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Optimization of Chromium and Copper Ions Uptake by *Aspergillus Terreus* Strain Using Different Techniques

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Abstract. The heavy metal resistant isolate FR1 was identified using phenotypic and genotypic characteristics; it was classified as *Aspergillus terreus* strain. Lactose and yeast extract were favorable for Cr^{+5} and Cu^{+2} ions biosorption by tested strain after 6 days incubation period. The positive significant variables (yeast extract, temperature & inoculum size or lactose, yeast extract, temperature & incubation period) affecting heavy metals biosorption using Placket-Burman statistical experimental design in presence Cr^{+5} and Cu^{+2} were further optimized by using response surface methodology. It was found that the highest growth of strain in presence of Cr^{+5} and Cu^{+2} (4.31 and 3.69 gL^{-1}) were attained at 0.15% yeast extract, 2% inoculum size & incubated at 28°C and 0.65 % lactose, 0.15% yeast extract, an incubation temperature of 30 °C and 8 days incubation period, respectively. There is no significant difference between absorption and adsorption mechanism for reduction of heavy metals from Egyptian phosphatic fertilizer sample.

Keywords: Biosorption; *Aspergillus terreus;* Placket-Burman design; response surface methodology; Cr^{+5} and Cu^{+2} ions.

1 Introduction

Metals are toxic to all biological systems from microbial to plant and animal, with microorganisms affected more so than other systems, due, in part, to their small size and direct involvement with their environment [1-2]. Metal toxicity negatively impacts all cellular processes, influencing metabolism, genetic fidelity and growth. Loss of bacterial populations in metal-contaminated soils impacts elemental cycling, organic remediation efforts, plant growth and soil structure. Human activities such as industrial production, mining, agriculture and transportation release a high amount of heavy metals to the biosphere. The primary sources of metal pollution are the burning of fossil fuels, smelting of metal like ores, municipal wastes, fertilizers, pesticides and sewage [3-5]. Heavy metals are accumulated into soils and plants and could have a negative influence on physiological activities of plants and cause for the reductions in plant growth, dry matter accumulation and yield [6]. Vegetables are vital to the human diet and in particular provide the well-known trace elements and heavy metals. Minor or trace elements are essential for good health if they come from an organic or plant source. In contrast, if they come from an inorganic or metallic source, they become toxic. The processes of plant growth depend on the cycle of nutrients including trace elements, from soil to plant. Vegetables, especially leafy vegetables, accumulate higher amounts of heavy metals because they absorb these metals in their leaves [7]. They showed that the Cd, Pb and Hg contents of the soils had increased significantly with the addition of fertilizer by the 14-60% over the control soil. Root and shoot, accumulation of the heavy metals by the plants had also increased after fertilizer application, with Cd and Pb being particularly high. From among the metals, Cd showed the highest transfer ratio from soil to plant tissues [8]. The rate at which heavy metals are accumulated in the soil depends on the physiochemical properties of the soil and the relative efficiency of crops to remove the metals from the soil. Heavy metals accumulated in cultivated soils can be transferred to humans through various exposure pathways causing adverse effects on human health [9]. Bioremediation can be separated into two categories, biosorption and bioaccumulation. Biosorption is a passive adsorption mechanism that is fast and reversible [10]. The metals are retained by means of physicochemical interaction (e.g., ion exchange, adsorption, complexation, precipitation, and crystallization) between the metal and the

functional groups present on the cell surface [11]. Both living and dead biomass can occur for biosorption because it is independent of cell metabolism. On the other hand, bioaccumulation includes both intra- and extracellular processes where passive uptake plays only a limited and not very well-defined role, therefore, living biomass can only occur for bioaccumulation [12]. The complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cell. The biosorption mechanisms are various and are not fully understood. They may be classified according to various criteria [13]. I. According to the dependence on the cell's metabolism, biosorption mechanisms can be divided into: a. Active metabolism-dependent and b. passive metabolism-independent. II. According to the location where the metal removed from solution is found, biosorption can be classified as: a. Extra- cellular accumulation/ precipitation, b. Cell surface sorption/ precipitation and c. Intracellular accumulation. The filamentous fungi, *Aspergillus* sp. and *Rhizopus* sp., *Fusarium* sp., *Penicillium* sp. and *Cephalosporium* sp. isolated from metal-contaminated agricultural soil were capability to biosorption of different heavy metals such as Cu,Cd, Pb and Cr [14-16]. The pH value of the solution is reported to influence the composition of the binding site on the surface of biomass as well as the chemical state of metal in solution [17]. The most of the microbial surfaces are negatively charged due to the ionization of functional group thereby contributing to metal binding [18]. The cell surface copper binding sites and the availability of copper in the solution are influenced by pH. Little biosorption of Cu(II) ions was observed at low pH due to the competition between hydrogen ions and copper ions on the biosorption active sites. The increase of pH resulted in an increased negative charge on the surface of the cell, which favored electrochemical attraction and adsorption of copper [19].

The present study aimed to screen and evaluate the factors affecting on the reduction of heavy metal ions by the identified fungal isolate using 18S rRNA sequence, and applied this strain for removal of some heavy metal from Egyptian phosphate fertilizer sample.

2 Materials and Methods

2.1 Microorganism and Molecular Identification

Aspergillus sp. FR1 was used in the present investigation as heavy metals removing isolate, which was previously isolated from Egyptian phosphatic fertilizers, and identified based on phenotypic characterization by Abd El Hameed *et al* [20]. The fungal culture slants were maintained at 5°C on potato-dextrose agar (PDA) [21] with the following composition (gL^{-1}): potatoes, 200; dextrose, 20; agar, 20 and adjusted to pH 5.0 after incubation at 30°C for 24 - 48 h. The fungal genomic DNA was extracted from the culture in PDA medium by using Gene Jet genomic DNA purification Kit (Thermo) according to the manufacturer's instructions. Amplification of 18S rDNA by PCR was done using universal fungi primer forward (ITS1) F (5`- TCC GTA GGT GAA CCT GCG G -3 ´ ) and reverse (ITS4) R (5`- TCC TCC GCT TAT TGA TAT GC -3 ´) described by [22]. Amplification was carried out in a 50 µl reaction volume. The thermal cycle (PCR) steps were applied as follows; 10 min initial denaturation at 95°C, followed by 40 cycles of 15 sec denaturation at 95°C, 30 sec primer annealing at 60°C, 30 sec extension at 72°C and a final 10 min extension at 72°C. The amplified DNA fragment was separated on 1.2% (w/v) agarose gel electrophoresis, eluted and purified using the Qiaquick gel extraction kit (Qiagen, Germany) following the manufacturer's protocol [23]. The purified PCR product was sequenced using the ABI 3730xl DNA sequencer (GATC Company). Sequence data of partial 16S rDNA was aligned and analyzed for finding the closest homologous microbes. The unknown query 18S rRNA nucleotide sequence was compared to nucleotide data bases using BLASTN program that is available from the National Center for Biotechnology Information [24] and retrieved from Gene Bank database. Then multiple sequence alignment was developed for these homologous sequences using the algorithm described in CLUSTAL Omega. A phylogenetic tree was then drawn using the Neighbor joining method.

2.2 Heavy Metals Biosorption by the Tested Strain

Batch experiments were performed in 250 ml plugged Erlenmeyer flasks, each containing 100 ml sterile modified Czapek Dox broth medium [20] with the following composition (gL^{-1}): Sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; KCl, 0.5; $FeSO_4$, 0.1; $MgSO_4.7H_2O$, 0.5; Heavy metal ion (Cr^{+5} or Cu^{+2}), 150 ppm and adjusted

to pH 5.0. *The above medium composition was modified by addition of different metal ions to heavy metals biosorption and inoculated with 2% of standard inoculum for the tested fungal strain which was incubated at 30°C on rotary shaker at 150 rpm for 6 days. The cultural medium was filtrated in order to determine the growth. All the experiments were carried out at least in triplicate.

2.3 Nutritional Factors

This experiment was performed to study the effect of different carbon sources on heavy metals biosorption by tested fungus. Therefore, the appropriate carbon source was selected by replacing the original carbon source of the used medium (sucrose) with equivalent carbon amount of each of the tested carbon source (glucose, sucrose, fructose and lactose) to eliminate errors which may occur as a result of differences in carbon concentrations in each source. The previous procedure of propagation was used. To detect the adequate nitrogen source for heavy metal removal by selected strain, the prescribed nitrogen source of the fermentation medium($NaNO_3$) was replaced by equivalent nitrogen amount of each of the tested organic nitrogen source (beef extract, peptone, yeast extract and tryptone) and inorganic nitrogen source ($(NH_4)_2SO_4$, $NaNO_3$ and urea). At the end of incubation period, the growth of fungal cultures was filtrated and determined as cell dry weight.

2.4 Statistical Experimental Designs for Evaluation of the Factors Affecting Growth of Tested Strain (Biosorption of Heavy Metals)

2.4.1 Screening of Most Significant Fermentation Parameters Using Plackett-Burman Design

The most heavy metal (Cr^{+5} or Cu^{+2}) removal by Plackett-Burman design. Plackett-Burman design using statistical software package Design-Expert software 9.0.0 (Stat-Ease, Inc., Minneapolis, MN 55413,USA 2014), was used to evaluate the relative importance of physical and nutritional factors for heavy metal absorption by *A. terreus* FR1. A total of 7 (n) variables including 2 nutritional (lactose concentration as a sole carbon source and yeast extract concentration as a nitrogen source) and 5 environmental factors (pH, temperature, inoculum size, incubation period and agitation speed) with 4 dummy variables (which will provide an adequate estimate of the error) were studied in 11 (n+1) experiments as shown in Table 1 for tested fungi [25]. All the trials were performed in duplicate and the average of observation was used as the response of the design. Each variable represented at 2 levels, high and low, was denoted by (+) and (-) signs, respectively. Each row represented a trial run and each column represented an independent variable.

Table 1. Plackett-Burman experimental design matrix and the actual values of cell dry weight in presence of Cr^{+5} and Cu^{+2} by *A. terreus* FR1.

Run	Variables											Cell dry weight (gL^{-1})	
no.	A	B	C	D	E	F	G	H	J	K	L	Cr^{+5}	Cu^{+2}
1	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	0.595	0.532
2	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	0.574	0.509
3	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	0.816	0.695
4	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	0.855	0.414
5	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	0.502	0.610
6	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	0.507	0.451
7	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.866	0.721
8	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	0.369	0.199
9	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	0.431	0.531
10	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	0.467	0.862
11	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	0.440	0.318
12	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	0.879	0.771

Variables		Real levels	
Symbol		−1	1
Lactose concentration (%)	A	0.85	0.9
Yeast extract concentration (%)	B	0.23	0.25
pH	C	6	7
Temperature (°C)	D	28	30
Agitation speed (rpm)	E	static	100
Inoculum size (%)	F	2	3
Incubation period (h)	G	6	7
Dummy 1	H	–	–
Dummy 2	J	–	–
Dummy 3	K	–	–
Dummy 4	L	–	–

A-G= Nutritional and physical variables, H-L= Dummy variables, -1 = low level of the variable & +1 = high level of the variable.

Plackett–Burman experimental design is based on first order model, which was determined by the following Eq. (1):

$$Y = B_0 + \Sigma B_i x_i \quad (1)$$

where Y is the response (Cell dry weight), B_0 is model intercept and B_i was variables estimates. Effect of each variable was determined by the following equation:

$$E\left(X_i\right) = 2(_+\Sigma M_i — \Sigma M_i^-) \ / N \quad (2)$$

where E (X_i) is tested variable effect and M_{i+} and M_{i-} represent heavy metals removal from trials when variables (X_i) measured were presently at high and low concentrations, respectively and N is the number of trials in Eq. (2).

Standard error (SE) of the concentration effect was square root of the variance of an effect, and the significance level (p-value) of each concentration effect was determined using Student's t-test t (X_i) in Eq. (3).

$$t(X_i) = E(X_i) \ / \ SE \quad (3)$$

where E (X_i) is variable X_i effect.

2.4.2 Central Composite Design (CCD) and Response Surface Methodology (RSM)

After identifying the significant variables for biosorption of both tested heavy metals by *A. terreus* FR1 through a Plackett-Burman design, a central composite design (CCD) was adopted to optimize the major variables. The three or four selected independent variables were studied at 3 different levels (as -1, 0 and +1), and sets of 20 or 30 experiments (batch experiments) were carried out in presence of Cr^{+5} or Cu^{+2} for tested fungi, respectively (Tables 2 and 3). All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in these tables.

Table 2. Central composite design matrix (CCD) of independent variables used in RSM studies and removal Cr^{+5} by *A. terreus* FR1.

Run no.	Variables			Cell dry weight (gL^{-1}) in presence of Cr^{+5}	
	A	B	C	Actual	Predict
1	−1	−1	+1	0.12	2.28
2	0	0	0	4.09	2.89
3	0	0	0	2.35	2.89
4	+1	+1	−1	0.40	0.18
5	0	+1	0	0.38	1.20
6	−1	0	0	4.31	4.07
7	−1	+1	+1	3.44	3.42
8	0	0	0	2.30	2.89
9	+1	−1	+1	3.24	3.46
10	−1	−1	0	2.05	1.33
11	0	0	0	3.31	2.89
12	0	0	−1	3.33	3.39
13	0	0	0	3.36	2.89
14	+1	−1	−1	1.53	1.56
15	−1	−1	−1	3.43	3.74
16	0	0	0	2.10	2.89
17	−1	+1	−1	4.03	3.81
18	+1	+1	+1	3.51	3.15
19	+1	0	0	2.36	2.70
20	0	0	−1	3.36	3.39

Variables	Symbol	Real levels		
		−1	0	+1
Yeast extract conc. (%)	A	0.15	0.19	0.23
Temperature (°C)	B	25	28	30
Inoculum size (%)	C	1	2	3

A-C= Nutritional and physical variables, -1 = low level of the variable, 0= medium level of the variable & +1 = high level of the variable.

The statistical software package Design-Expert software 9.0.0 (Stat-Ease, Inc., Minneapolis, MN 55413, USA 2014), was used to analyze the experimental data. The optimal values of the independent variables that gave theoretical maximum response in Eq. 4 were obtained by maximizing the equation within a definite boundary condition. Biosorption were taken as response (Y) and a multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The relationship of the independent variables and the response was calculated by the second order polynomial equation (Eq. 4).

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^{2} + b_{22}X_2^{2} + b_{33}X_3^{2} + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (4)$$

where Y_i is the predicted response, X_1, X_2, X_3 are independent variables, b_0, is the offset term, b_1, b_2, b_3 are linear effects, b_{11}, b_{22}, b_{33} are squared effects and b_{12}, b_{23}, b_{13} are interaction terms.

2.5 Preparation of Dead Fungal Biomass

The biomass of the *A. terreus* FR1 was prepared in PDA medium. After 48 h, the fungal biomass was separated from medium using filtered through Whattman filter paper. The biomass was dried by the process of drying on hot oven in order to absorb the moisture content at temperature of 120°C for about 3-4 days and was utilized in further biosorption studies [26].

Table 3. Central composite design matrix (CCD) of independent variables used in RSM studies and removal Cu^{+2} by *A. terreus* FR1.

Run no.	Variables				Cell dry weight (gl^{-1}) in presence of Cu^{+2}	
	A	B	C	D	Actual	Predicted
1	−1	+1	0	−1	2.91	2.83
2	0	0	+1	0	2.99	2.99
3	+1	0	+1	0	2.75	2.75
4	0	0	+1	0	2.99	2.99
5	0	0	+1	0	2.99	2.99
6	0	0	+1	0	2.99	2.99
7	0	1	+1	0	2.29	2.17
8	+1	−1	−1	+1	3.19	3.20
9	−1	1	−1	−1	3.42	3.42
10	0	0	+1	+1	3.17	3.17
11	−1	1	−1	+1	3.09	3.19
12	0	0	0	0	3.03	3.03
13	+1	1	−1	+1	3.33	3.33
14	−1	−1	0	−1	3.45	3.51
15	+1	1	−1	−1	3.11	3.06
16	+1	−1	−1	−1	2.88	3.04
17	0	0	+1	0	2.99	2.99
18	−1	−1	0	+1	3.44	3.41
19	0	−1	+1	0	3.69	3.80
20	0	0	+1	0	2.99	2.99
21	+1	1	+1	−1	1.29	1.48
22	0	0	+1	−1	2.63	2.63
23	0	0	+1	0	2.98	2.99
24	−1	−1	−1	+1	2.70	2.60
25	+1	1	0	+1	2.69	2.62
26	+1	−1	0	−1	3.56	3.26
27	−1	+1	0	+1	2.80	2.84
28	−1	+1	−1	−1	2.93	2.94
29	0	0	+1	0	2.99	2.99
30	+1	−1	0	+1	3.59	3.66

Variables	Symbol	Real levels		
		−1	0	+1
Lactose conc. (%)	A	0.45	0.65	0.85
Yeast extract	B	0.15	0.19	0.23
Temperature (°C)	C	25	28	30
Incubation period	D	7	8	9

A-D= Nutritional and physical variables, -1 = low level of the variable, 0= medium level of the variable & +1 = high level of the variable.

2.6 Application on Phosphate Fertilizer Sample by Using Heavy Metal Resistant Fungi

Living or dried dead biomasses of *A. terreus* FR1 were inoculated into 100 ml liquid medium containing tri-phosphate fertilizer sample or plates containing 5g phosphate fertilizer sample. The metal ions residual in samples were determined. The experiment was done in duplicate and the amount of metallic ion biosorbed per gram of biomass (q) and the efficiency of biosorption (E) were calculated.

2.7 Analytical Procedures

Determination of fungal growth (cell dry weight) was performed by filtrating using filter paper No.1, washing twice, and drying at 70°C until constant weight. The residual heavy metals in filtrated fungal culture (supernatant) and tri-phosphate fertilizer sample were determined using inductively coupled plasma-mass spectrometer (ICP-MS) atomic absorption spectrophotometer, CENTRAL lAB., FAC. OF AGRIC., AIN SHAMS UNIV., EGYPT.

2.8 Parameters Calculation

The specific growth rate (μ), doubling time (t_d), Multiplication rate (MR) and number of generations were calculated using the following equations [27-29]. The following formulas were used to calculate these parameters: specific growth rate (μ) (h^{-1}) = (ln X –ln X_0) (t - t_0)$^{-1}$, doubling time (t_d) (h) = ln(2) (μ)$^{-1}$, multiplication rate (MR) = 1 (t_d)$^{-1}$ and number of generation (N) = (t - t_0) (t_d)$^{-1}$
where:

X = Amount of growth after t time (t).

X0 = Amount of growth at the beginning time (t_0).

Specific heavy metal uptake (The amount of metallic ion biosorbed per gram of biomass (q)) was calculated using the following equation [30].

$$\left(\frac{\text{Ci-Cf}}{\text{m}}\right)\times \text{V}$$

where, Ci = initial concentration of the metallic ions (mg L^{-1}); Cf = final concentration of metallic ions (mg L^{-1}); m = dried mass of the biosorbent in the reaction mixture (g) and V = volume of reaction mixture (ml).

Efficiency of biosorption (E) was calculated using following equations [31].

$$\text{E}=\left(\frac{\text{Ci-Cf}}{\text{Ci}}\right)\times 100$$

where, Ci = initial concentration of the metallic ions (mg L^{-1}) and Cf = final concentration of metallic ions (mg L^{-1}).

2.9 Statistical Analysis

The collected data were statistically analyzed using IBM® SPSS® Statistics software [32].

3 Results and Discussion

3.1 Identification of the Most Efficient Heavy Metals Removing Isolates

According to the morphological characteristics (microscopic shape and color of conidia) of fungal isolate FR1, which was subjected to the preliminary classification to be the genus *Aspergillus* according to Barnett and Hunter [33], these isolates showed granular colonies on Czapek's Dox agar. The colonies were flat, with radial grooves. Microscopic observation of the fungal isolate indicated erect conidiophores with globose vesicles bearing chains of conidia (Fig. 1a).

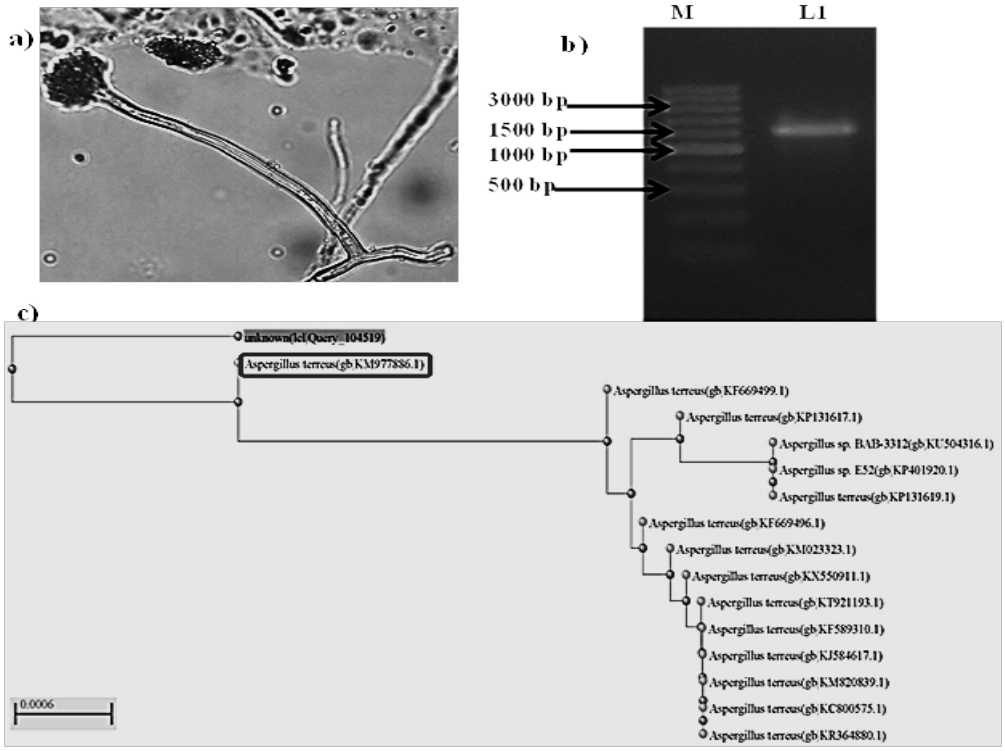

Figure 1. Identification of the most efficient heavy metal removing fungal isolate, a) Morphological identification, b) Agarose gel analysis of PCR amplification product using 18S rRNA primers for *Aspergillus* sp. FR1 isolate, c) Neighbor-joining tree based on 18S rRNA sequences of the genus *Aspergillus* sp. FR1 obtained from BLAST search showing the position of isolate and related strains. M= Markar, L1= *Aspergillus* sp. FR1 isolate.

In this respect, different species of *Aspergillus* have been reported as efficient heavy metals absorption [34]. Moreover, Dwivedi *et al* [35] found that many heavy metal tolerant isolates were identified as *A. niger* (Pb2, Cr10, Ni19, Ni27 and Ni33) and *A. flavus* (Pb7, Pb8, Ni35 and Ni36) by the laboratory culture. In addition, Iram *et al* [36] reported that among all tested fungal strains, few isolates of *Aspergillus flavus* and *A. niger* and *Fusarium* were tolerant to Cr and Pb. The molecular identification of fungal isolate *Aspergillus* sp. FR1 was performed by partial 18S rDNA sequencing. The apparent size of the PCR amplicon was ~ 1500 bp, shown in Fig 1b. The analysis of 18S rRNA gene of the isolate *Aspergillus* sp. FR1 was sequenced with R1 primer at the forward direction and produced 1020 bp which was compared with available 18S ribosomal sequences in the NCBI database site (Gen Bank) using BLASTN. The NCBI database showed the highest percentage of similarity being 99% with *Aspergillus terreus* isolate YF 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (accession number: KM977886.1). Multiple sequence alignment was developed using sequence of the isolate *Aspergillus* sp. FR1_Forw 1020 bp withe the sequence of the highest 15 previous homologous sequences, then a phylogenetic tree was drawn using clustal omega as shown in Fig. 1c. Neighbor joining analysis based on 18S rRNA gene sequences revealed that the isolate *Aspergillus terreus* strain (accession number: KM977886.1) and occupies a distinct pylogenctic position within the representative members of the genus *Aspergillus*, as depicted in Fig. 1c.

3.2 Time Course of Heavy Metal Removing Aspergillus Terreus FR1 Growth

Data illustrated by Fig. 2a show that all the tested strain grew exponentially during the first 6 days of incubation period on metal ions free Czapek Dox broth medium as a control and the same medium supplemented with different metal ions. The growth of the tested fungal strain (expressed as cell dry weight) at the end of exponential phase was found in metal ions free medium and same medium containing Cu^{+2} or Cr^{+5} ions, being 1.189 gL^{-1} and 0.120 or 0.132 gL^{-1}, respectively . The growth parameters were calculated at

the log phase of the growth curves and recorded in Fig. 2b.

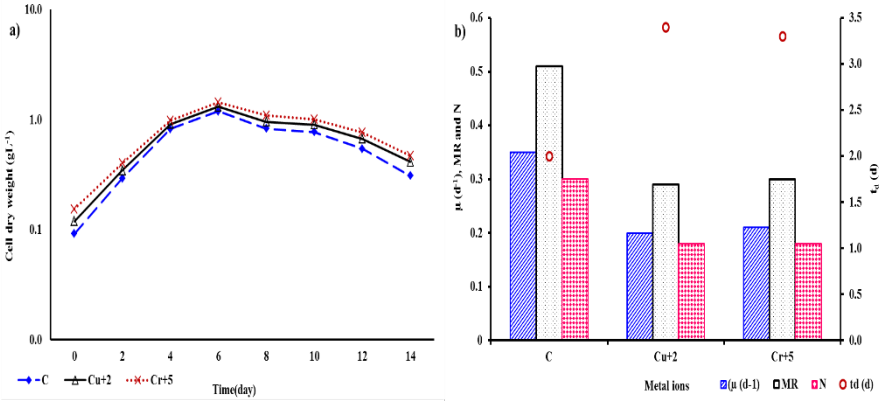

Figure 2. Growth curve a) and growth parameters b) of *Aspergillus terreus* FR1 on Czapek Dox broth medium containing different metal ions at concentration of 150 ppm during 14 days of incubation period at 28°C. C = Control (metal ions free medium), μ= specific growth rate, t_d= doubling time, MR= multiplication rate, N= the number of generation.

Results showed that the specific growth rate (μ) was ranged from 0.35, 0.20 & 0.21 h^{-1}, the doubling time (t_d) was 2.0, 3.4 & 3.3 h, multiplication rate (MR) was 0.51, 0.29 & 0.30 h and the number of generation (N) was 0.30, 0.18 & 0.18 on medium free metal ions and supplemented Cu^{+2} and Cr^{+5} ions, respectively. The statistical analysis revealed that the correlation coefficient (r) between incubation time and growth in log phase on control medium and medium containing different metal ions was positively higher, where it's ranged from 0.92 to 0.99 for tested strain *A. terreus* FR1. The obtained results could be explained by the results found by Leung *et al* [37] that bioaccumulation was generally less at later growth phases due to chemical changes in the mycelium. Also, it is reported that growth *A. niger* and *A. flavus* under suitable conditions produce spherical mycelia which have been used for heavy metals accumulation.

3.3 Optimization of Carbon and Nitrogen Sources for A. Terreus FR1 on Medium Containing Heavy Metal

Data in Fig. 3a clearly show that lactose was the best carbon source for growth *A. terreus* FR1 in presence of Cu^{2+} and Cr^{5+} with concentration of 150 ppm being 0.234 and 0.221 gL^{-1} followed by glucose being 0.203 and 0.211 gL^{-1}, respectively. Whereas the minimum growth of tested strain, was noticed in medium supplemented with fructose, as compared to other tested carbon sources. Generally, it could be stated that Cu^{2+} and Cr^{5} biosorption by *A. terreus* FR1 in presence of lactose were increased about 1.8 and 1.6 fold increase as compared to sucrose as control, respectively. From the obtained results it could be concluded that the biosorption of heavy metals ions from the growing media by the selected fungi were highly correlated to the carbon source, fungal strain, content of the media and the type state free or complex and concentration of the heavy metal ion in the growing media. These results are in agreement with those of Mapolelo and Torto [38] and Yoshida *et al* [39] who observed that glucose was increased the heavy metal reduction rate by *S. cerevisiae* and *C. sorokiniana.* Wang and Chen [40] also described the role of glucose in biosorption depending on the type of strains and the status of metal ions (free or complex), even for the same biomass and for the same metal ions. Maygaonkar and Permeswaran [41] also stated that biosorption of Na^{+} and Mg^{++} by *A. nidulans* in medium supplemented with 1% sucrose was more sources at 28°C for 6 days. cont.=Control, Different letters above the bars indicate significant differences between carbon or nitrogen sources, according to Duncan's [43] at 5% level.

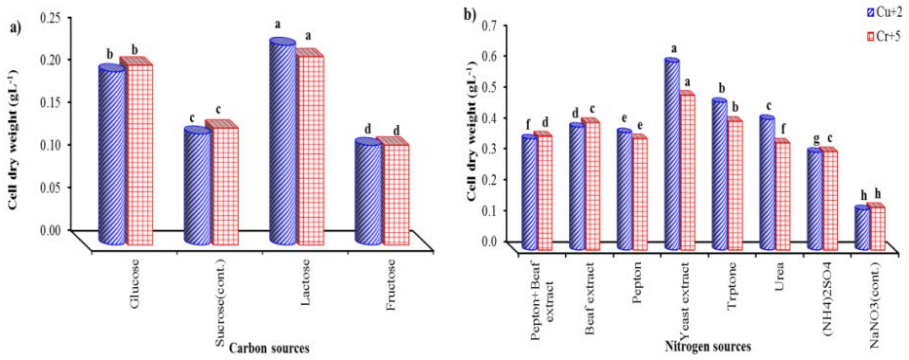

Figure 3. Biosorption of metal ions by *A. terreus* FR1 as influenced by different carbon a) and nitrogen b) favorable than 1% dextrose as carbon source. Whereas, Hirpara *et al* [42] reported that the highest reduction of chromium by *S. rubidaea* was recorded in broth medium supplemented with sucrose at 0.1% concentration.

Results in Fig. 3b clearly show that the sources of nitrogen greatly affected the growth (biosorption of heavy metal) by *A. terreus* FR1. Data showed that yeast extract was the best nitrogen source for *A. terreus* FR1 growth on modified medium supplemented with Cu^{2+} and Cr^{5+} being 0.607 and 0.500 gL^{-1} of cell dry weight followed by tryptone being 0.477 and 0.416 gL^{-1} of cell dry weight, respectively, followed by tryptone. These results could be interrupted on the basis organic nitrogen such as yeast extract and tryptone not only as an organic nitrogen sources but also a source of growth factors and protein which play a vital role in enhancement of the microbial growth. According to the results of Sayer *et al* [44], *A. niger* could adsorb Zn when growing on organic nitrogen (malt extract) with 4% (W/V) $Zn_3(PO_4)_2$. Moreover, Kumar and Riyazudin [45] added mixture of peptone and beef extract as organic nitrogen sources in medium containing different heavy metals (Co, Cd Ni and Pb) for biosorption by *Bacillus* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *A. niger*. So, it could be stated that organic nitrogen sources were more favorable than inorganic sources for growth of tested fungi on medium containing metal ions at 150 ppm. Also, it could be observed that increasing the growth activity of *A. terreus* FR1 on modified medium contains the best nitrogen source (yeast extract) in presences of Cu^{2+} and Cr^{5+} by 4.7 and 3.7 fold as compared to control medium ($NaNO_3$), respectively.

3.4 Statistical Experimental Designs for Evaluation of the Factors Affecting Heavy Metals Biosorption by A. Terreus FR1

3.4.1 Screening of Most Significant Fermentation Parameters Using Plackett-Burman Design

Plackett-Burman screening experimental design was used to determine the influence of independent variables (lactose concentration, yeast extract concentration, pH, temperature, inoculums size, incubation period and agitation speed) on biosorption of heavy metals by *A. terreus* FR1 as shown in Table 1. The design for 12 runs with two levels for each factor. Cell dry weight averages for different trials were given in gL^{-1}. Main effect of each variable on biomass was estimated as difference between both measurements averages made at high (+1) and low levels (−1) of that factor. Data showed a wide variation from 0.369 to 0.879 g CDW L^{-1} and 0.199 to 0.862 g CDW L^{-1} on growth in presence of Cr^{+5} and Cu^{+2}, respectively in 12 experiments due to the strong influence of variables on heavy metals biosorption. In presence Cr^{+5}, maximal biosorption (0.879 g CDW L^{-1}) was achieved during run number 12 with high levels of lactose concentration (0.90 %), pH (7.0), incubation temperature(30°C), inoculum size (3%) and incubation periods (7 d), and low levels of yeast extract concentration (0.23 %) and without agitation speed (static) followed by run number 7, 4 and then 3 (0.866, 0.855 and 0.816 g CDW L^{-1}), respectively. Meanwhile, the highest biosorption of Cu^{2+} (0.862 g CDW L^{-1}) was recorded at run number 10 with high levels of incubation temperature (30°C), inoculum size (3%) and incubation periods (7 d) and low levels of lactose concentration (0.85 %), yeast extract concentration (0.23 %), pH (6.0), without agitation speed (static) followed by run number 12 and 7 (0.771 and 0.721 g CDW L^{-1}), respectively. The lowest removal of all tested metal ions of Cr^{+5} and Cu^{+2} were observed in run number 8. To determine the main effect and the significance of each factor, the estimated effects, coefficients mean squares, F-values, and P-values for the

main effect of each variable, are shown in Table 4. The mean square (variance of effect) showed higher value for Cr^{+5} and Cu^{+2} removal in the presence of yeast extract (0.230 and 0.160). The significance of each coefficient was determined by F- and P-values which are listed in Table 4.

The Model F-value of 382.37 and 10.82 implies the model is significant for Cr^{+5} and Cu^{+2} removal, respectively. There is only a 0.01% chance that an F-value this large could occur due to noise. Variables having a probability value (p-value) less than 0.05 were considered significant.The analyzed data in Table 4 suggests out of 7 different independent variables which were likely to play important role for improving the tested fungal growth for Cr^{+5} and Cu^{+2} removal, 3 variables (yeast extract concentration, temperature and inoculum size) and 4 variables (lactose concentration, yeast extract concentration, temperature and incubation period) significantly affected Cr^{+5} and Cu^{+2} biosorption, which had p-values ranged from 0.014 - 0.029, 0.009 - 0.035, respectively. The coefficient of determination (R^2) was 0.99 and 0.97 (which means that 99 and 97 % of the total variation is explained by the model) for Cr^{+5} and Cu^{+2}, respectively which indicate a satisfactory.

Table 4. Statistical analysis of variance (ANOVA) of Plackett-Burman design for heavy metal biosorption by *A. terreus* FR1.

Variables	Metal ions							
	Cr^{5+}				Cu^{2+}			
	Mean square	df	F–Value	p–value (Prob>F)	Mean square	df	F–Value	p–value (Prob>F)
Model	0.040	11	382.37	0.040*	0.049	11	10.82	0.038*
A– Lactose	0.006	1	52.25	0.088	0.062	1	13.54	0.035*
B– Yeast extract	0.230	1	2163.63	0.014*	0.160	1	34.77	0.009*
C– pH	0.006	1	54.05	0.086	0.017	1	3.81	0.146
D– Temperature	0.081	1	769.06	0.023*	0.068	1	14.85	0.031*
E– Agitation speed	0.014	1	129.53	0.057	0.002	1	0.55	0.513
F–Inoculum size	0.052	1	490.41	0.029*	0.001	1	0.13	0.744
G– Incubation period	0.014	1	129.53	0.056	0.086	1	18.89	0.023*
H–Dummy1	0.0001	1	1.00	0.500	0.000	1	0.01	0.942
J–Dummy2	0.000	1	0.00	1.000	0.008	1	2.94	0.229
K–Dummy3	0.004	1	35.22	0.106	0.001	1	0.16	0.757
L–Dummy4	0.000	1	0.00	1.000	0.005	1	6.19	0.243
Std. Dev.			0.01				0.07	
Mean			0.61				0.55	
R^2			0.99				0.97	

df= degree of freedom, *Significant at 5% level ($P \leqslant 0.05$), Cr^{+5} Sign. = B- Yeast extract conc, D-Temperature and F-Inoculum size, Cu^{+2} Sign. = A- Lactose, B- Yeast extract, D- Temperature and G-Incubation period.

Representation of the process model and a high correlation between the experimental and predicted values. By using Design Expert, the equation obtained for Plackett-Burman design (first order model) was as follows:

$$\begin{aligned} Y\ Cr^{+5}{}_{removal} = {} & 0.609 + 0.021(lactoseconc.) - 0.138(yeast\ extractconc.) + \\ & 0.034\ \left(agitation\ speed\right) + 0.022\ \left(pH\right) - 0.082\ \left(temperature\right) - \\ & 0.066\ \left(inoculum\ size\right) - 0.034\ \left(incubation\ period\right) + 0.003\ \left(Dummy\ 1\right) - \\ & 0.0000003\ \left(Dummy\ 2\right) + 0.0000003\ \left(Dummy\ 3\right) - 0.018\ \left(Dummy\ 4\right) \end{aligned} \quad (5)$$

$$
\begin{aligned}
Y_{Cu^{+2}\,removal} = {} & 0.551 + -0.072(lactoseconc.) - \\
& 0.115(yeast\ extractconc.) - 0.014\ \left(agitation\ speed\right) \\
& - 0.038\ \left(pH\right)\ - 0.075\ \left(temperature\right)\ 0.007\ \left(inoculum\ size\right)\ + \\
& 0.085\ \left(incubation\ period\right)\ - 0.002\ \left(Dummy\ 1\right)\ + 0.020\ \left(Dummy\ 2\right)\ + \\
& 0.026\ \left(Dummy\ 3\right) + 0.0080\ \left(Dummy\ 4\right)
\end{aligned}
\tag{6}
$$

The positive coefficients for all factors suggest a linear effect on the increment in activity of Cr^{+5} biosorption, except yeast extract concentration, temperature, inoculum size, incubation period and Dummy 4, which were negative coefficients imply a linear effect on the decrement in Cr^{+5} biosorption, also 1 (incubation period, Dummy 2 and Dummy 3) and 5 (lactose conc., yeast extract conc., agitation speed, pH, temperature, Dummy 1) factors gave positive and negative impact on Cu^{+2} biosorption, respectively (Fig. 4).

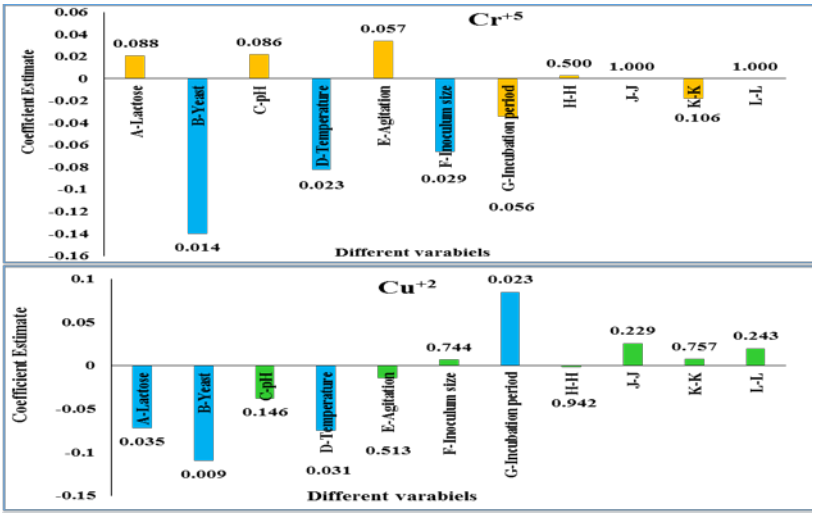

Figure 4. The extent of positive and negative effects of the eleven factors on heavy metals removal by *A. terreus* FR1 and the corresponding p-values (number above each bar) of the factors showing their significance (if $p < 0.05$) based on ANOVA according to the Plackett-Burman experimental results. The blue bars indicate the positively significant factors.

The pareto graph (Fig. 5) was used to show the effect of all variables on heavy metal biosorption in Plackett-Burman experimental design. The percentage of the main effect or contribution for twelve variables was ranging from 1.00 - 80.57% and 1.10 - 22% % for Cr^{+5} and Cu^{+2} removal, respectively. Among the twelve variables, yeast extract concentration, temperature and inoculum size showed the highest negative significant effect (80.6, 46.5 and 40 %), for Cr^{+5} biosorption, respectively. Whereas, yeast extract concentration, temperature and lactose showed the highest negative significance by 22, 14.78 and 13% and 15.15% and incubation period showed the highest positive significant (16.8%) for Cu^{+2} removal, respectively. These significant factors identified by the Plackett-Burman design are considered for the next stage in the medium optimization using response surface optimization technique for the future study. The results obtained are in concordance with Fujii and Fukunag [46] and Shivakumar *et al* [47] revealed that the optimal pH for *Aspergillus* and *Penicillium* grew in medium containing Cu was pH 5-6. Whereas the values of pH for maximum Cu^{2+} removal were 6- 6.5 for *A. niger* and *A. oryzae* and *P. squamosus* [31], 5 for *T. versicolor* and *F. oxisporum* and **5.5** for *P. hirsutum* [48]. The cell surface copper binding sites and the availability of copper in the solution are influenced by pH. Little biosorption of Cu(II) ions was observed at low pH due to the competition between hydrogen ions and copper ions on the biosorption active sites [19]. The increase of pH resulted in an increased negative charge on the surface of the cell, which favored electrochemical attraction and adsorption of copper. In addition, Simonescu and Ferdeş [31] observed that the optimal temperature for the Cu(II) removal by the tested fungal strains was 30°C.

Biosorptive removal of Cr^{+6} by *Rhizopus arrhizus* at 100 rpm and by *Rhizopus* sp. [**49**]. Generally, it could be concluded that 3 (yeast extract concentration, temperature and inoculum size) and 4 (yeast extract and lactose concentration, temperature and incubation period) variables were selected for fungal growth on Cr^{+5} and Cu^{+2}, respectively and their possible interactive effects on growth in presence of heavy metals (biosorption) were evaluated by response surface methodology.

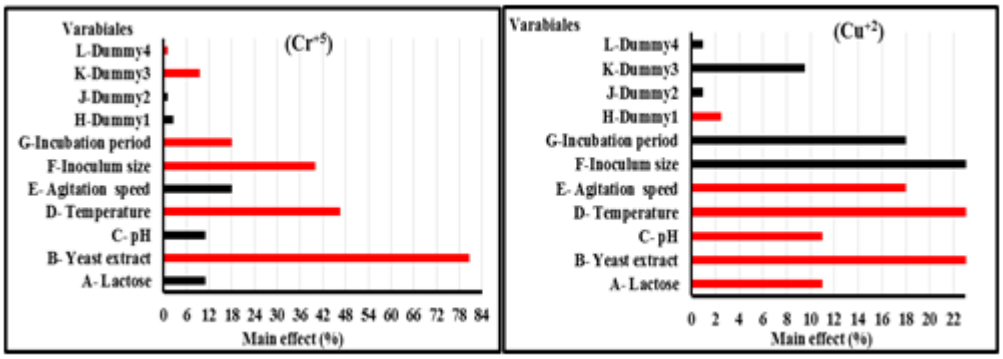

Figure 5. Pareto graph showing contribution effect % of different variables on heavy metals removal by *A. terreus* FR1 based on the observation of Plackett-Burman design (the black color represents positive effects and the red color represents negative effects).

3.4.2 Central Composite Design (CCD) and Response Surface Methodology (RSM)

After selecting the most significant variables influencing heavy metals biosorption (grow in presence of metal ions) by *A. terreus* FR1 showing confidence level 97 - 99% using Plackett–Burman design, a central composite design (CCD) was performed to determine the optimal levels and the interactions among the selected significant variables. In this study, a total of 20 and/or 30 experiments with 3 and/or 4 different combination of yeast extract concentration (A), incubation temperature (B) and inoculum size (C) and/or lactose concentration (A), yeast extract concentration (B), incubation temperature (C) and incubation period (D) and were performed and the results of experiments for studying the effects of three and /or four independent variables on growth in presence of Cr^{+5} and/ or Cu^{+2} at three different levels coded as -1, 0, and +1 are presented along with actual response, respectively (Table 2 and 3). The data showed great variation in growth activity. Data recorded in Table 2 showed that the highest growth activity (4.31 gL^{-1}) on Cr^{+5} was achieved at run number 6 (containing 0.15% yeast extract and inoculated with 2% inoculum size then incubated at 28°C) followed by run numbers 2 and 17. The minimum growth activity (0.12 gL^{-1}) was recorded in run number 1. While Data presented in Table 3 show that run numbers 9, 14, 18, 19, 26 and 30 showed a high growth activity in presence of Cu^{+2} ($\geqslant$3.69 gL^{-1}). The maximum growth (3.69 gL^{-1}) was achieved at run number 19 in the presence of 0.65 % lactose, 0.15% yeast extract, an incubation temperature of 30 °C and 8 days incubation period, while the minimum growth (1.29 gL^{-1}) was observed in run number 21. The statistical significance of the model was checked by F-test and ANOVA for the response surface quadratic model are summarized in Table 5. The model F-value of 47.10 and 4.22 implied the model for Cu^{+2} and Cr^{+5} were significant, respectively. A p-value was also less than 0.0001 demonstrate the model was highly significant and indicating that there was only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The determination coefficient R^2 of the model was 0.96 in presence of Cu^{+2} and 0.79 in presence of Cr^{+5}, indicated that 96% and 79% of the total variations were explained by the model and revealed good agreement between the experimental results and the predicted values calculated from the model. Therefore, the present R^2 value hinted that the model is reliable for growth activity in presence of metal ions in the present study. Interpretation of the data was based on the signs (positive or negative effect on the response) and statistical significance of coefficients ($P < 0.05$). Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient).

Table 5. Statistical analysis of variance (ANOVA) of C.C.D design for removal Cu^{+2} and Cr^{+5} by *A. terreus* FR1.

Variables	Cu^{+2}				Variables	Cr^{+5}			
	Mean square	df	F–Value	p–value (Prob>F)		Mean square	df	F–Value	p–value (Prob>F)
Model	0.55	10	47.1	< 0.0001*	Model	2.62	9	4.2	0.017*
A– Lactose conc.	0.01	1	1.2	0.296	A– Yeast extract conc.	1.62	1	2.6	0.137
B– Yeast extract conc.	0.32	1	27.4	< 0.0001*	B– Incubation temperature	0.19	1	0.3	0.591
C–Incubation temperature	0.06	1	5.1	0.036*	C–Inoculum size	0.13	1	0.2	0.657
D–Incubation period	0.03	1	2.5	0.130	AB	3.09	1	5.0	0.050*
AB	0.21	1	18.1	0.000*	AC	9.52	1	15.3	0.003*
AC	0.19	1	16.0	0.001*	BC	2.14	1	3.5	0.093
AD	0.25	1	21.1	0.000*	A^2	0.61	1	1.0	0.345
BC	2.39	1	203.4	< 0.0001*	B^2	6.76	1	10.9	0.008*
BD	0.01	1	1.0	0.343	C^2	1.24	1	2.0	0.189
CD	0.13	1	11.5	0.003*					
Residual	0.22					4.89			
Std. Dev.	0.11					0.79			
Mean	2.99					2.65			
R^2	0.96					0.79			

df= degree of freedom, *Significant at 5% level (P≤0.05), Cu^{+2} Sign. = B- Yeast extract conc., C-Temperature, AB, AC, AD, BC and CD, Cr^{+5} Sign. = AB, AC and B^2.

From the degree of significance, in presence of Cu^{+2}, the coefficients of model term, 2 variables yeast extract concentration (B) and incubation temperature (C) and interaction between two variables (AB, AC, AD, BC and CD) are significant influences on growth activity. The highest probability values were recorded in C variable (incubation temperature) and interaction between C (incubation temperature) and D (incubation period) being 0.003 and 0.036, respectively. The mathematical model describing the relationship between variables (A, B, C and D) and response (Y) for growth activity in medium supplemented with Cu^{+2} could be obtained by the following second order polynomial equation:

$$\begin{aligned} YCu^{+2} = {} & -14.6 + 5.5(Lactoseconc.) + 129.2(Yeastextractconc.) + \\ & 0.8(Temperature) - 1.5(Incubationperiod) - 14.5(Lactoseconc.^{Y}eastextractconc.) \\ & - 0.3(Lactoseconc.^{T}emperature) + 0.6(Lactoseconc.^{I}ncubationperiod) - \\ & 4.9(Yeastextractconc.^{T}emperature) + 0.7(Yeastextractconc.^{I}ncubationperiod) \\ & + 0.04(Temperature^{I}ncubationperiod) \end{aligned} \tag{7}$$

With respect to Cr^{+5} removal by tested fungi, the coefficients of model term, interaction between two variables (AB and AC) and quadratic of one variables (B^2) are significant influences on growth activity. The highest probability values were recorded in interaction between A (yeast extract concentration) and B (incubation temperature) being 0.05. The mathematical model describing the relationship between variables (A, B and C) and response (Y) for growth activity in medium supplemented with Cr^{+5} could be obtained by the following second order polynomial equation:

$$\begin{aligned} YCr^{+5} = {} & -190.1 - 76.5(Yeastextractconc.) + 15.4(Temperature) \\ & - 10.3(Inoculumsize) - 3.6(Yeastextractconc.^{T}emperature) \\ & + 21.0(Yeastextractconc.^{I}noculumsize) + 0.1(Temperature^{I}noculumsize) \\ & + 311.8(Yeastextractconc.^{2}) - 0.3(Temperature^{2}) + 0.9(Inoculumsize^{2}) \end{aligned} \tag{8}$$

Based on the model equation, three-dimensional response surface and two-dimensional contour plots graphically explain the interaction among variables and determine the optimum level of each factor for growth activity (Figs. 6 - 7). Data presented in Fig. 6, a-f revealed that growth activity was highly and interactively influenced by all selected variables. Cell dry weight predicted to be increased with high increase in concentrations of lactose (0.85%) and low in yeast extract concentration at 0.15% when incubation temperature is maintained in the selected medium level at 28°C and incubation period (Fig. 6a). Any further increases in these concentrations led to a decrease in cell dry weight. Optimum biomass yield could be possible with high lactose concentration (0.85 %) and in presence of incubation temperature 28°C, yeast extract concentration at 0.15% and incubation period 8 days (Fig. 6b). A similar response curve in Fig. 6c showed the interaction of lactose concentration (A) and incubation period (D) by yeast extract concentration (B) at low value; the maximum cell dry weight was attained beyond both high levels of lactose concentration and medium level of incubation period and incubation temperature. Cell dry weight predicted to be increased with high increase in concentrations of yeast extract concentration (0.15%) and medium in temperature for 28°C when incubation period is maintained in the selected low level at 32h (Fig. 6d). Fig. 6e illustrated the interaction of low incubation period and low yeast extract concentration by lactose concentration (A) at high value. The maximum cell dry weight was attained beyond medium level of incubation temperature and incubation period when lactose concentration is maintained in the selected higher level at (0.85%) (Fig. 6f). Data presented in Fig. 7, a-c revealed that growth activity was highly and interactively influenced by all selected cell dry weight predicted to be increased with low in concentrations of yeast extract (0.15%) and moderate increase in incubation temperature (28°C) when inoculum size is maintained in the selected moderate level at 2% (Fig. 7a). Any further increases in these concentrations led to a decrease in biomass. Optimum biomass yield could be possible with moderate inoculum size and in presence low concentration of yeast extract in medium concentration (Fig. 7b). A similar response curve in Fig. 7c showed the interaction of moderate inoculum size and temperature by low yeast extract concentration. From the previous results it could be concluded that optimized conditions (modified basal medium) were more favorable than un -optimized conditions (basal medium) for heavy metals biosorption (28 and 30 fold increase over control in presence of Cu^{+2} and Cr^{+5}, respectively). The cell dry weight was increased after using central composite design (CCD) and response surface methodology (RSM) for tested fungi in presence of Cu^{+2} and Cr^{+5} about 4.3 and 4.6 fold, as compared with Plackett-Burman design, respectively.

Obtained results are in accordance with those obtained by Kumar *et al* [50] they reported that the best incubation temperature for reduction of heavy metals (Zn and Cd) by *A. niger* was 30°C. Also, Shivakumar *et al* [47] who revealed that the highest removal heavy metals by *A. niger* (Ni, Cr, Cu, Zn and Pb) and *A. flavus* (Ni, Cu, Zn and Pb) was achieved at temperature ranging from 25-30°C. The decreased metal accumulation was noticed at low temperature between 10-20°C and high temperature between 35-40°C. Moreover Jha *et al* [51] revealed that the yeast extract was a vital component to simulate the rate of Cu^{+2} uptake by *A. lentulus* which enhanced about 18% of biosorption while increased about 21% of biosorption rate in presence mixture of glucose+ yeast extract. In Plackett-Burman design, the amount of metal ions (Cr^{+5} and Cu^{+2}) adsorbed per unit biomass (specific metal ions uptake) was 22.73 and 38.95 $ppmg^{-1}$ with 86.67 and 77.67% of efficiency at initial concentration of 150 ppm, respectively. While in respect of central composite design (CCD), the amount of metallic ion biosorbed per gram of biomass with efficiency % was 1.94 to 6.21 $ppmg^{-1}$ with 84.73 to 94.43% of efficiency, respectively (Data not shown). From previous results it could be summarized that the use of central composite design (CCD) led to increase the efficiency of heavy metals removed from media supplemented with metal ions by *A. terreus* FR1 (9.0 and 9.1% over increase of Cr^{+5} and Cu^{+2}, respectively) as compared to Plackett-Burman design. Similar results were obtained by Marandi *et al* [52] who found the highest heavy metal uptake efficiency by *P. chrysosporium* was 57% for Zn^{+2} and 87% for Pb^{+2} at 100 ppm of concentration. Moreover, Simonescu and Ferdeş [31] recorded the maximum Cu^{+2} removal capacity by *A. oryzae* ATCC20423 was ranged between 69 and 88%. In addition, Ekmekyapar *et al* [53] observed that the increase in biomass concentration led to the decrease in the metal specific uptake.

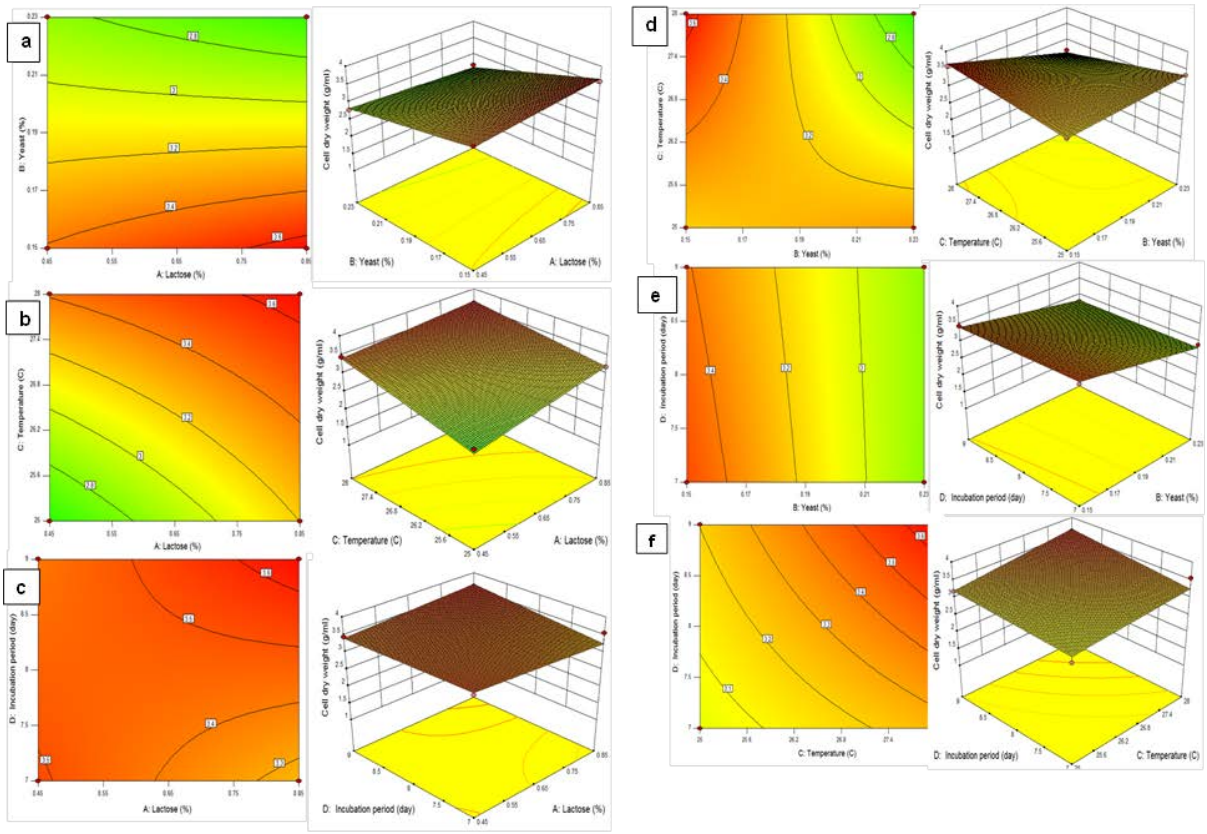

Figure 6. Three-dimensional response surface and two-dimensional contour plots showing the effect of lactose concentration, yeast extract concentration, incubation temperature and incubation period and their mutual effect on the growth of *A. terreus* FR1 in presence of Cu^{+2}. *a) Lactose concentration vs Yeast extract concentration with incubation temperature at 28° C for 8 day Incubation period. b) Lactose concentration vs Incubation temperature with yeast extract concentration at 0.15% for 8 day incubation period. c) Lactose concentration vs Incubation period with yeast extract concentration at 0.15 % for temperature at 28° C. d) Yeast extract concentration vs Incubation temperature with lactose concentration at0.85% for 8 day incubation period. e) Yeast extract concentration vs Incubation period with lactose concentration at0.85% for temperature at 28° C. f) Incubation temperature vs Incubation period with lactose concentration at 0.85% for yeast extract concentration at 0.15%.*

3.5 Some Application of Heavy Metals Removing Resistant Strain

Some applications of reduction of heavy metals from Egyptian phosphatic fertilizer sample (Tri-phosphate) which contains Co, Cu, Pb, Cr, Cd and Zn using living and dead *A. terreus* FR1 cells were tabulated in Table 6. The biosorption of heavy metals efficiency from tri-phosphate fertilizer by living *A. terreus* FR1 cells showed maximum removal of Zn (100%) followed by Cu (98.7%) > Cd (98.4%) > Co (97.0%) > Pb (95.3%) > Cr (90.5%), respectively. While, the adsorption efficiency of heavy metals from tri-phosphate fertilizer sample by dead fungal cells were in order of Zn (100.0 %) > Cu (98.7 %) > Cd (98.5 %) > Co (96.9%) > Pb (95.3%) > Cr (63.1%). Similar investigations were carried out and the present results were obtained after 6 days as incubation period may be, if the incubation period is longer then that trend of results could be changed. Same trend of results has been stated by Zaied *et al* [54] found that *Micrococcus* sp. has the ability to adsorb 79.22% of Pb. Moreover, the dead bacterial cells of *Bacillus* sp. showed 44.73% adsorption of Cu and *Pseudomonas* sp. adsorbed 86.66% of Cd [55]. In addition, Karakagh *et al* [56] revealed the inactivated bacterial cells increased with increasing initial concentrations of these metals from 50 mg L^{-1} to 400 ppm, Cd biosorption increased from 4.11 to 32.63 mg g^{-1} for *Actinomyces* sp., 3.39 to 33.18 mg g^{-1} for *Streptomyces* sp. and 3.96 to 38.71mg g^{-1} for *Bacillus* sp., respectively and Ni biosorption increased from 7.1 to 36.55 mg g^{-1} for *Actinomyces* sp., 5.47 to 34.82 mg g^{-1} for *Streptomyces* sp. and 4.08 to 32.8 mg g^{-1} for *Bacillus* sp. From the previous results, it could be noticed that no significant differences

between reduction of heavy metals in tri-phosphate fertilizer sample using adsorption (dead cells) and absorption (living cells) mechanisms by strain *A. terreus* FR1. Among of heavy metals were presented in phosphate fertilizer sample, Zn and Cu reached the highest reduction percentage by tested strain.

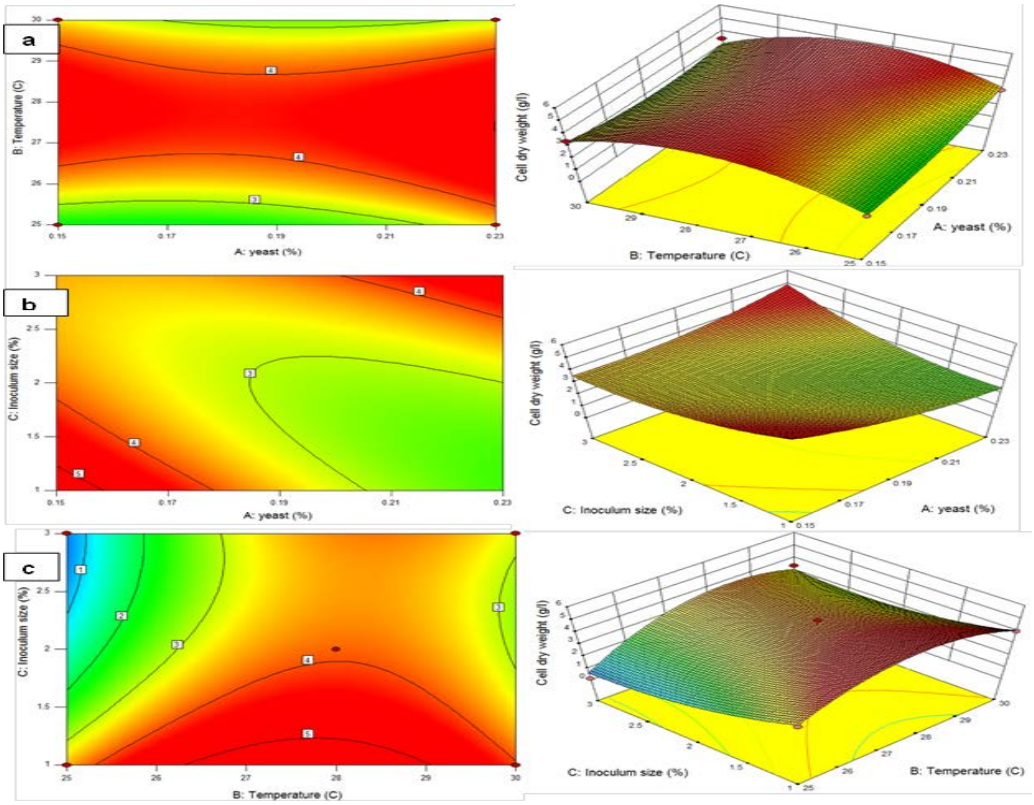

Figure 7. Three-dimensional response surface and two-dimensional contour plots showing the effect of yeast extract concentration, incubation temperature and inoculum size and their mutual effect on the growth of *A. terreus* FR1 in presence of Cr^{+5}. a) *Yeast extract concentration vs incubation temperature with inoculum size at 2%.b) Yeast extract concentration vs inoculum size with incubation temperature at 28° C. c) Incubation temperature vs inoculum size with yeast extract concentration at 0.15 %.*

Table 6. Absorption and adsorption of heavy metals from tri-phosphate fertilizer sample using living and dead cells of *A. terreus* FR1 after 6 days incubation period.

Heavy metal	Initial conc. (ppm)	Living cells of *A. terreus* FR1			Dead cells of *A. terreus* FR1		
		Res. (ppm)	Rem. (ppm)	E (%)	Res. (ppm)	Rem. (ppm)	E (%)
Co	**10**	**0.30**	**9.7^{d}**	**97.0^{c}**	**0.31**	**9.69^{d}**	**96.9^{c}**
Cu	**13**	**0.17**	**12.83^{c}**	**98.7^{bc}**	**0.168**	**12.83^{c}**	**98.7^{bc}**
Pb	**3**	**0.14**	**2.86^{e}**	**95.3^{d}**	**0.142**	**2.86^{e}**	**95.3^{d}**
Cr	**95**	**9.01**	**85.99^{b}**	**90.5^{e}**	**35.05**	**59.95^{b}**	**63.1^{e}**
Cd	**9**	**0.14**	**8.86^{d}**	**98.4^{bc}**	**0.137**	**8.86^{d}**	**98.5^{bc}**
Zn	**131**	**0.06**	**130.94^{a}**	**100.0^{a}**	**0.04**	**130.96^{a}**	**100.0^{a}**

conc.= concentration, Res.= Residual heavy metal (ppm), Rem.= Removal heavy metal (ppm), E= uptake efficiency, Removal heavy metals= initial heavy metal – residual heavy metal. Values in the same column followed by the same letter are not significantly different from each other, according to Duncan's [43] at 5%level.

4 Conclusions

From the present investigation, it could be summarized that strain *A. terreus* FR1 which is isolated from Egyptian phosphate fertilizer increases the efficiency in heavy metals (Cu^{+2} and Cr^{+5}) reduction after optimizing the nutritional and environmental factors using response surface methodology (RSM), which are increased by about 8.95% and 9.09% compared to control (Plackett-Burman design) for Cr^{+5} and Cu^{+2}, respectively. Some application of reduction of heavy metals from Egyptian phosphatic fertilizer sample (Tri-phosphate) uses living and dead fungal cells as absorption and adsorption mechanism, respectively. There is no difference signification between absorption and adsorption mechanisms for reduction of heavy metals from sample using strain of *A. terreus* FR1.

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