Isolation and Biochemical Characterization of Chromium Reducing Bacteria from Hudiara Drain Sludge

Nisar Numrah and Iqbal Hafsa
Department of Environmental Science, Lahore College for Women University, Jail road, Lahore PAKISTAN

Abstract

In the past few decades, the environmental pollution of toxic heavy metals is the major issue throughout the world since industrial evolution. Heavy metals found in wastewater are harmful to the environment and their effects on biological system are very severe. Chromium is one of these heavy metals whose concentration in the environment is increasing due to different industrial processes. There are two inorganic forms, Cr (VI) and Cr (III), in which chromium exists in nature. Cr (VI) is a serious environmental pollutant. A variety of microorganisms have been known for their ability to degrade these heavy metals. The current study aimed to isolate chromium reducing microorganism from Hudiara drain sludge. Among all the species diversity in sludge two isolated species (SP1 and SP2) were selected for further analysis based on their morphological characteristics. The isolated strains were identified through biochemical characterization. The species were characterized according to criteria given in Bergey's Manual of Determinative Bacteriology (1984). The efficiency of two isolates for chromium reduction was also determined, which shows that SP1 show 65% reduction and SP2 show 53% reduction of chromium from the medium in 12 hours. This shows that Pseudomonas putida was efficient in reducing Cr (VI) than Pseudomonas plecoglossicida.

Keywords: Environmental pollutant, chromium reduction, biochemical characterization, *Pseudomonas putida, Pseudomonas plecoglossicida.*

Introduction

In today's society, there is no escaping exposure to toxic chemicals and metals. Growing complex industrial clusters have profoundly affected the water resources since they are considered to be the dumping sites for most of the organic, inorganic and heavy metal waste discharged by them[1].

Hudiara drain is one of such an example of trans boundary water channel between India and Pakistan, whose water is utilized for various purposes such as irrigation of agricultural land and dumping of industrial effluents. The total length of the drain is 118 km, 55 km in India Territory and 63 km in Pakistan[2]. Almost 112 small industries located discharge their effluent on Pakistani side along 63 kilometers length in the Punjab before it merges into the Ravi. Out of the major polluting industries textile units are forty two, tanneries are four, pulp and paper five, pharmaceuticals are four, pesticides are three and steel melting units are two. The drain was originally a natural storm water channel; however, dumping of industrial discharges, municipal wastes, domestic wastewater and agricultural runoffs has turned it into a polluted drain[3].

Heavy metals pollution is one of the major pollution issues in the Drain. These toxic heavy metals cannot be recovered from the environment, and are remain ultimately indestructible. Chromium is one of the heavy metals whose concentration in the environment is still increasing. Cr (VI) in water and soil environment poses pressure on micro flora. In contaminated soils, the availability of chromium is affected by several processes such as organic and inorganic complexes formation, precipitation / dissolution, oxidation / reduction or adsorption / desorption[4]. In soil these processes are affected by the microbial activities[5], however higher chromium levels in soil are toxic enough to decrease microbial biomass, diversity and activities[6].

The conventional processes are usually ineffective and expensive from economical view point[7]. It is therefore, necessary to introduce a low-cost, innovative and eco-friendly method for the removal of toxic heavy metals from wastewater[8]. Some indigenous bacterial species are found highly resistant to Cr (VI) in chromium contaminated sites[9] and also been identified to reduce toxic and soluble Cr (VI) to less toxic and less soluble form Cr (III), e.g. *Ochrobactrum*, *and Arthrobacter*, *Pseudomonas* sp, *Serratiamarcescens*, *Ochrobactrum* sp, *Bacillus* sp, *Desulfovibrio vulgaris* and *Cellulomonas* sp. The potential use of these species for degradation purposes is much cheaper and ecofriendly in comparison with conventional degradation methods[10]. The present study was designed to explore the indigenous Cr resistant bacterial species from Hudiara drain sludge.

Material and Methods

Soil/Sludge Samples: The study area chosen was approximately 15km stretch of Hudiara Drain, of its total stretch

of approximately 55km in Pakistan. Three different sites of the Drain were selected, with a distance of about 5km approximately between adjacent sampling sites. The study was conducted for only the sludge of Hudiara Drain.

The total numbers of composite samples collected throughout the study area were decided to be three, after the survey, study requirement and feasibility of analytical tests. Each representative sample was composed of homogeneous mixture of five sub-samples.

Isolation and purification of bacteria: For isolation and purification of bacteria spread plate technique and streak plate technique were used. The sample solution was serially diluted to about 10^{-10} spread on agar plates. These plates were incubated at 37ºC (24 hrs). The sludge sample was streaked on the agar plates with further incubation at 37ºC (24 hrs).

Microscopic analysis of bacterial strains isolated from sludge sample: A drop of bacterial culture suspension was dissolved in the sterilized de-ionized water and observed under light microscope for cell shape and size.

Biochemical characterization of bacterial strains isolated from sludge sample: After Gram Staining Technique Biochemical tests were carried out for characterization purpose.

Oxidase test: Filter Paper Test Method was used. Selected bacterial cultures were rubbed onto filter paper treated with 1% Kovács Oxidase reagent. Change in color was observed.

Catalase test: Slide (drop) method was used. Selected bacterial cultures on microscopic slides were treated with 3% H_2O_2 and results were observed.

Citrate test: Bacterial cultures on citrate medium were incubated at 35ºC. After incubation of 18 to 48 hours the color of the medium was observed.

Urease test: Bacterial cultures on urea broth were incubated at 37ºC. After incubation of 24 to 48 hours the color of the medium was observed.

Indole production test: After incubation of 24 to 48 hrs, the drops (five) of Kovács reagent were directly added to the tube and colour change was observed.

Methyl Red and Voges Proskauer (MR-VP) Test: For MR-test after incubation of 48 hrs, drops (five) of Methyl Red were added in the test tube. The colour change was then observed. For VP-test after incubation of 48 hours Voges-Proskauer Reagent A (5% naphthol) and Voges-Proskauer Reagent B (40% KOH) were added and change in color was observed.

Hydrogen sulfide production test: Selected bacterial cultures on triple sugar iron agar (TSIA) slant were incubated (37ºC for 24 hrs) and results were determined on the basis of color change.

Determination Cr (VI) reducing activity of *Pseudomonas:* The LB broth was amended with the Cr (VI) concentrations of 10, 20, 30, 40 and 50mg/L of Cr (VI) ($K_2Cr_2O_7$), inoculated with bacterial cultures. A separate media with only LB agar media was inoculated with bacteria served as control. Here a positive control media without bacterial inoculum and only Cr (VI) was also used. The cultures along with the controls were incubated in shaking incubator for 12 hours at 30ºC at the speed of 100rpm. The bacterial growth was determined after every 2 hours for the duration of 12 hours, through determination of the optical density at 600nm by UV visible spectrophotometer. Cr (VI) in the culture supernatant was measured by Atomic Absorption Spectrophotometer[11].

Results and Discussion

Isolation and purification of bacteria: The white color colonies on the agar plates were further streaked and single particular colonies were selected. On the basis of the microscopic analysis, it was found that bacteria were rod shaped and could possibly be Clostridium, Salmonella, Escherichia, and Pseudomonas species.

Biochemical characterization of isolated bacterial strains: The isolated bacterial species were identified following the Bergey's Manual of Determinative Bacteriology. The identification criteria included the growth features (colony, shape and color), growth conditions (optimum temperature and suitable growth media), morphology of the cells (shape), physiological characteristics (motility, flagella and Gram reaction), production of enzymes (oxidase and catalase), and utilization of different carbon sources. Results of Biochemical tests are listed in table-1.

Table-1
Biochemical tests for SP1 and SP2

Test	SP1	SP2
Gram-staining	-	-
Oxidase	+	-
Catalase	+	+
Citrate	+	+
Urease	+	+
Indole production	-	-
Methyl Red	-	+
Voges Proskauer	-	-
Hydrogen sulfide production	-	-

*SP1, species 1 isolated; *SP2, species 2 isolated *+, positive; *-, negative

It was found that the bacteria were gram negative, so, after gram staining test it was confirmed that isolated bacteria belonged to genus Pseudomonas[11].

Oxidase test: Oxidase test was performed to determine cytochrome oxidase. Color of one of the bacteria culture

immediately turned to dark purple which indicated that they were Oxidase positive. While other species remained colorless, indicated that they were Oxidase negative[12].

Catalase test: To determine Catalase in bacteria, the catalase test was performed. The immediate effervescence (bubble formation) occurred on the slide which indicated that bacteria are Catalase positive. The reason of bubble formation is that; Catalase enzyme present in bacteria causes the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen[13].

$$2H_2O_2 + Catalase \rightarrow 2H_2O + O_2$$

Citrate test: The citrate test was conducted to check the potential of bacteria to utilize citrate as its carbon and energy source. After incubation the growth and intense blue in color indicated that cultures are citrate positive[12]. The reason of blue color production is that; citrate is a Krebs cycle intermediate generated by many bacteria[13] as described below.
Citrate = oxaloacetate + acetate
Oxaloacetate = pyruvate + CO_2
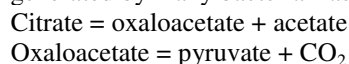
The alkaline condition metabolized pyruvate to acetate and formate[14];
Pyruvate = acetate + formate

Urease test: Urease test was conducted to identify the ability of bacteria to degrade urea into ammonia and carbon dioxide. After incubation the cultures in the test tubes appeared pink in color indicated that bacteria are Urease positive. The pink color indicated the production of ammonia which has caused the pH of the solution to rise to about 8.2. This increase in pH is responsible for color change[14];

$$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$$
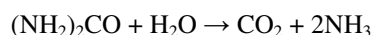
Pink color production in the solution confirmed that isolated species belong to genus *Pseudomonas*[15].

Indole production test: The indole test was conducted to check the potential of bacterial species to produce indole. After incubation few drops of kovacs reagent was added. The culture layer in the test tube became cloudy and appeared yellow in color indicated that cultures are indole negative and did not contained tryptophanase enzyme in them. So, it was confirmed that isolated species belong to genus *Pseudomonas*[16].

Methyl Red and Voges Proskauer Test: Methyl red test was performed to find out whether the bacteria perform acids fermentation. Color of the test tube 1 turned to yellow which indicated that bacteria were MR negative and color of the test tube 2 turned bright red indicated that bacteria were MR positive. The reason is that, after incubation when methyl red was added in the medium, change in pH was occurred. Methyl red is used as a pH indicator. The bacteria in test tube 2 showed the presence of the extreme acidity in acid fermentation[17].

Voges proskauer test was performed to determine whether the selected bacterial cultures produce 2,3-butanediol. No change in color was detected in both of the test tubes indicated that both bacterial cultures were VP negative[14].

Hydrogen sulfide production test: The hydrogen sulfide test was used to determine the production of sulfides during the metabolism of sulfur compounds. After incubation no black color was produced in each test tube indicated that bacteria did not have ability to cause reduction of sulfur containing compounds into sulfides i.e. they are H_2S negative and belong to genus *Pseudomonas*[18].

Determination of Cr (VI) reduction activity of *Pseudomonas:* From each conical flask the growth of bacteria was observed after every 2 hours by optical density of the solution at 600nm by using UV visible spectrophotometer. The reduction of Cr (VI) was measured after every 2 hours in the supernatant solution obtained after centrifugation, by using Atomic absorption spectrophotometer. The bacterial growth curve and reduction of Cr (VI) curve was plotted for each Cr (VI) concentration (10, 20, 30, 40 and 50mg/L) separately[19] figure-1 to 8.

The rate of Cr (VI) reduction increased with decreasing concentrations of Cr (VI) and increasing the time intervals. It was found that the reduction of Cr (VI) activity of the bacteria was dependent on the growth. The bacteria growth was determined by the initial lag phase as well as second exponential phase and stationary phase with the final death phase. It was found from the results that bacterial lag phase increased with the increase in Cr (VI) concentration. The reason for initial rapid Cr (VI) degradation rate was that; in the beginning there was high concentration of Cr (VI) in the medium which increased the Cr (VI) degradation. And as time passed the Cr (VI) levels in the solution started decline and degradation rate became slow. At the same time growth rate of bacteria started decline due to accumulation of toxic waste products[20].

The optimum temperature for both isolated species is 30^0C and optimum pH is approximately 7. At these optimum conditions maximum degradation of the Cr (VI) was observed. A graph was plotted for percentage degradation of each concentration at optimum temperature and pH. It was seen that maximum degradation occurred at lower concentration. At higher concentration the cellular growth started decline due to toxic effect of high Cr (VI) concentration[11] figure-9.

Conclusion

The present study concluded that indigenous bacterial species from Hudiara drain sludge have their naturally existing machinery to degrade pollutants (chromium), which is cost effective as compared to conventional methods. Two different bacterial species were isolated from sludge and identified that they have high degrading ability for Cr (VI) and have significant

potential to degrade the toxic Hexavalent chromium into less toxic Trivalent chromium. The species were biochemical characterized and results revealed close resemblance with *Pseudomonas putida* and *Pseudomonas plecoglossicida*. The degradation of chromium was calculated by the growth of *Pseudomonas putida* and *Pseudomonas plecoglossicida* in the culture medium amended with chromium salt. It was observed that the identified bacterial species can efficiently degrade Cr (VI) up to 65% and 50% respectively in 12 hours at the temperature of 30ºC. These findings are potentially useful because the species can possibly be harnessed to detoxify chromium contamination sites.
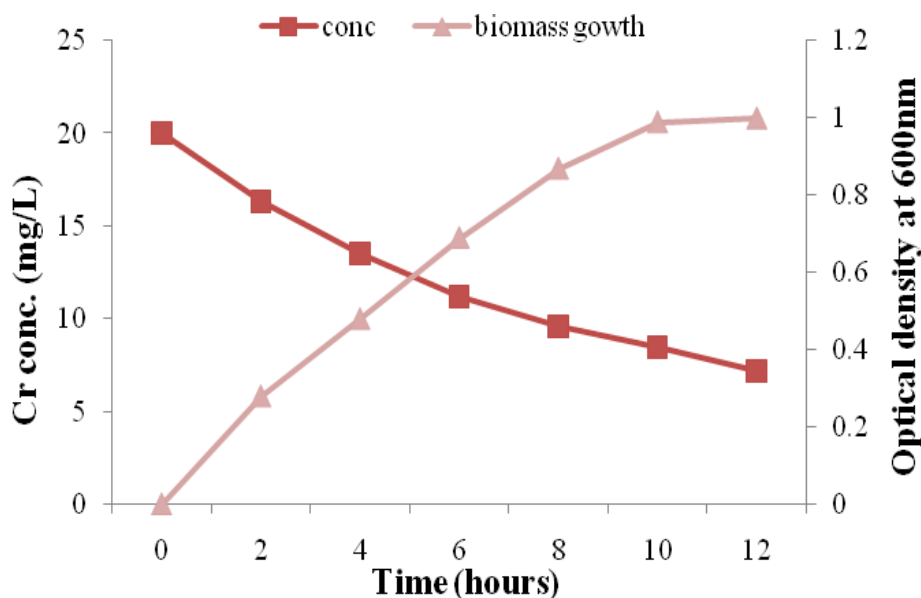
Figure-1
Cr (VI) reduction activity of isolated species (SP1) at initial Cr conc.20mg/L and optical density of the solution at 600nm for the duration of 12h
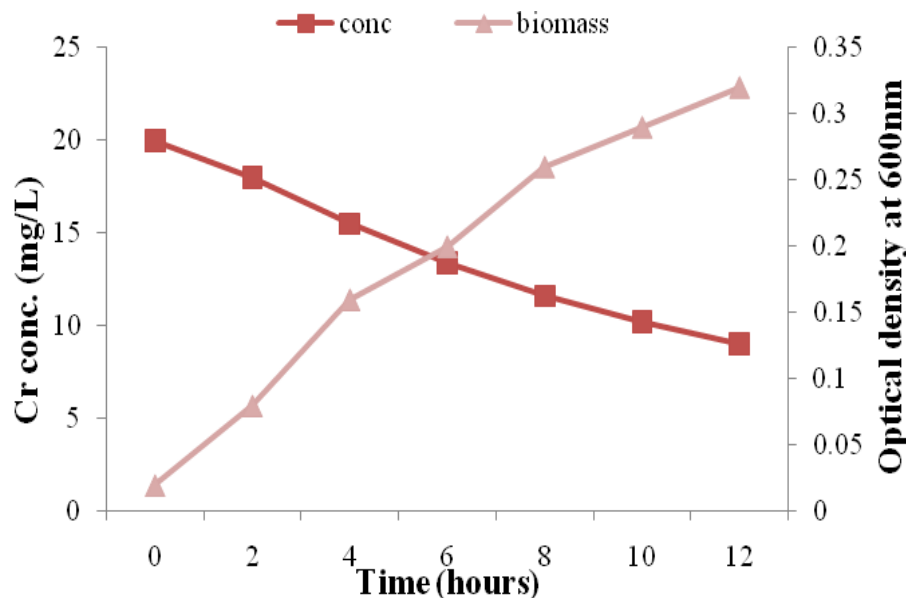

Figure-2
Cr (VI) reduction activity of isolated species (SP2) at initial Cr conc. 20mg/L and optical density of the solution at 600nm for the duration of 12h
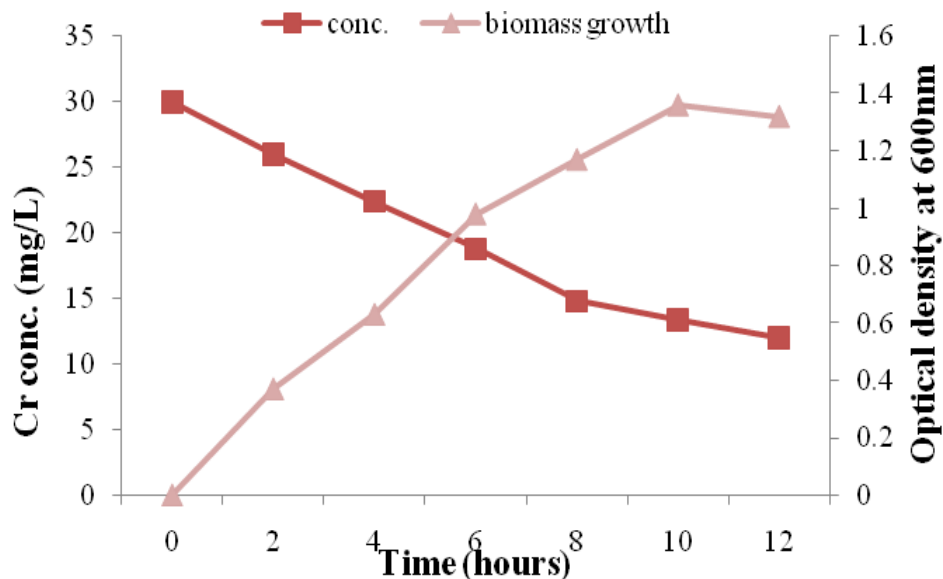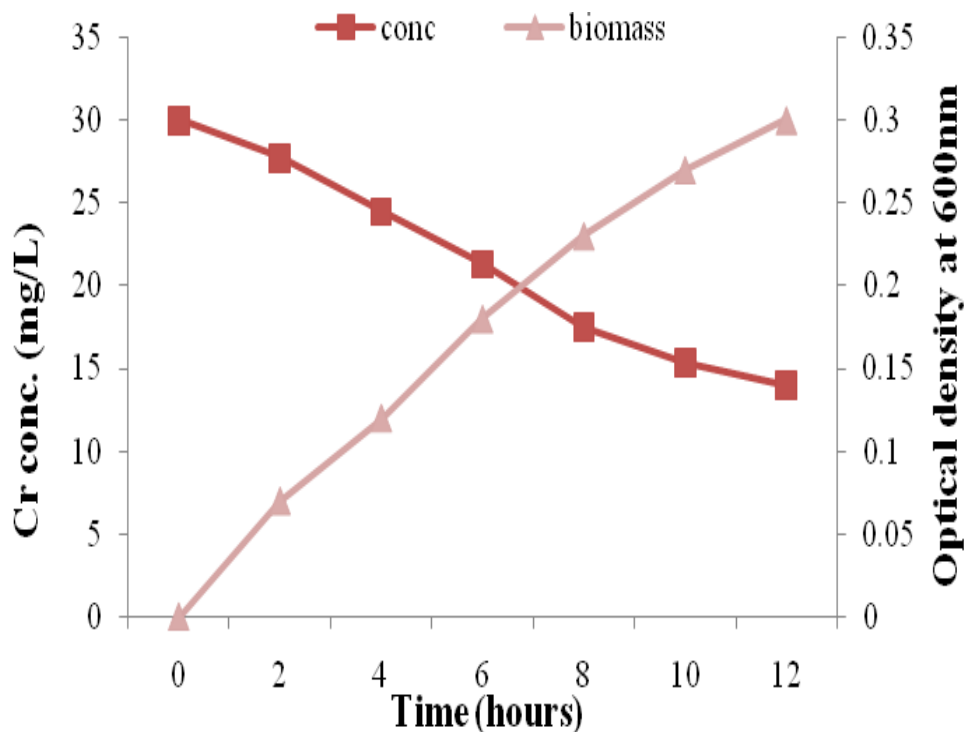
Figure-3
Cr (VI) reduction activity of isolated species (SP1) at initial Cr conc. 30mg/L and optical density of the solution at 600nm for the duration of 12h

Figure-4
Cr (VI) reduction activity of isolated species (SP2) at initial Cr conc. 30mg/L and optical density of the solution at 600nm for the duration of 12h
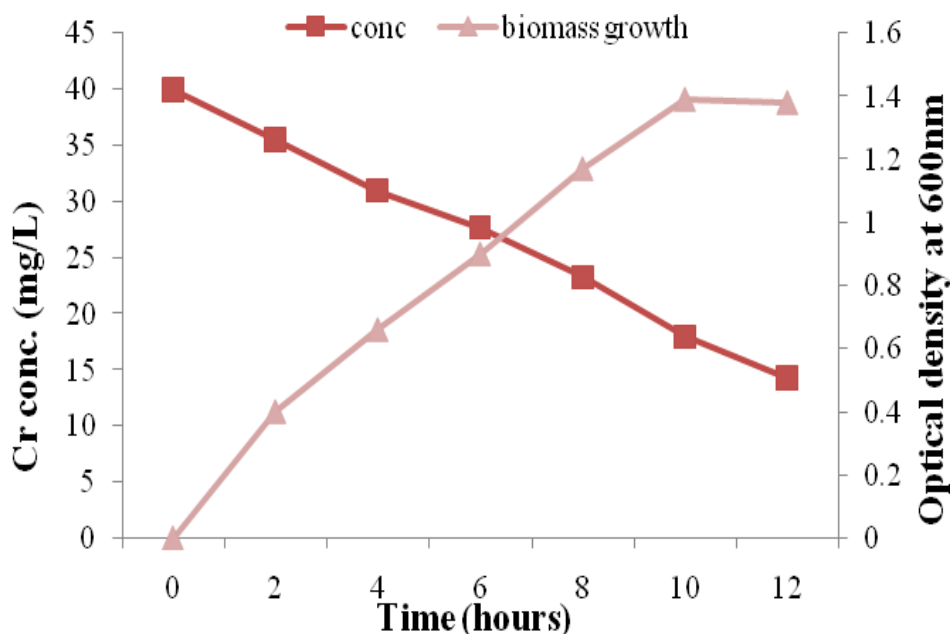
Figure-5
Cr (VI) reduction activity of isolated species (SP1) at initial Cr conc. 40mg/L and optical density of the solution at 600nm for the duration of 12h
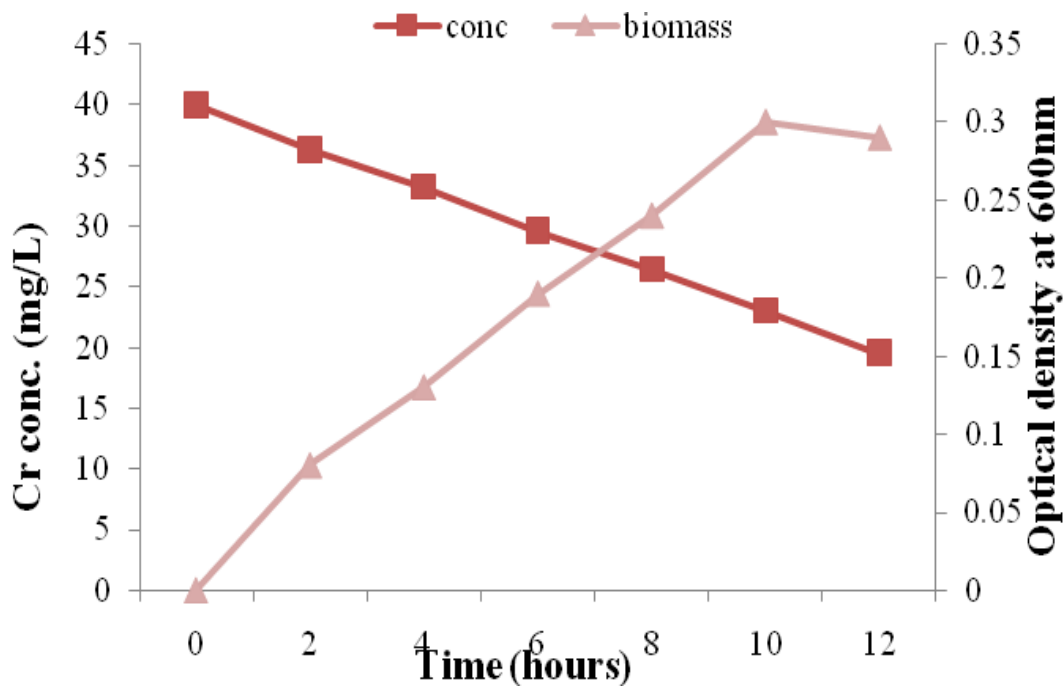

Figure-6
Cr (VI) reduction activity of isolated species (SP2) at initial Cr conc. 40mg/L and optical density of the solution at 600nm for the duration of 12h

Figure-7
Cr (VI) reduction activity of isolated species (SP1) at initial Cr conc. 50mg/L and optical density of the solution at 600nm for the duration of 12h

Figure-8
Cr (VI) reduction activity of isolated species (SP2) at initial Cr conc. 50mg/L and optical density of the solution at 600nm for the duration of 12h
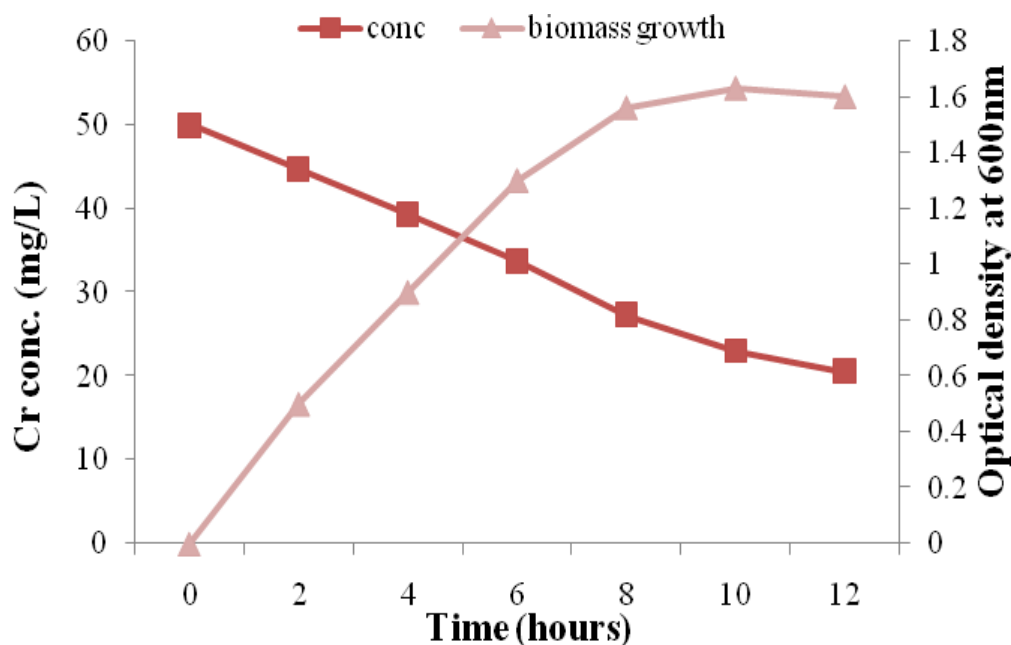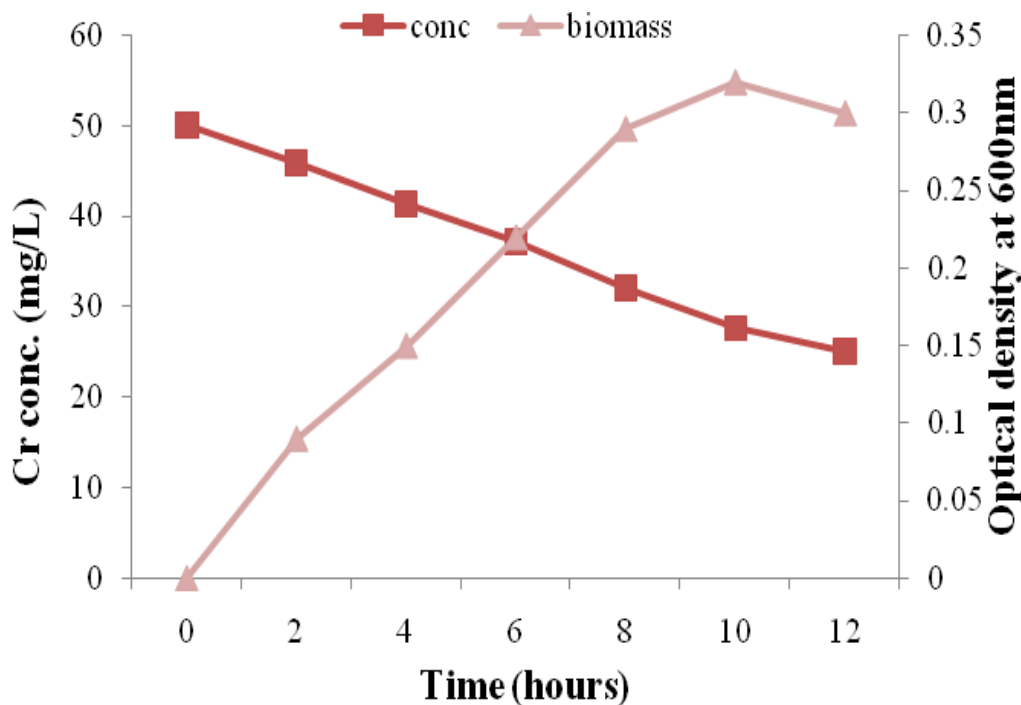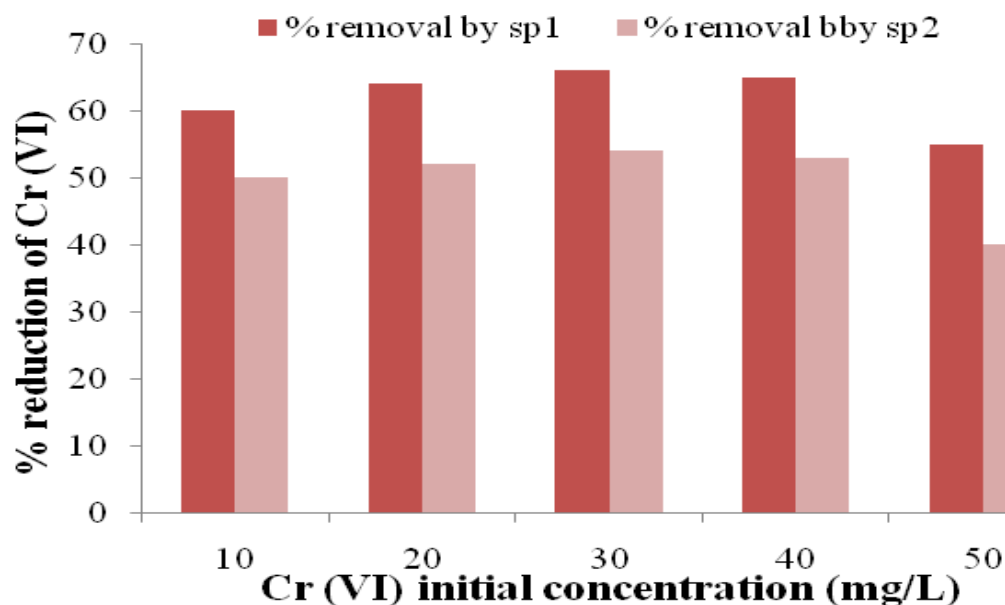
Figure-9
**Percentage reduction of Cr (VI) concentration at optimum temperature and pH by SP1 and SP2 where SP1 degraded 65%
and SP2 degraded 55% of Cr (VI)**

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