Optimization of Batch Process Parameters by Response Surface Methodology for Mycoremediation of Chrome-VI by a Chromium Resistant Strain of Marine *Trichoderma Viride*

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Abstract: Heavy metal resistant strain of a marine fungus, *Trichoderma viride,* was isolated from sea water samples collected from a heavy metal polluted area in the Mediterranean Sea, Alexandria, Egypt during February, 2009.The fungus effectively biosorb and accumulate Cr (VI) and Cu (II). Chromium resistant isolate could tolerate Cr (VI) toxicity at up to 1,000 mg L^{-1} and chromium accumulation did not affect the structure of mycelia and conidia. On the other hand, Cu (II) accumulation causes some changes in the conidial and mycelia structures. So removal of Cr (VI) by the non pathogenic marine fungus *Trichoderma viride* was investigated in a batch system. A2^4 full factorial central composite statistical design was employed for analysis of the experimental results. The combined effect of contact time, pH, biosorbent dosage and Cr (VI) concentration on the fungal removal efficiency was studied using response surface methodology. It was observed from this investigation that the percentage removal efficiency is significantly influenced by contact time, pH and biosorbent dosage as well as initial metal concentration. The contact time, pH, biosorbent dosage and metal concentration were found to be 45 min; 6; 3.75g/L and 175mg/L, respectively, for the maximum Cr (VI) removal (98 %). Regression equations were developed for removal efficiency using experimental data and solved using the statistical software Minitab 14. It was observed that the experimental values were in good agreement with the predicted values and the model developed was highly significant. The result indicates the applicability of the isolated *Trichoderma viride* to remove 4.66 mg /g at pH 6 after 45 min. These quantitative biosorbtion and accumulation capacities demonstrated the potential application of marine *Trichoderma viride* for the mycoremediation of Cr (VI) from water systems.

Key words: Mycoremediation · Response Surface Methodology · Chromium-*Trichoderma viride*

INTRODUCTION

The presence of toxic heavy metals in aqueous streams, arising from the discharge of untreated metal containing effluent into water bodies, is one of the most important environmental issues. Their presence in aquatic ecosystem posses human health risks and causes harmful effects to living organisms. Chromium is one of the toxic contaminants, which exists in hexavalent and trivalent forms. This pollutant is mainly introduced into the aquatic systems from the effluents of leather processing units as a result of chrome tanning of leather. Chromium's interaction with biological systems is very different and complex. Toxic kinetics of Cr (VI) show higher rate of penetration into biological membranes as compared to Cr (III). The carcinogenicity of Cr (VI) is well documented [1].

Copper is an essential micronutrient for plants and algae, it is a component of several proteins and enzymes involved in a variety of metabolic pathways [2] but it also can be a toxic element when applied in amounts higher than its particular level. Copper has high aquatic toxicity at environmentally relevant concentrations and increasing use as a replacement for tributyltin in antifouling biocides [3, 4].

Metal accumulation by micro-organisms has received much attention in recent years due to their potential application for both detoxification and metal recovery from industrial wastes. Filamentous fungi may be better

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suited for this purpose than other microbial groups, because of their high metals tolerance, wall binding capacity and intracellular metal uptake capabilities. Fungi can accumulate metal by physico-chemical and biological mechanisms. Strong biosorbent behavior of certain micro-organisms towards metallic ions is a function of the chemical make-up of the microbial cells [1, 5], this stage is very rapid and occurs in a short time after the biomass comes into contact with the metal solution [6].

Fungi may survive toxic effects of heavy metals by means of a detoxification mechanism and these are designated as resistant. Metal-resistant fungi are belonging to genera *Aspergillus, Penicillium, Alternaria, Geotrichum, Fusarium, Rhizopus, Monilia* and *Trichoderma* [7]. So far some studies have been conducted on the fungal biosorption of zinc, cadmium, copper, lead and mercury [8-10], but little of them investigated the biosorption of Cr (VI) from aqueous solutions [9].Conventional and classical methods of studying a process by maintaining other factors involved at an unspecified constant level does not depict the combined effect of all the factors involved. This method is also time consuming. These limitations of a classical method can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design such as Response Surface Methodology (RSM) [11].

Response Surface Methodology (RSM) consists of a group of mathematical and statistical methods that can be used to define the relationships between the response and the independent variables. RSM defines the effect of the independent variables, alone or in combination. Therefore, the present study was designed to investigate the potentialities of the marine fungal biomass as a resistant and a cost effective metal biosorbent for Cu (II) and Cr (VI) removal from aqueous solutions. The influences of individual parameters as well as the interactions between them were explained using RSM.

MATERIALS AND METHODS

Microorganisms and Growth Media: The fungi used in this study were isolated from sea water samples collected from a heavy metal polluted area in the Mediterranean Sea, Alexandria, Egypt during February, 2009. The most promising fungus in the term of heavy metal removal was identified based on morphology, physiology and biochemical characteristics. This identification was verified at Regional Center for Mycology and Biotechnology (RCMB), at Al-Azhar University -Cairo-Egypt. The fungus named *Trichoderma viride*. It was maintained on Malt Extract Agar medium (MEA).

Cultivation of *Trichoderma viride* for Biomass Production: The fungal strain was grown on MEA for approximately 4 days. The biomass production was carried out as described by Awofolu *et al.* [12].

Bioaccumulation and Biosorption Studies: One gram (1000 mg) L^{-1} of chromium and cupper standard stock solutions were prepared from reagent grade $K_2Cr_2O_7$ and $CuSO_4.5H_2O$. Working standard solutions used throughout the different experimental trials were prepared by dilution of the stock. Deionized water is used during the whole work. The basal metal solutions (100 mL) were containing 125 mg L^{-1} of Cu (II) or Cr (VI) taken in 250 ml Erlenmeyer flasks amended with 0.375 g of fungal pellets. The flasks were kept under agitation in a rotating orbital shaker at 150 rpm for 45 min and then the metal concentration was determined.

Metal Determination: Fungal suspension was filtered using 0.45 μm membrane filter and residual metal was determined using atomic absorption spectrophotometer. The amount of metal taken up by the biomass was calculated as the difference between the initial and final concentration of the metal in the aqueous solution.

Transmittion Electron Microscopy (TEM): The fungus was prepared for fixation and dehydration procedures using the programmable Electron Microscopy Science (EMS) tissue processor model (Lynx TMel) at the Regional Center for Mycology and Biotechnology (RCMB). at Al-Azhar University -Cairo-Egypt according to Zain [13]. Stained sections were examined with a JEOL 1010 Transmission Electron Microscope at 80 kv at the (RCMB) according to Reynolds [14]. Transmittion Electron Micrographs (TEM) of the control hyphae and conidia of *T. viride* were compared with those loaded with Cu (II) and Cr (VI).

Factorial Experimental Design: A 2^4 full-factorial experimental design with nine replicates at the center point, were employed in this study. Thus a total of 31 experiments were performed. The analysis focused on how the Cr (VI) removal efficiency is influenced by independent variables, i.e., time (X_1), pH (X_2), adsorbent dosage (X_3) and Cr (VI) concentration (X_4). The range and level of these independent parameters were summarized in Table 1. For statistical calculations, the variables Xi were coded as xi according to the following relationship:

$$\eta = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i X_i$$

Table 1: Experimental range and levels of independent process parameters affecting Cr(VI) removal efficiency by *Trichoderma viride*

Independent variable	Range and level				
	$-\alpha$	-1	0	$+1$	$+\alpha$
Time(X_1,min)	15	30	45	60	75
pH(X_2)	2	4	6	8	10
Biosorbent dosage(X_3,g/L)	1.25	2.5	3.75	5.0	6.25
Cr(VI), concentration(X_4,mg/L)	75	100	125	150	175

Where η is the dependent output variable (maximum removal efficiency of Cr (VI)), Xi are the levels of the independent variables; β_0 is the regression coefficient at center point; β_i are linear coefficients; β_{ii} are second-order interaction coefficients. The quality of the fit of the model equation was expressed by the coefficient determination of R^2. The results of the experimental design were studied and interpreted by statistical software MINITAB 14 (PA, USA) to estimate the response of the dependent variable.

RESULTS

Screening for Heavy Metal Removal Efficiency: In this study, several marine fungi were submitted to a preliminary experiment to screen their efficiency for Cu (II) and Cr(VI) removal.One of the isolates named *T. viride*, was selected for further investigations due to its highest activities in the term of heavy metal removal efficiency (62.14%).

Transmition Electron Microscopy (TEM) Studies: Transmition Electron Microscopy (TEM) micrographs of the control hyphae and conidia of *T. viride* and those loaded with Cu (II) and Cr (VI) are compared in Fig. 1. The fresh pellets have a highly porous hyphae and conidia. The appearance of the hyphae loaded with chromium is completely different from that loaded with copper. Moreover, Cr (VI) accumulated within the cells is higher than the accumulated Cu (II) and did not affect the cell structure. Therefore, Cr (VI) removal from aqueous solution by *T. viride* was optimized by (RSM).

Response Surface Methodology: The most important parameters, which affect the efficiency of a biosorbent, are contact time, pH, biosorbent dosage (bd) and heavy metal concentration. In order to study the combined effect of these factors, experiments were performed at different combinations of these parameters using statistically designed experiments. The ranges studied were given in
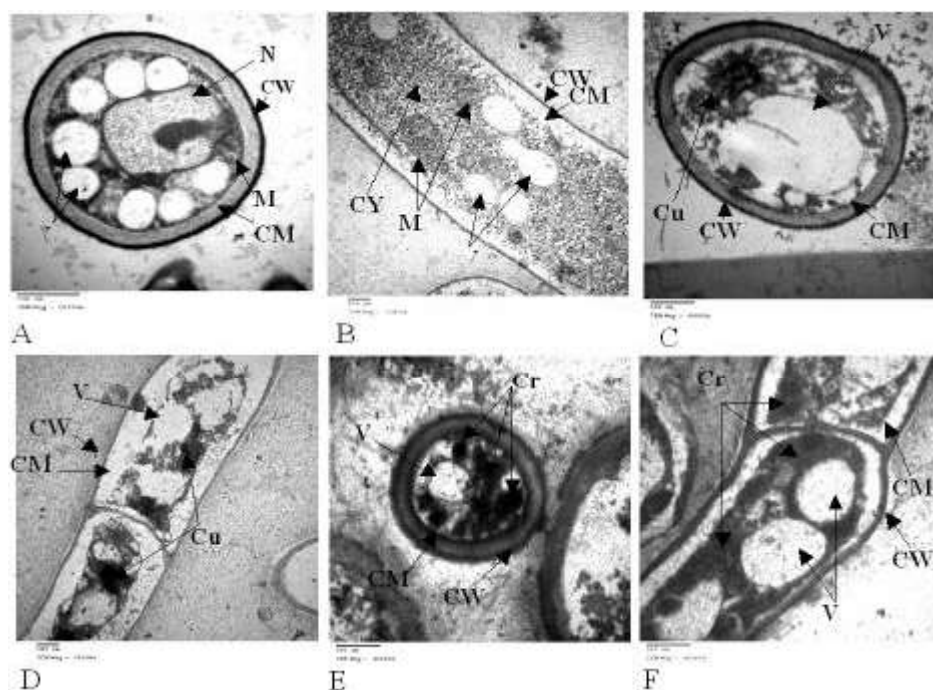

Fig. 1: Transmission Electron Micrograph of *Trichoderma viride* (A-control conidia*:* B-control hypha*);* (C-D) Electron micrographs of *Trichoderma viride* cells exposed to 125mgL^{-1} Cu (II). (E and F)Electron micrographs of *Trichoderma viride* cells exposed to 125mgL^{-1}Cr(VI). Note the changes in the shape of conidia and hypha. Also conidia and hypha loaded more Cr(VI) than Cu (II)
(CW): cell wall, (CM): cell membrane, (V): vacuoles, (N): nucleus, (M): mitochondria, (CY): cytoplasm, (Cu): copper and (Cr): chromium.
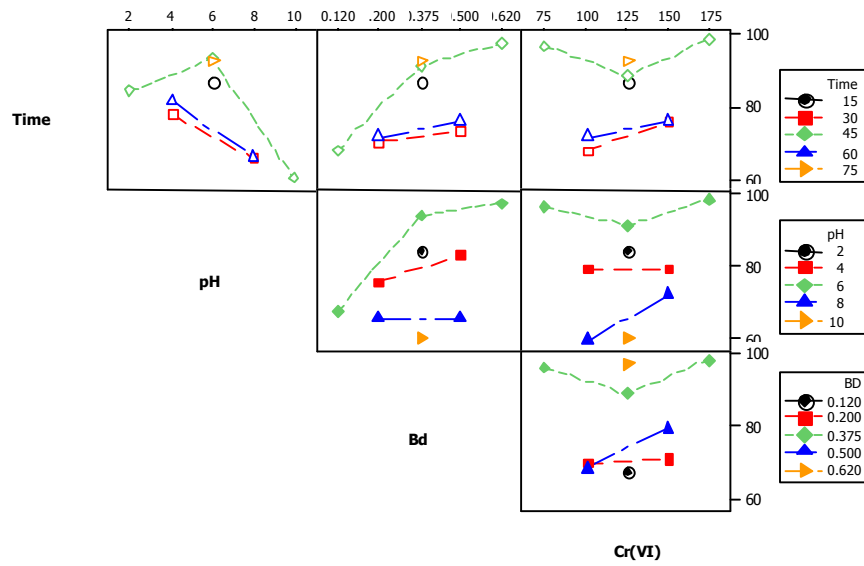
Fig. 2: Interaction between the different variables affecting Cr(VI) removal effeciency by *Trichoderma viride*
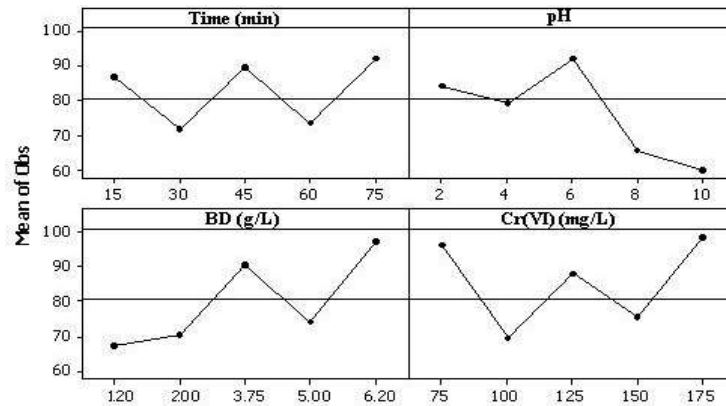

Fig. 3: Main effects plot of variables for maximum Cr(VI) removal effeciency by *Trichoderma viride*
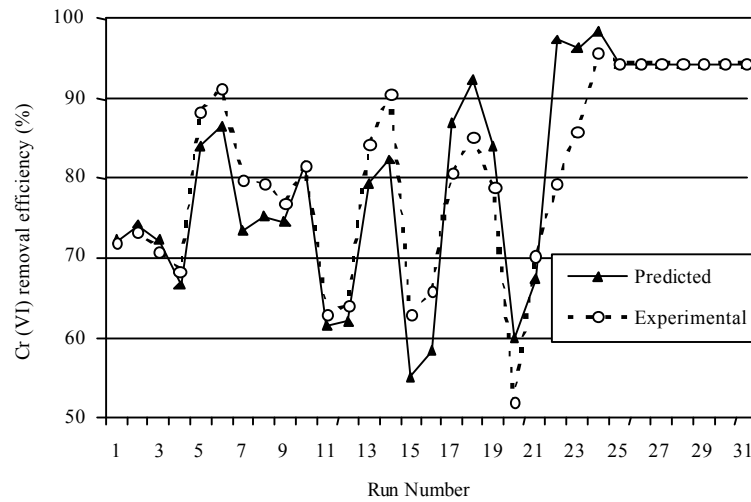

Fig. 4: Comparison of experimental and predicted removal efficiency of Cr(VI) by *Trichoderma viride*

Table 2: Values of the process parameters for maximum Cr(VI) removal effeciency by *Trichoderma viride*

Parameter	Values
η (Removal Efficiency, %)	98.44
X_1 (Time, min)	45.00
X_2 (pH)	6.00
X_3 (Biosorbent Dosage, g/L⁾	3.75
Cr (VI), concentration (X_4,mg/L)	175

Table 1. It was observed that the removal efficiency of Cr(VI) increased as the biosorbent dosage increased. Using the experimental results, the regression model equation relating the removal efficiency and process parameters was developed and expressed in the following equation:

$$\eta = 94.313 + 2.184\,X_1 - 13.407\,X_2 + 4.421\,X_3 + 4.813\,X_4 - 11.542\,X_1^2 - 29.017\,X_2^2 - 19.57\,X_3^2 - 3.622\,X_4^2 - 3.533\,X_1 X_2 + 1.423\,X_1 X_3 - 3.323\,X_1 X_4 - 6.024\,X_2 X_3 + 12.703\,X_2 X_4 + 7.43\,X_3 X_4.$$

Apart from the linear effect of the parameter for the Cr(VI) removal, the RSM also gives an insight into the quadratic and interaction effect of the parameters [15]. The significance of these interaction effects between the parameters (Fig. 2) would have been lost if the experiments were carried out by conventional methods. The values of the process parameters for the maximum removal efficiency are shown in Fig.3 and Table 2. These results were in close agreement with those obtained from the response surface analysis, confirming that the RSM could be effectively used to optimize the process parameters in complex processes using the statistical design of experiments. The predicted values (using the model equation) were compared with experimental result and the data are shown in Fig. 4 which depicted the experimental and model predicted removal efficiencies.

DISCUSSION

In recent years, there has been an increasing research interest in microorganisms that are able to transform the highly toxic and water-soluble hexavalent chromium to the less toxic and insoluble trivalent chromium. Cr (III) is more stable and is approximately 100 times less toxic and 1000 times less mutagenic than Cr (VI), Furthermore, Cr (III) is an essential trace element necessary for glucose, lipid and amino acid metabolism as well as a popular dietary supplement [1].

As mentioned previously, Cu (II) accumulation causes some changes in the conidial and mycelia structures of *T. viride*, so it was excluded and the study was carried out to optimize the Cr (VI) removal efficiency by a marine isolate named *T. viride*. Micro-organisms isolated from industrial processes and polluted environments with high metal concentrations exhibit considerable tolerance to these elements. This tolerance may be due to abiotic factors (pH, temperature, nutrients in the environment or growth media) or to the physiological and genetic adaptations of the micro-organism [16].

Heavy metal removal in a batch system usually depends on several abiotic parameters. The maximum removal of Cr (VI) was found to occur after 45 min which indicates that longer contact time between the metal and biosorbent resulted in higher metal removal efficiency. Similar trend was observed by Vankar and Bajpa [1]. It has been also reported that biosorption capacities for heavy metals are strongly pH sensitive. Maximum biosorption occurred at pH 6.00. This is due to the fact that sorption of heavy metals from aqueous solutions depends on properties of abiosorbent and molecules of abiosorbate transfer from the solution to the solid phase [17].

It was observed that the removal efficiency of Cr (VI) increases as the biosorbent dosage increases (3.75gL⁻¹). In living cells, the biosorption included both metabolism dependent and independent processes. Metabolism independent uptake process essentially involves cell surface binding through ionic and chemical interaction, while dependent process deals with the binding of both the surfaces followed by intracellular accumulation. This may be due to the increase in the available active surface area of the biosorbent. The removal efficiency of Cr (VI) decreases with the increase in Cr (VI) concentration, this could be attributed to unavailability of surface area of the biosorbent to the increasing number of Cr (VI) molecules. The associated *P*-value is used to judge whether *F* Statistics is large enough to indicate statistical significance. A *P*-value lowers than 0.05 indicates that the model is considered to be statistically significant [18]. The *P*-values for some of the regressions were lower than 0.05. A similar trend was observed for heavy metal removal using biosorbent by Gopal *et al.* [19].

The fit of the model is expressed by coefficient of determination, R^2, which was calculated to be 0.818. The closer the R^2 value to 1.00 the stronger the model is and the better it predicts response.Accordingly, our calculated R^2 value indicated that the model could explain

81.8 % of the variability in the response. Matching the predicted Cr (VI) removal (100%) and the experimental results (98.44%) under optimal conditions proved the accuracy and validity of the model. According to these results, it can be predicted that the optimum parameters for Cr (VI) bioremediation by the non pathogenic marine fungus *T. viride* are: Time 45 min; pH 6 and biosrbent dosage 3.75gL^{-1} as well as Cr (VI) concentration 175 mgL^{-1}.

In conclusion, the foregoing discussion indicated the highest potential of *T. viride* for Cr (VI) removal. Living mycelia of the non pathogenic fungus exhibited the highest chromium removal capacity of 4.66 mg/g at pH 6 after 45 min. Thus *T.viride* appears to be a good candidate organism for industrial application in mycoremediation of Cr (VI) from contaminated water systems.

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