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Studies on Selection of Efficient Bacterial Strain Simultaneously Tolerant to Hexavalent Chromium and Pentachlorophenol Isolated from Treated Tannery Effluent

M. Tripathi and S.K. Garg
Centre of Excellence in Microbiology,
Dr. Ram Manohar Lohia Avadh University, Faizabad-224001, UP, India

Abstract: Bioremediation is being viewed as a clean technology for decontamination of pentachlorophenol and chromium from tannery effluent. This study was conducted to isolate an efficient bacterial culture from treated tannery effluent which is tolerant to pentachlorophenol and Cr (VI) and could be employed for simultaneous bioremediation of both the toxic contaminants. Tannery effluent sample was collected from Common Effluent Treatment Plant, Kanpur (India). Fifty four bacterial cultures isolated by enrichment culture technique were screened for PCP and Cr (VI) tolerance on minimal salt medium supplemented with glucose as a cometabolite. The isolate B4 was found to be maximally tolerant to high concentration of both pentachlorophenol (500 mg L^{-1}) and chromium (VI) (200 mg L^{-1}) and was selected for further studies. It was identified as *Bacillus* sp. by morphological and biochemical analyses. The effect of various growth parameters such as carbon source at 0.2-1.0% (glucose, maltose, sucrose), pH (6.5-8.0), temperature (25-40°C) and inoculum size (0.5-2.5% v/v) were evaluated in minimal salt medium supplemented with 500 mg L^{-1} PCP and 200 mg L^{-1} Cr (VI). The best growth was exhibited at 0.4 % glucose, pH 7.0, 35°C and with 1.0% inoculum under shaking at 150 rpm. Thus, our isolate appears to have great potential for simultaneous bioremediation of pentachlorophenol and hexavalent chromium from the contaminated sites.

Key words: Bioremediation, chromium, pentachlorophenol, *Bacillus* sp., cometabolite

INTRODUCTION

The effluent released from tannery contains higher concentration of total dissolved solids, phenols, chlorides, chromium and other heavy metals, etc. (Armienta *et al*., 2001). Metals and chlorinated phenols pose serious threat to the ecosystem due to their hazardous impact on life forms. Contamination of Cr (VI) and pentachlorophenol (PCP) has become a serious concern for tannery effluent in India. The industrial effluents are released directly or indirectly into natural water resources, mostly without proper treatment thus posing a serious threat to the environment. The high BOD and COD indicates an elevated amount of organic compounds that are being discharged into the water bodies contributing to eutrophication. Both Cr (VI) and PCP are listed as priority pollutants by United States Environmental Protection Agency (USEPA). In the environment, chromium occurs mainly in trivalent and

Corresponding Author: S.K. Garg, Department of Microbiology, Dr. Ram Manohar Lohia Avadh University, Faizabad-224001, Uttar Pradesh, India

hexavalent forms (Horitsu *et al*., 1987). This metal is the second most common inorganic contaminant of ground water at hazardous waste sites (Horton *et al*., 2006). Cr (VI) is one of the major environmental pollutants which enters the ecosystem by metal finishing, leather tanning, chromate preparation and cooling towers of atomic power plants. Chromium sulphate [Cr (III)] is used as a tanning agent leading to its transformation in Cr (VI) which is more toxic than Cr (III). Cr (VI) is soluble, toxic and carcinogenic (Ackerley *et al*., 2004). On account of its rapid permeability, interaction with proteins and nucleic acids and indirectly generating oxygen radicals, Cr (VI) poses severe health risk in humans, animals and plants (Upreti *et al*., 2004). The maximum permissible levels of Cr (VI) in potable and industrial waste water are 0.05 and 0.1 mg L^{-1}, respectively (Goyal *et al*., 2003). Several bacteria such as *Pseudomonas ambigua*, *Serratia marcescens*, *Bacillus* sp., *Bacillus cereus* have been reported for Cr (VI) reduction (Horitsu *et al*., 1987; Mondaca *et al*., 2002; Camargo *et al*., 2004; Faisal and Hasnain, 2006; Iftikhar *et al*., 2007). Serious concern about the toxicity of chromium compounds necessitates recovery and reuse of chromium from tannery effluent as well as other industrial wastes and/or rendering it to a less toxic form (Yamamoto *et al*., 1993).

Pentachlorophenol is used as a biocide in the tanning process. Besides Cr (VI), PCP is highly toxic and a recalcitrant organic compound in tannery effluent. This polychlorinated aromatic compound is toxic to numerous aquatic organisms at a concentration as low as 0.5 mg L^{-1} (Borthwick and Schimmel, 1978). Because of its stable aromatic ring system and high chloride content, PCP persists in the environment (Copley, 2000). High level of PCP causes uncoupling of oxidative phosphorylation, inhibition of oxidative phosphorylation, inactivation of respiratory enzymes and damage to mitochondrial structure (Bevenue and Beckman, 1987). Different researchers have reported PCP degrading microorganisms such as *Arthrobacter* sp., *Sphingobium chlorophenolicum*, *Serratia marcescens* from the natural environment (Edgehill, 1994; Dams *et al*., 2007; Singh *et al*., 2007).

Only a limited research work has been done towards simultaneous bioremediation of Cr (VI) and phenolics in the tannery effluent, particularly by native microbes (Chirwa and Wang, 2005; Srivastava *et al*., 2007; Tziotzios *et al*., 2008). Most of the researchers have employed either coculture or microbial consortium for simultaneous bioremediation of PCP and Cr (VI). If a single potent indigenous strain is available, its nutritional requirement, growth and maintenance are likely to be more conveniently managed than a coculture or a consortium. In view of the above facts, the present study was aimed to isolate an efficient indigenous bacterial strain that has ability to grow under varied environmental conditions, particularly in the presence of PCP and chromium at high concentrations.

MATERIALS AND METHODS

Sampling

The treated tannery effluent samples were collected in the year 2008 from different sites in sterile glass bottles from common effluent treatment plant (CETP), Kanpur (India), transported on ice to the laboratory and used for analyses.

Isolation and Screening of PCP and Cr (VI) Resistant Bacteria

The Cr (VI) and PCP resistant bacteria were isolated and enumerated by standard plate technique (Baldi *et al*., 1990; APHA/AWWA/WEF, 1998), respectively on Nutrient Agar (NA) and minimal salt agar (MSM) medium containing (g L^{-1}): KH_2PO_4, 6.0; Na_2HPO_4, 7.0;

$MgSO_4.7H_2O$, 0.2; NH_4Cl, 2.0 and agar 18.0 supplemented with glucose as a cometabolite. The MSM and NA plates were amended with different concentrations (mg L^{-1}) of PCP (50-550) and Cr (VI) (50-550) and incubated at 35±1°C for 60 and 48 h, respectively. The Minimum Inhibitory Concentration (MIC) of PCP and Cr (VI) at which no growth occurred, was determined by both agar and culture broth methods (Luli *et al*., 1983; Calomoris *et al*., 1984). The bacterial colonies on MSM agar (4 isolates) and NA plates (50 isolates) were picked and purified by repeated streaking on the same medium. The above bacterial isolates (54) were screened on MSM agar plates amended with varying concentrations of both PCP and Cr (VI) and incubated at 35±1°C for 60 h for isolation of the most efficient bacterial strain likely to be employed for further studies.

Culture Identification

The selected bacterial strain was characterized in our laboratory by morphological, physiological and biochemical tests as per Bergey's Manual of Determinative Bacteriology (Holt, 1994). The tests included Gram's staining, spore formation, motility, catalase, cytochrome oxidase, arginine dihydrolase, lysine and ornithine decarboxylase, nitrate reduction, starch and casein hydrolysis, urea hydrolysis, growth on MacConkey, gelatin liquefaction, indole, methyl red and Voges Proskauer test, anaerobic growth, acid production from carbohydrate catabolism and oxidation/fermentation. The results were authenticated by Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India in the year 2009.

Effect of Various Growth Parameters

Influence of Carbon Source

The effect of carbon source (glucose, maltose, sucrose) was studied on the growth of bacterial isolate. Various concentrations of glucose, sucrose and maltose as an additional carbon and energy source @ 0.2-1.0 % (w/v) was supplemented in MSM broth amended with maximum tolerable concentration of PCP (500 mg L^{-1}) and Cr (VI) (200 mg L). The broth was inoculated with fresh culture of exponentially growing organism (@ 1% v/v) of 0.86 absorbance having cell density of 3.0×10^6 cfu mL^{-1} and incubated at 35±1°C on a rotatory shaker (New Brunswick Scientific, USA) at 150 rpm for 60 h. The growth was evaluated periodically at 12 h interval up to 60 h by measuring the absorbance at 600 nm.

Effect of Inoculum Size, pH and Temperature on Bacterial Growth

Inoculum Size

The above medium was adjusted to pH 7±0.2 using 0.1 N NaOH or 0.1 N HCl, inoculated with 0.5-2.5% (v/v) exponentially growing culture and was incubated at 35±1°C on an incubator shaker at 150 rpm for 60 h. The samples were withdrawn at an interval of 12 h and analyzed for growth.

Initial pH and Incubation Temperature

The pH of the MSM broth amended with 500 mg L^{-1} PCP and 200 mg L^{-1} Cr (VI) supplemented with optimized concentration of glucose at 0.4% (w/v) in 250 ml an Erlenmeyer flask was adjusted prior to sterilization, in the range of pH 6.5-8.0. The sterilized culture flasks were inoculated with optimized dose i.e., at 1% (v/v) of log phase bacterial inoculum. The above flasks were then incubated at different temperatures in the range of 25-40°C on an incubator shaker at 150 rpm so as to determine the combined effect of pH and temperature on growth during the course of 60 h incubation.

Statistical Analysis

The statistical calculation was performed according to the standard method (Steel and Torrie, 1980). The results are given as Mean±SD values.

RESULTS AND DISCUSSION

Isolation and MIC Determination

In this study, we describe the isolation and screening of Cr (VI) and/or PCP tolerant bacterial isolates and the characterization of most efficient culture for simultaneous bioremediation of both the contaminants. The results presented in Table 1 reveal that 50 bacterial isolates (>92%) were resistant to Cr (VI) at >50 mg L^{-1}, while 4 (~8%) were resistant to PCP concentration of >50 mg L^{-1} in the presence of glucose supplemented at 0.4% (w/v) as an additional carbon and energy source. There was no growth in both agar as well as broth media which were not supplemented with glucose, indicating the phenomenon of co-metabolism in which microorganisms do not obtain energy from the transformation reaction, rather require another substrate for growth. Dehalogenation and oxidative dehalogenation reactions are important cometabolism reactions, which may make chlorinated xenobiotic molecule accessible for further breakdown (Cruger and Cruger, 1989). Further, the number of bacterial isolates was inversely proportional to increasing concentration of PCP and Cr (VI) at >50 mg L^{-1} concentration. There was only one isolate designated as B4 which was tolerant to simultaneous presence of very high concentration of PCP at 500 mg L^{-1} and Cr (VI) at 200 mg L^{-1} (Table 1). This isolate was selected for further studies. Chandra *et al.* (2006) reported the isolation of two bacterial strains *Bacillus cereus* and *Serratia marcescens* from pulp-paper mill effluent which were able to tolerate individual PCP at 300 mg L^{-1} concentration. Srivastava *et al.* (2007) have also isolated *Acinetobacter* sp. from pulp-paper industries which was simultaneously tolerant to PCP only at 50 mg L^{-1} and Cr (VI) at 500 mg L^{-1} concentrations. Contrary to that, our isolate was tolerant to high PCP concentration at 500 mg L^{-1} but lower Cr (VI) level at 200 mg L^{-1}. The literature availability on simultaneous tolerance of bacteria to PCP and Cr (VI) is scanty and so far no one has reported the native strain from tannery effluent.

Culture B4 is an endospore forming Gram-positive rod, motile, catalase positive, fermentative, nitrate reducer producing acids from sugars viz., dextrose, lactose, maltose,

Table 1: Isolation of hexavalent chromium (Cr VI) and/or pentachlorophenol (PCP) tolerant bacterial isolates from treated tannery effluent and determination of their minimum inhibitory concentration (MIC) in search to find efficient strain for simultaneous bioremediation of both the toxic contaminants

Cr (VI)	Bacterial isolates from			Bacterial isolates from		PCP+Cr (VI)		Bacterial isolates from	
(mg L^{-1})	NA	NB	PCP (mg L^{-1})	MSMA	MSMB	-----(mg L^{-1})-----		MSMA	MSMB
50	50	50	50	4	4	50	200	3	3
100	36	29	100	3	3	100	200	3	2
150	24	20	150	3	2	150	200	2	2
200	17	13	200	2	1	200	200	2	1
250	06	5	250	1	1	250	200	1	1
300	4	2	300	1	1	300	200	1	1
350	2	2	350	1	1	350	200	1	1
400	2	1	400	1	1	400	200	1	1
450	1	1	450	1	1	450	200	1	1
500	1	-	500	1	1	500	200	1	1
550	-	-	550	-	-	550	200	-	-

NA: Nutrient agar, NB-Nutrient broth, MSMA: Minimal salt agar medium, MSMB: Minimal salt broth medium

Table 2: Morphological, physiological and biochemical tests on selected efficient bacterial isolate B4 simultaneously tolerant to pentachlorophenol (500 mg PCP/l) and hexavalent chromium (200 Cr VI/l)

		Acid production from carbohydrates	
Name of the test	Observation	Carbohydrate	Result
Gram's reaction	+	Adonitol	-
Shape	Moderate rod	Arabinose	-
Colony configuration	Round	Cellobiose	+
Margin	Wavy	Dextrose	+
Elevation	Convex	Dulcitol	-
Surface	Rough	Fructose	-
Endospores	+	Galactose	-
Motility	+	Inositol	-
Fluoroscence (UV)	-	Lactose	+
Anaerobic growth	+	Maltose	+
Growth on MacConkey agar	-	Mannitol	-
Indole test	-	Melibiose	+
Methyl Red test	-	Raffinose	+
Voges Proskauer test	-	Rhamnose	-
Citrate utilization	-	Salicin	+
Gas production from glucose	-	Sorbitol	+
Casein hydrolysis	-	Sucrose	+
Starch hydrolysis	-	Trehalose	+
Urea hydrolysis	-	Xylose	+
Nitrate reduction	+		
H_2S production	-		
Cytochrome oxidase	±		
Catalase test	+		
Oxidation/fermentation (O/F)	F		
Gelatin hydrolysis	-		
Arginine dihydrolase	-		
Lysine decarboxylase	-		
Ornithine decarboxylase	-		

'+', reaction or property present; '-', reaction or property absent, '±', result not clear, i.e., non definable; 'F', fermentative

melibiose, raffinose, sucrose, salicin, sorbitol, trehalose, xylose and cellobiose. It showed no growth on MacConkey agar and negative test for H_2S production, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrolysis of casein, urea, starch and gelatin (Table 2). The morphological, physiological and biochemical identification revealed the identity of our efficient isolate as *Bacillus* sp. as per Bergeys Manual of Determinative Bacteriology which was assigned MTCC number 9777 at Institute of Microbial Technology, Chandigarh (India).

Effect of Carbon Source

The effect of carbon source as cosubstrate on growth of *Bacillus* sp. isolate was studied in MSM broth containing maximum tolerable concentration of PCP (500 mg L^{-1}) and Cr (VI) (200 mg L^{-1}). Although, sucrose and maltose supported the growth of this organism, the glucose was a better additional source of carbon and energy. The maximum growth of our isolate was noted with glucose cometabolite at 0.4 % (w/v) level (Fig. 1). Among the sugars tested, glucose is the most easily metabolizable sugar and therefore supported the maximum growth. Furthermore, the enzymes required for glucose metabolism are constitutive type which are always synthesized by the cells independent of environmental stimulation. Any deviation in glucose concentration from optimum 0.4%, the growth rate was adversely affected (Fig. 1). Our findings are in agreement with the results of Premlatha and Rajkumar (1994) who have also found glucose as the best carbon source at 0.5% level for supporting

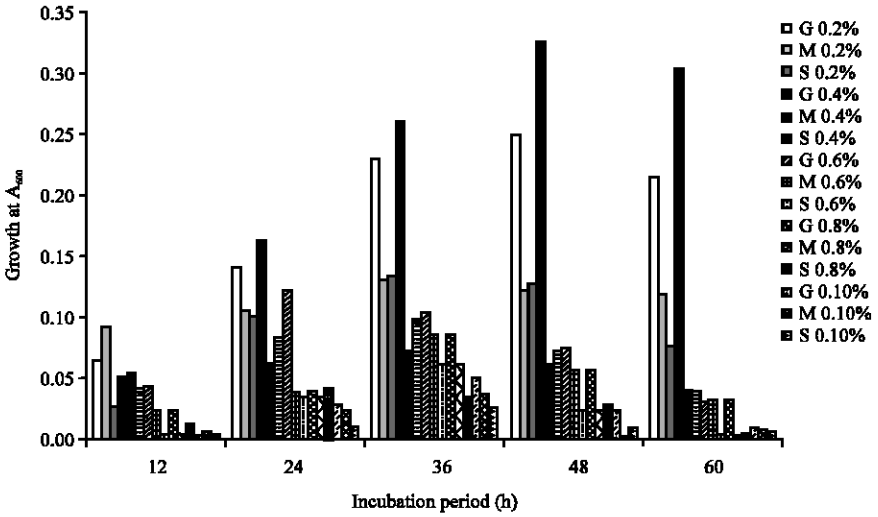

Fig. 1: Influence of carbon source (glucose-G, maltose-M, sucrose- S) on growth of bacterial isolate in MSM broth containing PCP 500 mg L^{-1} and Cr (VI) 200 mg L^{-1} supplemented with 0.4% (w/v) glucose

the optimum growth of *Pseudomonas aeruginosa* in the presence of pentachlorophenol alone. Murialdo *et al.* (2003) have reported that glucose and glutamate had positive effects on the population density of *Pseudomonas* sp. when employed for individual PCP degradation. Singh *et al.* (2007) have also used MSM broth amended with glucose for the isolation of individual PCP tolerant bacterial strains which could not grow in the absence of cometabolite glucose. The best bacterial isolate was identified by them as *Serratia marcescens* ITRC S7 strain which exhibited best growth and PCP degradation at 1.0% (w/v) glucose cometabolie concentration.

Influence of Inoculum Size

At each dose of inoculum employed, the growth rate increased with the course of time during 0 to 48 h incubation followed by decline up to 60 h. The growth of our isolate in the presence of PCP and Cr (VI) was noted at all the cell densities tested. However, maximum growth was evident when culture broth was inoculated with 1.0% inoculum and therefore employed in future experiments (Fig. 2). Wolski *et al.* (2005) have also reported similar results on PCP degradation by *Pseudomonas* sp. Further, they noted that with high cell density, the PCP degraded rapidly, while with low inoculum density, PCP degradation was progressive.

Combined Effect of Temperature and pH on Growth of the Isolate

The cultural factors such as pH and temperature in the environment affect the growth of microorganisms. The influence of pH and temperature on growth of *Bacillus* sp. was evaluated. This isolate could grow on high concentration of PCP and Cr (VI) at a wide range of temperature and pH (Table 3). The results further reveal that maximum growth was evident at pH 7.0, 35±1°C under shaking conditions (150 rpm) at 48 h of incubation. Present results on pH optimization for the growth of Cr (VI) and PCP resistant isolate are in accordance with the findings of Losi *et al.* (1994) who have also found pH 7.0 to 7.8 optimum for the growth

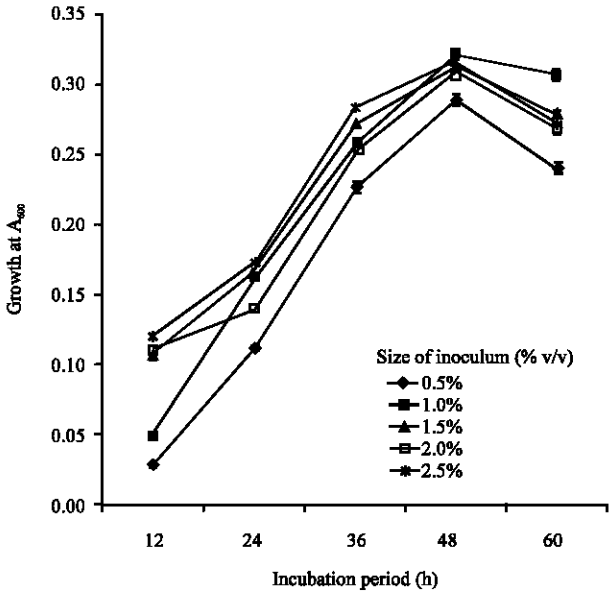

Fig. 2: Effect of inoculum size (0.5-2.5 % v/v) on bacterial growth in MSM broth containing PCP 500 mg L and Cr (VI) 200 mg L^{-1} supplemented with 0.4% (w/v) glucose

of test bacteria. Camargo *et al.* (2004) have reported that the growth of *Bacillus* sp. isolate was pH, temperature and Cr (VI) concentration dependent and maximum growth was noted at pH 7.0 to 9.0 and temperature 30°C. Premlatha and Rajkumar (1994) have reported a narrow range of pH between 7.0 to 7.8 for growth and PCP degradation by *Pseudomonas aeruginosa* strain. On the other hand, Wolski *et al.* (2005) have reported that *Pseudomonas* sp. isolate could degrade PCP at a wide range of pH, from 5.5 to 8.0, with an optimum pH of 6.3. The pH and temperature optima for the growth of *Bacillus cereus* strain in the presence of individual chromate have been reported to be 7.0 and 37°C, respectively which are in accordance with our findings (Faisal and Hasnain, 2006). Present results show that with increase in time from 12 to 48 h, there was concomitant increase in bacterial growth irrespective of pH and incubation temperature. With further increase in incubation time from 48 to 60 h, there was either constancy or decline in growth of the bacterial culture (Table 3). The pH and temperature of the medium are important parameters for cell survival and its function. Any deviation in pH and temperature from optimum attribute to detrimental effect on growth and metabolism (Miller *et al.*, 2004).

The pH of medium strongly affects enzymatic processes involved in bacterial metabolism (Wolski *et al.*, 2005). As proton motive force in chemiosmosis is affected by the medium pH value, it is possible that under optimum pH range the relative metabolic efficiency is high. The effect of different temperatures on growth rate could be predicted in terms of activation energy required for growth. Above the optimum temperature, cell degradation probably becomes dominant over the growth process and with sub-optimal temperature, the regulation of metabolism may fail. Hence, under optimum temperature, the bacterium could utilize the substrate better, with other optimum cultural and nutritional conditions. Since our isolate exhibited growth at wide temperature and pH range, it reveals that this strain can be used efficiently for bioremediation process applicable to various geographical locations.

Table 3: Combined effect of pH and temperature on growth of efficient strain *Bacillus* sp. during 60 h incubation

Incubation temperature (°C)	*Incubation period (h) 12				24			
	6.5	7.0	7.5	8.0	6.5	7.0	7.5	8.0
25	0.015±0.002	0.023±0.005	0.021±0.003	0.025±0.002	0.029±.004	0.077±0.009	0.081±0.007	0.042±0.005
30	0.019±0.003	0.029±0.005	0.027±0.002	0.019±0.003	0.047±.006	0.089±0.005	0.092±0.004	0.054±0.002
33	0.025±0.002	0.054±0.007	0.051±0.005	0.013±0.003	0.034±.003	0.119±0.009	0.105±0.002	0.062±0.003
35	0.028±0.003	0.049±0.005	0.034±0.007	0.017±0.002	0.053±.009	0.163±0.007	0.130±0.003	0.071±0.004
37	0.017±0.002	0.031±0.007	0.026±0.003	0.023±0.005	0.035±.007	0.128±0.009	0.121±0.011	0.060±0.003
40	0.024±0.001	0.035±0.005	0.027±0.003	0.019±0.003	0.037±.002	0.131±0.004	0.119±0.006	0.040±0.001
Incubation temperature (°C)	*Incubation period (h) 36				48			
	6.5	7.0	7.5	8.0	6.5	7.0	7.5	8.0
25	0.120±0.014	0.109±0.010	0.143±0.018	0.065±0.003	0.139±0.012	0.148±0.007	0.157±0.004	0.071±0.006
30	0.125±0.009	0.134±0.008	0.157±0.009	0.089±0.003	0.143±0.007	0.163±0.010	0.189±0.014	0.093±0.002
33	0.117±0.012	0.205±0.011	0.162±0.005	0.115±0.003	0.150±0.007	0.287±0.015	0.203±0.009	0.130±0.005
35	0.139±0.002	0.257±0.013	0.169±0.005	0.117±0.009	0.158±0.003	0.321±0.008	0.215±0.013	0.147±0.003
37	0.128±0.008	0.214±0.013	0.154±0.007	0.109±0.003	0.146±0.005	0.269±0.012	0.190±0.003	0.127±0.006
40	0.079±0.003	0.130±0.007	0.138±0.009	0.056±0.006	0.084±0.005	0.151±0.008	0.178±0.006	0.085±0.009
Incubation temperature (°C)	*Incubation period (h) 60							
	6.5		7.0		7.5		8.0	
25	0.105±0.003		0.102±0.011		0.130±0.004		0.045±0.007	
30	0.113±0.006		0.117±0.007		0.143±0.012		0.081±0.009	
33	0.120±0.007		0.201±0.003		0.189±0.008		0.093±0.005	
35	0.093±0.005		0.307±0.009		0.174±0.007		0.102±0.012	
37	0.104±0.003		0.203±0.008		0.157±0.020		0.090±0.002	
40	0.052±0.001		0.127±0.003		0.135±0.004		0.039±0.005	

*Becterial growth at A_{600}

CONCLUSIONS

Microorganisms able to tolerate and remediate PCP as well as Cr (VI) can be employed for simultaneous detoxification of environments contaminated with both the pollutants.

In the present study, an efficient indigenous bacterial strain, simultaneously tolerant to high concentration of both PCP (500 mg L^{-1}) and Cr (VI) (200 mg L^{-1}), was isolated from treated tannery effluent. The advantage of selecting indigenous potent isolate from contaminated sites may be the minimization of inhibitory effects of other pollutants that may be present along with PCP and Cr (VI) in the tannery effluent. The effective bacterial inoculum should be able to tolerate high level of pollutants/toxicants while maintaining a level of activity to provide efficient bioremediation. The efficient bacterial isolate B4 was identified as *Bacillus* sp. by morphological and biochemical analyses. The isolate appears to have greater potential to grow under wide environmental conditions indicating its possible exploitation for *in situ* simultaneous remediation of Cr (VI) and PCP from contaminated sites including tannery effluent. Further research on simultaneous chromium reduction/biosorption and degradation of PCP are in progress.

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