	Capsule staining	Non-capsulated
	Endospore staining	Non-spore forming
	Pigment	Bluish-green on Nutrient agar
Biochemical characterization	Test	Result
	Catalase	+
	Oxidase	+
	Citrate	+
	Coagulase	-
	Gelatin hydrolysis	+
	Indole	-
	Methyl red (MR)	-
	Voges Proskauer (VP)	-
	Urease	-
	Nitrate reduction	+
	TSI	Alkali
	Cetrimide	+
	Fructose	+
	Lactose	-
	Mannitol	+
	Sucrose	-
	Xylose	-

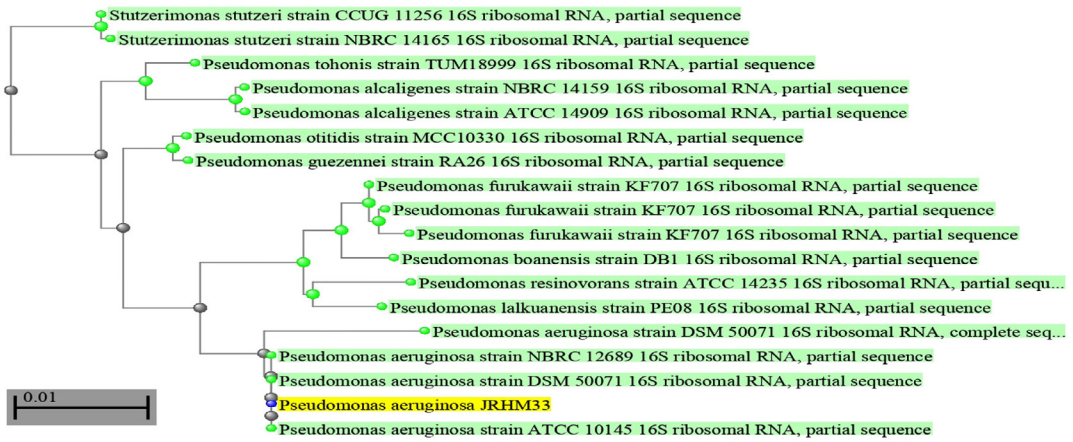

Figure 2. Phylogenetic analysis of *Pseudomonas aeruginosa* JRHM33

were identified as *Pseudomonas aeruginosa* and labeled as *Pseudomonas aeruginosa* JRHM33. The sequence was submitted to the GenBank database and obtained accession number OR105012. The distance tree result revealed the relatedness between the bacterial isolates (Figure 2).[27] Prior research has also used BLAST and NCBI to identify bacteria and fungi by DNA sequencing, allowing for species-level identification of the organisms.[28,29,30] Similar identification found by *Patel et al.*[18]

Metabolic profiling using Phenotypic Microarray (PM) technology

The PM technology enables us to step-by-step examine the unique metabolic pathways of various isolates. Research on the carbohydrates by

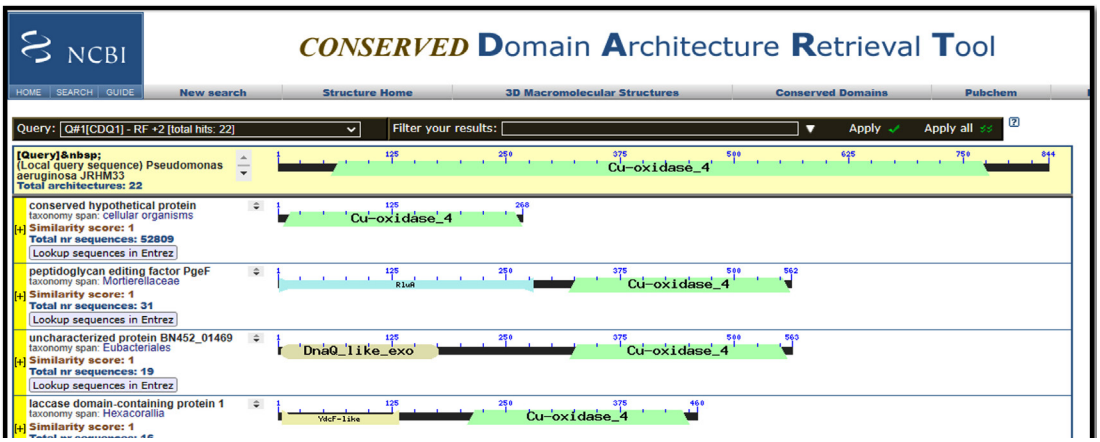

Figure 3. Conserved domain of laccase gene in *Pseudomonas aeruginosa* JRHM33

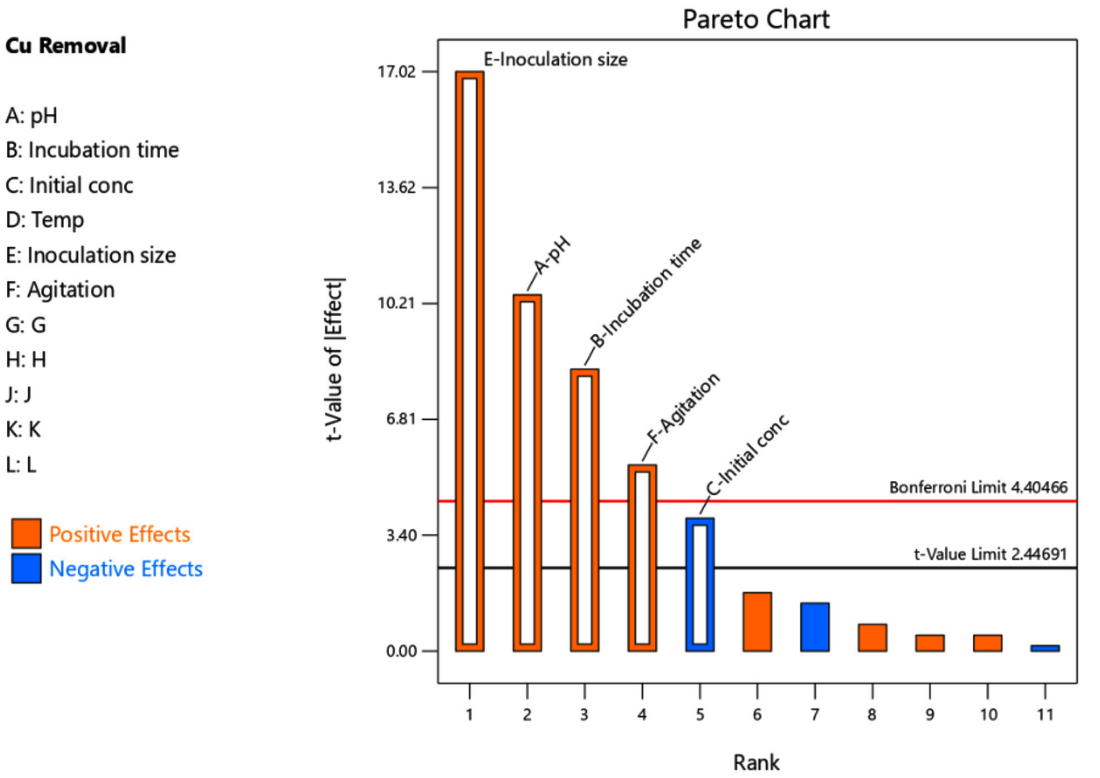

Figure 4. Pareto chart illustrating the significance order for eleven elements

Table 4. Coded independent variables for Cu removal by Placket – Burman experimental design

Run	Factor 1 A: pH	Factor 2 B: Incubation time Hrs	Factor 3 C: Initial conc ppm	Factor 4 D: Temp C	Factor 5 E: Inoculation size ml	Factor 6 F: Agitation rpm	Factor 7 G: G	Factor 8 H: H	Factor 9 J: J	Factor 10 K: K	Factor 11 L: L	Response Cu Removal %
1	-1	-1	-1	1	-1	1	1	-1	1	1	1	38
2	-1	1	1	-1	1	1	1	-1	-1	-1	1	60
3	1	1	-1	1	1	1	-1	-1	-1	1	-1	74
4	1	-1	1	1	-1	1	1	1	-1	-1	-1	45
5	-1	-1	1	-1	1	1	-1	1	1	1	-1	52
6	-1	1	-1	1	1	-1	1	1	1	-1	-1	61
7	1	1	-1	-1	-1	1	-1	1	1	-1	1	60
8	1	1	1	-1	-1	-1	1	-1	1	1	-1	47
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	31
10	1	-1	-1	-1	1	-1	1	1	-1	1	1	60
11	1	-1	1	1	1	-1	-1	-1	1	-1	1	59
12	-1	1	1	1	-1	-1	-1	1	-1	1	1	36

all the distinct bacteria has demonstrated that, in addition to basic carbohydrate metabolism, these bacterial isolates have a specific capacity to utilize complex carbohydrates. The metabolic processes of *Pseudomonas aeruginosa* JRHM33 utilized all of the complex sources like D-salicin, Gentibiose, cellobiose, dextrin, α-D-glucose, D-mannose, D-mannitol, etc. Tohsato *et al.* findings showed that Glyceraldehyde-3-P played a significant role as a molecular switch of the focal metabolic system.[31] Baumler also investigated how different enterobacteria might distinguish between species that had been preserved via the PM technique by using carbon, nitrogen, and sulfur. The data demonstrated a phylogenetic link between the tested organism and enterobacteria but also revealed differences in systems biology and the metabolic networks of several catalysts.[32] The PM technology was greatly helpful in understanding the central metabolic lignocellulose degradation network with a unique metabolic pathway in *Bacillus* sp. NAULH2 in banana pseudostem degradation also.[17]

Characterization of laccase gene

The best temperature for annealing Lac CF and Lac CR primers was found to be between 60.7°C and 64.7°C, according to research on the primer sequence. An exact band of the laccase gene was extracted and purified from the gel. using a specialized column provided by the Thermo Scientific Genjet kit to sequence it. The final sequences for Laccase *Pseudomonas aeruginosa* JRHM33 were 844 bp. The same software's homology search revealed 99% similarity between the *Pseudomonas aeruginosa* genes for copper oxidase, which is correlated with the laccase gene family.

The determined amino acid sequences of the laccase genes from *Pseudomonas aeruginosa* were compared with known characterized laccase of the multicopper oxidase family using the CLUSTAL-W multiple alignment tools. The Conserved domain of the cupredoxin superfamilies was found by ORF-BLAST analysis of the three laccase amino acid sequences. In the *Pseudomonas aeruginosa* JRHM33 isolate, the multicopper polyphenol oxidoreductase laccase domain was found at amino acid positions 59–788. Data study shows that this domain contains a trinuclear

copper binding site with the copper oxidase 4 superfamily (Figure 3). Many researchers found that removing heavy metals from contaminated water and soil is bioremediation using laccase enzymes. The heavy metal ions are changed from their soluble and toxic form to their insoluble and non-toxic form during laccase-mediated oxidation.[33]

Screening of important variables using Plackett-Burman method for Cu removal

For the selection of significant variables for Cu removal, the Plackett–Burman design was used to examine six process variables over 12 experimental runs with five dummy variables. Table 4 shows the experimental design as well as the recorded response (Cu removal). According to Table 5 statistical analysis of the Cu removal data, among the chosen process variables Inoculum size, pH, incubation time and agitation have a positive effect, whereas Initial concentration has a negative effect on the Cu removal. The same result found by Kumar *et al.*[34] is that more ions compete for those binding sites as concentration increases, and more binding locations become available for ion complexation

Table 5. Statistical study of the Plackett – Burman experimental strategy

Source	Co-efficient estimate	F- value	P-value	
Model		104.58	< 0.0001	significant
A-pH	5.58	109.49	< 0.0001	
B-Incubation time	4.42	68.51	0.0002	
C-Initial conc	-2.08	15.24	0.0079	
E-Inoculation size	9.08	289.78	< 0.0001	
F-Agitation	2.92	29.88	0.0016	

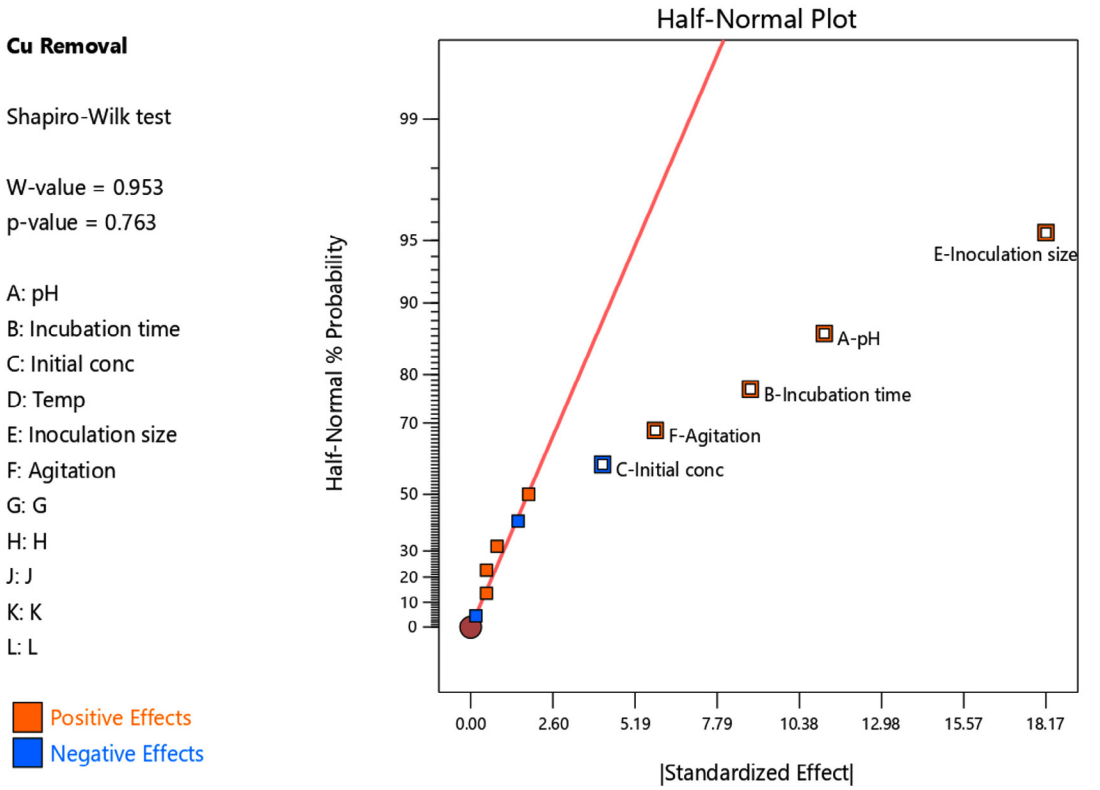

Figure 5. Half-normal plot displays the important effect of the outliers on the Cu removal

at the starting concentration, the percentage clearance is reduced as the initial concentration of metal ions increases. The elements' relative importance is shown by the Pareto chart (Figure 4), where the orange component (dark color bar) indicates a positive influence on Cu elimination and the blue component (light color bar) indicates a negative effect. Inoculum size, pH, incubation time, and agitation had a much stronger influence on Cu removal than the other parameters, as seen by the half-normal plot of effect (Figure 5). All four variables are outliers that sit beneath or to the right of the straightness; however, because they are typically distributed along a straight path, all other variables have less of an impact on the removal of Cu. The ANOVA table's model F-value of 94.81 indicated that the model is significant. Due to noise, there is only a 0.01% coincidence that an F-value this large will happen.[35] Inoculum size, pH, incubation time, and agitation are all significant model terms with p-values less than 0.05. (95 percent confidence). According to the model, these four process parameters have a substantial impact on Cu removal using JRHM33 isolates (Table 5).

Optimization of selected variables for maximizing Cu removal using CCD-RSM

From the Plackett-Burman design, process factors with a substantial effect on Cu removal were chosen, and the CCD-RSM was utilized to evaluate their combined influence and

Table 6. Four coded factors with predicted and experimental responses make up the CCD design layout

Run	Factor-1 A: Inoculum size min	Factor-2 B: pH N	Factor-3 C: Incubation N	Factor-4 D: Agitation time %	Experimental Answer Copper reduction %	Predicted Answer %
1	5	8	120	0	42	41.75
2	3	2	72	75	10	11.17
3	5	8	24	150	44	45.08
4	7	6	72	75	47	45.00
5	1	4	120	0	23	20.42
6	3	6	72	75	62	67.50
7	3	6	168	75	41	45.67
8	3	10	72	75	30	31.83
9	5	4	24	0	25	26.75
10	5	4	24	150	30	29.75
11	3	6	72	75	67	67.50
12	5	4	120	0	26	25.92
13	3	6	72	0	31	30.50
14	3	6	72	75	67	67.50
15	1	4	24	150	19	17.75
16	1	8	120	150	44	40.75
17	3	6	72	225	45	48.50
18	1	4	120	150	36	33.42
19	3	6	72	75	71	67.50
20	5	8	120	150	63	61.25
21	5	8	24	0	39	40.08
22	0	6	72	75	12	17.00
23	1	8	120	0	27	25.75
24	3	6	72	75	69	67.50
25	3	6	72	75	69	67.50
26	1	8	24	0	23	22.08
27	5	4	120	150	44	43.42
28	3	6	00	75	30	28.33
29	1	4	24	0	19	19.25
30	1	8	24	150	24	22.58

determine the ideal proportion of each variable. Thirty-run experimentation was conducted using 5 levels (rotatable) of each of the 4 variables with 6 replicates at the centre point (Table 6).

Based on the model statistics data, a quadratic model was suggested by the software. Wherein, the F-value was 64.47. There is just a 0.01 percent chance of a better F-value due to noise. The determination coefficient shows how fine the

Table 7. ANOVA for quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	9457.13	14	675.51	64.47	< 0.0001	significant
A-Inoculum size	1176.00	1	1176.00	112.24	< 0.0001	
B-pH	640.67	1	640.67	61.15	< 0.0001	
C-Incubation time	450.67	1	450.67	43.01	< 0.0001	
D-Agitation	486.00	1	486.00	46.38	< 0.0001	
AB	110.25	1	110.25	10.52	0.0055	
AC	4.00	1	4.00	0.3818	0.5459	
AD	20.25	1	20.25	1.93	0.1848	
BC	6.25	1	6.25	0.5965	0.4519	
BD	4.00	1	4.00	0.3818	0.5459	
CD	210.25	1	210.25	20.07	0.0004	
A^2	2283.86	1	2283.86	217.97	< 0.0001	
B^2	3627.43	1	3627.43	346.20	< 0.0001	
C^2	1594.71	1	1594.71	152.20	< 0.0001	
D^2	1344.00	1	1344.00	128.27	< 0.0001	
Residual	157.17	15	10.48			
Lack of Fit	109.67	10	10.97	1.15	0.4641	not significant
Pure Error	47.50	5	9.50			
Cor Total	9614.30	29				
R^2=0.9837	PredR^2 = 0.9272		AdjR^2 = 0.9684		AdeqPrec = 24.61	

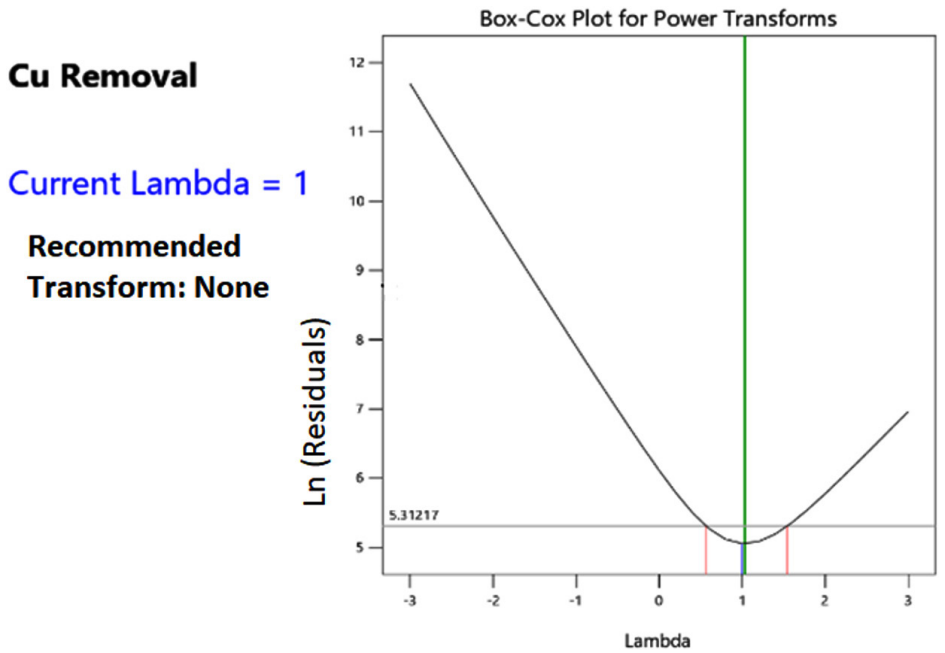

Figure 6. Box-cox plot

model fits the statistics, which is nearer to 1 (R^2 = 0.9837), indicating a stronger relationship between actual and expected responses. Similarly, there is less than a 0.2% difference between the predicted R^2 (0.9272) and the adjusted R^2 (0.9684) which is highly desirable for a good model. Secondly, the proposed model has a 24.61 signal-to-noise ratio, suggesting that it is precise enough for the study. According to the F-value of 0.4641, Compared to the pure error, the lack of fit is negligible (a non-significant lack of fit is desirable for the model). The appropriateness of the suggested quadratic model for enhanced Cu removal is supported by all of these fit statistical results (Table 7). In addition, the appropriateness of the suggested paradigm was tested using Design-Expert software's diagnostics and influence tools. There was a straight line in the normal probability plot, indicating that the residuals were normally distributed. To check for constant variance, a plot of the ratio of residuals to expected responses was examined, which revealed a random scatter plot, indicating that variance

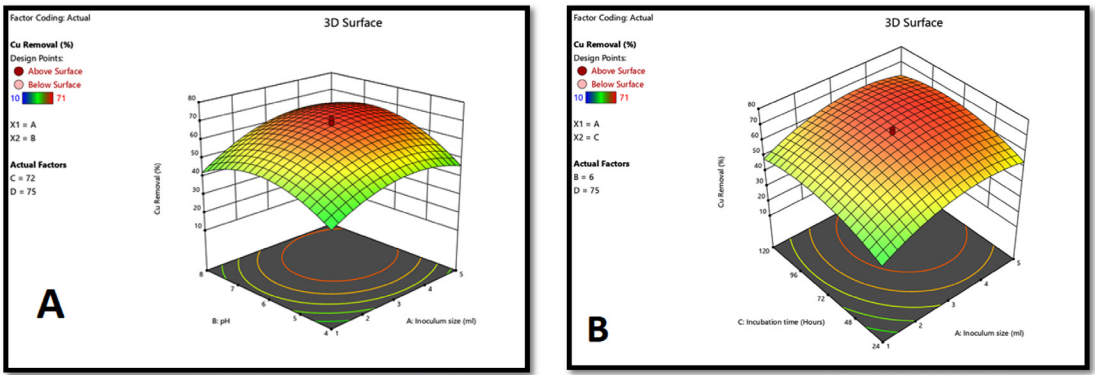

Figure 7. The action of Cu removal as a function of two-parameter interaction is displayed using three-dimensional surface plots

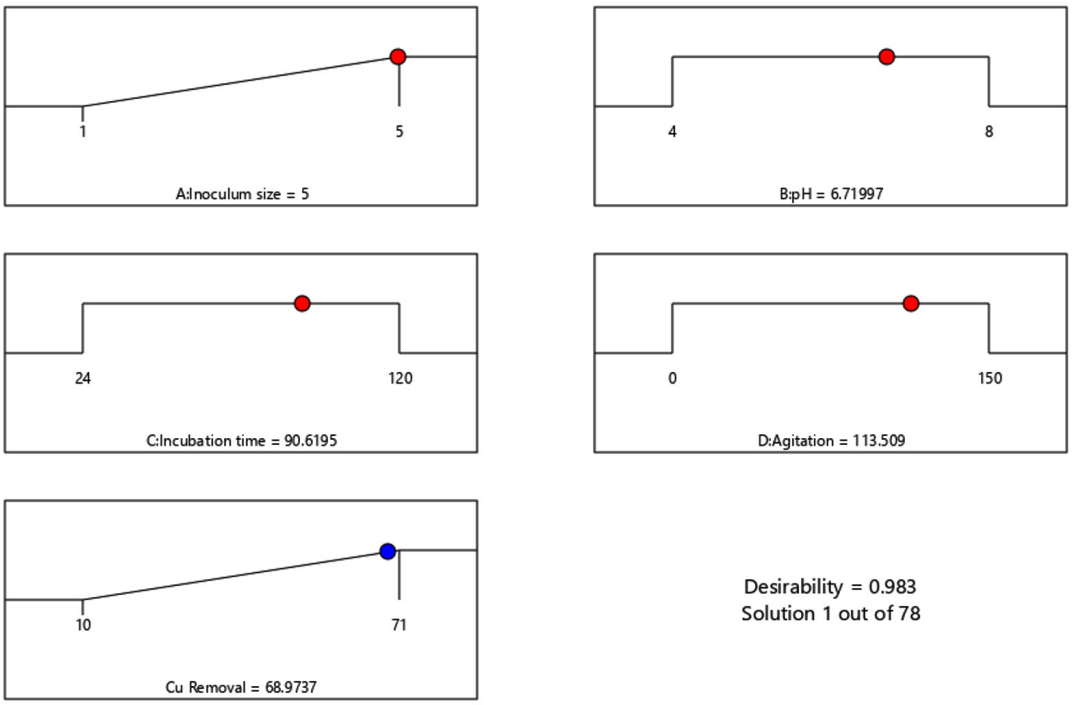

Figure 8. The software's recommended optimal solution is shown on the desirability plot

did not expand from lower to higher probable scores. The residual versus run plot revealed no discernible trend. Using a box-cox plot, the required power transformation was found. The graphic shows a lambda value range of 0.56 to 1.54 at the 95 percent confidence level, with the optimum lambda at 1.03 (Figure 6). Given that the recommended model's lambda value is within the given range and is quite close to the optimum lambda value, there was no suggestion for power transformation. All of these diagnostics data points suggest the model's suitability.[23]

The second-order polynomial equation was used to accomplish it with each component in this model to predict the response for given values of each component. The relative influence of the component may be determined by comparing the factor coefficients:

Cu removal= +67.50+7.00 A-5.17B+4.33 C+4.50 D+ 2.63 AB-0.500 AC+1.13 AD+0.6250 BC+0.500 BD+3.63 CD-9.12 A^2-11.50 B^2-7.62 C^2 -7.00 D^2

To investigate how interactions affect the identified control factors, a three-dimensional surface plot and a two-dimensional contour plot were produced utilizing the response plotted against another two numeric components while the other parameters were held constant at their mid-values (Figure 7). The result shows that the curvature effect was obtained and that revealed the interaction effect of selected independent variables for the maximum removal of Cu heavy metal. Maximum Cu removal was noticed at around the midpoint value of inoculum size, pH, incubation time, and agitation. The graph shows that Cu removal increases initially and reaches maximum at mid-point value and then it decreases (Figure 7a & 7b).

According to the Design-Expert software, the optimal solution for maximum Cu elimination (68.97%) is shown in Figure 8. Validation trials were supported in triplicates using shake flask studies as per the optimized solution wherein, 5 ml of 10^8/ml bacterial cell inoculum size, 6.7 pH, 113 rpm agitation speed, and 90.61 min incubation time. The results of the validation experiment were compared to the predicted response data, revealing that the mean (71 percent) elimination of Cu was within the 95 percent prediction interval of 63.99 percent to 73.95 percent. Abou *et al.* [24] has achieved Cu removal up to 83.3% from industrial wastewater using *B. mojavensis* C6, similarly; around 85% Cr removal efficiency was found by Chang *et al.* Cheng *et al*[36] by bacterial isolates using RSM BBD. Another study by *Tarangini et al.*[37] found 60.5% Cu removal by *pseudomonas aeruginosa*.

Based on RSM using CCD found that a combination of Inoculum size, pH, agitation, and Incubation time have significant impact on the removal of Cu ions.[34,38]

One important factor in the elimination of Cu is time. The ability of *P. aeruginosa* to remove various additional metal ions was time-dependent. Removal gets better with passing time, and metal ions in certain situations reveal that they work best at a particular moment in time. As a result, optimization is necessary because the impact of time varies depending on the metal and the bacterium. For *P. aeruginosa* JRHM33, maximum Cu removal occurs at 90.61 minutes of incubation time; however, if contact time exceeds 90.61, Cu removal declines. Similar results were observed by Deepali[12] where maximum Cu removal (80%) was achieved at 96 hours incubation time using *P. putida* in optimum conditions. According to Bandela *et al.*[39] reported higher heavy metal removal at 72 hours of incubation time. Furthermore, it was found that the incubation period has a significant effect on the process of metal removal from industrial effluent.

One of the key elements influencing both the metabolic activity of bacteria and the chemical behaviour of metal ions in solution is pH. According to the current research, Cu elimination was higher at pH 6.71 a noticeable drop in Cu removal as the medium's pH changes. The bioremediation process may vary depending on pH variations, which may be caused by variations in *Pseudomonas* ligand protonation on the cell surface. The degree of ligand protonation that includes metal binding can be strongly influenced by variations in the external pH of the medium.[40] Similar results were observed by Sarin *et al.*[41] At pH 6.8, it was discovered that *Pseudomonas fluorescence* had a great effectiveness in removing Cd. The ideal pH for bacterial isolates in heavy metal bioremediation was found to be 7.0 in a study, which was higher than the current results.[10] The metal ion's solubility in the medium was modified by pH throughout the bioremediation process, which is mostly

dependent on the functional groups present on the bacterial cells' surfaces.[42] Functional groups including hydroxyl, carboxyl, phosphate, and amino play a significant role in the uptake of heavy metals in microorganisms, and their behaviour varies depending on the pH level.[43] At pH 7, Ahokkumar *et al.*[44] found that *Sphaerotilus natansin* removed 48%, 75%, and 52% of Cu, Pb, and Cr, respectively.

Bioremoval of Cr (Ahmaruzzaman and Gupta[45]) by *P. aeruginosa* was greatly influenced by inoculum size. The wastewater's Cu content decreased by 68.97% in just ninety hours, despite the inoculum's 5 milliliters (10^8 cells per milliliter). Additionally, as the inoculum size was reduced, the time for Cu reduction increase, and at the optimal inoculum size, the time decreased. According to reports from Mackey and Kerridge[46] and Robinson *et al.*,[47] the inoculum size had a minor impact on the eradication of Cr.

Another important factor for Cu removal is the agitation and fluid medium oxygen concentration could be greatly affected by it,[48] where the growth of microorganisms can be influenced by the dissolved oxygen. Figure 7 illustrates the impact of agitation on *P. aeruginosa's* bio-reduction of Cu. Agitation at 113 rpm resulted in a 68.97% reduction in Cu content. A decrease in Cu was discovered in static conditions, however, it was not as noticeable as under shaking conditions. These findings suggested that *P. aeruginosa* might be used in both shaking and static circumstances to decrease Cu in the wastewater. However, the rate of Cu reduction under static conditions was lower than that under shaking conditions. As agitation speed increases, copper is removed more efficiently. However, the removal standing of Cu was best after reaching an optimum speed at 113 rpm. Due to Cu high solubility in water, copper residues or soil contaminated by Cu can easily be dissolved into liquid. Therefore, *P. aeruginosa* has a large deal of potential to remove Cu pollution.[10,49] Tarangini & Satpathy[50] found that agitation time is a crucial factor in establishing Cr biosorption by the resistance organisms of *Bacillus subtilis* and *Pseudomonas aeruginosa*.

CONCLUSION

Pseudomonas aeruginosa JRHM33, the most promising bacterial isolate found thus far, has been shown to grow efficiently, tolerating up to 10,000 parts per million of copper, and to successfully remove copper from an aqueous solution. *Pseudomonas aeruginosa* was identified using conventional microbiological techniques such as 16S rRNA sequencing, and its metabolic system was validated by Biolog analysis. Additionally, a bioinformatics study revealed that the bacterial laccase gene has a multicopper oxidase superfamily conserved domain, providing additional proof that the gene has a laccase conserved domain, which helps to transform more toxic to less toxic form of copper heavy metal. The PM metabolic investigation demonstrated that bacterial isolates had a significant capacity to consume most of the complex carbohydrates. The optimization of various factors affecting bacterial growth using a statistical technique, PBD, and CCD model seems to be a helpful resource for forecasting and comprehending the way independent variables interact. like pH, Inoculum size, Incubation time, agitation, temperature, and Initial concentration of metal ion for the maximal Cu removal. This model was used to identify the ideal conditions: pH 6.71, 90.61 min of incubation time, 5 ml of inoculum size, and agitation 113 rpm. In these situations, the effectiveness of the removal of copper was achieved by 68.97%. The current study has demonstrated that *Pseudomonas aeruginosa* JRHM33 is highly efficient at removing Cu from electroplating effluents. As a result, it may be used to treat industrial effluents containing Cu before discharging it into bodies of water, which could spur the development of wastewater remediation technologies that are more effective, economical, and ecologically friendly.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

REFERENCES

1. Moghaddam MH, Nabizadeh R, Dehghani MH, Akbarpour B, Azari A, Yousefi M. Performance investigation of Zeolitic Imidazolate framework-8 (ZIF-8) in the removal of trichloroethylene from aqueous solutions. *Microchem J.* 2019;150:104185. doi: 10.1016/j.microc.2019.104185
2. Dehghani MH, Nikfar E, Zarei A, Esfahani NM. The effects of US/H2O2 processes on bisphenol-A toxicity in aqueous solutions using Daphnia magna. *Desalination and Water Treatment.* 2017;68:183-189. doi: 10.5004/dwt.2017.20415
3. Czikkely M, Neubauer E, Fekete I, Ymeri P, Fogarassy. Review of heavy metal adsorption processes by several organic matters from wastewaters. *Water.* 2018;10(10):1377. doi: 10.3390/w10101377
4. Shamsollahi HR, Alimohammadi M, Momeni S, et al. Assessment of the health risk induced by accumulated heavy metals from anaerobic digestion of biological sludge of the lettuce. *Biol Trace Elem Res.* 2019;188(2):514-520. doi: 10.1007/s12011-018-1422-y
5. Ihsanullah, Abbas A, Al-Amer AM, et al. Heavy metal removal from aqueous solution by advanced carbon nanotubes: critical review of adsorption applications. *Separation and Purification Technology.* 2016;157:141-161. doi: 10.1016/j.seppur.2015.11.039
6. Nghiem LD, Mornane P, Potter ID, Perera JM, Cattrall RW, Kolev SD. Extraction and transport of metal ions and small organic compounds using polymer inclusion membranes (PIMs). *J Membr Sci.* 2006;281(1-2):7-41. doi: 10.1016/j.memsci.2006.03.035
7. Harikumar PP, Megha T. Treatment of heavy metals from water by electro-phytoremediation technique. *J Ecol Eng.* 2017;18(5):18-26. doi: 10.12911/22998993/76208
8. Demim S, Drouiche N, Aouabed A, Benayad T, Couderchet M, Semsari S. Study of heavy metal removal from heavy metal mixture using the CCD method. *J Ind Eng Chem.* 2014;20(2):512-520. doi: 10.1016/j.jiec.2013.05.010
9. Garba ZN, Bello I, Galadima A, Lawal AY. Optimization of adsorption conditions using central composite design for the removal of copper (II) and lead (II) by defatted papaya seed. *Karbala International Journal of Modern Science.* 2016;2(1):20-28. doi: 10.1016/j.kijoms.2015.12.002
10. Congeevaram S, Dhanarani S, Park J, Dexilin M, Thamaraiselvi K. Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. *J Hazard Mater.* 2007;146(1-2):270-277. doi: 10.1016/j.jhazmat.2006.12.017
11. Su C, Jiang L, Zhang W. A review on heavy metal contamination in the soil worldwide:Situation, impact and remediation techniques. *Environmental Skeptics and Critics*. 2014;3(2):24.
12. Deepali A. Bioremediation of Chromium (VI) from textile industry's effluent and contaminated soil using *Pseudomonas putida*. *Iranica Journal of Energy and Environment.* 2011;2(1):24-31.
13. Choinska-Pulit A, Sobolczyk-Bednarek J, Laba W. Optimization of copper, lead and cadmium biosorption onto newly isolated bacterium using a Box-Behnken design. *Ecotoxicol Environ Saf.* 2018;149:275-283. doi: 10.1016/j.ecoenv.2017.12.008
14. Cowan ST. Cowan and Steel's manual for the identification of medical bacteria, Cambridge university press.1993.
15. Deng X, Wang P. Isolation of marine bacteria highly resistant to mercury and their bioaccumulation process. *Bioresour Technol.* 2012;121:342-347. doi: 10.1016/j.biortech.2012.07.017
16. Cicatelli A, Guarino F, Enrico B, Castiglione S. Genetic and biochemical characterization of rhizobacterial strains and their potential use in combination with chelants for assisted phytoremediation. *Environ Sci Poll Res.* 2017;24(9):8866-8878. doi: 10.1007/s11356-016-7982-5
17. Patel H, Khimani M, Vekariya R, Mahatma L, et al."A sustainable production of lignocellulolytic enzymes and value added metabolites from banana pseudostem waste by Bacillus wakoensis NAULH-4." *Process Biochemistry,* 2021;105:42-49. doi: 10.1016/j.procbio.2021.03.021
18. Patel BY, Patel HK. Current approaches toward the removal of methylene blue dye from synthetic textile effluent using bacterial treated agricultural waste absorbent through statistical design. *Heliyon*. 2023;9(9):19857. doi: 10.1016/j.heliyon.2023.e19857
19. Plackett RL, Burman JP. The design of optimum multifactorial experiments. *Biometrika*. 1946;33(4):305-325. doi: 10.1093/biomet/33.4.305
20. Baskar G, Renganathan S. Optimization of L asparaginase production by *Aspergillus terreus* MTCC 1782 using response surface methodology and artificial neural network linked genetic algorithm. *Asia Pacific J Chem Eng.* 2012;7(2):212-220. doi: 10.1002/apj.520
21. Montgomery DC. Design and analysis of experiments, John wiley & sons. 2017.
22. Marshall WE, Champagne ET, Evans WJ. Use of rice milling byproducts (hulls & bran) to remove metal ions from aqueous solution. *J Environ Sci Health A Tox Hazard Subst Environ Eng.* 1993;28(9):1977-1992. doi: 10.1080/10934529309375990
23. Chaudhary P, Chhokar V, Choudhary P, Kumar A, Beniwal V. Optimization of chromium and tannic acid bioremediation by *Aspergillus niveus* using Plackett-Burman design and response surface methodology.

AMB Express. 2017;7(1):1-12. doi: 10.1186/s13568-017-0504-0

24. Abou-Shanab R, van Berkum P, Angle JS. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. *Chemosphere.* 2007;68(2): 360-367. doi: 10.1016/j.chemosphere.2006.12.051
25. Alam MZ, Malik A. Chromate resistance, transport and bioreduction by *Exiguobacterium* sp. ZM 2 isolated from agricultural soil irrigated with tannery effluent. *J Basic Microbiol.* 2008;48(5):416-420. doi: 10.1002/jobm.200800046
26. Kalsoom, Batool A, Din G, et al. Isolation and screening of chromium resistant bacteria from industrial waste for bioremediation purposes. *Braz J Biol.* 2021;83:e242536. doi: 10.1590/1519-6984.242536
27. Smily JRMB, Sumithra PA. Optimization of chromium biosorption by fungal adsorbent, *Trichoderma* sp. BSCR02 and its desorption studies. *HAYATI J Biosci.* 2017;24(2):65-71. doi: 10.1016/j.hjb.2017.08.005
28. Sharma S, Malaviya P. Bioremediation of tannery wastewater by chromium resistant novel fungal consortium. *Ecol Eng.* 2016;91:419-425. doi: 10.1016/j.ecoleng.2016.03.005
29. Lotlikar NP, Damare SR , Meena RM, Linsy P, Mascarenhas B. Potential of marine-derived fungi to remove hexavalent chromium pollutant from culture broth. *Indian J Microbiol.* 2018;58:182-192. doi: 10.1007/s12088-018-0719-z
30. Kumar V, Dwivedi S. Hexavalent chromium reduction ability and bioremediation potential of *Aspergillus flavus* CR500 isolated from electroplating wastewater. *Chemosphere.* 2019;237:124567. doi: 10.1016/j.chemosphere.2019.124567
31. Tohsato Y, Mori H. Phenotype profiling of single gene deletion mutants of *E. coli* using Biolog technology. *Genome Informatics.* 2008;21:42-52. doi: 10.1142/9781848163324_0004
32. Baumler DJ, Ma B, Reed JL, Perna NT. Inferring ancient metabolism using ancestral core metabolic models of enterobacteria. *BMC Syst Biol.* 2013;7:46. doi: 10.1186/1752-0509-7-46
33. Alengebawy A, Abdelkhalek ST, Qureshi SR, Wang M-Q. Heavy metals and pesticides toxicity in agricultural soil and plants:Ecological risks and human health implications. *Toxics.* 2021;9(3):42. doi: 10.3390/toxics9030042
34. Kumar R, Singh R, Kumar N, Bishnoi K, Bishnoi NR. Response surface methodology approach for optimization of biosorption process for removal of Cr (VI), Ni (II) and Zn (II) ions by immobilized bacterial biomass sp. *Bacillus brevis*. *Chem Eng J.* 2009;146(3):401-407. doi: 10.1016/j.cej.2008.06.020
35. Dhingani R, Shah G, Joshi BH. Sequential optimization of bioprocess nutritional parameters for maximum l-asparaginase production from *Pseudomonas aeruginosa* BGR1I1. *Biocatalysis and Biotransformation.* 2023;41(3):198-207. doi: 10.1080/10242422.2021.2018420
36. Cheng J, Gao J, Zhang J, et al. Optimization of hexavalent chromium biosorption by *Shewanella putrefaciens* using the Box-Behnken design. *Water, Air, & Soil Pollution.* 2021;232(3):1-14. doi: 10.1007/s11270-020-04947-7
37. Tarangini K. Biosorption of heavy metals using individual and mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*. 2009.
38. Neethu P, Chinnamma M. Natural adsorbents for agricultural waste water treatment. *Int J Adv Eng Manage Sci.* 2017;3(4):239823. doi: 10.24001/ijaems.3.4.15
39. Bandela N, Gmais SA, Mehta T, Kaushik G. Heavy metal removal by isolates from domestic and industrial waste water. *J Mater Environ Sci.* 2016;7(12):4589-4595.
40. Singh V, Chauhan PK, Kanta R, Dhewa T, Kumar V. Isolation and characterization of Pseudomonas resistant to heavy metals contaminants. *Int J Pharm Sci Rev Res.* 2010;3(2):164-167.
41. Sarin C, Sarin S. Removal of cadmium and zinc from soil using immobilized cell of biosurfactant producing bacteria. *Environment Asia.* 2010;3(2):49-53.
42. Palanivel TM, Sivakumar N, Al-Ansari A, Victor R. Bioremediation of copper by active cells of *Pseudomonas stutzeri* LA3 isolated from an abandoned copper mine soil. *J Environ Manage*. 2020;253:109706. doi: 10.1016/j.jenvman.2019.109706
43. Chergui A, Bakhti MZ, Chahboub A, Haddoum S, Selatnia A, Junter GA. Simultaneous biosorption of Cu^{2+}, Zn^{2+} and Cr^{6+} from aqueous solution by Streptomyces rimosus biomass. *Desalination.* 2007;206(1-3):179-184. doi: 10.1016/j.desal.2006.03.566
44. Ashokkumar P, Loashini VM, Bhavya V. Effect of pH, Temperature and biomass on biosorption of heavy metals by Sphaerotilus natans. *Int J Microbiol Mycol.* 2017;6(1):32-38.
45. Ahmaruzzaman M, Gupta VK. Rice husk an its ash as low-cost adsorbents in water and wastewater treatment. *Ind Eng Chem Res.* 2011;50(24):13589-13613. doi: 10.1021/ie201477c
46. Mackey B, Kerridge A. The effect of incubation temperature and inoculum size on growth of salmonellae in minced beef. *Int J Food Microbiol.* 1988;6(1):57-65. doi: 10.1016/0168-1605(88)90085-2
47. Robinson TP, Zahoor A, Muneer B, Sasnain S. The effect of inoculum size on the lag phase of Listeria monocytogenes. *Int J Food Microbiol.* 2001;70(1-2):163-173. doi: 10.1016/S0168-1605(01)00541-4
48. Hay JXW, Wu TY, Teh CY, Jahim JM. Optimized growth of Rhodobacter sphaeroides OU 001 using response surface methodology (RSM). 2012;71(2):149-154.
49. Rehman A, Zahoor A, Muneer B, Hasnain S. Chromium tolerance and reduction potential of a Bacillus sp. ev3 isolated from metal contaminated wastewater. *Bull Environ Contam Toxicol.* 2008;81(1):25-29. doi: 10.1007/s00128-008-9442-5
50. Tarangini K, Kumar A, Satpathy GR, Sangal VK. Statistical optimization of process parameters for Cr (VI) biosorption onto mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*. *Clean-Soil, Air, Water.* 2009;37(4-5):319-327. doi: 10.1002/clen.200900033