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Optimization of Culture Conditions for Growth Associated with Cr(VI) Removal by *Wickerhamomyces anomalus* M10

Pablo Marcelo Fernández[1] · Elías Leonardo Cruz[1] · Silvana Carolina Viñarta[1] · Lucía Inés Castellanos de Figueroa[1,2]

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Abstract Chromate-resistant microorganisms with the ability of reducing toxic Cr(VI) to less toxic Cr(III), are candidates for bioremediation. An alternative culture medium to reduce Cr(VI) using *Wickerhamomyces anomalus* M10 was optimized. Using the Plackett-Burman design, it was determined that sucrose, K_2HPO_4 and inoculum size had significant effects on chromate removal (i.e., reduction) at 24 h. Concentrations of these significant factors were adjusted using a complete factorial design. In this case, only the K_2HPO_4 effect was significant at 12 h of culture, with greater Cr(VI) removal at low concentration (1.2 g L^{-1}). The optimum medium was validated at the fermenter scale level. Optimal culture conditions for complete removal of Cr(VI) (1 mM) were 400 rpm agitation and air flow of 1 vvm. Moreover, *W. anomalus* M10 completely removed consecutively added pulses of Cr(VI) (1 mM). These results show interesting characteristics from the standpoint of biotechnology because the development of a future remediation process using *W. anomalus* M10 can represent an efficient and highly profitable technology for removing the toxic form of Cr.

Keywords Cr(VI) · Bioremediation · Cr(VI)-resistant yeasts · Plackett-Burman · Factorial design · Bioreactor

✉ Pablo Marcelo Fernández
pablomfernandez79@gmail.com; pfernandez@proimi.org.ar

[1] PROIMI-CONICET, Av. Belgrano y Caseros (T4001MVB), Tucumán, Argentina

[2] Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

Chromium (Cr) is a naturally occurring element found in rocks, animals, plants, soil, volcanic dust and gases, but the largest contribution to the deposition of Cr is the result of anthropogenic activities. Chromium is utilized in various industrial activities, including electroplating, steel, wood treatment, leather tanning, pigments, dyes and paints (Alam and Ahmad 2012; Lu et al. 2013).

Cr(VI) compounds are known to be extremely toxic to living organisms, causing allergies, irritations and respiratory tract disorders (Poljsak et al. 2010). Along with the strong oxidizing nature of Cr(VI), the intracellular reduction to Cr(III) gives rise to reactions with nucleic acids and other cell components in biological systems with consequent alterations of DNA and toxic effects (Poljsak et al. 2010; Zhitkovich 2011). On the other hand, Cr(III) at low concentrations constitutes an essential micronutrient for many higher organisms, being related to carbohydrate metabolism, synthesis and conformational stability of proteins and nucleic acids (Poljsak et al. 2010; Di Bona et al. 2011).

Biological treatment of heavy metal containing wastewater by using microorganisms is one of the most active research fields. Compared to conventional chemical treatment methods (e.g. chemical reduction plus precipitation, ion exchange, adsorption on activated coal), biological treatment methods have many advantages, which include (a) low operation cost, (b) steady performance, and (c) easy recovery of some valuable metals. Biological systems employing processes such as bioreduction, bioacumulation or biosorption with living cells have been extensively examined for their Cr removal abilities (Krishna and Philip 2005; Sultan and Hasnain 2005; Villegas et al. 2008; Poljsak et al. 2011).

Several microorganisms have the exceptional ability to adapt to and colonize the noxious metal polluted

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environments, which are inhabitable by higher organisms. Among eukaryotic microorganisms, *Candida utilis, Schizosaccharomyces pombe* and *Candida intermedia*, have been found to be effective in accumulating aggressive Cr-compounds (Paš et al. 2004; Poljsak et al. 2010), whilst others (*Candida maltosa, Candida* sp., *Lecytosphora* sp. NGV1, *Candida* sp. NGV9, *Cyberlindnera jadinii* M9, *Aureobasidium pullulans* VR-8, *Wickerhamomyces anomalus* M10) have developed the ability to bioconvert them into stable, non-toxic and bioavailable forms (Ramírez-Ramírez et al. 2004; Juvera-Espinosa et al. 2006; Cheung and Gu 2007; Villegas et al. 2008; Poljsak et al. 2010; Fernández et al. 2013). Among them, *W. anomalus* M10 Cr(VI)-resistant yeast isolated from a site receiving textile dye-effluents is a promising candidate for bioremediation of waters contaminated with chromate. Previous studies have demonstrated that the main resistance mechanism described for this yeast is the reduction of Cr(VI) to Cr(III) (Martorell et al. 2012a; Fernández et al. 2013).

Bioremediation process optimization may involve the study of many biochemical and physical parameters, including media formulation and culture parameters. The classical method of changing one medium variable at a time in order to optimize performance is impractical. The need for efficient methods for screening a large number of variables has led to adoption of statistical experimental designs. The methodology based on the Plackett-Burman design (Plackett and Burman 1946) provides an efficient way of screening a large number of variables and identifying the most important ones. Such designs have already been used in many research projects (Mabrouk 2008; Martorell et al. 2012b; Romero et al. 2012).

In the present study, the main objective was to obtain an alternative culture medium with similar nutritional characteristics of a commercial medium to remove Cr(VI) by tolerant yeast employing statistical experimental designs. The validation of these results in terms of field applicability was also tested by scaling-up the process to a bioreactor.

Materials and Methods

The experiments described in this paper were performed with yeast cells isolated from a textile-dye factory drainage channel in the proximity of a textile factory (Famaillá, Tucumán, Argentina). Using molecular, physiological and morphological analysis the isolate was identified as *W. anomalus* M10 (Fernández et al. 2013). The strain was maintained by periodic subculturing on Petri plates with YM agar. Incubation was performed at 25°C for 3 days and stored at 5°C. The medium contained (in g L^{-1}): glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; agar, 20. For inoculum preparation, active cells from YM-plates were transferred to liquid YM medium and incubated at 25°C and 250 rpm for 24 h.

A $K_2Cr_2O_7$ stock solution containing 5.2 mg Cr(VI) mL^{-1} (100 mM) in bidistilled water served as the hexavalent Cr source for our culture medium. A filter-sterilized Cr(VI)-stock solution was added to each sterile culture medium to a desired final concentration of Cr(VI) with minimal dilution of the medium.

The Cr(VI) removal potential of M10 strain was assessed in different culture media according to YNB medium composition, previously designed for Cr(VI) removal (Fernández et al. 2009). The media tested were (g L^{-1}): a-M1: Yeast Nitrogen Base (DIFCO-YNB w/o aminoacids and ammonium sulfate, 10×) 10 % v/v with sucrose 50 and $(NH_4)_2SO_4$ 0.6 (Fernández et al. 2009), and b-M5: sucrose, 50; $(NH_4)_2SO_4$, 0.6; yeast extract, 1; K_2HPO_4, 1; $MgSO_4$, 0.5; NaCl, 0.1; CaCl, 0.1. The media were adjusted at pH 5.0 with 0.1 M HCl and autoclaved at 121°C for 15 min. YNB solution 10× was filter sterilized using a sterile disposable filter with a 0.22 µm pore diameter (Sartorius, Goettinger, DE) (Fernández et al. 2009). All the experiments were performed in 500 mL Erlenmeyer flasks containing 100 mL of media and inoculated to 10 % v/v. Inoculated flasks were supplemented with the desirable Cr(VI) final concentration (1 mM) and incubated at 25°C for 72 h on a rotary shaker at 250 rpm. Samples periodically withdrawn were analyzed for pH, Cr(VI) concentration and biomass. Uninoculated controls were included to determine the Cr(VI) loss by the components of the culture medium.

A nine-factor Plackett–Burman design was applied (Plackett and Burman 1946). Sucrose, $(NH_4)_2SO_4$, yeast extract, K_2HPO_4, $MgSO_4$, NaCl, CaCl, inoculum size and inoculum medium were the variables under study. A total of 24 different experiments were employed to evaluate their final effects on Cr(VI) removal (i.e. reduction to Cr(III)) by *W. anomalus* M10. Each variable was investigated at a high (+1) and low (−1) level, which in the present investigation means two different nutrient concentrations, inoculum size or medium used to prepare the inoculum (Table 1). A complete factorial design was used to optimize the concentration of the variables selected according to Plackett-Burman analysis. This experimental design consisted of each variable at the high and low levels in eight runs. These experiments were run for 72 h. At the end of this period, the residual Cr(VI) concentration in the media was measured. The accuracy and fitness of the above models were evaluated by determination of the coefficient (R^2) and F value. The optimum values of the variable parameters for metal removal were obtained by solving the regression equation, analyzing the contour plots and constraints for the variable parameters. The statistical software package MiniTab 17 (MiniTab Inc., State College, PA, USA) was

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Table 1 Variables showing medium components (g L^{-1}), inoculum size (%) and medium used to prepare the inoculum at two test levels used in Plackett-Burman design

	Sucrose	$(NH_4)SO_4$	Yeast extract	K_2HPO_4	$MgSO_4$	NaCl	$CaCl_2$	Inoculum size	Inoculum medium
Low level (−1)	25	0.3	0.5	0.5	0.25	0.05	0.05	5	YM
High level (+1)	75	0.9	1.5	1.5	0.75	0.15	0.15	15	M5

used for regression analysis of experimental data and to plot response surface.

The optimized medium setting by statistical techniques was validated by conducting an experiment in a bioreactor. These assays were conducted in an instrumented fermenter (LH Serie 210, Inceltech, Tolouse, FR) equipped with a working volume of 1 L. The medium was adjusted at pH 5.0 with 0.1 M HCl, and autoclaved. It was then inoculated with active cells growth in liquid YM medium at 25°C and 250 rpm for 24 h, and a stock solution of Cr(VI) was added to achieve a final metal concentration of 1 mM. Antifoam (0.1 % v/v, DOWFAX 63N10L nonionic surfactant) was used. To determine appropriate aeration conditions, two tests were carried out: (a) 400 rpm and 0.5 vvm (volume of air/volume of medium/min) and (b) 400 rpm and 1 vvm.

To assess whether *W. anomalus* M10 presented tolerance and the capacity to remove additional pulses of Cr(VI), the same culture was re-contaminated with volumes of stock solution of Cr(VI) (to again achieve a final concentration of 1 mM). Samples were aseptically taken at periodic intervals and dry weight of biomass, pH, and metal concentration were monitored at regular time intervals.

Aliquots of culture medium periodically withdrawn were centrifuged at 10,300×*g*/25°C/15 min, and supernatants were subsequently analyzed for cell concentration, pH and Cr(VI) removal.

Biomass dry weight (BDW) was estimated after double washing of the resulting pellets from centrifugation with distilled water followed by drying at 85°C to constant weight.

Cr(VI) in solution was determined by colorimetric measurement of the pink-violet colored complex formed after reaction with 1,5-diphenylcarbazide (DPC) in acid solution. Spectrophotometric determinations were performed at 540 nm using a Multiskan® Go instrument (Thermo Fisher Scientific, Waltham, MA, USA) against a reagent blank (Urone 1955; Fernández et al. 2009). Cr(VI) concentrations were proportional to their absorbance and were quantified by the use of an external $K_2Cr_2O_7$ standard (1 mM) with a seven-point calibration curve. The reaction is very sensitive, the absorbance index per gram atom of chromium being about 40,000 at 540 nm (APHA and WPCF 1975). A wide range of Cr(VI) concentrations can be effectively determined with DPC (Limit of Detection (LOD): 41.5 µg L^{-1} Cr(VI)/Limit of Quantification (LOQ): 125 µg L^{-1} Cr(VI)). In the concentration region 40–950 µg L^{-1} the calibration lines exhibited a very good linearity ($R^2 = 0.9986$). Evaluation of calibration and performance of sample analyses were performed to ensure the quality of procedures used. All experiments were conducted in triplicate, and the averages of the results were taken as response values. All the chemicals used in the analysis were analytical grade reagent (AR).

Results and Discussion

Yeast Nitrogen Base (YNB) medium is a synthetic growth medium widely used for culturing yeast, is rich in nutrient and contains nitrogen, vitamins, trace elements and salts. But, it is an expensive commercial medium that affects the economy of the process in view of a further scaling-up to remove Cr(VI) from media. In previous reports, the YNB medium was widely used for removal of heavy metals with good performance (Villegas et al. 2008; Fernández et al. 2009, 2013). Therefore, design and optimization of an alternative medium, as substitute option to YNB medium was studied. The objective was to obtain a culture medium with similar nutritional characteristics and special interest to not interfere on Cr(VI) removal and analytical determination. For that reason, salts present in higher concentrations within the YNB manufacturer's specifications (K_2HPO_4, $MgSO_4$, $CaCl_2$ and NaCl) plus carbon and nitrogen source (sucrose and $(NH_4)_2SO4$, respectively) were used for alternative culture medium design. Sucrose and $(NH_4)_2SO_4$ concentration were applied according to a medium composition previously optimized (M1) (Fernández et al. 2009). Yeast extract was also included as source of vitamins, amino acids and other growth factors. Based on these components, a new culture medium (arbitrarily called M5) was formulated to study the Cr(VI) removal with *W. anomalus* M10.

Data in Fig. 1 show the kinetics of chromate reduction and growth of the strain in both media M1 and M5. Percentage of Cr(VI) removal in M5 medium were similar to the results obtained when M1 was used. Complete removal of Cr(VI) using M1 medium was reaching after 48 h. But, Cr removal was close to 90 % at 24 h and the maximum

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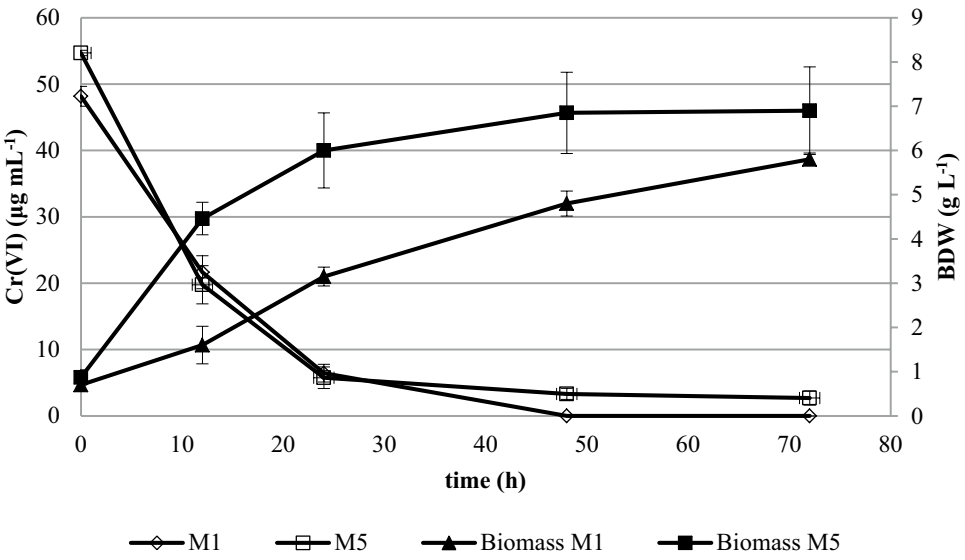

Fig. 1 Growth curves of *W. anomalus* M10 including determination of Cr(VI) during 120 h of cultivation in M1 and M5 medium plus 1 mM Cr(VI) at 25°C and 250 rpm. Not visible standard deviation bars indicate that these do not exceed symbol size

value (95 %) was achieved at the end of cultivation (72 h) in medium M5. Biomass concentration reached 6.93 g L^{-1} after 72 h of growth in medium M5, which was higher than that for M1. It was also observed that Cr(VI) removal was in accordance to biomass production. Growth-related chromate removal was also reported previously (Sultan and Hasnain 2007). No removal of Cr was observed in non-inoculated controls included in the experiment even after prolonged incubation up to 72 h. The results confirmed that Cr(VI) removal was not associated with substances existing in the formulated medium M5 or M1, and that removal strictly required the presence of yeast cells with the potential to detoxify hexavalent Cr. These results are in good agreement with previous reports (Pazouki et al. 2007; Thacker et al. 2007).

The results of 12 Plackett-Burman runs exhibited a wide variation in Cr(VI) removal percentages (from 27.89 % to 98.02 %). Analysis of variance (ANOVA) showed that sucrose (*p* 0.003; E 9.47), K_2HPO_4 (*p* 0.001; E 11.32) and inoculum size (*p* 0.000; E 12.11) within the test range had a positive effect on Cr(VI) removal, whereas yeast extract, $MgSO_4$, NaCl, $(NH_4)_2SO_4$, $CaCl_2$ and inoculum medium contributed negatively. The regression coefficients (R^2) and *t* and *p* values for all the variable linear effects are given in Table 2. The significance of each factor was determined using statistical parameter *p*. The *p* value is the indicator of the significance of the test, whose value <0.05 indicates that a test parameter is significant at the 5 % level of significance. In general, the larger the magnitude of *t* and the smaller the value of *p*, the more significant is the corresponding coefficient term (Montgomery 1991). These results are in good agreement with previous reports. Mabrouk (2008) observed that yeast extract, peptone, glucose and inoculum size had statistical significance in

Table 2 Effects of variables and statistical analysis on the removal of Cr(VI) at 24 h of culture by *W. anomalus* M10 using Plackett-Burman ($R^2 = 82.49$)

	E	Coef.	SE-coef.	*t*	*p*
Sucrose	9.47	4.74	1.35	3.5	0.003
$(NH_4)_2SO_4$	5.03	2.52	1.35	1.86	0.082
Yeast extract	3.87	1.93	1.35	1.43	0.172
K_2HPO_4	11.32	1.93	1.35	4.18	0.001
MgSO4	3.47	6.05	1.35	1.28	0.219
NaCl	−0.4	−0.2	1.35	−0.15	0.883
$CaCl_2$	3.5	1.75	1.35	1.29	0.214
Inoculum volume	12.11	6.05	1.35	4.47	0
Inoculum medium	−4.03	−2.01	1.25	−1.61	0.128

E effect, *Coef.* coefficient of the regression equation, *SE-coef.* standard error of coefficient, *t* student test, *p* value associated with the statistical test

Cr(VI) removal by *Streptomyces* sp. M-S2. Organic compounds are considered as essential supplements for NADH regeneration since they act as efficient electron donors for the reduction of chromium (Pal and Paul 2004). Inoculum size added to the culture in the range studied (5 %–15 %) indicates that an increase in cell mass promoted chromate removal. This finding is consistent with other researchers (Sultan and Hasnain 2007; Mabrouk 2008) on the increase in the reduction percentage with increase in the inoculum size. With respect to K_2HPO_4, Venil et al. (2011) reported that concentration increments (from 0.2 to 0.6 g L^{-1}) resulted in positive effects on Cr(VI) removal using *Bacillus* spp. REP02.

Concentrations of the significant factors selected by Plackett-Burman design were adjusted using a complete (2^3) factorial design. The removal of Cr(VI) at 12 and 24 h,

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are shown in Table 3. From the results obtained at 12 h of culture, it was observed that incomplete removal was attained (Table 3). A large share (78.32 %) of the variance (R^2) was explained by the model used. In this case, only potassium hydrogen phosphate effect was significant, with greater Cr(VI) removal at low concentration (1.2 g L^{-1}). The effect of the three major variables affecting Cr(VI) removal after 12 h of incubation are shown in Fig. 2. After 24 h of culture, a low R^2 value (39.81 %) indicated that the model could not explain the capacity of yeast for removal of Cr(VI). According to an analysis of significant effects at $p = 0.05$, none of the factors had a significant effect upon Cr(VI) removal. This may have been due to the fact that the concentrations of variables were already within a range where the average was optimized for the desired response.

A response optimizer tool helps to identify the combination of variable settings that jointly optimize a response. The impact of the variables (sucrose, K_2HPO_4, and inoculum) on the removal of Cr(VI) (response) at 12 and 24 h were evaluated using response optimization. The software estimated 80.91 and 100 % of metal removal after 12 and 24 h respectively, with concentrations of sucrose, 90 g L^{-1}; K_2HPO_4, 1.2 g L^{-1} and inoculum, 20 %. Within media optimization, one of the objectives was to reduce the concentrations of the provided components that did not affect the response. For that reason, a new adjustment was made. Results indicate that decreasing the concentration of the three factors, Cr(VI) removal close to 100 % at 24 h and 68.42 % at 12 h is reached. This represents a lower response with respect to the previous case, but considering the benefit obtained with lower concentrations, decreasing response is considered amortized. According to this, an analytically adjusted-medium was formulated (in g L^{-1}): sucrose, 60; $(NH_4)_2SO_4$, 0.6; yeast extract, 1; K_2HPO_4, 1.2; $MgSO_4$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1 and inoculum size, 10 %.

The maximum experimental response for chromium removal at 1 mM initial concentration was 90 % at 12 h and 100 % after 16 h of cultivation, whereas the predicted

Table 3 Complete factorial design results, used for identification of most important effects on Cr(VI) removal by *W. anomalus* M10. SD: standard deviation

Order	Sucrose (g L^{-1})	K_2HPO_4 (g L^{-1})	Inoculum (%)	Cr(VI) removal 24 h (%)	SD	Cr(VI) removal 12 h (%)	SD
1	60	1.2	10	96.585	±4.82	68.42	±4.82
2	60	1.2	20	97.085	±4.12	74.05	±4.12
3	60	1.8	10	89.755	±12.50	37.71	±12.50
4	60	1.8	20	97.935	±2.92	61.91	±2.92
5	90	1.2	10	100	±0	65.7	±4.82
6	90	1.2	20	100	±0	80.91	±4.82
7	90	1.8	10	94.705	±7.48	49.27	±4.82
8	90	1.8	20	97.62	±3.36	55.54	±4.82

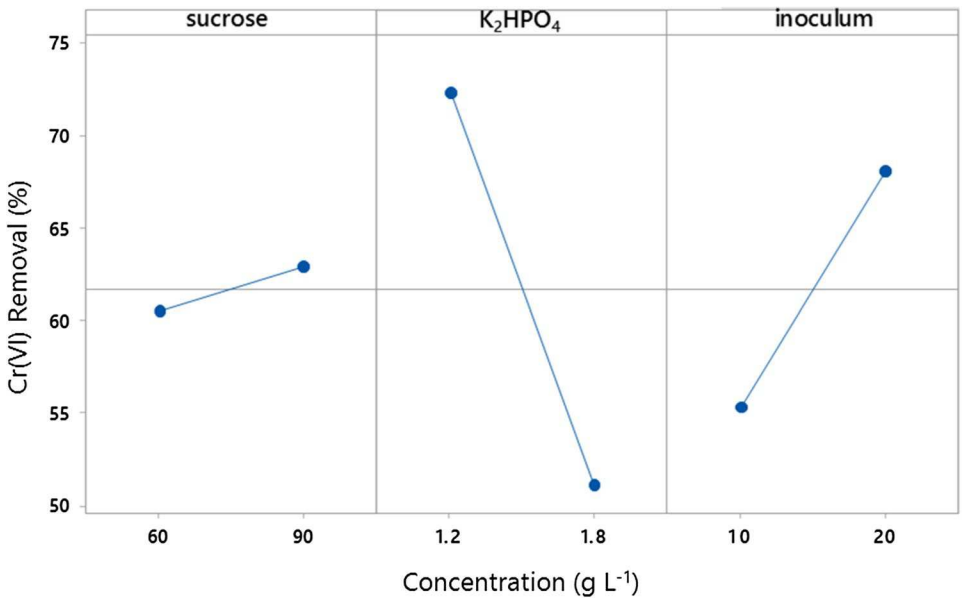

Fig. 2 Main effects plot of parameters for Cr(VI) removal after 12 h of cultivation (*y* axis). The concentrations of the factors analyzed are in the *x* axis

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response was 68.42 % and 96.59 % at 12 and 24 h respectively, indicating a metal removal improvement by using analytically adjusted-medium. An increased rate of Cr(VI) removal was achieved that was threefold higher than that recorded with the referent YNB medium. Biomass production at the end of cultivation was similar to concentration obtained in medium M5 (~6.0 g L^{-1}).

For experimental validation, a scale-up study was carried out in a 1 L reactor. In order to optimize operating conditions, various speeds of agitation and airflow were setting. When working with an agitation of 200 rpm and an airflow rate of 0.5 vvm, the biomass obtained at 24 h of culture (4.33 g L^{-1}) was lower than that achieved in shake flasks assays (6.33 g L^{-1}). This might be related to the limiting effect of dissolved oxygen in the culture medium. This directly affected Cr(VI) removal, which was slower and less efficient. Under these conditions, complete removal (100 %) was reached after 21 h of culture compared to 16 h in shake flasks test. Using high aeration (400 rpm and 1 vvm), biomass production was 6.76 g L^{-1} at 24 h, with a total reduction of hexavalent chromium within 16 h of cultivation, similar to reductions in shake flasks assays. These results showed that by altering certain parameters of culture, it was possible to modify the rate of Cr(VI) removal. Thus, the time required for total removal of Cr(VI) decreased with increasing agitation and aeration. Biomass production accompanied Cr(VI) removal.

Once operational culturing conditions were established, the behavior of the strain *W. anomalus* M10 to additional pulses of 1 mM Cr(VI) was evaluated. Complete removal of Cr(VI) was reached after two metal pulses (at 0 and 16 h). Immediately afterwards, a third pulse was added, but total Cr(VI) removal was incomplete (Fig. 3). On the same graph, the kinetics of cell growth is presented. Complete Cr(VI) removal could be explained by the initial high level of biomass growth. After 24 h of culture, biomass growth slowed, which could explain the slow and incomplete Cr(VI) removal. At the end of culture, biomass concentration of 5.89 g L^{-1} was reached. In agreement with our results, Sen et al. (2014) showed that during different operational strategies of cultivation, the main constraint of closed cultivation systems was the inhibition of cell growth by accumulation of Cr in the medium. In the same publication it was concluded that growth in continuous culture was the best strategy for the system to remove Cr(VI) over a long period at a maximum specified speed. Kaszycki et al. (2004) demonstrated that exponentially growing cultures were more tolerant with increasing concentrations of metal in relation to studies of yeasts in early or late stages of growth. The report by Xu et al. (2009) seems to agree with the previously mentioned study. Moreover, a significant increase in bacterial growth was evident when there was a more efficient reduction of Cr(VI), indicating that the reduction of Cr(VI) and bacterial growth are mutually affected. Therefore, the growth of cells stimulates the reduction of Cr (VI), at the same time that the efficient reduction of Cr (VI) promotes bacterial growth.

The results of this study have indicated that the statistical design was a powerful tool for the determination of the more relevant variables in reducing and detoxifying Cr(VI) by *W. anomalus* M10. The new medium that was developed showed a much higher level of Cr(VI) removal as compared to a commercial medium previously used. The strain studied in this work showed interesting characteristics from a biotechnology point of view. The results obtained after cultivation in a bioreactor, with several pulses of Cr(VI) at high concentration, provided evidence that *W. anomalus* M10 may be a viable candidate for use in the biological remediation of environmental media contaminated with Cr(VI).

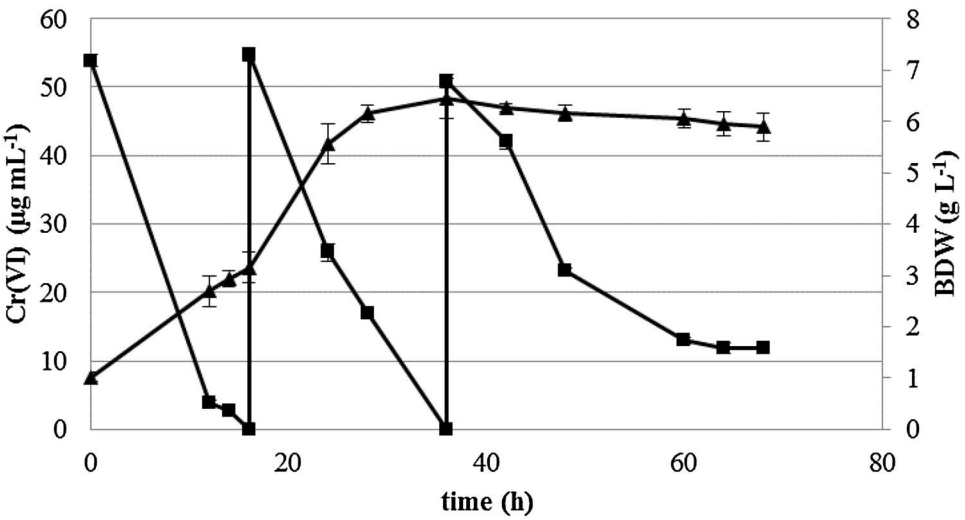

Fig. 3 Variation of Cr(VI) concentration and biomass production by *W. anomalus* M10 during time with pulses of Cr(VI) 1 mM at 25°C, 400 rpm and 1 vvm in bioreactor. *Filled square* Cr(VI) concentration, *filled triangle* biomass concentration, *asterisk* indicate additional Cr(VI) pulses

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