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BIOSORPTION OF CHROMIUM (VI) BY A MUTATED STRAIN OF *Bacillus cereus* M$^1_{16}$

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ABSTRACT

The removal of chromium, a highly toxic metal causing environmental pollution from dilute aqueous solution, was studied in the present work using growing and washed cells of a mutant strain of *Bacillus cereus* (M$^1_{16}$) isolated from tannery waste. Particularly, the effects of pH, temperature, metal ion concentration and contact time on removal of chromium were studied. About 40% chromium removal, was observed by growing cells of the selected strain at pH 6.5, temperature 30º±1ºC, inoculum size 3%, medium volume 50 ml/250 ml Erlenmeyer flask, and initial chromium concentration 50 ppm. Using resting cells 98.02% and 78.34% chromium removal was possible with initial chromium concentration of 25 and 50 ppm, respectively, at pH 3.0, temperature 25-35ºC and 2.73 g/L biomass concentration. It was found that the overall adsorption process was best described by pseudo-second order kinetics. Freundlich and Langmuir adsorption models were found suitable for describing the short-term biosorption of chromium (VI). IR spectral analysis of the biomass was carried out to find out the functional groups responsible for chromium (VI) biosorption.

Key words: lead biosorption, *Bacillus cereus* M$^1_{16}$, immobilization, pseudo second order, Freundlich isotherm.

Introduction

Environmental pollution due to development of industries in the recent time period is a great threat to living beings. Heavy metal contamination in aqueous waste systems occurs from electroplating, mining, pigments, ceramics, metallurgy, and tanning industries. Chromium is one of the heavy metal contaminants present in trivalent and hexavalent forms. Hexavalent chromium is also more toxic than trivalent and carcinogenic also (Nishioka, 1975; Mearus et al., 1976; Ptrilli and Flora, 1977). Tannery waste contains 80-250 ppm chromium, whereas safe value for drinking purposes is 0.05 ppm and the recommended value for discharge is less than 5 ppm (Directive 98/83/EC). The metallic species are nonbiodegradable and therefore persist indefinitely, accumulating in living tissues throughout the food chain (Sag et al., 1994). Conventional methods such as lime or caustic soda treatment, oxidation–reduction, ion exchange, filtration, and evaporative recovery can be used for removing heavy metals from wastewater but are expensive, insufficient for metal removal, and require costly equipment and high-cost operation and energy. In this context, biological processes may offer a more effective alternative (Akhtar and Mohan, 1995; Akhtar et al., 1995; Bonaventura and Johnson, 1997). Many aquatic species such as fungi (Tobin et al., 1984; Hang et al., 1990), yeast (Hang et al., 1990; Volesky, 1993), algae (Crist et al., 1981; Ozer, 1994) and bacteria (Strandberg et al., 1981; Aksu et al., 1991; Schatt and Karasykar, 1992; Sag and Kustal, 1995) are capable of accumulating large amounts of heavy metals. Instead of living organisms dried and killed algae, fungi and bacteria were used for removal of heavy metals. A few investigators used living organism for this purpose (Volesky, 1987; Volesky, 1994; Karna et al., 1996). The advantage of using a growing culture in biosorption is in avoiding the need for a separate biomass production process for cultivation, harvesting, drying, processing, and storage prior to use.

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This paper describes biosorption of chromium using growing and resting cells of a mutant strain of *Bacillus cereus* M$^1_{16}$. Previously, Mullen et al., 1989 reported sorption of cadmium and copper by *B. cereus*. We also studied Langmuir and Freundlich adsorption isotherm. The paper also describes the functional groups responsible for biosorption using IR analysis.

Methods and materials

Isolation and identification of microorganisms

A bacterial strain capable of chromium biosorption was isolated from tannery waste collected from a tannery near Park Circus, Kolkata, using the plate and dilution technique. Nutrient agar containing 50 ppm chromium (VI) ion was used as a plate medium (Bera et al., 2003). The organism was maintained in nutrient agar by monthly subculturing and stored at 4°C. Identification of the organism as *Bacillus cereus* was performed according to Bergey's Manual of Determinative Bacteriology, 1983 (Holt et al., 1994).

Removal of chromium using growing cells of *Bacillus cereus*

Composition of the fermentation medium and inoculum medium was as follows: (g/L), beef extract, 10; yeast extract, 2.0; peptone, 5.0; sodium chloride, 5.0, pH 6.0. Inoculum was prepared by transferring one loop full of cells from the slant culture to the inoculum medium (50 ml/ 250 ml Erlenmeyer flask) and incubating the flask at 30 ± 1°C in a rotary shaker at 120 rpm for 24 hours. A 50 ml fermentation medium containing 50 ppm Cr(VI) ion in a 250 ml Erlenmeyer flask was inoculated with 4% (V/V) 24-hour cell growth, and incubated for 48-hour under the same conditions. Samples were collected at 24 and 48 hours. Culture fluid was centrifuged at 4000 rpm for 15 min. Concentration of residual chromium present in the supernatant was estimated. The percent of metal bound was taken to be the difference between the control and the final concentration of metal in the supernatant (Gardia-Torresdey and Arenas, 1998).

Estimation of chromium

Residual concentration of chromium in the culture fluid was estimated using an atomic absorption spectrophotometer (Varian, 1656).

Induced mutation and selection of high-yielding strain

Mutation of the selected strain was carried out using N- methyl-N^1- nitro - N- nitroso guanidine at a concentration range of 10-100 microgram/ml in 15 ml of 24-hour culture broth, and incubating and shaking at 30± 1°C and 120 rpm. Samples were collected at different time intervals viz., 1,2,3,4,5, and 24 hours. Isolates were collected using the plate and dilution technique using nutrient agar. Isolates were tested for their activities regarding biosorption of Cr(VI), using above-mentioned process.

Preparation of resting cells

The same growth medium and the environmental conditions were used to grow the selected strain without chromium. After 24 hours, the biomass was separated by centrifugation at 5500 rpm for 15 min. After washing twice with normal saline, 350 mg of wet cell mass was suspended in 50 ml

normal saline containing 50 ppm Cr (VI) ion in a 250 ml Erlenmeyer flask and incubated at 30ºC using a rotary shaker at 120 rpm. The experiments were carried out with wet biomass of the selected strain, but results were calculated with dry biomass basis. According to Volesky, for scientific interpretations, the sorbent material dry-weight basis is preferred (Volesky, 1999).

Preparation of dry cells

Washed biomass (wet) from a measured amount of whole-cell broth was taken in a previously weighed aluminium cup and dried at 70ºC overnight and was again weighed. The weight of the dry cell was calculated by the difference.

Biosorption isotherm

The Langmuir model (Langmuir, 1916) is described by the following equation:

$q_e = q_m b.C_e/(1+b.C_e)$. . . (1)

The above equation may be rearranged to the following linear form:

$C_e/q_e = 1/b.q_m + C_e /q_m$…..(2),

where C_e is the equilibrium concentration (mg/L) and q_e is the adsorbed amount of metal ion per gram of biomass at equilibrium (mg/gm). q_m is the maximum amount of metal ion per unit weight of biomass to form a complete mono layer on the surface bound at high Ce (mg/L). b is a constant related to the affinity of the binding sites (L/mg). A plot of C_e / q_e vs C_e should indicate a straight line of slope $1/q_m$ and an intercept of $1/bq_m$. The Freundlich model equation (Freundlich, 1926) is of the following form :

$q_e = k.C_e^{1/n}$. . . (3).,

where k and n are the Freundlich constants characteristics of the system (Selatnia et al., 2004). k is the relative indicator of adsorption capacity (L/g), and n indicates the intensity of adsorption. Equation (3) is conveniently used in the linear form by taking the logarithm of both sides as

$\ln q_e = \ln k + (1/n) \ln C_e$ ….(4).

Kinetic modeling

The order of adsorbate–adsorbent interactions has been described using various kinetic models. The first-order rate expression of Lagergren (1898), Ho, and McKay (1999a), Aksu (2001), based on solid capacity, is generally expressed as follows:

$- \log_{10}(q_e-q_t)/q_e = k_1 t/2.3$ . . . (5),

where k_1 is the rate constant of first-order biosorption (min^{-1}). The pseudo second-order equation is also based on the sorption capacity of the solid phase (Aksu, 2001; Ho and Mckay, 1999b). The integrated form of the equation is

$1/(q_e-q_t) = 1/q_e + k_2 t$ . . . (6)

Here, k_2 is the second–order rate constant. The linear form of the Equation (6) is

$t / q_t = 1/h + (1 / q_e)t$ . . . (7) ,

where $h = k_2.q_e^2$ can be regarded as the initial sorption rate. If the pseudo second–order kinetics is applicable, the plot of t/q_t versus t gives a linear relationship, which allows computation of q_e, k_2 values.

Results and discussion

Isolation and identification of the selected strain

Of the three strains isolated from the tannery waste, only one strain (S_1) was found to be capable of removing Cr (VI) ion from solution. For example, 27.75 ppm was adsorbed out of 50 ppm present initially (55.5% removal).

Identification of the selected strain was carried out according to Bergey's Manual of Determinative Bacteriology, 1983 (Holt et al., 1994). The media for different biochemical tests were prepared according to Harrigan and McCance, 1976. Results are shown in Table 1. On the basis of these taxonomical studies, the strain S_1 was identified as *Bacillus cereus* (Fig. 1).

Induced mutation

During this experiment, 33 isolates were collected of which (24 strains were low in activity, four strains were similar in activity to the parent strain, and five strains showed high activity) isolate M^1_{16} showed maximum activity (75.5% chromium (VI) ion removal) compared to the parent strain S_1 [55.5% removal of chromium (VI)] ion. The mutant strain *B. cereus* M^1_{16} was selected for further studies (Fig. 1).

Effect of Environmental conditions on biosorption of Cr (VI) ion by *Bacillus cereus* M^1_{16} in growing conditions

Effect of aeration

Biosorption of Cr (VI) ion was carried out using different volumes viz, 40, 50, 60, 70 ml of medium (pH 6.5) in a 250 ml Erlenmeyer flask containing 50 ppm Cr (VI) ion at 30 ± 1^oC for 48 hours. 1% 24-hour cell suspension was used as inoculum. After 24 and 48 hours the fermentation broth was centrifuged at 5500 rpm for 15 min and the concentration of Cr (VI) ion in the supernatant in each case was estimated. Cr(VI) ion sorption was more or less the same in 24 and 48 hours. The maximum consumption of Cr (VI) ion was observed with a 50 ml medium (Fig. 2).

Effect of temperature

The effect of temperature on the uptake of chromium during growth of the organism was studied using 50 ml medium in 250 ml Erlenmeyer flask varying the temperature viz, 25, 30, 35°C , other conditions remaining the same. Fig. 3 shows that optimum temperature for biosorption was 30°C.

Effect of pH

Initial pH of the growth medium varied from 4.5 to 7.0, keeping other conditions constant, and pH 6-6.5 was found to be the superior rate (Fig. 4) for Cr(VI) ion biosorption.

Effect of inoculum size

A 50 ml medium containing 50 ppm Cr (VI) ion in a 250 ml Erlenmeyer flask was inoculated with different amounts of inoculum (1-5 ml), other parameters remaining the same. Chromium biosorption increases with increase in inoculum size, and then remains constant (Fig. 5).

Effect of chromium concentration

Initial chromium concentration of the fermentation medium varied from 10–100 ppm during

removal of chromium using growing cells of the selected strain. Maximum accumulation was observed using 50 ppm. Cr (VI) ion; other conditions of the experiment were constant (Fig. 6).

Experiments with washed cells of *Bacillus cereus* M$^1_{16}$

Effect of initial pH on Cr (VI) biosorption

Experiments were carried out in a 50 ml 0.1-M citrate buffer (pH range 2-6) in a 250 ml Erlenmeyer flask containing 25 ppm chromium (VI) ion and cell biomass concentration 4.5 g/l (dry basis) at 30°C for 24 h. From Fig. 7, it was observed that 92% removal was possible at pH 3.0.

Influence of biomass concentration

Fig. 8 shows the % metal ion removal as a function of biomass concentration. The graph shows an initial increase in the elimination of the metal ions with the increase in concentration of the biomass from 1.3 g/L to 4.5 g/l. When biomass concentration was 2.73 g / L (Fig. 8) at q = 14 mg/g, % removal was 78.36%.

Influence of the initial Cr(VI) concentration

Chromium removal was carried out varying the initial concentration of chromium from 25 ppm to 200 ppm at 30° C, other conditions remaining the same. Maximum removal was observed using 2.73 g/l biomass and 25 ppm chromium initially. Removal rate is very fast during first 10 min and equilibrium was attained at 7 h in each case. Percent removal of chromium decreases with an increase in chromium concentration (Fig. 9).

Effect of temperature

Experiments with biosorption of chromium were carried out as usual using chromium (VI) concentration of 50 ppm at different temperatures of 20, 25, 30, and 35°C. Fig. 10 shows that there is initial increase in chromium biosorption up to 25 °C, and then it remains constant.

Time course of biosorption

Time-dependency batch experiments were performed with varying contact times of biomass to Cr(VI) ion removal from 0-200 minutes using 25 ppm initial Cr(VI) ion concentration, other conditions remaining the same. Table 4 shows that removal rate is fast during first 120 min, and metal binding sites are saturated within 240 min.

Adsorption isotherm analysis

Taking into account the fact that the solution pH was constant (pH 3.0) during the biosorption process, and in order to optimize the biosorption process parameters, we have modeled the equilibrium curve (Fig. 11 and Fig. 12). The Langmuir and Freundlich isotherms were tested. Fig. 11 and Fig. 12 are the transformed forms of these models, which permit calculations of Langmuir's constant (q$_m$ and b) and Freundlich constants (k and n). Table 2 shows the values of the computed constants. Values of correlation parameter R^2 show that the Langmuir model fits best with our experimental data.

IR spectral analysis

In order to find out which functional groups were responsible for the chromium adsorption, IR (Perkin Elmer-297) analysis of the biomass was carried out. Fig. 13 shows the IR spectra and the

various functional groups corresponding to the adsorption bands. The frequencies of vibrations and their corresponding groups are presented in Table 3.

Different authors reported the effect of pH on biosorption of chromium. Some reports are similar to our results and some are different. Increased removal of CrO_4^{2-} by complexation on bacteria, algae, fungi and yeast at pH 2.0 was reported by several authors (Crawford and Crawford, 1996; Domez et al., 1999). Kovacevic et al., 2000 reported that pH values from 3 to 7 had no influence on sorption of chromium using fungal pellets of *Aspergillus niger* 405 from solution. Increased sorption of chromium (VI) was observed as the pH increased from 2-6 using dried and lyophilized biomass of *Synechococus* sp. PCC 7942 by Gardea Torresdey and Arenas in 1998 . Previous research has demonstrated that under acidic conditions (pH 2.5) *Bacillus coagulans* biomass biosorbed maximum of 62.11 mg Cr/g dry weight. Our results showed maximum adsorption of Cr (VI) at pH 3.0 (Fig. 7).

In 2003, Ozodemir et al. studied the biosorption of Cr(VI) at a concentration of 100 mg/L using pH range 1-8. The greatest capacity of biosorption was observed at pH 2.0 for Cr(VI) ions. Biosorption increases with increase in pH from pH 1.0 to 2.0 and thereafter decreases with increase in pH. In the present study, using resting cells of *B. cereus* M^1_{16}, the removal of chromium was observed at pH 3.0. In this case biosorption increases with increased in pH from pH 2 to pH 3.0 then it decreased with increase in pH (Fig. 7). The maximum absorption at low pH values may be attributed to the large number of H^+ ions present at low pH values which might neutralize the negatively charged surface of the adsorbents or convert a neutral group to a positively charged group there by reducing the hindrance to the diffusion of dichromate ions. Cr(VI) occurs in the form of oxy-anion as $HCrO_4^-$, $Cr_2O_7^{2-}$ CrO_4^{2-}, $Cr_4O_{13}^{2-}$, $Cr_3O_{10}^{2-}$ (Tewari et al., 2005). At higher pH, the reduction in adsorption may be possibly due to the abundance of OH^- ions creating increased hindrance to diffusion of dichromate ions (Jasuja et al., 1997).

Experiments were carried out at different temperatures viz. 20, 25, 30, 0°C using 1.38 g/ L biomass, Co=50 mg/l (ppm), pH 3.08; it was observed that at first biosorption increased with temperature i.e from 20°C to 25°C then it remained constant with increase in temperature. Several authors (Kovacik et al., 2000, Srinath et al., 2002, Gupta et al., 2001) reported the maximum removal of chromium occurred at pH 2-2.5.

In 2003, Ozdemir et al. reported that the biosorption capacity of the biomass increased first with increase in initial concentration of metal ions and then reached a saturated value. When the initial Cr (VI) ion concentration was increased from 30 to 280 ppm, the loading capacity increased from 6.4 to 57.8 mg/g of *Ochrobacterium anthropy*. In our study Cr (VI) ion concentration was varied from 25 to 200 ppm and the loading capacity increased from 7.84 to 35.18 mg/g.

In 2003, Ozdemir et al. reported same type of adsorption equilibrium. Using initial Cr (VI) ion concentration 200 ppm, they observed metal concentration decreased rapidly during first 5 min and saturation point reached within 2 hours. Amount of adsorption increases with initial metal ion concentration due to increasing driving force (C_i-C), where C_i and C are initial Cr (VI) ion concentration and Cr (VI) ion concentration at any time respectively. After saturation it remained constant.

Amount of chromium (VI) ion adsorption increases with amount of adsorbent (cell mass) due to

increase in surface area initially. Then due to clump formation of cell mass, surface area decreases and total usable surface area is adjusted, hence amount of adsorption remains almost constant.

The equilibrium of biosorption of chromium was modeled using adsorption type isotherms. The Fraundlich and Langmuir models were used to describe the biosorption equilibrium (Donmez, 1999, Aksu et al., 1996, Aksu, 1998). The linearized Langmuir adsorption isotherm of chromium (VI) ion for *B. cereus* M$^1_{16}$ are shown in Fig.11. After obtaining the value of C_e, residual metal ion concentration at equilibrium (mg/L) and q_e, adsorbed metal ion quantity per gm of cell at equilibrium (mg/g) from experimental data, the plot of $1/C_e$ vs $1/q_e$ was employed. The Freundlich and Langmuir adsorption constants evaluated from the iostherms with the correlation coefficients are also given in Table 2. The value of q_m 70.25 mg/g (maximum adsorption coefficient) appears to be significantly higher for Cr (VI) using *B. cereus* mutant M$^1_{16}$ system. It indicated a high adsorption capacity. The magnitude of k (10.187), 1/n (0.347) (Freundlich constants) showed easy uptake of Cr (VI) from solution with a high adsorptive capacity of *B. cereus* M$^1_{16}$. Keskinkan in 2004 reported that *Ceratophyllum demersum* adsorbed zinc, lead and copper and maximum adsorption capacity were 13.98 mg/g, 44.80 mg/g and 6.17 mg/g for zinc, lead and copper respectively (based on the Langmuir coefficients). IR analysis describes the functional groups responsible for chromium biosorption. Selatnia et al. in 2004 reported IR adsorption bands and corresponding possible groups responsible for Cd^{2+} adsorption by *Streptomyces rimosus* biomass.

Conclusions

A mutated strain of *Bacillus cereus* M$^1_{16}$ in growing and resting cells is an efficient adsorbent of chromium (VI) in dilute solution. Up to 98.08% and 55% chromium (VI) removal were possible when initial chromium (VI) concentration were 25-200 mg/L. The external mass transfer is the controlling step in the overall sorption process. The cell wall of this biomass contains anionic groups (Table 3) whose adsorbance towards chromium ions is fairly high. Moreover adsorption was influenced by various parameters such as initial pH, initial chromium concentration, biomass concentration and temperature. The results obtained during this study show that this method of eliminating Cr (VI) ion is very promising compared to the more conventional processes.

Acknowledgement

Nomenclature

b : Constant related to the affinity of the binding sites (L/mg).

C_e: Metal ion concentration at equilibrium (mg/L).

H: Constant value.

k : Fruendlich constant (L/gm).

k_1 : First-order rate constant.

k_2: Pseudo second-order rate constant (min.gm/mg).

n : Fruendlich constant related to binding affinity.

q_e : Amount of adsorbed metal ion per gram of biomass at equilibrium (mg/gm).

q_m: Maximum metal ion adsorbed per gram of biomass (mg/gm).

q_t : Metal ion adsorbed at any time by biomass (mg/gm).

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Table 1. Taxonomical characteristics of the wild strain.

Parameters	Characteristics
A) Cellular characteristics	
Morphology	Small rod, occurring singly or in pairs, non-motile
Size	2.85 X 1.2 micron
Staining characteristics	Gram positive, spore former
B) Cultural characteristics	
Nutrient agar colonies	Round (2.2 mm), opaque, smooth, slightly raised, undulate edge, white with greenish pigmentation
Nutrient broth	
Stationary condition	Moderate growth, deposits, pellicle formation,
Shake flask	Moderate growth, ring formation (deep brown), light yellowish-green broth
Growth in anaerobic condition	Positive
C) Physiological and biochemical characteristics	
Growth factors	Optimum growth at 30^0C; range 28-37^0C, PH 7.5
Tolerance	No growth at 6.5% Nacl at PH 9.6. small growth after 5 days
Ammonia from arginine	Positive
Arginine used as sole source of energy	Positive
Protein liquefaction (gelatine)	Negative
Catalase reaction	Positive
Litmus milk test	Positive
Hydrolysis of starch	Negative
Nitrate reduced to nitrite	Positive
Phenol red	Negative
Methylene blue	Positive
Tyrosine decomposition	Positive
Egg yolk reaction	Positive
Citrate utilization test	Positive

Fermentation characteristics

Sugar	Acid Production	Gas Production
Glucose	+	-
Mannose	+	-
Fructose	-	-
Galactose	-	-
Xylose	+	-
Lactose	-	-
Maltose	-	-
Sucrose	-	-
Starch	-	-
Dextrin	-	-
Glycerol	-	-
Sorbitol	+	-
Mannitol	-	-
Salicin	-	-

Table 2. Sorption isotherm coefficients of Langmuir and Freundlich models.

Langmuir			Freundlich		
q_m (mg/g)	b (L/mg)	R^2	k(L/g)	1/n	R^2
70.25	0.026	0.9744	10.18687	0.3474	0.943

Table 3. Absorption bands and corresponding possible groups.

Frequency (Cm^{-1})	Functional group
2930	-CH
1654	- COO^-, - C = O
1520	- COO^-
1000	- C≡-O, - C≡-N, - P≡-OH, - P≡-OC
1340	-OH

Table 4. Time course of biosorption.

Time (min)	% removal of Cr(VI) ion
5	0.32
12	9.16
15	11.08
20	14.56
30	20.84
40	26.24
50	29.16
60	34.96
120	44.28
180	45.44
240	51.80
300	50.76
360	49.36
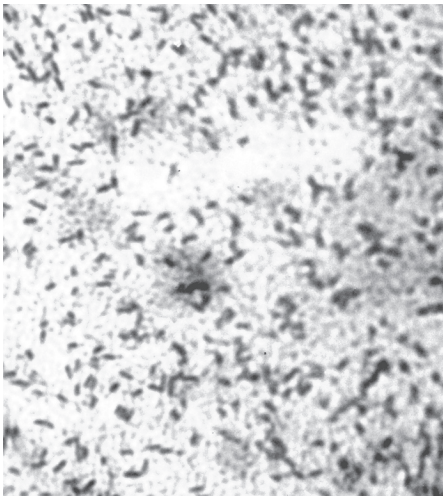
Figure 1. Morphological characteristics of the isolated strain *B. cereus.*
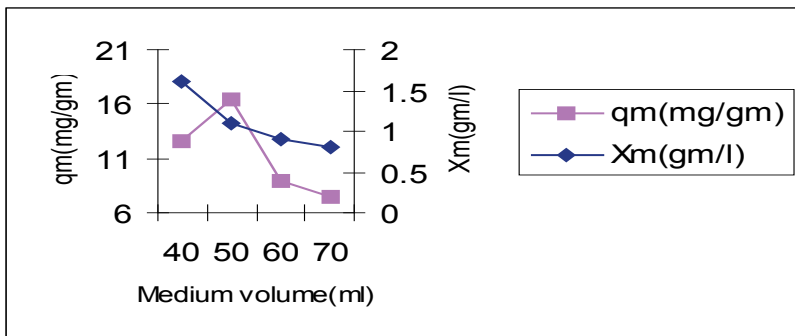
Figure 2. Effect of medium volume on chromium consumption by growing cells of *B. cereus* M^1_{16}.
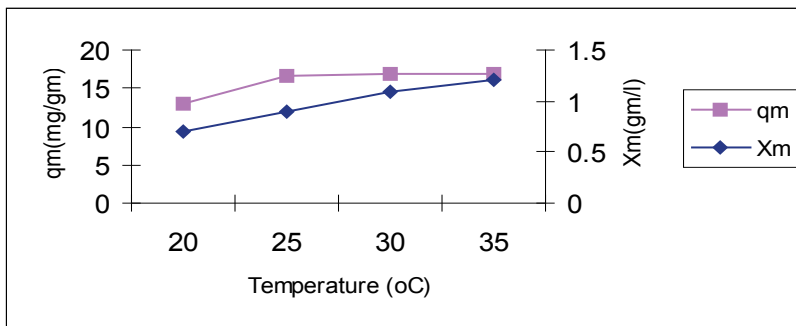
Figure 3. Effect of temperature on biosorption of chromium using growing cells of *B. cereus* M^1_{16}.
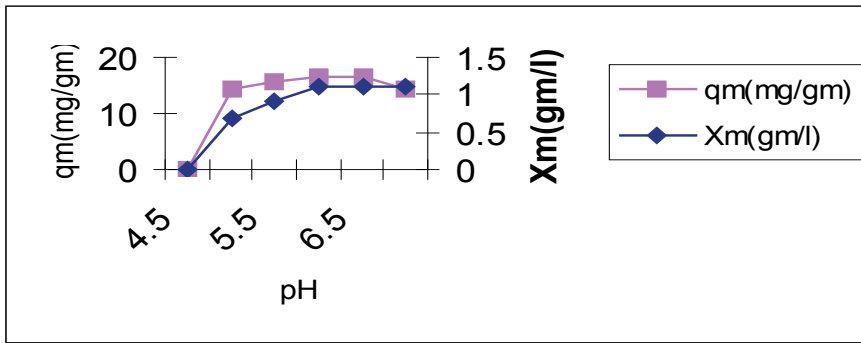
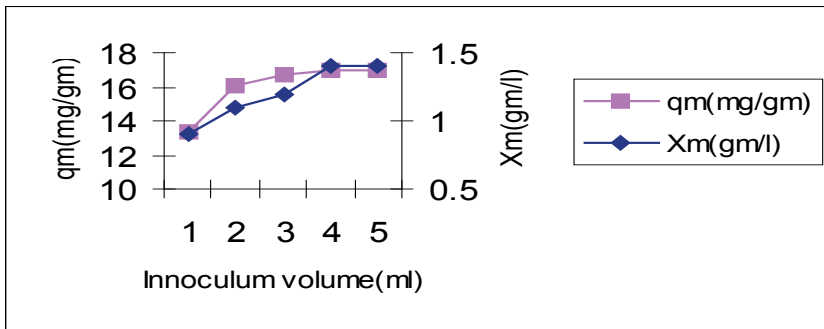**Figure 4.** Effect of initial pH of the medium on chromium consumption.

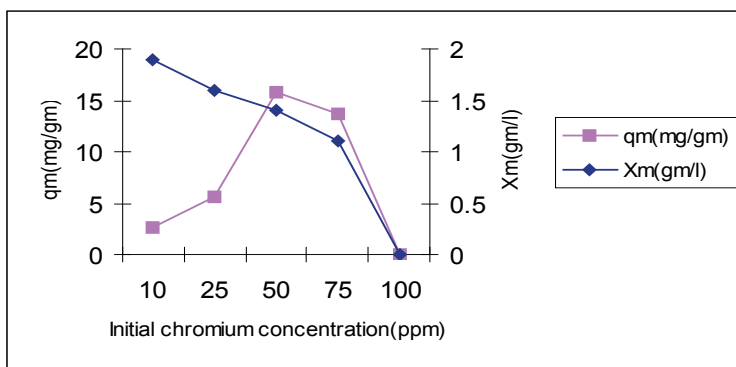**Figure 5.** Effect of inoculum size for chromium removal by growing cells of *B. cereus* M^1_{16}.

Figure 6. Effect of chromium concentration on chromium removal using growing cells of *B. cereus* M^1_{16}
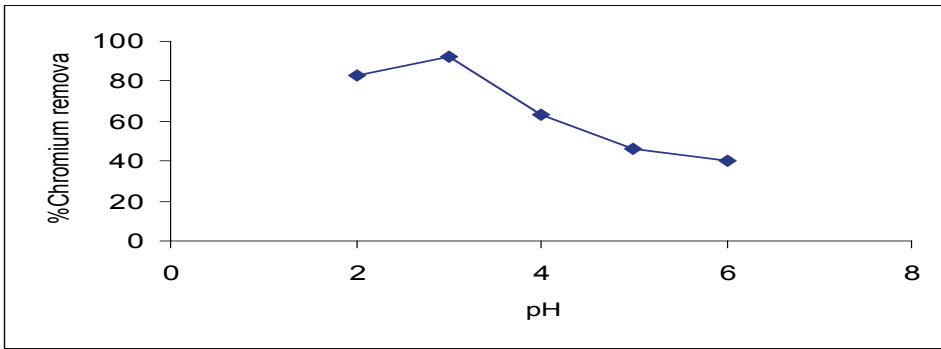
Figure 7. Effect of pH on chromium consumption using resting cells of the selected strain.
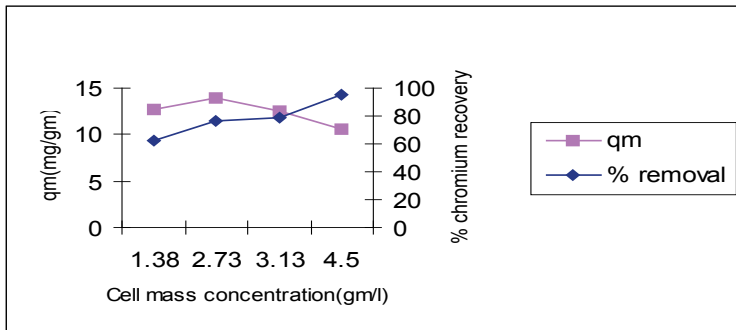
Figure 8. Effect of biomass concentration on chromium bioaccumulation.
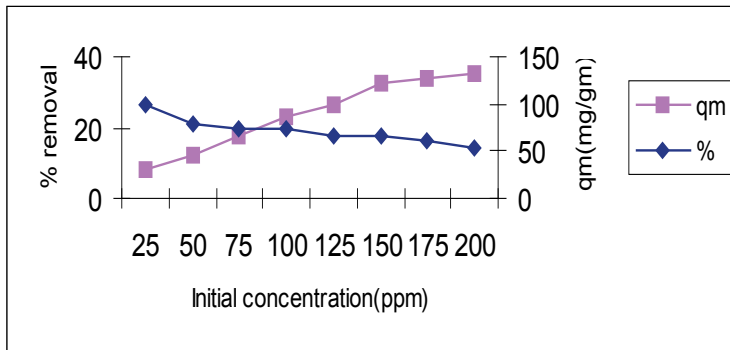
Figure 9. Effect of initial chromium concentration on chromium biosorption using washed cells of *B. cereus* M^1_{16}.
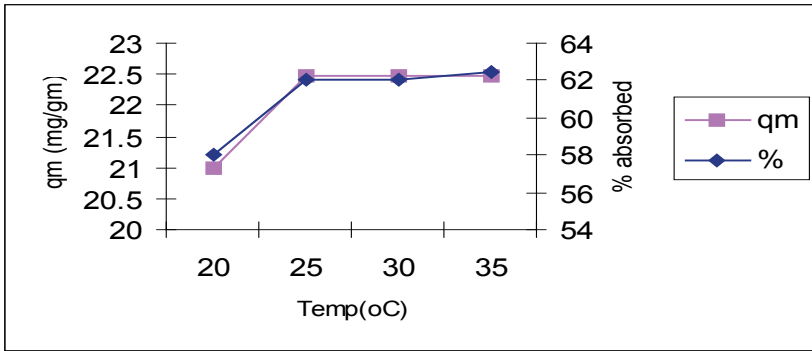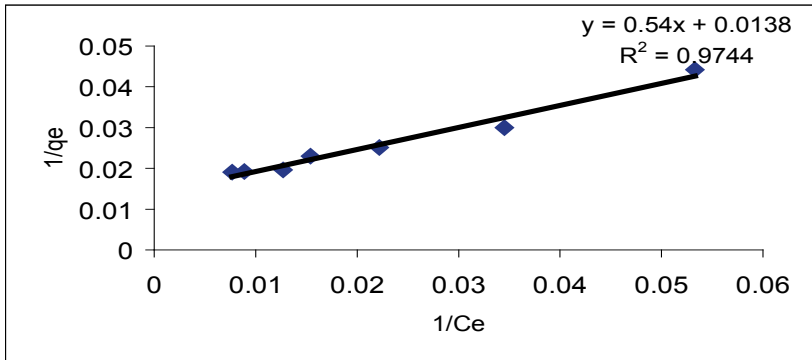
Figure 10. Effect of temperature on chromium removal.

$$y = 0.54x + 0.0138$$
$$R^2 = 0.9744$$

Figure 11. Langmuir adsorption isotherm.

$$y = 0.3474x + 2.3211$$
$$R^2 = 0.943$$
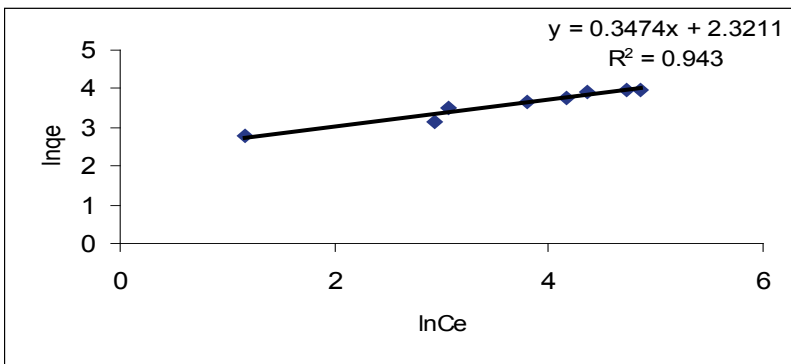
Figure 12. Freundlich adsorption iostherm.
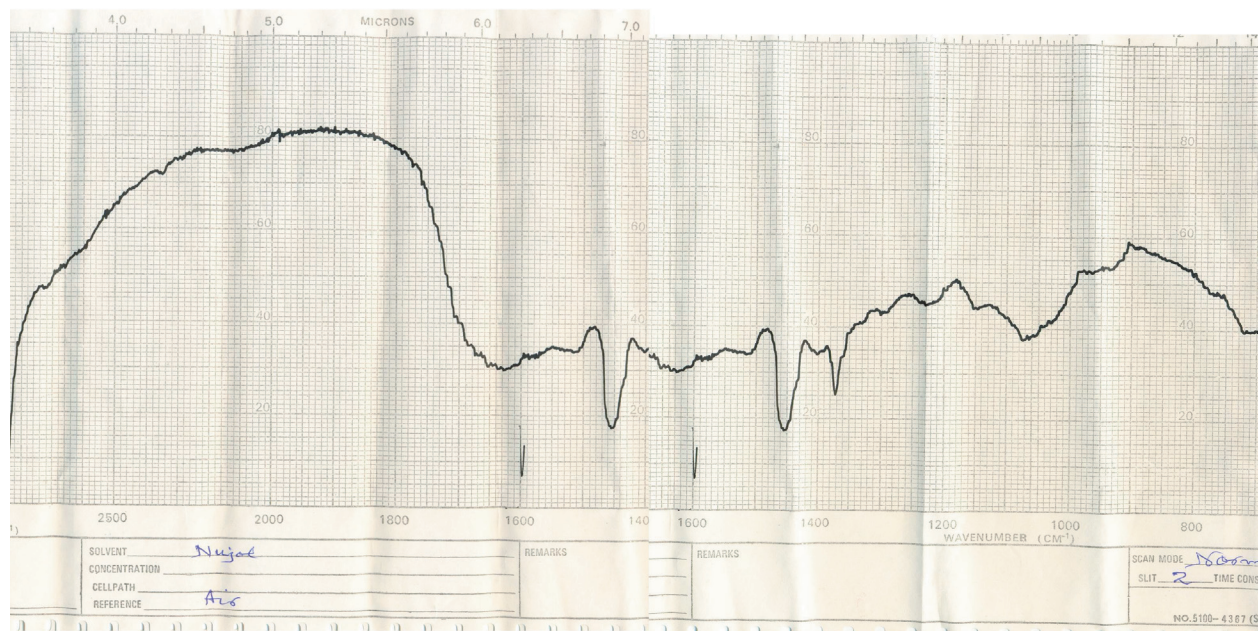
Figure 13. IR spectral analysis of the biomass.