Isolation and Characterization of Chromium Reducing Bacteria

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Abstract. Removal of highly toxic Cr (VI) using bioremediation can start a new way for effective treatment of chromium contamination. Therefore, the chromate-reducing strain G-13 under alkaline conditions was isolated from chromium factory. G-13 was identified as *Micrococcus sp.*.The strain of G-13 was selected to study the resistance of Cr (VI) and its ability to reduce Cr (VI) under different culture conditions. The results showed that the optimum temperature and pH for the strain were found to be 30℃ and 7.0 or 8.0, respectively. The percent reduction of Cr(VI) for 50,100, 200, 400 and 500 mg/L of initial concentration at 96 h of incubation were 82.1%, 63.6%, 34.5%, 21.2% and 9.1%, respectively. The *Micrococcus sp.*G-13 strain was remarkable under the condition of glycerol and sodium lactate as the electron donor, which could reduce 50 mg/L Cr (VI) to 0 within 36 h, and the reduction rate was 100%. Consequently, the isolation of bacteria can be exploited for the bioremediation of Cr (VI) pollution. It is hoped that this study can provide theoretical basis for the treatment of environmental chromium pollution.

1. Introduction

Chromium is one of the most widely and heavily used heavy metals in various industrial processes, such as metallurgy, wood preservation, nuclear power plants and so on[1]. The widespread use of chromium compounds can result in large quantities of chromium being released into the environment [2]. Therefore, chromium contamination has been often reported in many industrial sites due to improper disposals, poor storage and accidental leakages measures. In natural systems, chromium generally exists in two stable oxidation states, hexavalent chromium (Cr (VI)) and trivalent chromium (Cr (III))[3].Soluble hexavalent chromium [Cr (VI)], such as [CrO_4^{2-}, $HCrO_4^-$] is highly toxic, and shows mutagenic and carcinogenic effects on biological systems due to its strong oxidizing nature [4].While trivalent chromium (Cr (III)) like [$Cr(OH)_3$] is an essential micronutrient for organisms (50-200 ug/day). It is 100-fold less toxic than Cr (VI) due to its lower cell permeability and insolubility [5].

Since Cr (VI) poses a great threat to humans, cleaning up this contaminant from soil and water is

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essential and reduction to Cr (III) may be considered a satisfactory solution in eliminating the toxicity of Cr (VI)[6]. Over the past few decades, several technologies based on this reason have been developed to remove chromium from the environment. Far-ranging conventional methodologies have been used for the removal of Cr (VI) from industrial wastewaters including ion-exchange resins [7], filtration [8], chemical precipitation [9], chemical oxidation or reduction and so on [10]. However, these methods tend to generate large amount of secondary waste products, resulting in secondary pollution of the environment. Thus, to overcome these disadvantages, it is important to develop an innovative, cost-effective and eco-friendly methods for extraction of hazardous materials present in environment.

Microbial remediation, is a potential alternative for the removal of chromium [Cr (VI)] in environmental studies because of the advantages of its environmental friendliness, cost effectiveness compared with conventional methods [11]. Soil microorganisms play a dominant role in reducing the toxic charge of heavy metals through conversion of toxic Cr(VI) to relatively nontoxic Cr(III)[12].In some cases, the microorganisms remediation occurs spontaneously that reduces the toxic Cr(VI) to Cr(III) because the essential materials required for bacterial growth are naturally present at the contaminated sites[13,14].Hence, the present study is aimed at the isolation of potential hexavalent chromium reducing bacteria from a contaminated soil and to study their hexavalent chromium reduction characteristics.

2. Materials and methods

2.1. Sample collection

Soil sample was collected from the surface horizon (0-10 cm) of the chromium pollution sites of a chromate factory in Changsha, China. Soil samples collected in sterilized plastic bags were brought to the laboratory and stored at 4 ℃ refrigerated condition until use.

2.2. Preparation of media

The medium selected was Luria Broth (LB) medium to isolate and the reduction characteristics of chromium-reducing bacteria. The medium specific ingredients [15] was tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L,$MgSO_4 \cdot 7H_2O$ 0.2 g/L,K_2HPO_4 0.05 g/L, distilled water 1 L, Agar 2%(w/v).The pH value of the medium was adjusted to 8.0 by adding aliquots of either 1 mol/L H_2HSO_4 or 1 mol/L NaOH. The medium were autoclaved at 121 ℃ for 20 min.

2.3. Isolation of Cr (VI) - tolerant bacterial strains

Isolation of the bacterial culture was done by an enrichment culture technique [16]. Luria Broth was amended with filter sterilized 200 mg/L $K_2Cr_2O_7$ as Cr(VI) and 10 g soil which was incubated at 30 ℃,170 r/min for 12 h on a rotary shaker. After 12 h enriched bacterial strains were isolated by plating on Luria agar plate amended with 200 mg/L of $K_2Cr_2O_7$.This process was serially diluted with sterile water of soil sample and plated onto Luria Broth (LB) agar and incubated at 30 ℃ for 24–36 h to isolate Cr (VI)-resistant bacteria. Bacterial colonies of different morphologies were obtained through many round streaking and purification on the same agar medium. From this preliminary screening strains showing resistance to chromium were selected for further studies. The ability of the isolates to reduce Cr (VI) was determined by Cr (VI) reduction experiments.

2.4. Screening of Cr (VI)-reducing strain

The selected Cr (VI)-resistant strain were inoculated into 100 mL of liquid LB medium containing 50 mg/L of Cr(VI) as $K_2Cr_2O_7$ and incubated in an orbital shaker(170 r/min) at 30 ℃.1 mL liquid of culture were taken out in sterilized tube at regular time interval and were centrifuged at 10,000 r/min for 5 min at room temperature. The supernatant was used to measure Cr (VI) concentration at 540 nm

using UV 754 N model spectrophotometer.

2.5. Identification of the isolated strain

The isolated strain was identified based on standard biochemical tests [17]. In addition, molecular identification was done by 16S rRNA analysis using universal bacterial 16S rRNA gene primers 27F (5'-AGAGTTTGATCCTGGCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for polymerase chain reaction (PCR) amplification of the 16S rRNA gene [18]. Purified PCR products were conducted by Platinum Sequencing Company, Shanghai, China. The resulting 16S rRNA gene sequences were initially analyzed with known 16S rRNA sequences in the GenBank database to identify the most similar sequence alignment and to download the corresponding sequence. The phylogenetic tree was then constructed by neighbor-joining method using the MEGA 7.0 package version.

2.6. Determination of optimum growth temperature and pH of the isolated strain

For optimum growth of the bacterial isolates, two parameters, i.e., temperature and pH were considered [19]. For the determination of optimum temperature, the logarithmic strain seed solution were inoculated into sterilized 100 mL LB medium at 5% inoculum concentration (250 mL culture flasks, pH 9.0) and incubated at 25 ℃, 30 ℃, 35 ℃, 40 ℃ and 45 ℃, respectively. In addition, to determine the optimum pH of the strain, a set of liquid LB medium of the same specifications was prepared and the pH values were set to 3.0, 5.0, 7.0, 8.0, 9.0, 10.0 and 11.0, respectively. Then inoculated with 5% inoculum concentration into the sterilized LB medium, placed in 30 ℃, 170 r/min incubated on a rotary shaker, observed at regular time intervals the OD_{600} value of the culture medium, each experiment was carried out in triplicate. Data are the mean of three replications.

2.7. Determination of chromium reduction experiments

2.7.1. Effects of initial chromium concentration on chromium reduction. To determine the effect of initial chromium concentration on Cr (VI) removal of strain, the logarithmic strain seed solution of G-13 was inoculated into sterilized 100 mL LB liquid medium at a 5% inoculum concentration (250 mL culture flasks, pH 9.0) amended with variable concentration of Cr (VI) as K_2CrO_4 [20], respectively 50, 100, 200, 400 and 500 mg/L and incubated on a rotary shaker at 30 ℃, 170 r/min. The chromium removal was measured at regular time intervals by measuring the residual Cr (VI) in the cell-free supernatant. Each experiment was carried out in triplicate. Data are the mean of three replications.

2.7.2. Effects of various electron donors on chromium reduction. To determine the effect of carbon source on Cr (VI) removal of strain, the logarithmic strain seed solution of G-13 was inoculated with 5% inoculum concentration into 100 mL LB liquid medium (250 mL culture flasks, 50 mg/L Cr (VI), pH 9.0) amended with a certain concentration of carbon source as electron donors *[21], r*espectively glucose, sucrose, sodium lactate, glycerol and incubated on a rotary shaker at 30 ℃, 170 r/min. The chromium removal was measured at regular time intervals by measuring the residual Cr (VI) in the cell-free supernatant following centrifugation. Each experiment was carried out in triplicate. Data are the mean of three replications.

2.8. Analytical methods

Cr (VI) concentration in the supernatant was determined colorimetrically with a spectrophotometer using diphenylcarbazide reagent in acid solution method. The reagent was prepared by adding 0.2 g of diphenylcarbazide to 50 mL of acetone and then made up to a final volume of 100 mL with distilled water. To the cooling solution were added 12.5 mL H_2SO_4© and H_2PO_4©, respectively. The

reagent was stored in a brown bottle at 4℃ until used. The residual Cr (VI) concentration in the culture was measured at 540 nm by UV 754N model spectrophotometer. The growth of cells was routinely monitored by measuring optical density (OD) at 600 nm.

3. Results and discussion

3.1. Identification of the isolated Cr (VI)-reducing strain

The colony morphology, cell morphology and biochemical test results of the isolated strain are presented in Table 1 and the partial amplification sequences was sequenced by 16S rRNA gene. The result was compared using the BLSAT function provided by NCBI database to identify the most similar sequence alignment. The results revealed that the strain was 99 % homologous to *Micrococcus luteus strain* ATCC 4698 (NR 114673.1) (Figure 1).Hence, the isolates strain was identified from this result as *Micrococcus sp*.

Table 1. Cell morphology and biochemical test results for the isolate strain.

Biochemical characteristics	Results
Cell / Colony morphology	Round
Colony Color	Yellow
Colony edge	Smooth
Motility	+
Gram staining	+
V-P test	−
Citrate test	+
Indole test	+
Voges-proskauer	−
Lactose fermentation test	+

Note: positive : +; negative : −

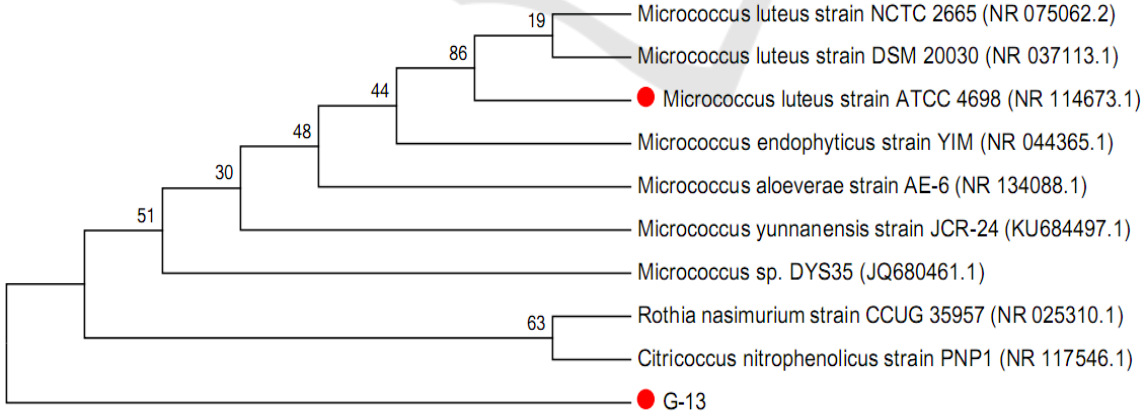

Figure 1. Phylogenetic tree based on 16S rRNA gene sequence for *Micrococcus sp*.G-13.

3.2. Determination of optimum growth temperature and pH of the isolated strain

Temperature and pH play a crucial role in the growth rate and chromium reduction of the *Micrococcus sp*.G-13 strain. The variation in temperature and pH of the LB medium affects the

viability of the cells, the activity of chromium reductases and causes changes in the ionic form of active sites [22].Therefore, the optimum growth temperature and pH of the isolated bacteria *Micrococcus sp*.G-13 were studied and the results are shown in Figure 2.In the temperature range of 25 ~ 45 ℃, the *Micrococcus sp*. G-13 has the best growth trend at 30 ℃. However, the *Micrococcus sp*. G-13 strain can also grow in other temperature ranges but its growth is significantly inhibited at 45 ℃, which may be due to the decrease of membrane fluidity hindered cell growth. In addition, it was found that the optimum pH of *Micrococcus sp*. G-13 was pH 7.0 or pH 8.0, but it cannot tolerate extreme acid and extreme alkali environments (Figure 3).

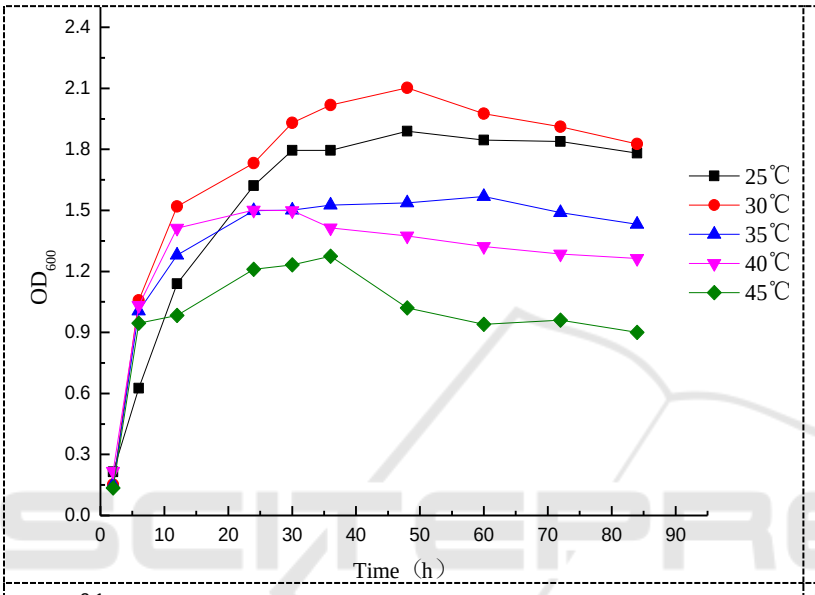

Figure 2. Growth curve of the *Micrococcus sp*. G -13 strain at different temperatures.

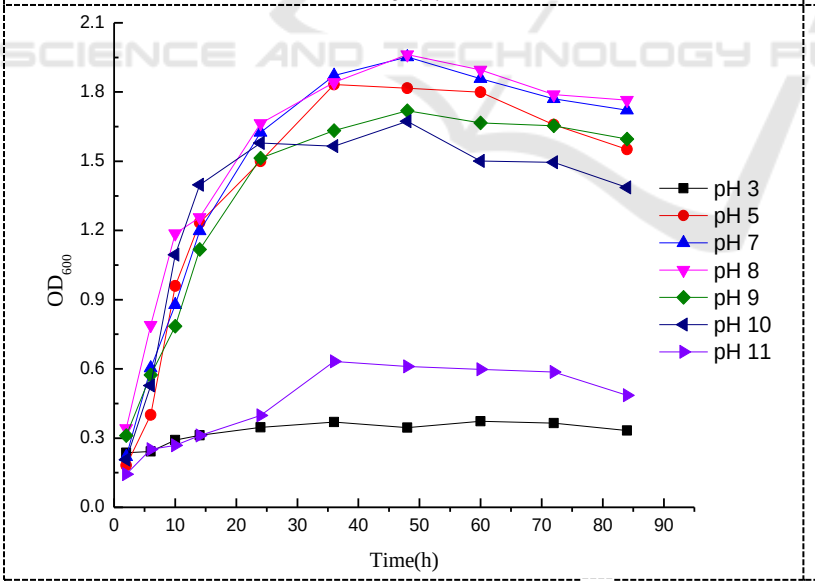

Figure 3. Growth curve of the *Micrococcus sp*. G -13 strain at different pH .

3.3. Effects of initial chromium concentration on chromium reduction

Research reported that the concentration of Cr (VI) in the process of chromium reduction can affect the growth and chromium reduction of chromium reduction strain [23].With the increase of chromium concentration, the reduction rate of chromium gradually decreased and the total reduction

time of chromium increased. The effect of initial concentration on biomass growth and Cr (VI) reduction by *Micrococcus sp.*G-13 was studied over an initial concentration range of 50 mg/L~500 mg/L and the results are shown in Figure 4. Higher amount of biomass concentration was observed at an initial concentration of 50~200 mg/L and increase in initial concentration of Cr (VI) decreased the biomass concentration. The growth of the *Micrococcus sp.*G-13 strain was significantly delayed at 500 mg/L Cr (VI) concentration compared to 400 mg/L, it may be that when the initial concentration of chromium in the culture medium exceeded a certain limited concentration, the toxic effects on the cells of the strain and Cr (VI) reducing strain is irreversibly inactivated.

Bacterial Cr (VI) reduction is enzyme mediated. Rate of this enzyme-catalyzed reaction increases with the increase in the number of active collisions, as Cr (VI) occupies more enzyme active sites [20]. Consequently, Cr (VI) reduction increases with reflecting increased enzyme activity until Cr (VI) concentration saturates the enzyme, but the rate of reduction was decreased with increase in incubation time and Cr (VI) concentration. The trend observed in the present study was the maximum Cr (VI) reduction obtained for 50,100,200,400 and 500 mg/L of initial concentration at 96 h of incubation were 82.1%, 63.6%, 34.5%, 21.2% and 9.1%, respectively (Figure 5).

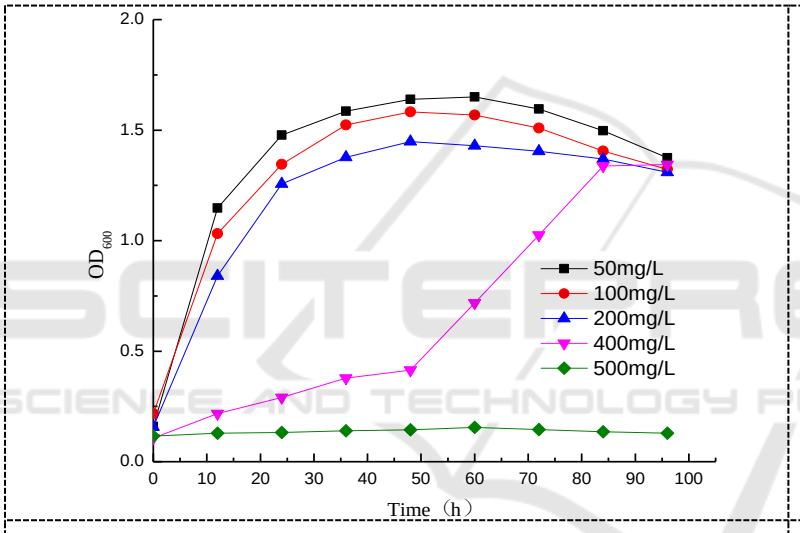

Figure 4. Growth curve of the *Micrococcus sp.* G -13 strain at different initial Cr (VI) concentrations.

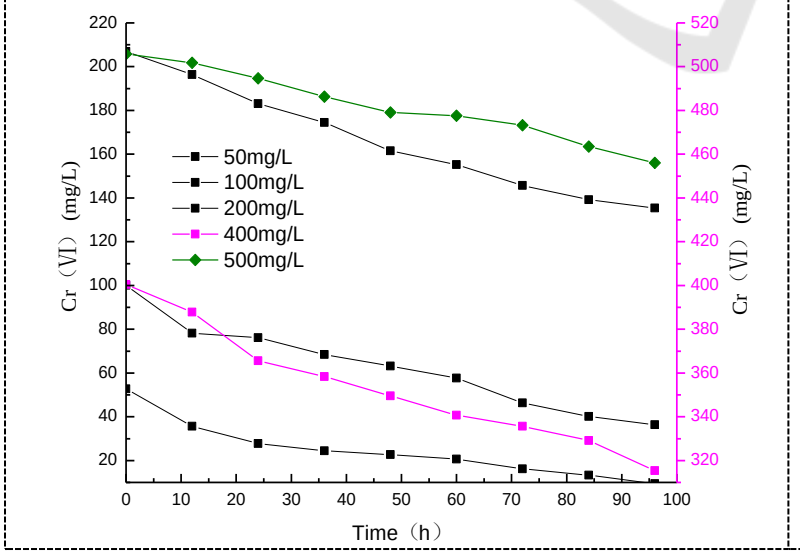

Figure 5. Chromium reducing ability the *Micrococcus sp.* G -13 strain at different initial Cr (VI) concentrations.

3.4. Effects of various electron donors/carbon sources on chromium reduction

The types of carbon sources include four types of carbohydrates, organic acids and lipids which can be used as electron donors in the process of oxidative reduction to participate in the transmission of electrons in the process of microbial growth. However, Cr (VI) reducing strains often preferentially utilize energetically more favorable electron donors in the process of Cr (VI) reduction [24]. The commonly of known electron donors are low molecular weight carbohydrates, organic acids, and fatty acids. Since microbes will give priority to the use of electron donors that are more conducive to growth, it is extremely important to select the appropriate electron donor to improve the reduction capacity of Cr (VI). Hence the influence of electron donors such as glucose, sucrose, sodium lactate and glycerin on chromium reduction of the strain G-13 were studied (Figure 6).

The study found that the growth of the cells was affected in the medium of different carbon source types with the increase of time and the growth trend was: sodium lactate> glycerol> sucrose> glucose. The results showed that the growth tendency of the *Micrococcus sp.*G-13 strain was the best in the culture medium containing sodium lactate and glycerol. Simultaneously, the results obtained from the Figure 7 that the reduction rate of Cr (VI) in the *Micrococcus sp.*G-13 strain was significantly increased under the condition of glycerol and sodium lactate as the electron donor, which can be 50 mg/L Cr (VI) reduced to 0 in 36 h, the reduction rate of 100%.In the presence of four electron donors, the chromium reduction rate was glycerol> sodium lactate> sucrose> glucose. The results indicated that low molecular weight carbohydrates as electron donors may inhibit the growth of the strain, thereby inhibiting the ability of Cr (VI) reduction.

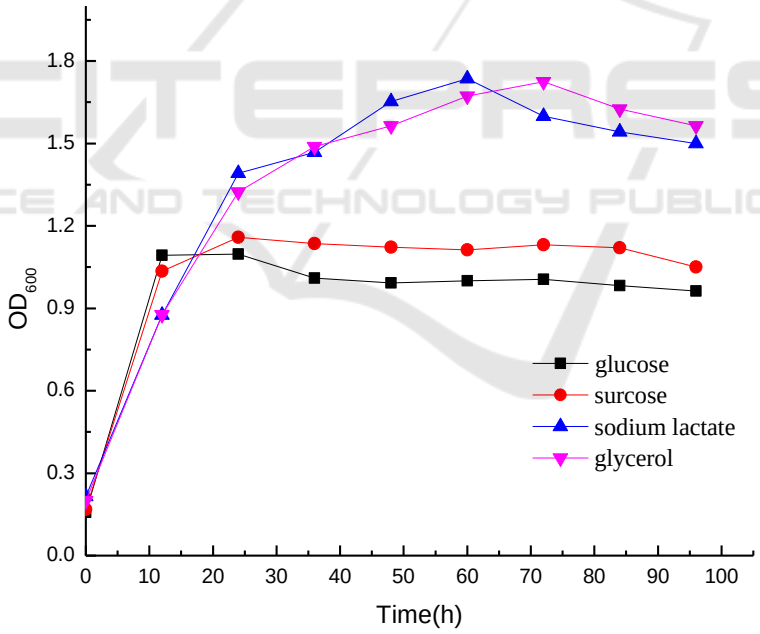

Figure 4. Growth curve of the *Micrococcus sp.* G -13 strain at different electron donor.

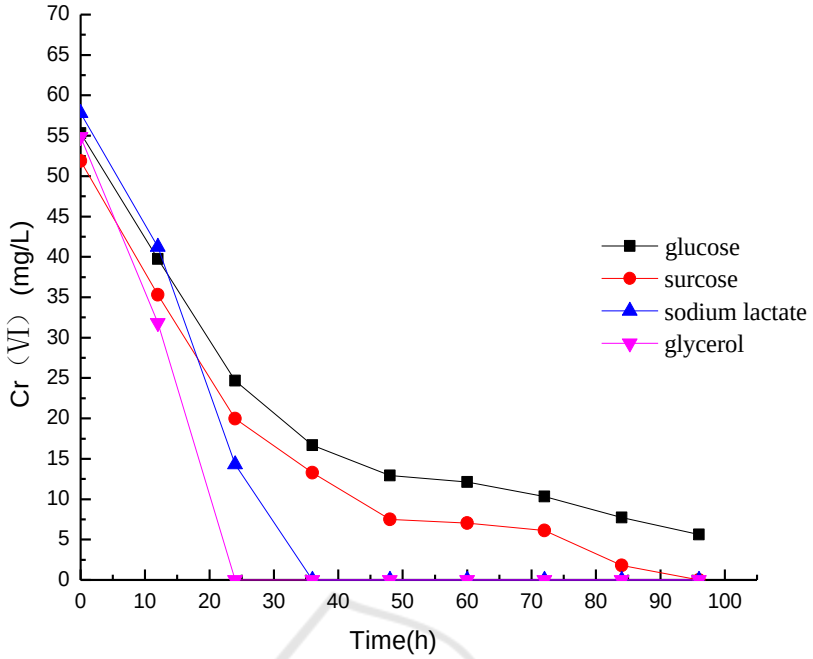

Figure 5. Chromium reducing ability the *Micrococcus sp.* G -13 strain at different electron donor.

4. Conclusions

At present, chromium is widely used in industrial activities, resulting in a large area of environmental chromium contamination. Therefore, search for alternative techniques for treatment of Cr (VI) contaminated environment came into existence. Microbial remediation as a technology with enormous potential that has attracted much attention to remedy chromium contaminated environment, So in the present study, the microbial reduction of Cr (VI) using *Micrococcus sp.*G-13 strain isolated from chromium-contaminated environment was investigated. Simultaneously, the effects of parameters such as temperature, pH, initial chromium concentration and carbon sources on the growth and chromium reduction ability of the *Micrococcus sp.*G-13 strain were studied and the optimum conditions for the reduction of chromium were achieved. This study found that the optimum temperature and pH for the strain were found to be 30 ℃ and 7.0 or 8.0, respectively. Furthermore, the reduction of Cr (VI) increased with increased biomass and decreased with increase in initial Cr (VI) concentration. At the same time, research found that the great increase reduction rate of Cr (VI) for the *Micrococcus sp.*G-13 strain when the addition of glycerol and sodium lactate to do electronic donor. All the results indicated the necessity to study the various parameters of the chromium reduction rate of the strain. Therefore, it can be concluded that future studies can focus on how to use advanced biotechnology to optimize the culture conditions of strains and reduction mechanism of chromium bacteria to improve the reduction rate of Cr (VI).

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