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Full Length Research Paper

Statistical analysis and optimization of copper biosorption capability by *Oenococcus oeni* PSU-1

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Lactic acid bacteria (LAB) have been reported to remove heavy metals. The removal of heavy metals, from aqueous solution by LAB has been observed to be strain dependent. In this study, statistical designs were performed to optimize the conditions required for metal biosorption by *Oenococcus oeni* PSU1. A screening experiment revealed that *O. oeni* PSU-1 biosorp Cu^{2+} with a high percentage (28%), in comparison to other heavy metals. The Plackett-Burman design was applied and eleven variables were examined. The increase in Cu^{2+} biosorption was mainly affected by Cu^{2+} concentration, cell immobilization and mixing speed. These three variables were further adopted by the three-level Box–Behnken design to correlate between the three variables and to estimate the optimal conditions required for Cu^{2+} biosorption. Although the maximum value for Cu^{2+} biosorption was 85%, the calculated optimum percentage was 72%, which was a 2.57-fold increase over that in basal medium. The estimated optimum values for Cu^{2+} biosorption are as follows: metal concentration (8.6 mg/ml), mixing speed (136 rpm) and immobilization (2.27%).

Key words: *Oenococcus oeni* PSU-1, metal biosorption, Plackett-Burman, Box-Behnken.

INTRODUCTION

Heavy metal pollution has become one of the serious environmental problems. With the rapid development of many industries, wastes containing metals has been directly or indirectly discharged into the environment causing serious environmental pollution and affecting human health (Das et al., 2008). Heavy metal pollution represents an important problem due to its toxic effects and accumulation throughout the food chain, which leads to serious ecological and health hazards as a result of their solubility and mobility. Metal remediation through common physico-chemical techniques is expensive and not eco-friendly (Lyer et al., 2005).

Although a vast array of biological materials, especially bacteria, algae, yeasts and fungi have received increasing attention for heavy metal removal and recovery due to their good performance, low cost and large available quantities (Wang and Chen, 2009), little is known about metal ion uptake by lactic acid bacteria (LAB) (Mrvcic et al., 2009). Some *Lactobacillus* species have already been successfully tested for the removal of toxic compounds (Haskard et al., 2001; Poltonen et al., 2001) and an effective tool for heavy metal biosorption (Halttunen et al., 2007; Yilmaz et al., 2010). LAB are non-pathogenic, food safe microorganisms commonly used in the food industry to produce fermented foods.

Oenococcus oeni (formerly called *Leuconostoc oenos*) is a lactic acid bacterium that occurs naturally in fruit mashes and related habitats. The objective of this study is to use statistical designs to optimize the conditions for heavy metal biosorption by *O. oeni* PSU-1. To the best of our knowledge, *O. oeni* PSU-1 strain have not been investigated regarding this matter.

MATERIALS AND METHODS

Bacterial strain and heavy metals

O. oeni PSU-1 was obtained from the Laboratory of David Mills (Department of Viticulture and Enology, University of California, Davis). The strain was maintained on AR medium (2% tryptone,

Table 1. Different levels of the eleven independent variables used in the Plackett-Burman design.

Factor	Abbreviation	High level (+)	Low level (-)
Metal concentration	M	8.6 mg/ml	6mg/ml
Washing cells	W	Washed	Unwashed
Incubation time	I	48 h	24 h
Temperature	Tm	30°C	25°C
Dry weight	D	0.36 mg/ml	0.13 mg/ml
Glucose	G	0.42%	0%
Ethanol(absolute)	E	10%	0%
Malic acid	ML	0.04%	0%
Viability	V	Viable	Dead
Immobilization	IM	2% Alginate	0%
Mixing speed	A	150 rpm	Static

0.5% glucose, 0.5% yeast extract, supplemented with 20 ml/L apple juice) with 15% glycerol. The heavy metals used in this work were obtained from the Microbiology Laboratory, Faculty of Science, Alexandria University, Egypt. Stock solutions of analytical grade metals (Cu, Ni, Co, Cr, Cd, Pb, Mg and Zn) were prepared in water and autoclaved for 15 min at 121°C.

Cultivation

A seed culture of *O. oeni* PSU-1 was prepared by developing aliquots (5 ml) of AR media in test tubes and autoclaved for 20 min at 121°C. The tubes were inoculated with stock culture of *O. oeni* PSU-1 and incubated for 10 days at 30°C under static conditions. A 100 ml Erlenmeyer flask containing 25 ml of AR fermentation media was inoculated with 100 µL of seed culture and grown under the same conditions.

Screening for different heavy metals

Since the metal removal percentage decreased with the increase of the initial metal concentration (Parameswari et al., 2010), a low concentration of 8.6 mg/ml of the above mentioned heavy metals were prepared for examination of biosorption efficiency by *O. oeni* PSU-1. The metal with the highest biosorption efficiency by *O. oeni* PSU-1 was selected for further experiments.

Measurement of metal biosorption

The cultures previously prepared were first centrifuged at 10,000 rpm for 15 min, after which cell pellets were suspended in 600 µL water and 600 µL of 8.6 mg/ml heavy metal in Eppendorf tubes. The tubes were kept at 30°C while shaking at 150 rpm/ 24 h. The mixture was then centrifuged and the remains of the metal concentration in the metal solution were measured by the atomic absorption spectrophotometry (Perkin Elmer-2380) located in Central Laboratory in Mohrem bek, Faculty of Science. The Metal biosorption efficiency can be described in terms of percentage.

$$\text{Percentage of cell biosorption} = \frac{I - F}{I} \times 100$$

Where, F is the final reading after biosorption and I is the initial reading before biosorption. The initial reading is the amount of metal concentration in the basic solution before being biosorption by cells and the final reading is the amount of metal in the solution after biosorption. The subtraction of the final reading from the initial reading results in the amount of metal concentration biosorped by the cell pellets.

A concentration of 8.6 mg/ml of the abovementioned heavy metals were prepared for examination of biosorption efficiency by 0.3 mg/ml dry weight of *O. oeni* PSU-1. The metal with the highest biosorption efficiency by *O. oeni* PSU-1 was selected for further experiments.

Statistical analysis

Plackett-Burman design

The effect of some variables on metal biosorption was studied by applying the Plackett-Burman experimental design (Plackett and Burman, 1946). In this experiment, 11 factors (metal concentration, viability, washed cells, dry weight, time of incubation, temperature, immobilization, mixing speed, addition of ethanol, glucose and malic acid) were screened in 12 combinations organized according to the Plackett-Burman matrix. A 50% increase of the original component level of metal concentration is represented by the"+" sign, while 50% decrease of the original component level is represented by the "-" sign. Variable levels are represented in Tables 1 and 2. Glucose, ethanol, alginate and malic addition are represented by "+" sign, while their absence by "- "sign. The viable cells, temperature of 30°C, dry weight of 0.36 mg/ml, incubation time of 48 h, mixing speed of 150 rpm and washed cells were represented by "+" sign, while dead cells, temperature of 25°C, dry weight of 0.13 mg/ml, incubation time of 24 h, static incubation and unwashed cells were represented by "-" sign. The pH was not as among the examined factors since AR media was adjusted at pH = 5 and it was proven previously by Yilmaz et al. (2010), that maximum copper biosorption capacities were attained at pH 5.0 and 6.0 .

A control was performed using immobilized alginate only without cells. The efficiency of biosorption by immobilized cells was measured by subtracting the result from the control. The main effect of each factor was determined using the following equation:

$$Exi = (\Sigma Mi+ - \Sigma Mi-) / N$$

Where, Exi is the variable main effect, Mi+ and Mi- are the sum of

Table 2. Response to Plackett Burman design of 11 variables and 12 trials

Trial no	Variables											
	Metal conc (M)	Washing Cells (W)	Incubation time (I)	Temperature (Tm)	Dry weight (D)	Glucose (G)	Ethanol (E)	Viability (V)	Malic acid (ML)	Immobilization (IM)	Mixing speed (A)	Response (Percent of biosorption)
1	+	+	-	+	+	+	-	-	-	+	-	78.0
2	+	-	+	+	+	-	-	-	+	-	+	58.0
3	-	+	+	+	-	-	-	+	-	+	+	63.6
4	+	+	+	-	-	-	+	-	+	+	-	72.0
5	+	+	-	-	-	+	-	+	+	-	+	68.0
6	+	-	-	-	+	-	+	+	-	+	+	72.0
7	-	-	-	+	-	+	+	-	+	+	+	52.4
8	-	-	+	-	+	+	-	+	+	+	-	16.0
9	-	+	-	+	+	-	+	+	+	-	-	00.0
10	+	-	+	+	-	+	+	+	-	-	-	61.0
11	-	+	+	-	+	+	+	-	-	-	+	1.3
12	-	-	-	-	-	-	-	-	-	-	-	00.0

the recorded results of biosorption percentage by trials which contain + and – levels of independent variables (xi), respectively, and N is the number of trials divided by two. A main effect figure with a positive sign indicates that the + level of this variable is nearer to optimum metal biosorption percentage, while a negative sign indicates that the - level of this variable is nearer to optimum. Using the Microsoft Excel program, statistical t-values for equal unpaired samples were calculated for the determination of variable significance.

Box-Behnken design

In order to describe the nature of the response surface in the experimental region and elucidate the optimal concentrations of the most significant independent variables, a Box–Behnken design (Box and Behnken, 1960) was applied. Three variables, namely treated copper concentration (X1), immobilization (X2) and mixing speed (X3), were included in the model. Each factor was examined at three different levels (-, 0, +). According to the applied design, 15 combinations were executed and their observations were fitted to the following second order polynomial model:

$$Z = b_o + b_1X1 + b_2X2 + b_3X3 + b_{11}X1^2 + b_{22}X2^2 + b_{33}X3^2$$

Where, Z is the dependent variable (Percentage of metal biosorption); X1, X2 and X3 are the independent variables (copper concentration, immobilization and mixing speed respectively); b_0 is the regression coefficient at center point; b_1, b_2 and b_3 are linear coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients. The values of the coefficients as well as the optimum concentrations were calculated using Statistica software. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination, R^2.

RESULTS

Screening experiment

O. oeni PSU-1 was screened for metals (Cu, Ni, Co, Cr, Cd, Pb, Mg and Zn) biosorption efficiency (Figure 1). The highest biosorption efficiency (28%) was achieved with copper concentration at (8.6 mg/ml), which was selected for further study.

Elucidation of different factors affecting metal biosorption by O. oeni PSU-1

Plackett-Burman design

The design was applied with 11 different fermentation conditions as shown in Table 1. All experiments were performed in duplicate and the average results as percentage of copper biosorption are presented in Table 2. The main effect of each variable was calculated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that factor. The results of main effect are presented in Table 2 and Figure 2. It was deduced from Figure

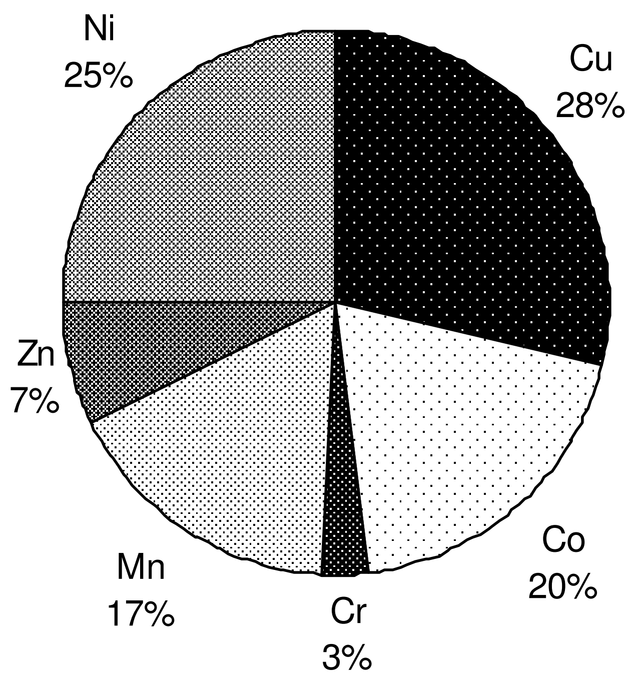

Figure 1. Biosorption percentage of different heavy metals by *O. oeni* PSU-1.

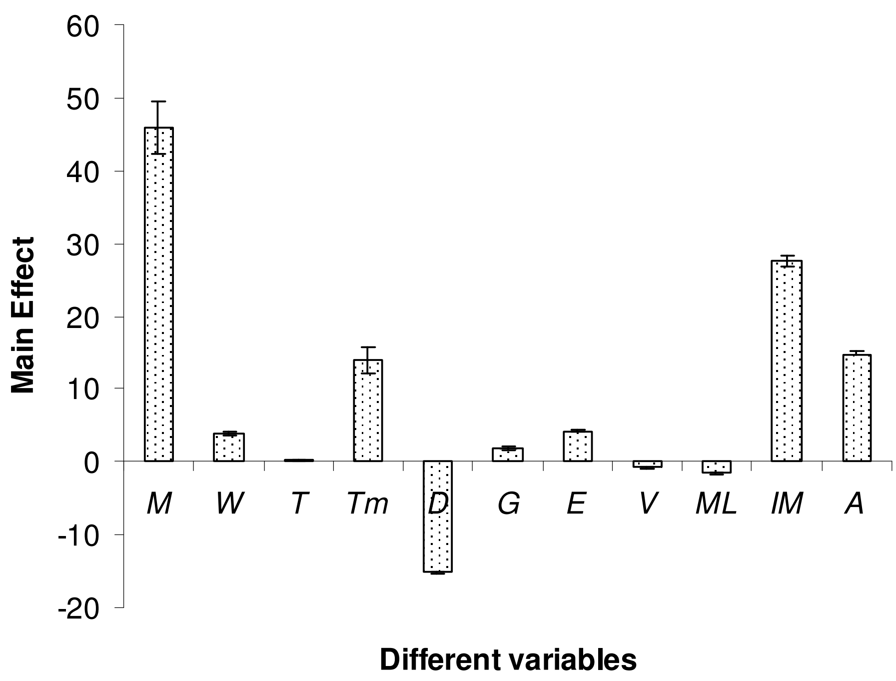

Figure 2. Main effect of Cu biosorption by *O. oeni* PSU-1 according to Plackett-Burman design.

2 that the copper concentration was the most significant variable that affected biosorption efficiency. Immobilization of the cells and mixing speed of the culture also had a very high effect, followed by the temperature, glucose and ethanol addition, washing cells and time of incubation. On the other hand, the other variables such as dry weight, viability of cells, and malic acid, had negative effects on the response. Overall,

Table3. Main effect values for each variable based on the Plackett Burman experiment

Variables	M		W		T		Tm		D		G		E		V		ML		IM		A	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	78.0	63.6	78.0	58.0	58.0	78.0	78.0	72.0	78.0	63.6	78.0	58.0	72.0	78.0	63.6	78.0	58.0	78.0	78.0	58.0	58.0	78.0
	58.0	52.4	63.6	72.0	63.6	68.0	58.0	68.0	58.0	72.0	68.0	63.6	72.0	58.0	68.0	58.0	72.0	63.6	63.6	68.0	63.6	72.0
	72.0	16.0	72.0	52.4	72.0	72.0	63.6	72.0	72.0	68.0	52.4	72.0	52.4	63.6	72.0	72.0	68.0	72.0	72.0	00.0	68.0	16.0
	68.0	0.00	68.0	16.0	16.0	52.4	52.4	16.0	16.0	52.4	16.0	72.0	00.0	68.0	16.0	52.4	52.4	61.0	72.0	61.0	72.0	0.00
	72.0	1.3	0.00	61.0	61.0	0.00	0.00	1.3	0.00	61.0	61.0	00.0	61.0	16.0	00.0	1.3	16.0	1.3	52.4	1.3	52.4	61.0
	61.0	0.00	1.3	0.00	1.3	0.00	61.0	0.00	1.3	0.00	1.3	00.0	1.3	00.0	61.0	00.0	00.0	00.0	16.0	00.0	1.3	0.00
Mean	68.1	22.2	47.2	43.2	45.3	45.1	52.2	38.2	37.6	52.8	46.1	44.3	43.1	47.3	45.3	46.1	44.4	46	59	31.4	52.6	37.8
Main Effect	**45.9**		4		0.2		14		-15.2		1.8		4.2		-0.8		-1.6		**27.6**		**14.8**	

M, Metal concentration; W, washing cells, I, incubation time; Tm, temperature; D, dry weight; G, glucose; E, ethanol (absolute) ; ML, malic acid; V, viability; IM, immobilization; A, mixing speed.

the results of the Plackett–Burman experiment represented by the main effect values of each variable (Table 3) reveal that the three factors which showed high biosorption efficiency are copper concentrations, immobilization and mixing speed. These were examined more thoroughly by applying the Box–Behnken design.

Box–Behnken design

In order to approach the optimum response region of copper biosorption, the three independent variables with the highest main effect (copper concentration, immobilization and mixing speed) were further explored, each at three levels according to Box and Behnken (1960). Table 4 represents the design matrix of the variables together with the experimental results of biosorption. All cultures were performed in duplicate and the average of the observations was used. Experimental results in the form of surface plots show that there is a slight increase in percentage of biosorption by changing the mixing speed; a notable effect was obtained after 160 rpm. However, the percentage of biosorption increased rapidly (up to 90%) by increasing immobilizing material and then a slight decrease was obtained. The mixing speed appeared to have an insignificant influence on the biosorption process (Figure 3A).

The biosorption is inversely proportional to copper concentration in reaction mixture. Immobilization showed the same behavior as previous (Figure 3B). For predicting these optimal points mathematically, a second order polynomial function was fitted to the experimental results of copper biosorption efficiency by the help of Statistical software;

$$Z= 60.42 - 0.044\ X_1 + 0.00016\ (X_1)^2 - 0.48(X_2) + 0.0093\ (X_2)^{2} + 14.58\ (X_3) - 2.1\ (X_3)^2$$

Where, Z is the efficiency of copper biosorption (%); and X1, X2, and X3 are mixing speed, concentration of copper and immobilization, respectively. At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation coefficients R and the determination coefficient R^2. The closer the value of R is to 1, the better is the correlation between the measured and the predicted values. In this experiment the value of R^2 was 0.93 for the efficiency of copper biosorption. This value indicates a high degree of correlation between the experimental and the predicted values. Furthermore, the optimal levels of the three variables were estimated using Statistica software and found to be: metal concentration (8.6 mg/ml), mixing speed (136 rpm) and immobilization (2.27%), with a predicted biosorption percentage of 72% (Figure 4).

DISCUSSION

Several methods have been used for heavy metal removal (chemical precipitation, ion exchange, etc), and each of these methods have its own merits and demerits. Moreover, these methods are expensive and results in great amount of sludge needed to be treated. Therefore the search for a new cost effective technology has been directed toward biosorption. Algae, bacteria, fungi and yeast have been proved to be potential metal biosorbents (Das et al., 2008). In this study, different metals were screened for heavy metal biosorption efficiency by *O. oeni* PSU-1 and the highest biosorption efficiency was achieved with copper concentration, which was selected for further study. Copper has been one of the most

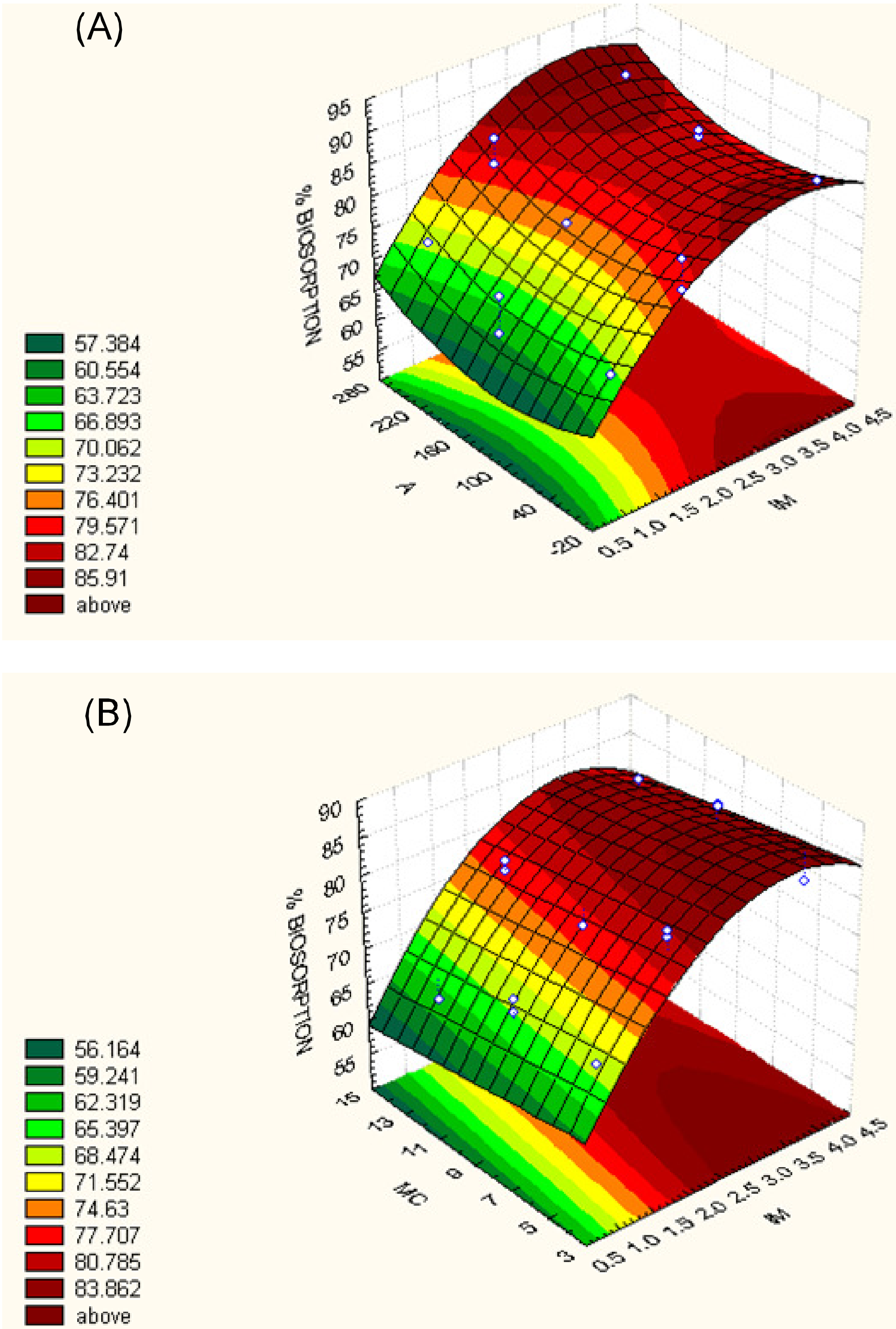

Figure 3. Response of immobilization and mixing speed (A) and response of immobilization and copper concentration (B) on percentage of biosorption based on Box-Behnken experiment.

widely used metals for centuries and is mainly employed in electrical and electroplating industries. Due to its toxicity to living organisms, its presence in the environment causes serious toxicological concerns. Wastewaters of especially the electrical and electroplating industries contain high levels of Cu^{2+} ions and treatment of such waters to remove Cu^{2+} ions is needed before disposal (Yilmaz et al., 2010). The Plackett-Burman

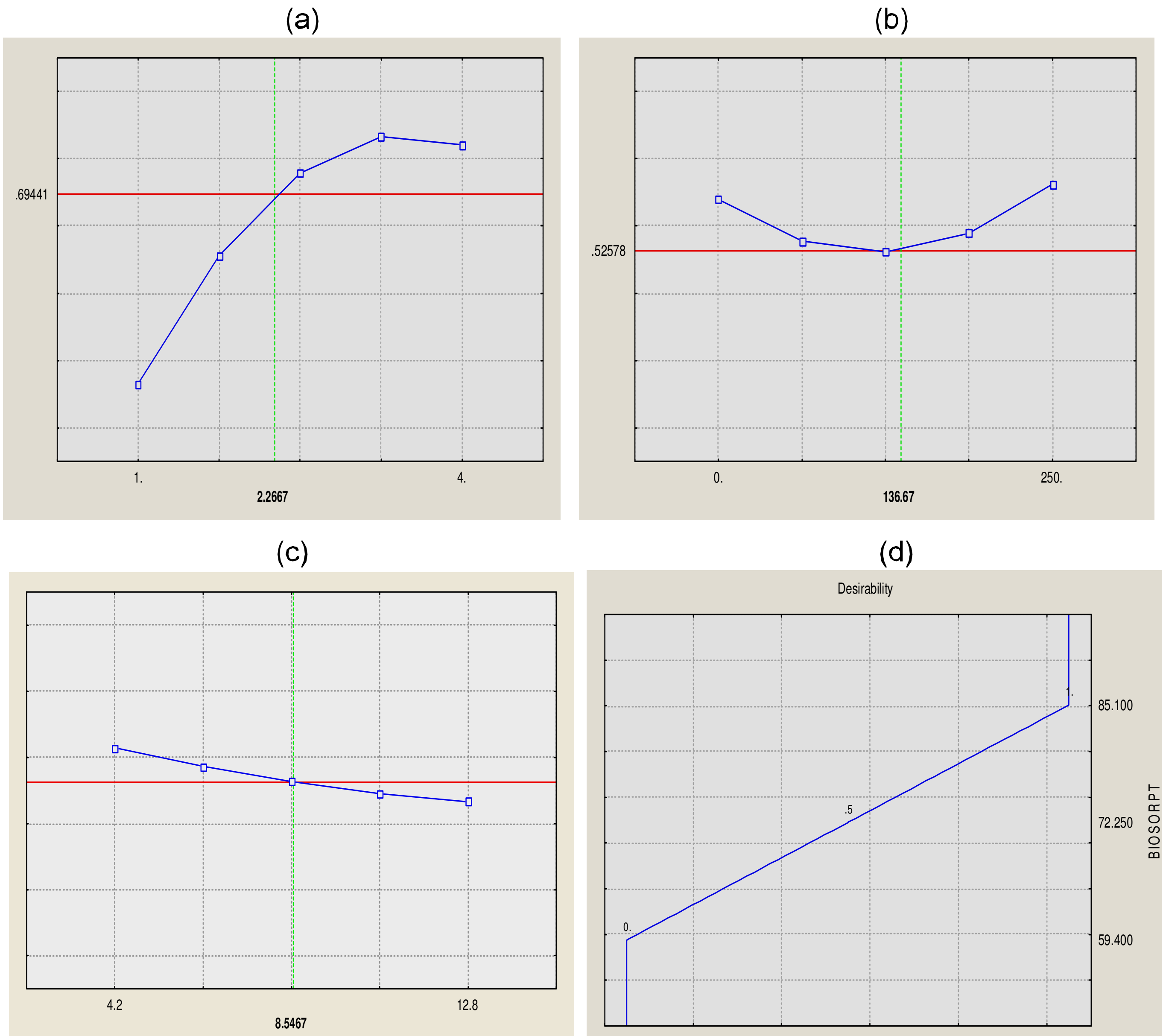

Figure 4. Calculated optimum values of different variables: (a) immobilization; (b) mixing speed; (c) Cu concentration; (d) biosorption efficiency based on Box- Behnken experiment.

statistical design examined 11 factors affecting copper bioremoval (biosorption) (Andreazza et al., 2010). These factors affect general microbial metabolism and specifically promote or decrease copper biosorption (Chen et al., 2005; Ozer et al., 2009; Tunali et al., 2006; Umrania, 2006).

Copper is an essential but toxic heavy metal that negatively impacts living systems at high concentration. Here, the results show that the efficiency of metal biosorption increased by increasing the metal concentration up to 8.6 mg/ml, but further increase up to 12.8 mg/ml led to a decrease of biosorption efficiency. The initial metal ion concentration seems to have an impact on biosorption, with a higher concentration resulting in a high solute uptake (Binupriya et al., 2007; Ho and McKay, 2000). An increase in copper concentration had a negative impact on biosorption efficiency (Das et al., 2008). Another factor which is the immobilization showed a high impact on biosorption efficiency. Biomass immobilization in a polymeric matrix may improve biosorption capacity and facilitate the separation of biomass from metal-bearing solutions. Ivnova et al. (2010) showed that the sorptions of copper ions on gel immobilized beads are the most suitable. The immobilized biomass offers many advantages including better reusability, high biomass loading and minimal clogging in continuous flow

Table 4. Box-Behnken design and response for the three variables, Cu2$^+$concentration immobilization, and mixing speed.

Trial	Variable			
	M	IM	A	Response = Biosorption %
1	+ (12.8mg/L)	+ (4%)	0 (150 rpm)	82.0
2	+	- (1%)	0	59.4
3	- (4.2 mg/ml)	+	0	81.0
4	-	-	0	66.6
5	+	0 (2%)	+ (250 rpm)	77.6
6	+	0	- (Static)	75.9
7	-	0	+	82.6
8	-	0	-	81.6
9	0 (8.6 mg/ml)	+	+	84.9
10	0	+	-	85.1
11	0	-	+	68.0
12	0	-	-	65.9
13	0	0	0	75.0

M, Metal concentration; IM, immobilization; A, mixing speed.

systems (Holan and Volesky, 1994). The immobilization of the biomass on solid structures creates a material with the right size, mechanical strength and rigidity and porosity necessary for metal accumulation (Ahalya et al., 2003).

Data also reveals that mixing speed had a high effect on biosorption efficiency. Bacterial cells retain metals when grown under shaking conditions (Mago and Srivastava, 1994). Hossian and Anantharaman (2005) investigated copper biosorption by bacterial cells and found that increasing the shaking speed increases copper biosorption. The Plackett-Burman results showed that temperature also had a positive effect on copper biosorption. This behavior is a metabolic advantage in bio-treatment of contaminated natural environments in which the temperature can vary (Andreazza et al., 2010). Hossian and Anantharaman (2005) also investigated the effect of temperature on copper biosorption and it was found that increasing temperature increases copper biosorption up to 40℃, beyond which biosorption decreases. Biosorption efficiency also increases on the addition of ethanol (Darvishi et al., 2009). Ethanol activates the functional groups for binding heavy metal ions (Pamukoglu and Kargi, 2006).

On the other hand, the data reveals that copper biosorption efficiency increase on decreasing biomass. An increase in biomass concentration leads to the interference between the binding sites (Gadd et al., 1988). Biosorption efficiency increased with the used of dead cells. The use of heat inactivated or dead biomass in industrial application may offer some advantages over living cells, such as less sensitivity to heavy metal ions concentration and adverse operating conditions (Tunali et al., 2002; Wang and Chen, 2009). Therefore, it can be stated that dead biomass can be used as an efficient biosorbent system for the biosorption of metals from combined industrial effluent. The results also reveal that biosorption efficiency decreases with the use of organic acids (e.g., malic acids), which may chelate toxic metals and thereby resulting in the formation of metallo-organic molecules. Organic acids help in the solubility of metal compounds and their leaching from their surfaces (Ahalya et al., 2003), thus decreasing the efficiency of metal biosorption.

The Plackett–Burman and Box–Behnken designs have been successfully applied in many recent biotechnological applications (Lotfy, 2000; Mohamed, 2000). The Box-Behnken design reflects the accuracy and applicability of the model in the optimization processes (Bloor and England, 1991; Kimmel et al., 1998).

Conclusion

Different metals were screened for metal biosorption efficiency by *O. oeni* PSU-1 and the highest biosorption efficiency (28%) was achieved with copper concentration at 8.6 mg/ml, which was selected for further study. Plackett–Burman design was applied to determine the factors affecting biosorption efficiency. The results represent by the main effect values of each variable revealed that the three factors which showed high biosorption efficiency are copper concentrations, immobilization and mixing speed. These were examined more thoroughly by applying the Box–Behnken design. In general, the optimal levels of the three variables estimated using Statistica software were found to be: metal concentration (8.6 mg/ml), mixing speed (136 rpm) and immobilization (2.27%), with a predicted biosorption percentage of 72%.

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