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Optimization of copper reduction from solution using *Bacillus pumilus* PD3 isolated from Marine water

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ABSTRACT

Copper is one of the essential trace nutrients in all high plants and animals. But it can be toxic in high concentration. In this study a Copper resistant bacteria, *Bacillus pumilus* was isolated from Marine water. A multi-step response surface methodology was successfully applied for the maximum removal of Cu(II) from the aqueous solutions using the live bacteria to optimize the following factors : pH of the solution(3.50-5.50), concentration of the copper solution(15-50mg l^{-1}), incubation time (14-46 hours) and to evaluate the effects and interactions of process variables. Based on the statistics analysis the optimum conditions were obtained from pH=6.18, copper concentration=32.50mg l^{-1} of the media, contact time=30 hours and the copper removal was 60.264%. The higher value coefficient of determination (R^2=0.9991) justified an excellent correlation between the process parameters. Finally the difference between the theoretical value and statistical value was very low.

Introduction

Maximum Industrial effluent is consists of various heavy metals which cause hazardous toxic effects to environment [1-2]. Copper is one of the heavy metal which is essential in all high plants and animals, but very toxic in high concentration [3-5]. Waste water contains several hazardous organic and inorganic components according to United States Environmental Protection Agency (USEPA, 1978), among them copper is one of the hazardous components. Copper is present in maximum industrial effluents include metal cleaning and plating baths, pulp, paper board mills, wood pulp production, the fertilizer industry etc [6]. It has been found that marine life is damaging due to high copper concentration in water.[3,7].It has been observed that high copper uptakes may cause liver, kidney, brain damage, Schizophrenia and even death in human beings [5].

There are some old formal methods of copper (II) removal from wastewaters, such as precipitation, ion-exchange, electrolysis, adsorption etc. But these methods require high capital and operating costs [7]. Bioremediation is an important tool for environmental remediation of heavy metals using microorganisms. Biosorption is an effective process for removal of copper and other toxic heavy metals from the environment [8-16]. Biosorption is two types- active and passive biosorption. Live bacteria metabolically uptake and degrade heavy metal in active biosorption whereas dead bacterial biomass used in passive biosorption to remove heavy metal from aqueous solution by cell-surface adsorption methods [17].

The objective of the present study was to investigate the capacity of copper biosorption using the copper resistant bacteria isolated from marine water of Pattaya. The batch study involves changing one independent parameter (pH, heavy metal concentration, time) while maintaining others at a fixed level which is very much time consuming and expensive for a large number of parameters [18]. To overcome the difficulty Response surface methodology was used for the copper reduction study. Response Surface Methodology (RSM) is a classic combination of statistical and mathematical techniques useful for developing, improving, and optimizing processes [19-20]. Using RSM the adsorption study requires very less time and easy operating methods [10]. The graphical representation in RSM may be used to describe the individual and combined effect of the parameters on the response and determine the common interaction between the parameters and their incidental effect on the response [21-22].

Design-Expert 7.0.0 trial software was used for this study. In this study, 2^3 full factorial central composite design 20 experiments including 6 replicates using response surface methodology was employed.

Based on the statistics analysis the optimum conditions were obtained at pH=6.18, copper concentration=32.50 mg l^{-1} of the media, contact time 30 hours and the copper removal was 60.264%.

Materials and methods:-

Bacteria isolation and cultivation:-

Marine water was collected from pattaya of Thailand, and serial dilution was followed upto 10^{-3} times. The nutrient agar plates containing various copper concentration was inoculated by the sea serial dilution's solution of 10^{-3}. These plates were incubated at 30°C for 24 hours. Maximum copper resistance of the bacteria was obtained at 150 mg l^{-1} Cu(II) , using $CuSO_4{,}5H_2O$ in nutrient agar plate. Cu(II) concentration was varied from 10 - 150 mg l^{-1} in the nutrient agar plates. Nutrient agar was obtained from Himedia.

Characterization of the bacteria:-

The isolated bacteria was a gram-negative bacteria. On the basis of nucleotide homology and phylogenetic analysis the

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micro-organism was detected as *Bacillus pumilus* PD3.The bacterial 16s RNA sequence had been submitted to the GenBank under Accession Number: JQ809230 and had published also in the GenBank.

Preparation of Metal solution:-

A stock solution of copper was prepared using copper sulfate pentahydrate ($CuSO_4,5H_2O$) dissolving in distilled water. 1000 mg l^{-1} Cu(II) stock solution was prepared by diluting 3.93 g of $CuSO_4,5H_2O$ (obtained from Merck) in 1 liter distilled water. Using this stock different copper concentration solution was prepared.

Bacterial growth-medium Preparation:-

Nutrient broth consists of different concentration of Cu solution & different pH was prepared for the copper reduction experiment. For pH adjustment 0.1(N) HCl and 0.1(N) NaOH solution was used and for pH calibration pH meter (ELICO) was used. After that media was autoclaved in 250 ml conical flasks containing 100 ml. media.

Biosorption experiments:-

The media was inoculated with the isolated bacteria maintaining aseptic condition in laminar-hood. Then the conical flasks were kept in rotary shaker incubator at 30°C, 120 rpm. After different time intervals sample was collected and centrifuged at 6000 rpm for 10 minutes. Supernatant fractions was analyzed for the remaining copper ions in the media after reduction, were determined spectrophotometrically at 460 nm by using sodium diethyl dithiocarbamate (obtained from Loba) as the complexing agent [23].Copper reduction percentage by biosorption was obtained by using the formula-

$$\%reduction = (C_0 - C_1)/C_0 * 100\% \qquad \text{-----------------------(1)}$$

Where, C_0 is initial copper concentration (mg l^{-1}) and C_1 is remaining copper concentration. Standard graph was used for the calculation of remaining copper concentration, obtained from optical density (OD). All OD was taken by the Techcomp UV 2300 Spectrophotometer.

Results and discussions:-

The effect of copper reduction parameters namely initial copper concentration, pH of the solution and contact time on percentage removal of copper was studied by statistically designed experiments and optimization by response surface methodology. The ranges and levels of variables investigated in the research are given in Table 1. The percent removal of copper was taken as response of the system.

Table 1:- Experimental range and levels of independent process Variables:-

Independent Variables	Range and levels(coded)				
	$-\alpha$	-1	0	+1	$+\alpha$
pH(A)	2.81821	3.50	4.50	5.50	6.18179
Copper concentration, mg l^{-1} (B)	3.06863	15.00	32.50	50.00	61.93
Time, hours(C)	3.09131	14.00	30.00	46.00	56.9087

The quadratic equation model for predicting the optimal point was expressed according to Eq. (2).

$$Y = \beta o + \sum_{i=1}^{K} \beta_i X_i + \sum_{i=1}^{K} \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j + \in \qquad \text{------------------- (2)}$$

Where Y is the predicted response, Xi refers to the coded variables,b_0,b_i,b_{ii},b_{ij} are the regression coefficients and £ is the statistical error. Three factors were studied and their low and high levels are given in Table 1. Percentage removal of copper was studied with a standard RSM design called the central composite design (CCD). A total twenty experiments were conducted according to 2^3 full factorial central composite design consisting of factorial points (coded to the usual $\pm$1 notation), axial points ($\pm\alpha$) and six replicates at the centre points (0), and each of the experiment was conducted in duplicates in Table 2. The results of each experiment performed as per the software are given in Table 2. An empirical relationship between the response and the independent variables has been expressed by the following quadratic model (Equation 3). The design expert 7.0.0 trial software was used for regression analysis of the data obtained and to estimate the coefficient of the regression equation.

$$R_1 = 111.81270 - 63.63219\, A + 0.76459\, B + 0.14797\, C - 0.15882\, AB - 0.016639\, AC - 0.00559692\, BC + 9.14735\, A^2 + 0.00077177\, B^2 + 0.00288541\, C^2 \qquad \text{------------------------ (3)}$$

Where,

A=pH of the solution,

B=copper concentration (mg l^{-1}),

C=Time (hours).

Table 3 Analysis of variance for the response surface quadratic model for copper removal:-

Source	Sum of squares	Degree of freedom (df)	Mean square	F value	Probablity>F
Model	3648.15	9	405.35	1208.77	<0.0001
Residual	3.35	10	0.34		
Lack of fit	2.47	5	0.49	2.80	0.1415
Pure error	0.88	5	0.18		
Cor Total	3651.50	19			

R^2=0.9991, Adjusted R^2=0.9983, Predicted R^2=0.9945

Table 4 Regression analysis by using central composite design:-

Model Term	Coefficient estimate	Standard error	F value	P value
A	13.03	0.16	6917.90	< 0.0001
B	-1.19	0.16	57.37	< 0.0001
C	1.03	0.16	43.13	< 0.0001
AB	-2.78	0.20	184.27	< 0.0001
AC	-0.27	0.20	1.69	0.2227
BC	-1.57	0.20	58.59	<0.0001
A^2	9.15	0.15	3595.91	< 0.0001
B^2	0.24	0.15	2.40	0.1523
C^2	0.74	0.15	23.45	0.0007

The Model F value (1208.77) with a low probablity (P<0.0001) shows that the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, BC, A^2, C^2 are significant model terms. The "Lack of Fit F-value" of 2.80 implies the Lack of Fit is not significant relative to the pure error. There is a 14.15% chance that a "Lack of Fit F-value" this large could occur due to noise.

The multiple corelation coefficient (R^2) also demonstrates the goodness of the model. Moreover,R^2 value is 0.9991 implies that more than 99% of the data deviation can be explained by the developed quadretic model, and the predicted R^2 values are in agreement with adjusted R^2, which means all the terms depicted in the model are significant.

Effect of initial solution pH and initial copper concentration:-

Bioremediation of copper onto the surface of a microorganism is affected by several factors such as initial pH, initial copper ion concentration, time and temperature etc. From the contour plot (Fig 1) it can be observed that copper removal percentage increased with increase of pH and initial copper concentration. Initially, copper reduction percentage increased with increasing copper concentration upto 32.50 mg l^{-1}, after that with increasing copper concentration percentage reduction decreased. That signified the bacterial growth and copper uptake capacity is maximum at 32.50 mg l^{-1} copper concentration. The copper reduction was increased with increasing pH and copper concentration of the media.

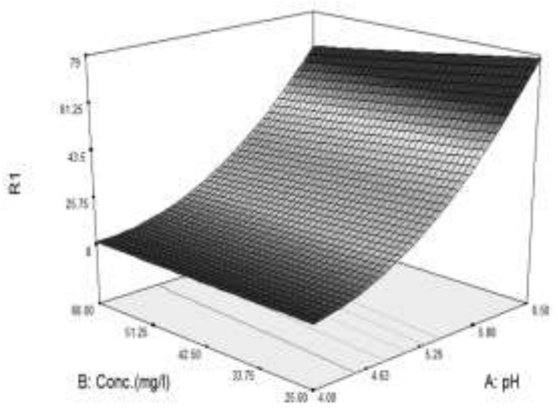

Fig 1: The 3D-contour plot shows the relationship between pH and the concentration with the R1

Effect of initial solution pH and contact time :-

The combined effect of initial solution pH and contact time on copper reduction was shown in the contour plot of Fig. 2. It was observed that initially % reduction getting increase with increasing pH. With increasing time the reduction of copper gets increase using the live bacteria.

Bacterial population in the culture gets increase with increasing time, so bacterial metabolic uptakes of copper gets increase with time [17]. After a certain time copper reduction goes to the equilibrium. It seems that the food in the media gets finished upto a certain times and bacteria go to the death phase after uptaking the copper. It was evident from contour plots (Fig: 2) that both the independent variables had a strong influence on the copper reduction process. At pH 6.18 and contact time 30 hours the copper degradation was 60.264% where copper concentration was fixed at 32.50 mg l^{-1} of the media. At high pH copper removal increased, it means that the isolated bacteria are more efficient for the biosorption in basic pH. But copper salt's solution starts precipitating above pH 6 as observed in the lab. Due to this problem in this experiment high pH range was kept at 5.5.

The percentage of reduction was a function of the initial metal concentration, pH, time etc. The combined effect of initial copper concentration and contact time on copper degradation was shown in the contour plot of Fig. 2. At copper concentration 32.50 mg l^{-1} of the media, contact time 30 hours the copper reduction was 60.24 % when pH was fixed at 6.18.

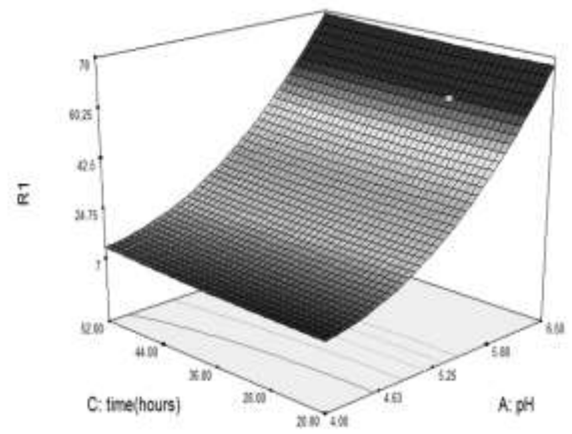

Fig 2: The 3D-contour plot shows the relationship between pH and the time with the R1

Effect of initial copper concentration and contact time:-

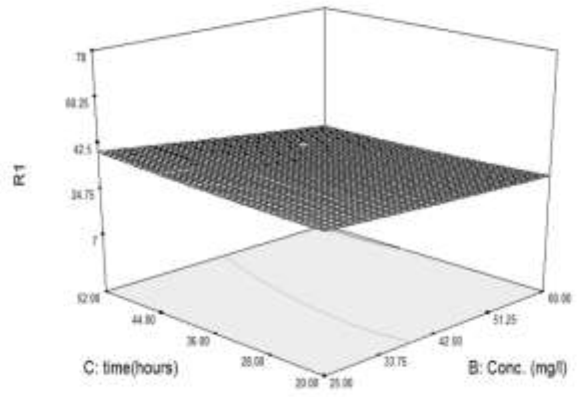

Fig 3: The 3D-contour plot shows the relationship between initial concentration and time with the R1

Optimization using the desirability functions:-

In numerical optimization process the desired goal for each process variables and response from the menu was chosen: maximize, minimize, target, within range, none (for responses only) and set to an exact value (factors only) [24]. A minimum and a maximum level must be provided for each process variables included in the study.

A multiple response method was applied for optimization of any combination of four goals, namely initial solution pH, initial copper concentration, contact time and percentage reduction of copper. The numerical optimization found a point that maximizes the desirability function.

Level of initial copper concentration (15-50 mg l^{-1}), contact time (14-46 hours) were in between the range, and initial solution pH (5.50), percentage degradation of copper (60.264%) were set for maximum desirability. Fig. 4 showed a ramp desirability that was generated from 10 optimum points via numerical optimization.

From the contour plot we can seen copper removal percentage increases with increase of pH and initial copper concentration.

It is evident from Fig. 4 that both the independent variables had a strong influence on the copper reduction process. Solution pH effects on biosorption using the bacteria.

Table 2:- 2^3 factorial experimental setup and percent removal response

Run	Initial pH of the solution	Copper concentration(mg/l)	Contact time(hours)	%Reduction
1	4.50	61.93	30.00	11.4385
2	4.50	32.50	56.91	17.06
3	6.18	32.50	30.00	60.2426
4	3.50	15.00	14.00	5.58571
5	5.50	50.00	46.00	30.408
6	4.50	32.50	30.00	12.246
7	4.50	32.50	30.00	13.18
8	5.50	50.00	14.00	32.6802
9	5.50	15.00	14.00	37.7433
10	3.50	15.00	46.00	10.6469
11	4.50	32.50	30.00	12.1678
12	4.50	32.50	3.09	12.02
13	4.50	32.50	30.00	12.8
14	5.50	15.00	46.00	41.7396
15	3.50	50.00	14.00	11.6397
16	2.82	32.50	30.00	16.4041
17	3.50	50.00	46.00	10.4324
18	4.50	32.50	30.00	12.278
19	4.50	32.50	30.00	12.1678
20	4.50	3.07	30.00	14.8

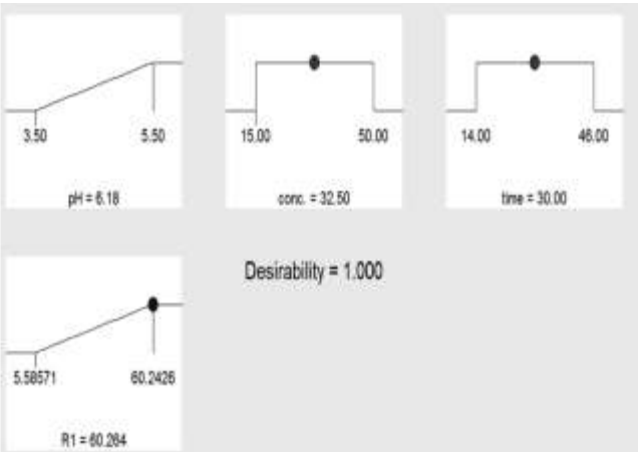

Fig 4: Desirability ramp for optimization of three goals, namely initial solution of pH, concentration of copper and time on maximum removal of copper (R1) .

Comparison between experimental and theoretical results:-

Theoretically optimum condition for copper adsorption was pH 6.18, initial copper concentration of 32.50 mg l^{-1},contact time 30 hours and the copper removal was 60.264%. Experimentally result was 60.551% at pH 6.18, initial copper concentration of 32.50 mg l^{-1},contact time 30 hours. The deviation between experimental and theoretical was 0.47%.

Conclusion:-

The present study was taken with the aim of scale-up of the copper biosorption process using the isolated bacteria and to investigate the combined effect of various process parameters on copper removal using response surface methodology. The initial solution pH, initial copper concentration and time significantly influenced the copper removal efficiency. Optimization conditions for the maximum removal efficiency of copper were obtained by applying a desirability function in RSM. The level of the three variable, initial solution, pH 6.18; initial copper concentration, 32.50 mg l^{-1}; time,30 hours, were found to be optimum for maximum copper removal. The corresponding removal in optimum condition was found to be 60.264%. The results indicated that the isolated bacteria can be used for copper reduction in Industrial waste water treatment plant at low costs.

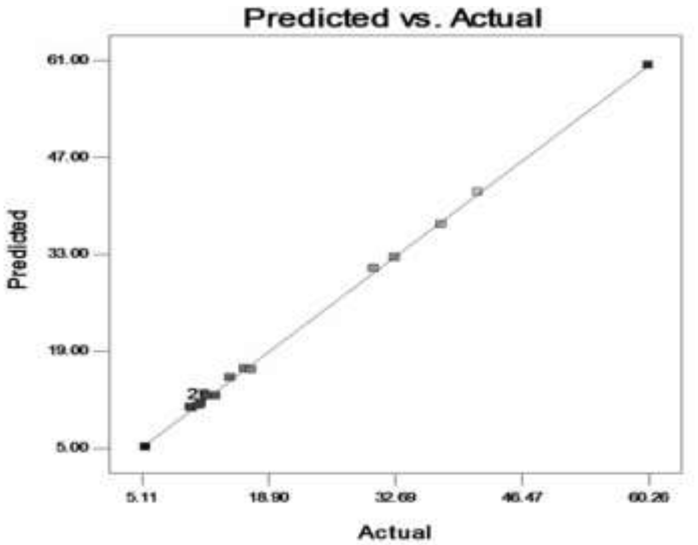

Fig 5: Comparison of the predicted and experimental results

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