

BIOLOGICAL REMOVAL OF CR(VI) AND PHENOL FROM SINGLE AND BINARY SUBSTRATE SOLUTION PURE AND CONSORTIUM CULTURE OF *ESCHERICHIA COLI* AND *BACILLUS SP.*

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ABSTRACT: In this study bioaccumulation and biodegradation of Cr(VI) and phenol was carried out using bacterium *Bacillus sp.* (MTCC No. 3166) and *Escherichia coli* (NCIM No. 5051) from single and binary substrate solution of Cr(VI) and phenol. Shake flask batch experiments were performed in an incubator cum shaker at 37°C temperature. The bacterium *Escherichia coli* was acclimatized to the various concentrations of Cr(VI) and phenol in single and binary substrate solution. The binary substrate solution contains Cr(VI) and phenol in (1:2) ratio. The growth of bacterium in the presence of toxic pollutant Cr(VI) and phenol was analysed in UV spectrophotometer at 600 nm. The initial concentrations of Cr(VI) was varied from 5-25 mg/L for single substrate solution of Cr(VI) for pure culture of *Escherichia coli*, 10-50 mg/L for single substrate solution of Cr(VI) and 20-100 mg/L of phenol for pure and consortium culture of *Escherichia coli* and *Bacillus sp.*

Key words: *Escherichia coli*, *Bacillus sp.*, single, binary, substrate, acclimatization, bioaccumulation, biodegradation.

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INTRODUCTION

The pollution created by the organic compounds and heavy metals have harmful effects to the environment including man. Out of various toxic heavy metals, Cr(VI) is most mutagenic and carcinogenic to the living organism [1]. Chromium is found in environment in the two forms Cr(III) and Cr(VI), in which Cr(VI) is most toxic and mobile. Cr(III) (Chromium sulphate) is used in the tanning process in tanneries can be converted into Cr(VI) which caused drastic groundwater contamination around tanneries [2]. According to guidelines set by WHO (World Health organization) the maximum permissible limit for the discharge of chromium from various industries is 0.05 mg/L [3]. Inhalation of Cr(VI) via mouth and dermal contact can cause cancer of digestive tract and lungs and may cause epigastric, nausea, vomiting, severe diarrhea and hemorrhage [4]. Various methods are used for the removal of toxic metals and organic compounds such as oxidation-reduction, electrochemical, reverse osmosis, evaporation and ion exchange, but these methods have several disadvantages such as high energy and chemical requirements, low efficiency and usually produce a large amount of sludge [5,6,7]. Therefore there is essential to develop a biological method which is ecofriendly and cost effective. Biosorption is a process which use sorbent of biological origin having low cost, less sludge disposal problem, and can be regenerated [8]. The biological removal of Cr(VI) had a great interest during the last decades [9]. Biological removal of Cr(VI) is attached with cell multiplication phase when there is no limitation of carbon and nitrogen source [10,11,12]. Bacteria can be used as biosorbent due to advantages of their smaller size, ubiquity, ability to grow under controlled conditions and resilience to a wide range of environmental situations [13].

MATERIALS AND METHODS

Bacterial strain and medium

The microorganism *Bacillus* sp. (MTCC No. 3166) was purchased from IMTECH Chandigarh and the bacterium *Escherichia coli* (NCIM No. 5051) was purchased from National Collection of Industrial microorganisms (NCIM) Pune. The *Bacillus* sp. was grown in nutrient broth containing Beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g in 1 L of distilled water in an incubator cum shaker at 30°C and the *Escherichia coli* was grown in ATCC 57 Nutrient broth (K₂HPO₄ 7.0 g, KH₂PO₄ 3.0 g, glycerol 5.0 g, (NH₄)₂SO₄ 1.5 g, L-lysine 0.1 g, MgSO₄ 0.1 g, CaCl₂ 0.01 g, and FeSO₄.7H₂O 0.5 mg in 1 L of distilled water, pH 7.0) (Chulbae et al. 2000).

Preparation of Single and binary substrate solution of Cr(VI) and Phenol

All chemicals used for the batch experiment were of AR grade having more than 99% purity. Stock solutions of Cr(VI) and phenol were prepared by dissolving adequate amount of phenol and potassium dichromate in 1 L of distilled water. To avoid photo-oxidation of phenol, stock solution was stored in a brown glass bottle [14]. Different initial concentrations of batch experiments of Cr(VI) and phenol were prepared by diluting their respective stock solution.

Batch experiments

Batch experiments were performed in an incubator cum shaker at 30°C. Six flasks of 250 mL containing various concentrations of Cr(VI) (2.5, 5, 10, 15, 20 and 25 mg/L) and phenol (5, 10, 20, 30, 40, and 50 mg/L), working volume of 200 mL was used for the growth of *Bacillus* sp. and *Escherichia coli* using single substrate solution of Cr(VI) and phenol. *Bacillus* sp. and *Escherichia coli* were grown at different concentrations of Cr(VI) and phenol in an incubator cum shaker. The *Escherichia coli* was grown at different concentrations of Cr(VI). Single and binary solution (1: 2) of Cr(VI) and phenol was used for the acclimatization of *Bacillus* sp. 5 mL samples of different concentration of Cr(VI) and phenol from both single and binary solution were taken out at fixed interval of time from shaker and centrifuged at 10,000 rpm. All the batch experiments were carried out in properly cleaned UV chamber and all the glassware's used for the batch experiments were sterilized to avoid the contamination. For the measurement of biomass concentration, 5 mL samples of different concentrations of Cr(VI) and phenol were centrifuged at 10, 000 rpm and the bacterial biomass attached to the centrifuge tube was dissolved in 2 mL millipore water and its O D was taken at 600 nm [15,16]. The residual concentration of Cr(VI) and phenol was analyzed using U V spectrophotometer (HACH DR 5000) after centrifugation at 10,000 rpm. Calorimetric method 1, 5 diphenyl carbazide (E.M. Contreras et al. 2011) and 4 amino antipyrene (H. Song et al. 2009) method was used for the analysis of Cr(VI) and phenol, respectively (APHA, 2005). The wavelength used for the analysis of Cr(VI) and phenol was 540 nm and 510, nm respectively.

RESULTS AND DISCUSSION

Effect of concentration of Cr(VI) and phenol on growth of *Bacillus* sp. and *Escherichia coli* in single substrate solution

In this investigation, experiments were carried out in batch reactor to study the effect of Cr(VI) and phenol onto the growth pattern of bacterial strain *Bacillus* sp. and *Escherichia coli*. *Bacillus* sp. was used for both the reduction of Cr(VI) and biodegradation of phenol while *Escherichia coli* was used for the reduction of Cr(VI). The microorganism *Bacillus* sp. MTCC No. 3166 was purchased from Industrial microbial culture collection (IMTECH) Chandigarh and *Escherichia coli* NCIM No. 5051 was obtained from National Collection of Industrial microorganisms (NCIM) pune. The bacterium *Bacillus* sp. was acclimatized to the 50 mg/L of Cr(VI) and 100 mg/L of phenol. The bacterium *Escherichia coli* were acclimatized to the 25 mg/L of Cr(VI). Initially, the growth pattern of bacterium *Bacillus* sp. with and without 10 mg/L of Cr(VI) and 20 mg/L of phenol in minimal media was studied for single substrate solution. The growth pattern of *Escherichia coli* was studied with and without Cr(VI) at 5 mg/L of Cr(VI) for single substrate solution. The minimal media used for the growth of *Bacillus* sp. was Beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g, in 1 L of Millipore water. The *Escherichia coli* was purchased from NCIM pune and grown in ATCC 57 medium. ATCC 57 medium consists of (K₂HPO₄ 7.0 g, KH₂PO₄ 3.0 g, glycerol 5.0 g, (NH₄)₂SO₄ 1.5 g, L-lysine 0.1 g, MgSO₄ 0.1 g, CaCl₂ 0.01 g, and FeSO₄.7H₂O 0.5 mg in 1 L of distilled water, pH 7.0) [17].

The pH of nutrient medium was kept 7.0. An incubation time of 66 h and 60 h at temperature 30°C and pH 7.0 were considered for *Bacillus* sp. and *Escherichia coli*, respectively. The change in optical density (O.D.) of the growth media (for the experiment) with time is shown in Fig. 1(a) and Fig. 1(b) for *Bacillus* sp. and *Escherichia coli* respectively. The percentage removal obtained at different concentrations of Cr(VI) and phenol is given in Table 1.

Table 1: Percentage removal obtained at different concentrations of Cr(VI) and phenol.

Single substrate solution of Cr(VI) using <i>Escherichia coli</i>	Initial concentration (mg/L)		Percentage Removal	
	5		98.99	
	10		78.67	
	15		65.43	
	20		45.67	
Single substrate solution of Cr(VI) using <i>Bacillus</i> sp.	25		20.00	
	10		95.68	
	20		80.67	
	30		71.01	
	40		56.78	
Single substrate solution of phenol using <i>Bacillus</i> sp.	50		35.35	
	20		99.99	
	40		85.34	
	60		76.67	
	80		64.32	
Binary substrate solution of Cr(VI) and phenol using pure culture of <i>Bacillus</i> sp.	100		50.93	
	Initial concentrations of Cr(VI)	Initial concentrations of phenol	Percentage removal of Cr(VI)	Percentage removal of phenol
	10	20	98.99	90.56
	20	40	85.45	80.56
	30	60	75.67	71.21
	40	80	67.89	63.67
Binary substrate solution of Cr(VI) and phenol using consortium culture of <i>Escherichia coli</i> and <i>Bacillus</i> sp.	50	100	50.90	49.89
	10	20	99.99	95.67
	20	40	89.68	86.56
	30	60	80.99	79.67
	40	80	72.56	66.66
	50	100	60.98	53.45

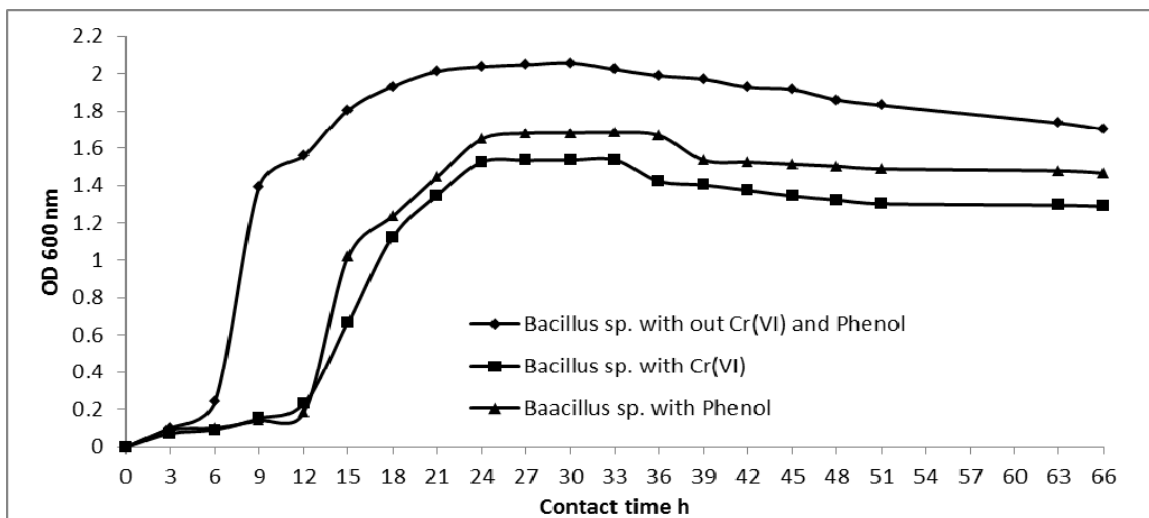


Figure 1(a): Effect of Cr (VI) and phenol onto the growth of *Bacillus* sp. under optimum conditions at temperature 37°C and pH 7.

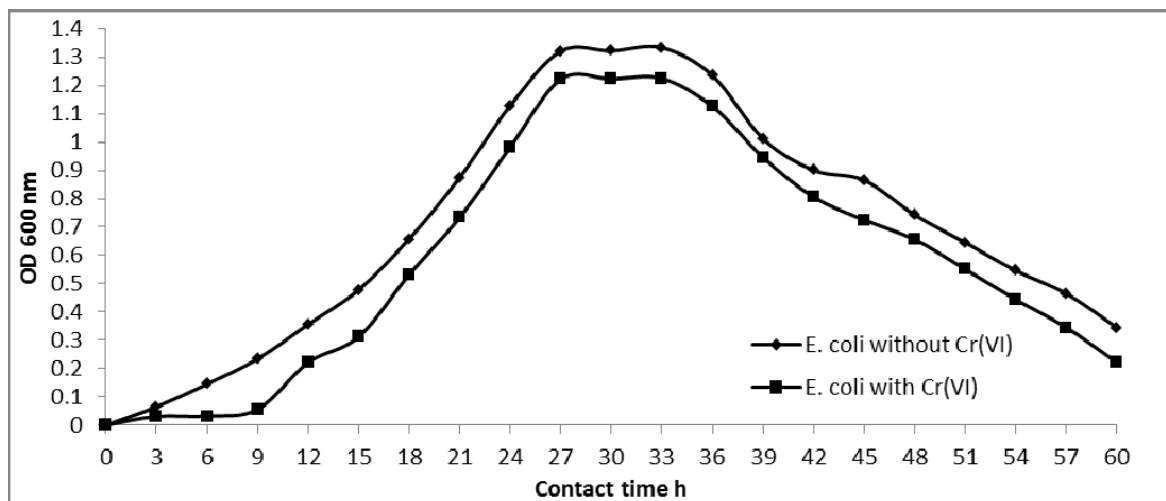


Figure 1(b): Effect of Cr (VI) onto the growth of *Escherichia coli* under optimum conditions as temperature 37°C and pH 7.

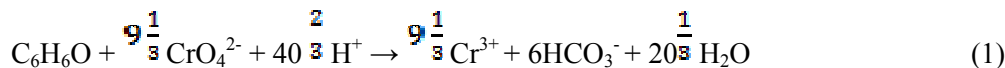
From Fig. 1(a) and Fig. 1(b), it is evident that the value of O.D. decreases slightly in the presence of 10 mg/L of Cr(VI) and 20 mg/L phenol in the minimal media, it is due to the inhibition effect of Cr(VI) and phenol. Moreover, duration of lag phase was increased in the acclimatization of bacterium cell to the Cr(VI) and phenol containing environment.

With the course of incubation period, the concentration of nutrient in the minimal decreases resulting in the consumption of Cr(VI) and phenol by the bacterium. Therefore, after the lag phase, the presence of Cr(VI) and phenol in the media which is toxic to bacterium cell, delayed lag phase and shortened the stationary phase. Generally, Cr(VI) and phenol resistant bacterium consume Cr(VI) and phenol ions in the late lag phase of growth curve after the exhaustion of first carbon source provided with minimal media [18,19].

Simultaneous reduction and biodegradation of phenol using pure and combined culture of *Escherichia coli* and *Bacillus* sp. in binary substrate solution:

Both pure and combined culture of *Escherichia coli* and *Bacillus* sp. were tested for the reduction of Cr(VI) and biodegradation of phenol. It was found that *Escherichia coli* was only capable of reducing Cr(VI) while *Bacillus* sp. was capable of both reducing Cr(VI) and biodegrading the phenol. The reduction of Cr(VI) and biodegradation of phenol using pure culture of both bacterium is discussed above. Both bacterial strain were acclimatized to the toxic concentrations of Cr(VI) and phenol. As *Bacillus* sp. was capable of both the reduction of Cr(VI) and biodegradation of phenol therefore simultaneous reduction of Cr(VI) and biodegradation of phenol was performed using this bacterium from binary solution.

The consortium culture of *Escherichia coli* and *Bacillus* sp. was grown in nutrient broth solution containing Beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g, in 1 L of Millipore water. When pure culture of *Bacillus* sp. was used for the simultaneous reduction of Cr(VI) and biodegradation of phenol it was capable of reducing highest concentration of Cr(VI) as lag phase was comparatively shorter in comparison to the single component solution of Cr(VI). The possible reason behind this fact that phenol was used as carbon source and helps in the reduction of Cr(VI). The simultaneous reduction of Cr(VI) and biodegradation of phenol was also achieved using consortium culture of *Escherichia coli* and *Bacillus* sp., in this case the reduction of Cr(VI) was more in comparison to the pure culture exposed to both single and binary solution. It can be due to the kinetics of Cr(VI) reduction using *Escherichia coli* was improved when coupled to the biodegradation of phenol using *Bacillus* sp. because the metabolites formed during biodegradation of phenol was utilized by *Escherichia coli* for the reduction of Cr(VI). The following reaction describes the kinetics of Cr(VI) reduction and degradation of phenol [20].



The above reaction depicts that phenol is used as electron donor for the reduction of Cr(VI) to Cr(III) which is no or less toxic to the living organism. Fig. 2(a) and Fig. 2(b) shows the growth curve of pure culture of *Bacillus* sp. and consortium culture of *Escherichia coli* and *Bacillus* sp. for the simultaneous reduction of Cr(VI) and biodegradation of phenol from binary solution. It was observed that O.D. at 600 nm was slightly less in the presence of 10 mg/L of Cr(VI) and 20 mg/L phenol in the minimal media than both pure culture of *Bacillus* sp. and consortium culture of *Bacillus* sp. and *Escherichia coli*, it is due to the inhibition effect of Cr(VI) and phenol. The lag phase was longer in the acclimatization of bacterium cell to the Cr(VI) and phenol containing environment.

It was observed that co-culture of *Bacillus* sp. and *Escherichia coli* show the less toxic effect as the value of OD obtained in this case was more than pure culture of *Bacillus* sp. and *Escherichia coli* for single substrate solution. The value of OD investigated for the simultaneous reduction of Cr(VI) and biodegradation of phenol using *Bacillus* sp. in binary substrate solution was more than a single substrate solution but less than co-culture of *Bacillus* sp. and *Escherichia coli* for the simultaneous reduction of Cr(VI) and biodegradation of phenol. The possible reason behind this fact was phenol used as carbon source like glucose by the bacterium which inhibits the toxic effects of Cr(VI). In the case of co culture metabolites are formed during biodegradation of phenol by *Bacillus* sp. which is used by *Escherichia coli* for the reduction of Cr(VI). It is evident in the case of co-culture of *Bacillus* sp. and *Escherichia coli* biodegradation of phenol by *Bacillus* sp. initialized the energy flow to the co-culture and served as energy source for both bacterium therefore amount of phenol consumed was more than stoichiometric amount of phenol.

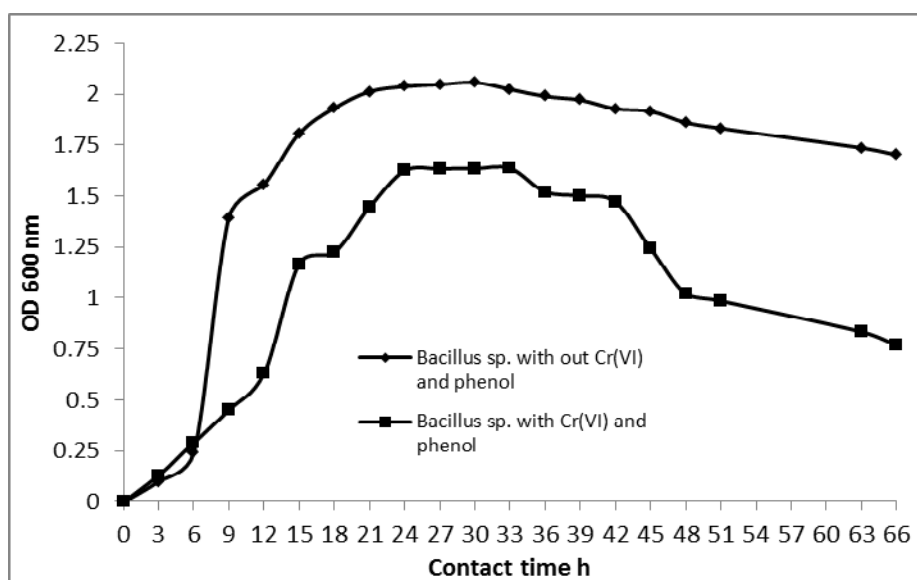


Figure 2(a) Growth curve for simultaneous reduction of Cr (VI) and biodegradation of phenol using pure culture of *Bacillus* sp.

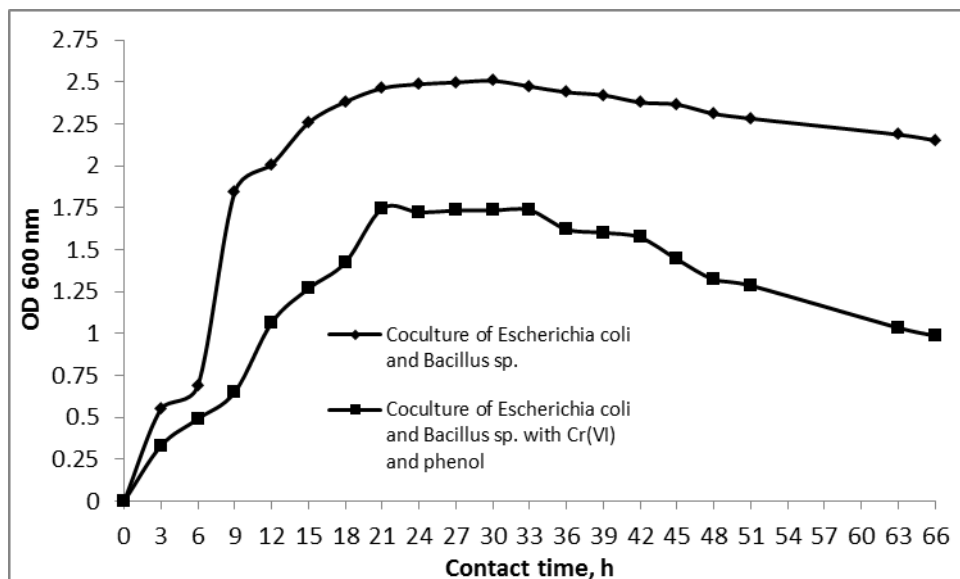


Figure 2(b) Growth curve for simultaneous reduction of Cr(VI) and biodegradation of phenol using consortium culture of *Escherichia coli* and *Bacillus sp.*

Cr(VI) reduction and phenol biodegradation tendency using pure culture of *Escherichia coli* and *Bacillus sp.* from single substrate solution

Batch experiments were performed to investigate the Cr(VI) and phenol bioaccumulation and biodegradation tendency on the bacterium strain *Bacillus sp.* and reduction of Cr(VI) on the bacterium strain *Escherichia coli* at different initial concentrations (mg/L) of Cr(VI) and phenol. The concentrations of Cr(VI) and phenol in the solution were in the range of 10-50 mg/L and 20-100 mg/L, respectively for bacterial strain *Bacillus sp.* and 5-25 mg/L of Cr(VI) using bacterial strain *Escherichia coli*. The same conditions were maintained as pH 7 and temperature 37°C for the experimentation. The change in optical density (O.D.) of the growth media at various Cr(VI) and phenol concentrations with time is depicted in Fig. 3(a) and Fig. 3(b) respectively for Cr(VI) and phenol respectively. The change in optical density (O.D.) of the growth media at various concentrations of Cr(VI) using bacterial strain *Escherichia coli* is shown in Fig. 3(c).

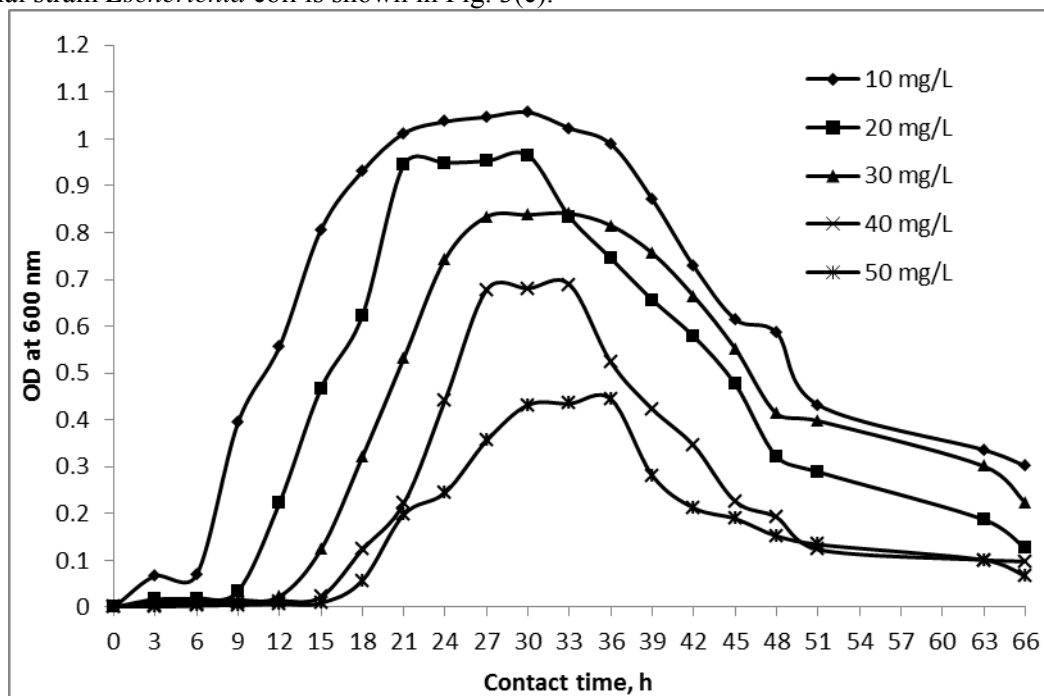


Figure 3(a): The growth of *Bacillus sp.* at various initial Cr(VI) concentrations under optimum conditions as temperature 37°C and pH 7.

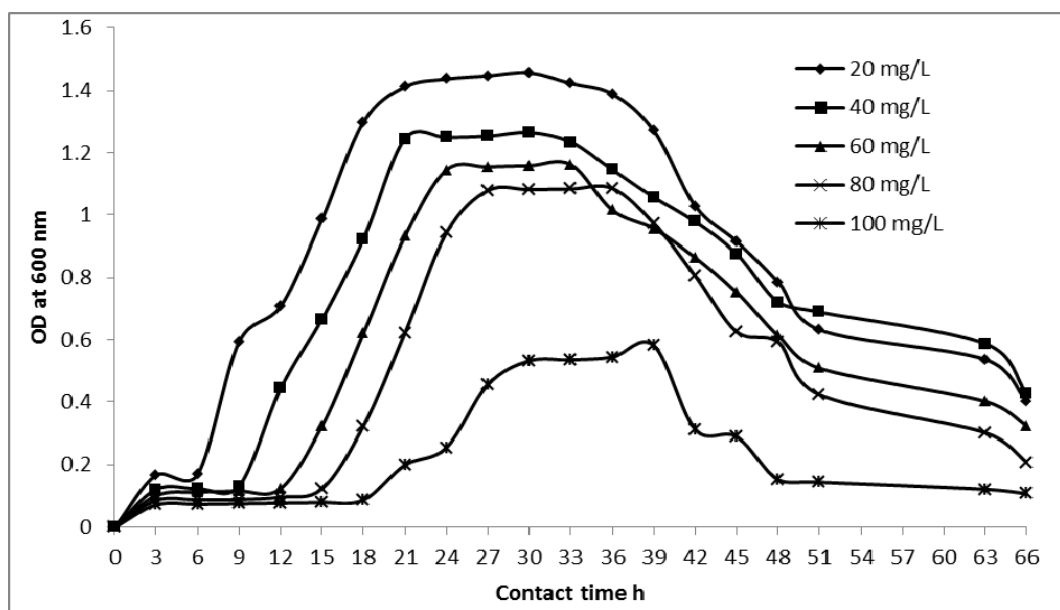


Figure 3(b): The growth of *Bacillus* sp. at various initial Phenol concentrations under optimum conditions as temperature 37°C and pH 7.

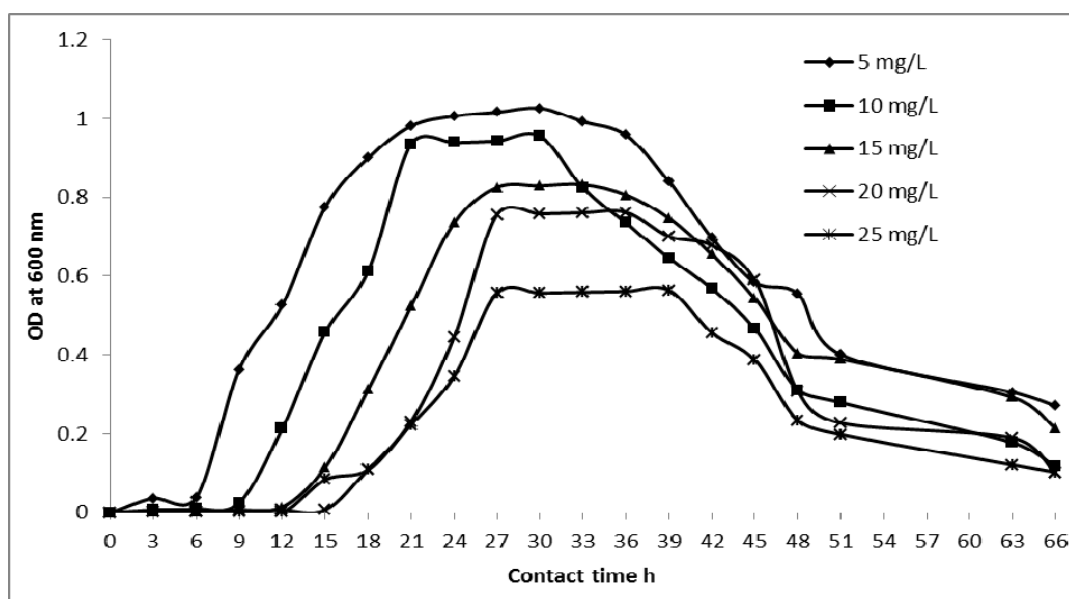


Figure 3(c): The growth of *Escherichia coli* at various initial Cr(VI) concentrations under optimum conditions at temperature 37°C and pH 7.

At initial stage, Cr (VI) and phenol accumulation rate was slow and steady but gradually the growth rate of bacterium cell increased with time and rate of Cr(VI) reduction and phenol biodegradation also increased with the acclimatization for Cr(VI) and phenol. However, with the increase in Cr(VI) and phenol concentrations in the liquid media, the growth rate of bacterium cell was decreased for both bacterial strain *Bacillus* sp. and *Escherichia coli*. The maximum Cr(VI) accumulation rate of Cr(VI) and biodegradation rate of phenol were obtained at a 50 mg/L of Cr(VI) and 100 mg/L of phenol, respectively by bacterial strain *Bacillus* sp. after increase in concentration of Cr(VI) and phenol no or very less growth of bacterium was observed. The bacterial strain *Escherichia coli* was capable of accumulating 25 mg/L of Cr(VI). The Cr(VI) concentration above 50 mg/L and phenol above 100 mg/L were found to be toxic to bacterium *Bacillus* sp. and bioaccumulation and biodegradation rate were decreased. Similarly Cr(VI) concentration above 25 mg/L shows toxic effects for bacterial strain *Bacillus* sp. The lag phase of the bacterium cell ranges from 3-6 h at lower concentrations and increased up to 12 h at higher concentration for both bacterial strain *Bacillus* sp. and *Escherichia coli*.

The exponential and stationary phase for bacterium growth at Cr(VI) concentration ranging from 10-50 mg/L and phenol concentrations 10-100 mg/L were obtained in between 12 to 36 h for *Bacillus* sp. For bacterial strain *Escherichia coli* the lag phase and exponential phase were obtained between 12 to 30 h. After 36 to 66 h for both bacterium *Bacillus* sp. and *Escherichia coli*, there was no increase in growth of bacterium was observed in the medium containing Cr(VI) and phenol. This is due to the saturation of Cr(VI) and phenol accumulation by the bacterium and then the cell enters into death phase [21]. In case of Cr(VI) concentration of 50 mg/l and 100 mg/l of phenol, the bacterium adopted less exponential phase, followed by stationary phase and ultimately the cells start to die (death phase). This short-term growth of bacterium cells may be due to the maximum inhibitory concentration (MIC) of Cr (VI) and phenol [22]. MIC is the concentration at which no or very less growth of bacterium were observed.

The experimental results reveal that the highest rate of Cr (VI) and phenol accumulation was achieved in the exponential phase of bacterium cell growth. Due to the toxicity of Cr (VI) and phenol to the cells, reduction in Cr(VI) accumulation and biodegradation of phenol were obtained at higher Cr (VI) and phenol concentrations, respectively. Moreover, after 21 h of agitation time, stationary phase was reached resulting in reduction of Cr (VI) accumulation and biodegradation of phenol by the both bacterium strain *Bacillus* sp. and *Escherichia coli*. Similar results were obtained by the various researchers for removal of toxic elements from contaminated water [23,24, 25].

Tendency of simultaneous reduction of Cr(VI) and biodegradation of phenol using pure and combined culture of *Escherichia coli* and *Bacillus* sp. from binary substrate solution

Batch experiments were performed to investigate the tendency of simultaneous reduction of Cr(VI) and biodegradation of phenol using pure culture of *Bacillus* sp. and consortium culture of *Bacillus* sp. and *Escherichia coli* at different initial concentrations of Cr(VI) and phenol (2:1) according to the composition of waste water discharged by various industries. The concentrations of Cr(VI) and phenol in the solution were in the range of 20-100 mg/L and 10-50 mg/L, respectively for both pure culture of *Bacillus* sp. and consortium culture of *Bacillus* sp. and *Escherichia coli*. In this study an incubation time of 66 h at a temperature of 30°C and pH 7.0 were considered. The change in optical density (O.D.) of the growth media with time at various Cr(VI) and phenol concentrations is depicted in Fig. 4(a) and Fig. 4(b) for pure culture of *Bacillus* sp. and consortium culture of *Bacillus* sp. and *Escherichia coli*, respectively. For both pure culture of *Bacillus* sp. and consortium culture of *Bacillus* sp. and *Escherichia coli* the lag phase was increased with the increase in toxic concentrations of Cr(VI) and phenol and shortened the stationary phase in case of co culture the stationary phase was longer than pure culture of *Bacillus* sp.

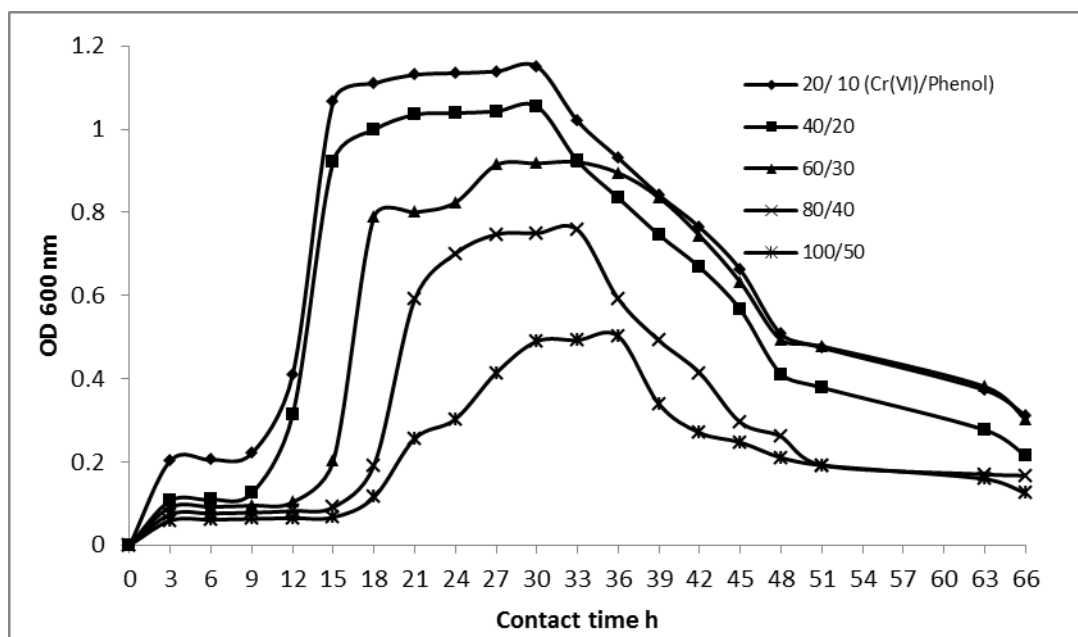


Figure 4(a) Growth curve for simultaneous reduction and biodegradation of phenol using pure culture of *Bacillus* sp. at various concentrations of Cr(VI) and phenol (2:1).

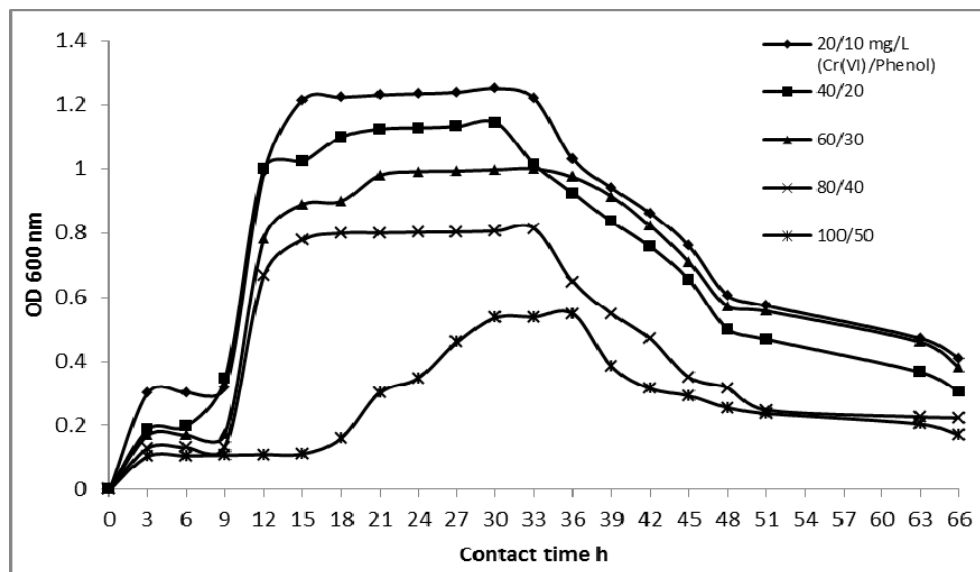


Figure 4(b) growth curve for simultaneous reduction and biodegradation of phenol using consortium culture of *Bacillus* sp. and *Escherichia coli* at various concentrations of Cr(VI) and phenol (2:1).

CONCLUSION

In this study *Escherichia coli* and *Bacillus* sp. was acclimatized to the various toxic concentrations of Cr(VI) and phenol in single and binary substrate solution. The bacterium *Escherichia coli* was acclimatized up to 25 mg/L of Cr(VI) in single substrate solution while *Bacillus* sp. was acclimatized to the 50 mg/L of Cr(VI) and 100 mg/L of phenol in single and binary substrate solution. Simultaneous reduction of Cr(VI) with biodegradation of phenol was also carried out using consortium culture of *Escherichia coli* and *Bacillus* sp. It was observed that the growth of bacterium *Bacillus* sp. was maximum in the presence of phenol in binary substrate solution of Cr(VI) and phenol in comparison to single substrate solution of Cr(VI). The approx. 99% removal was obtained at 5 mg/L of Cr(VI) for single substrate solution of Cr(VI) using *Escherichia coli* and 10 mg/L of Cr(VI) and 20 mg/L phenol for binary substrate solution of Cr(VI) and phenol using pure culture of *Bacillus* sp. and consortium culture *Bacillus* sp. and *Escherichia coli*. The probable reason behind this fact as phenol was used as carbon source which inhibits the toxic effect of Cr(VI). In case of simultaneous reduction of Cr(VI) with biodegradation of phenol, the metabolites formed during the biodegradation of phenol by *Bacillus* sp. was utilized by *Escherichia coli* for the reduction of Cr(VI) in binary substrate solution. Therefore mixed culture of *Escherichia coli* and *Bacillus* sp. was more efficient for the removal of Cr(VI) and phenol than pure culture of *Bacillus* sp.

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