Bacterial Reduction of Toxic Cr (Ⅵ) into Cr (Ⅲ)

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Abstract Two chromium-resistant bacterial strains CrT-1 and CrT-13, which can tolerate K_2CrO_4 up to 40 mg·mL^{-1} on nutrient agar, 25 mg·mL^{-1} K_2CrO_4 in nutrient broth, and up to 10 mg·mL^{-1} in acetate-minimal media, were used in this study. On the basis of 16S rRNA, strain CrT-1 was identified as *Ochrobactrum intermedium* and CrT-13 as *Brevibacterium* sp.. Uptake of chromate was greater in living cells than in heat-killed cells. *Ochrobactrum intermedium* CrT-1 reduced 73% and 41% of Cr (Ⅵ) while *Brevibacterium* CrT-13 reduced 62% and 48% Cr(Ⅵ) at an initial chromate concentration of 750, and 1500 μg·mL^{-1}, after 96 hours with an inoculum size of 9.6×10^7 cells·mL^{-1}. Different heavy metals at low concentrations did not affect the reduction potential of the strains significantly. *Ochrobactrum intermedium* CrT-1 reduced 84% and 65% while *Brevibacterium* CrT-13 reduced 60% and 44% of Cr (Ⅵ) at an initial Cr (Ⅵ) concentration of 250 and 500 μg·mL^{-1}, respectively, in an industrial effluent sample.

Key words *Brevibacterium*, chromate reduction, *Ochrobactrum*, bioremediation

Over the last few decades environmental contamination with heavy metals has increased drastically. Heavy metals found in wastewaters are harmful to the environment and their effects on biological system are very severe. An efficient and cheap treatment for their removal and reuse of spent metals from wastewater needs to be deve-loped. The removal of toxic metals from the environment by microorganisms has potential as an effective means of remediating heavy metals wastes. Microbe-based technologies can provide an alternative to conventional methods for metal removal[1]. Chromium is generated from different industries. It occurs in different oxidation states but Cr (Ⅲ) and Cr (Ⅵ) are the most significant. Trivalent chromium occurs naturally in the environment and is an essential nutrient for animals[2]. Hexavalent chromium is a well-known human carcinogen[3]. It can also cause skin ulcer, convulsions, kidney and liver damage. To avoid such toxic effects with the Cr (Ⅵ), it is necessary to convert it into Cr (Ⅲ). The accumulation and reduction of hexavalent chromium has been observed in many bacterial genera such as *Pseudomonas*[4], *Bacillus*[5], *Escherichia*[6], and *Desulfovibrio*[7]. Bacterial accumulation and reduction do well in comparison to sorption on commercial ion exchange resin, activated carbon and metal oxides. The objective of the present study was to measure the uptake and reduction of toxic Cr (Ⅵ) from industrial wastewater by two indigenous bacterial strains (CrT-1 and CrT-13) into less mobile Cr (Ⅲ).

1 Materials and Methods

1.1 Bacterial strains and culture conditions

Strains CrT-1 and CrT-13 used in this study were isolated from wastewater of tannery and ICI Chemicals, Lahore, Pakistan. Chromate tolerance in these strains was determined both in the nutrient broth and acetate-minimal media[8] supplemented with different concentrations of K_2CrO_4.

1.2 Strains characterization

Isolated strains were characterized morphologically, biochemically and physiologically following Gerhardt *et al*[9]. To evaluate the heavy metals resistance of these strains following concentrations of different heavy metals ($NiSO_4$ 500 μg·mL^{-1}; $ZnSO_4$ 700 μg·mL^{-1}; $MnSO_4$ 1500 μg·mL^{-1}; $CuSO_4$ 1000 μg·mL^{-1}; $CoCl_2$ 500 μg·mL^{-1}; $HgCl_2$ 50 μg·mL^{-1} and $Pb(NO_3)_2$ 1000 μg·mL^{-1}) were used.

1.3 16S rRNA gene sequencing

To confirm taxonomic identity, both strains CrT-1 and CrT-13 were selected for 16S rRNA gene sequencing. To this end, DNA was extracted. A part of the 16S rRNA gene (500 bp) was amplified and the amplicon sequenced using fluorescent di-deoxy terminator cycle sequencing chemistry. The extension product was then separated on an ABI PRISM® (automated DNA sequencer) and the data was compared to the MicroSeq® databases (ACCUGENIX™ Newark DE 19702).

1.4 Chromium accumulation experiments

To monitor chromium biosorption, strains were grown in nutrient broth at 37℃ with 150 r/min shaking for 24 hours. After 24 hours cultures were harvested and centrifuged at 13 000 × g for 10min. The pellet was washed twice with sterile-distilled water and stored at 4℃. Two different initial K_2CrO_4 concentrations (750 and 1500 μg·mL^{-1}) were used. The inoculum was prepared as follows: i) one gram of

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fresh cell pellet was dried at 60℃ for 48 hours (dried cells); ii) the same amount of pellet was taken and was heat-killed at 121℃ for 20 min(heat killed); iii) same amount was used untreated (live cell mass). All experiments were conducted in 250 mL conical flasks containing 100 mL of metal solution. Before adding the bacteria, the pH of the metal solution was adjusted to the required value with 0.01mol/L NaOH. The bacterial cell suspensions were added to metal solutions and incubated at required temperature and shaking. At regular time intervals samples were taken aseptically and were centrifuged at 10 000 r/min for 10 min at 4℃. Pellets obtained were digested and the amount of total chromium accumulated both trivalent and hexavalent, first by oxidizing any trivalent chromium into hexavalent with $KMnO_4$ and then was determined spectrophotometrically at 540 nm in a spectrophotometer using diphenylcarbazide as the complexing agent[10]. All experiments were conducted in triplicate.

1.5 Cr (Ⅵ) reduction experiments

In this experiment two initial K_2CrO_4 concentrations (750 and 1500 $\mu g \cdot mL^{-1}$) and two cell concentrations (2.4×10^7 and 9.6×10^7 cells $\cdot mL^{-1}$) were used. For reduction experiment, DeLeo and Ehrlich[11] medium (grams per litre: tryptone 10, yeast extract 5, NaCl 5, citric acid 1, Na_2HPO_4 6.9) was used. Cultures were kept in an incubating shaker with 150 r/min at 37℃. At regular time intervals i.e., after 24, 48, 72 and 96 hours, samples were taken aseptically and were analyzed for Cr (Ⅵ) reduction. Chromium (Ⅵ) and chromium (Ⅲ) were determined colorimetrically as before.

1.6 Effects of heavy metals on Cr (Ⅵ) reduction

Effects of different heavy metals on chromium reduction by the two bacteria were also studied. For this purpose cultures were also separately amended with Zn, 200 $\mu g \cdot mL^{-1}$; Ni, 200 $\mu g \cdot mL^{-1}$; Mn, 200 $\mu g \cdot mL^{-1}$; Cu, 200 $\mu g \cdot mL^{-1}$; Co, 50 $\mu g \cdot mL^{-1}$. After 24 hours cultures were harvested and were processed as above to check the amount of Cr (Ⅵ) reduced in to Cr (Ⅲ).

1.7 Cr (Ⅵ) reduction in an industrial effluent

Samples from metal finishing industry Lahore-Sheikhupura Road, heavily polluted with Cr (Ⅵ) were collected in sterilized bottles. Physico-chemical parameters of the sample were determined. pH and temperature of the sample was recorded on-site. Strains were exposed to the polluted sample and their subsequent dilutions for which initial Cr (Ⅵ) was known. Two different dilutions of effluent sample were used: sample Ⅰ and sample Ⅱ that contain 250 $\mu g \cdot mL^{-1}$ and 500 $\mu g \cdot mL^{-1}$ of Cr (Ⅵ), respectively. Cultures were harvested after 40 hours and the amount of Cr (Ⅵ) reduced was measured as described above.

1.8 Statistical analysis

Standard errors of the means and LSD were calculated following Steel and Torrie[12].

2 Results

2.1 Characteristics of strains

On the basis of 16S rRNA gene sequencing analyses (500 bp), strains CrT-1 and CrT-13 were identified as *Ochrobactrum intermedium* (98.58% homology with C32413) and a *Brevibacterium* sp. (95.51 homology with C32414), respectively. *O. intermedium* CrT-1 resisted very high concentration of K_2CrO_4 in nutrient broth (up to 25 $mg \cdot mL^{-1}$) as well as on nutrient agar (up to 40 $mg \cdot mL^{-1}$) while *Brevibacterium* CrT-13 could resist up to 20 $mg \cdot mL^{-1}$ on nutrient agar (Fig-1a). Both strains could also tolerate up to 10 $mg \cdot mL^{-1}$ K_2CrO_4 in acetate-minimal medium (Fig-1b). The resistance of *Brevibacterium* CrT-13 to Cr (Ⅵ) in liquid media was relatively low as compared to *O. intermedium* CrT-1. In acetate-minimal medium (Fig-1b) both strains exhibited initial sharp decrease in growth up to 1.5 $mg \cdot mL^{-1}$ K_2CrO_4 followed by steady state decrease up to 10 $mg \cdot mL^{-1}$ K_2CrO_4. As other metals can also be present in industrial effluents, resistance of the two isolates to other metallic salts was also determined. Both strains displayed multiple metals resistance (Table-1). $HgCl_2$ was found to be most toxic metal tested. *Brevibacterium* CrT-13 could not grow at 50 $\mu g \cdot mL^{-1}$ of this metal. *O. intermedium* CrT-1 could resist very high concentration of majority of heavy metals except $HgCl_2$, which was again tolerated only up to 50 $\mu g \cdot mL^{-1}$.

Table 1 MIC's of chromium resistant bacterial isolates against different heavy metals

Strains	Heavy metals/($\mu g \cdot mL^{-1}$)						
	$NiSO_4$	$ZnSO_4$	$Pb(NO_3)_2$	$CuSO_4$	$CoCl_2$	$HgCl_2$	$MnSO_4$
O. intermedium CrT-1	1000	1000	1000	700	400	50	1500
Bravibacterium CrT-13	400	500	1000	1000	200	-	1500

2.2 Chromium accumulation

Living cells of *O. intermedium* CrT-1 accumulated more chromate than heat killed and dried cell mass. When the chromate concentration in the solution was 750 $\mu g \cdot mL^{-1}$, live cells of *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 accumulated (11.7 ± 0.07) and (8.1 ± 0.14) $mg \cdot g^{-1}$ cells dry weight, respectively, in the first fifteen minutes (Fig-2). But after 120 minutes contact time, live cells of *O. intermedium* CrT-1 was able to take up (39 ± 0.15) $mg \cdot g^{-1}$ cells (dry weight) whereas live cells of *Brevibacterium* CrT-13 took up only (43 ± 0.18) $mg \cdot g^{-1}$. At this lower initial metal concentration, heat killed cells of strain *O. intermedium* CrT-1 were able to accumulate (10.2 ± 0.09) and (24 ± 0.16) $mg \cdot g^{-1}$ cells dry weight after 15 and 120 minutes of contact time period while dried cells mass of this strain accumulated only (5.2 ± 0.09) and (9.3 ± 0.18) $mg \cdot g^{-1}$ (dry weight) of cells, respectively. At high initial Cr (Ⅵ) concentration (1500 $\mu g \cdot mL^{-1}$) after 120 minutes live cells of *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 accumulated (95 ± 1.68) and (79 ± 1.74) $mg \cdot g^{-1}$ cells (dry wt), respectively. After 120 minutes, live cells accumulated much more chromium, especially at higher concentration, relative to heat-killed and dried cells. Regardless of whether live, heat-killed or dried cells were used, the amount of chromium accumulated increased with contact time, but heat-killed and dried cells took up less chromate in 120 minutes than live cells. This effect was more pronounced at high initial chromate concentration (1500 $\mu g \cdot mL^{-1}$) at which the difference in chromium

accumulated in dried cells and living cells was much higher.

2.3 Cr(Ⅵ) reduction

The results of Cr (Ⅵ) reduction by *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 at initial K_2CrO_4 concentrations of 750 and 1500 $\mu g \cdot mL^{-1}$ are presented in Fig. 3. Each of these two organisms was tested at inoculum concentrations of 2.4×10^7 and 9.6×10^7 cells$\cdot mL^{-1}$. *O. intermedium* CrT-1 was able to reduce 73% Cr (Ⅵ) from the medium after 96 hours while *Brevibacterium* CrT-13 reduced 62% when the initial Cr (Ⅵ) was 750 $\mu g \cdot mL^{-1}$ (Fig.3). At high initial Cr (Ⅵ) concentration (1500 $\mu g \cdot mL^{-1}$), *O. intermedium* CrT-1 reduced 19% of total chromium within 24 hours contact period while *Brevibacterium* CrT-13 reduced 11% in this length of time. At this initial Cr (Ⅵ) concentration *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 reduced 19% and 18% after 72 hours when initially inoculated with 2.4×10^7 cells$\cdot mL^{-1}$ and 21%, 18% with 9.6×10^7 cells$\cdot mL^{-1}$, respectively.

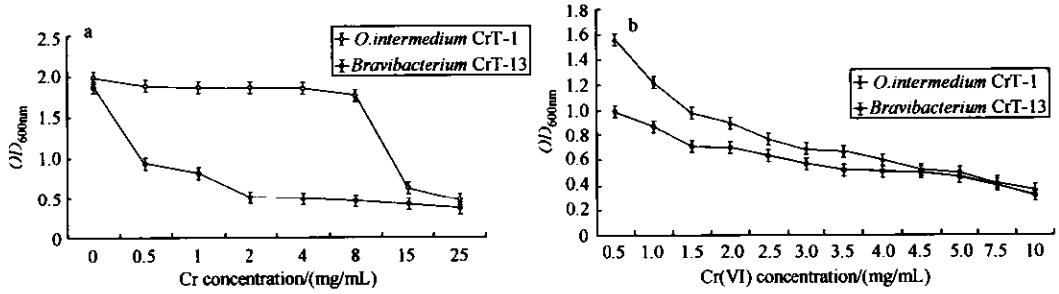

Fig. 1 (a). Growth of *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 in the presence of different concentrations of K_2CrO_4 mg$\cdot mL^{-1}$ in nutrient broth. (b). Growth of bacteria in the presence of different concentrations of K_2CrO_4 mg$\cdot mL^{-1}$ in acetate-minimal medium

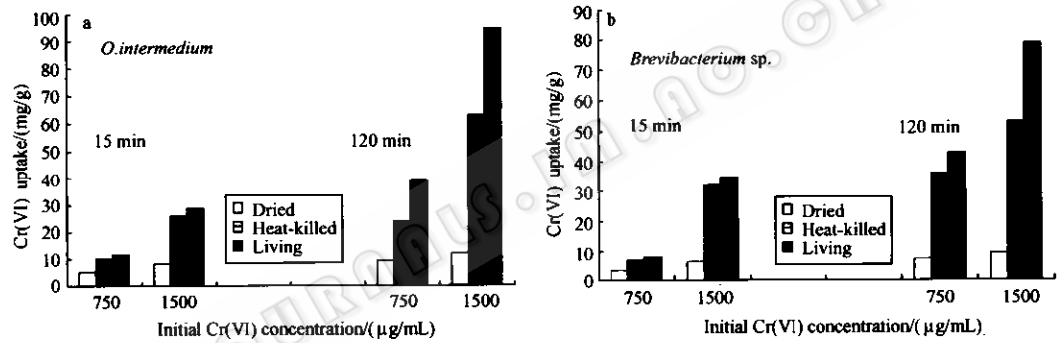

Fig. 2 Uptake of potassium chromate at two initial K_2CrO_4 concentrations (750 and 1500 $\mu g \cdot mL^{-1}$)

Cells were used as dried, heat killed and as live. Contact time (after 15 and 120 minutes). a: *O. intermedium*; b: *Brevibacterium* sp.

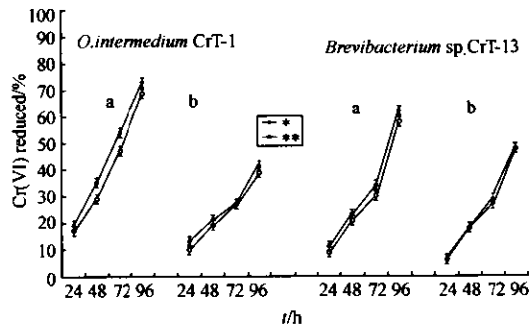

Fig. 3 Reduction of K_2CrO_4 by *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 at two level of chromate

Reduction was monitor at after 24, 48, 72 and 98 hours of growth incubation. Open circle (○) represent 2.4×10^7 cells$\cdot mL^{-1}$ and solid circle (●) represent 9.6×10^7 cells$\cdot mL^{-1}$ used for initial inoculation. a) Cr (Ⅵ) 750 $\mu g \cdot mL^{-1}$; b) Cr (Ⅵ) 1500 $\mu g \cdot mL^{-1}$

2.4 Effect of different heavy metals on Cr (Ⅵ) reduction

The presence of others heavy metals did not affect the Cr (Ⅵ) reduction potential of either strain. With *O. intermedium* CrT-1, 200 $\mu g \cdot mL^{-1}$ $CuSO_4$ and 200 $\mu g \cdot mL^{-1}$ $MnSO_4$ augment reduction potential of the strain up to 4% ~ 5%, as compared to their respective control while 200 $\mu g \cdot mL^{-1}$ $ZnSO_4$ and 50 $\mu g \cdot mL^{-1}$ $CoCl_2$ cause 3% ~ 4% inhibitions in this respect. Interestingly 200 $\mu g \cdot mL^{-1}$ $ZnSO_4$ improves the reduction potential of *Brevibacterium* CrT-13 to 14% as compared to the control, whereas 50 $\mu g \cdot mL^{-1}$ $CoCl_2$ exhibited some inhibition of Cr (Ⅵ) reduction ability.

2.5 Cr(Ⅵ) reduction in industrial effluent

The pH and temperature of the industrial sample were 5 ~ 6 and 26℃, respectively. Other sample parameters were also determined (Cr (Ⅵ) 800 $\mu g \cdot mL^{-1}$; Fe 121 $\mu g \cdot mL^{-1}$; Cu 65 $\mu g \cdot mL^{-1}$; Zn 4 $\mu g \cdot mL^{-1}$; Ni 109 $\mu g \cdot mL^{-1}$; Co 2 $\mu g \cdot mL^{-1}$; Pb < 1 $\mu g \cdot mL^{-1}$; Mn < 1 $\mu g \cdot mL^{-1}$). When the Cr (Ⅵ) concentration in industrial effluent sample (Ⅰ) was adjusted to 250 $\mu g \cdot mL^{-1}$, *O. intermedium* CrT-1 reduced 84 % of total Cr (Ⅵ) in to Cr (Ⅲ) after 40 hours but in effluent sample Ⅱ which contained an initial Cr (Ⅵ) concentration of 500 $\mu g \cdot mL^{-1}$, this strain reduced 65% within the same time period. *Brevibacterium* CrT-13 converted 60% and 44% of Cr (Ⅵ) in to Cr (Ⅲ) after 40 hours when initially supplied with 250 and 500 $\mu g \cdot mL^{-1}$ of Cr (Ⅵ) (Table 2).

Table 2 A. Effects of different heavy metals on Cr(Ⅵ) reduction by *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 (Initial chromate concentration 750 $\mu g \cdot mL^{-1}$); B. Chromate reduction in an industrial effluent

Source	Reduction/%	
	O. intermedium CrT-1	*Brevibacterium* CrT-13
A. Metals/($\mu g \cdot mL^{-1}$)		
Control	30 ± 0.85	21 ± 0.65
Ni(200)	31 ± ±0.90	22 ± 1.20
Mn(200)	32 ± 0.80	23 ± 0.75
Zn(200)	29 ± 1.08	24 ± 0.60
Cu(200)	33 ± 1.16	22 ± 1.15
Co(50)	29 ± 0.95	21 ± 1.0
B. Industrial effluents		
a*	84 ± 1.80	60 ± 2.15
b**	65 ± 1.56	44 ± 1.65

a*: Initial Cr(Ⅵ) concentration 250 $\mu g \cdot mL^{-1}$; b**: Initial Cr(Ⅵ) concentration 700 $\mu g \cdot mL^{-1}$ (means of three replicates)

3 Discussion

The present work focused on the uptake and reduction of toxic hexavalent chromium into trivalent chromium by the bacterial strains *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 which showed very high-level resistance to chromate. The chromate resistance level of these strains is very high in minimal medium relative to strains reported by other workers. Chromium-resistant bacteria isolated from effluent of tanneries could resist up to 250 $\mu g \cdot mL^{-1}$ of hexavalent chromium in the medium[13]. Megharaj *et al*[14] observed that strains isolated from chromium-contaminated soil could grow at concentrations of Cr (Ⅵ) up to 100 $\mu g \cdot mL^{-1}$ in minimal medium. The strains reported here could grow at 10 $mg \cdot mL^{-1}$ K_2CrO_4 (2.68 $mg \cdot mL^{-1}$ Cr) in acetate-minimal medium. Results of uptake experiments shows that the chromium uptake capacity of living cells of both strains was greater than that of heat killed and dried cell mass in the presence of different K_2CrO_4 concentrations (750 and 1500 $\mu g \cdot mL^{-1}$). The reduction in K_2CrO_4 accumulation capacity of dried cell mass in comparison with heat-killed and living cells may be attributed to the loss of intracellular uptake, as the cell organelles may be damage or deformed.

Beside chromium accumulation, strains *O. intermedium* CrT-1 and *Brevibacterium* CrT-13 also have capacity to reduced handsome amount of Cr (Ⅵ) into Cr (Ⅲ). In both strains the rate of Cr (Ⅵ) reduction was fast in the first 24 h than subsequently it declined. The initial K_2CrO_4 concentrations used in this experiment were much higher than in others studies reported in the literature. Middleton *et al*[15] observed that *Shewanella oneidensis* MR-1 could grow and reduce Cr (Ⅵ) when supplied with 100 μmol/L Cr (Ⅵ) (5.2 $\mu g \cdot mL^{-1}$ Cr) but was inhibited at a concentration of 150 μmol/L. In cultures which were initially supplied with 750 $\mu g \cdot mL^{-1}$ K_2CrO_4 (187.6 $\mu g \cdot mL^{-1}$ Cr) *O. intermedium* CrT-1 reduced more than 17% of Cr (Ⅵ) into Cr (Ⅲ) aerobically in the first twenty-four hours contact time when initially inoculated with 2.4×10^7 cells $\cdot mL^{-1}$. In compa rison to *O. intermedium* CrT-1, *Brevibacterium* CrT-13 removed almost 9% of total Cr (Ⅵ) supplied within first twenty-four hours at same inoculum size.

Different heavy metals (Ni, Mn, Zn, Cu, Co) did not affect the chromate reduction capability of these strains. Addition of these heavy metals at low concentration did not significantly affect the reduction potential of either strain. Shen and Wang[16] reported 16% to 33% decreases in chromate reduction with Zn (200 $\mu g \cdot mL^{-1}$) and Cu (100 $\mu g \cdot mL^{-1}$) but not at lower concentrations. Both strains were also able to convert Cr (Ⅵ) into Cr (Ⅲ) present in industrial sample after 40 hours incubation period. Many other workers also reported the reduction of toxic Cr (Ⅵ) in industrial wastewaters. Ganguli and Tripathi[17] observed the reduction of Cr (Ⅵ) in industrial effluent by a *Pseudomonas aeruginosa* strain isolated from effluent of tannery, but they diluted industrial effluent used in their test to contain only 15 $\mu g \cdot mL^{-1}$ Cr (Ⅵ) whereas we diluted our industrial effluent to contain as much as 250 and 500 $\mu g \cdot mL^{-1}$ of Cr (Ⅵ). Even at this high initial Cr (Ⅵ) concentration along with others pollutants, both strains reduce surprising amount of toxic Cr (Ⅵ) in to Cr (Ⅲ), which suggest that a hexavalent chromium reduction system of this nature can be economical to run.

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利用细菌还原有毒 Cr(Ⅵ)为 Cr(Ⅲ)

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