Optimization of Cr(VI) Adsorption by *Bacillus amyloliquefaciens* and Its Mechanism Study

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Abstract: In recent decades, with the rapid development of social economy, heavy metal pollution has become increasingly serious. With laboratory preservation a strong resistant *Bacillus amyloliquefaciense* was used as experiment strains to verify whether Ca^{2+} could improve the tolerance of the experimental strains to metal and explore the adsorption characteristics of the experimental strains to Cr^{6+} as well as optimize the adsorption conditions. This experiment used the single factor experiment combined with dibenzoyl hydrazine method to optimize *Bacillus amyloliquefaciens* of Cr^{6+} adsorption conditions. The experimental results showed that when calcium chloride (0.1 g/L) was in the medium, the tolerance was increased by 21.26%, 76.21% and 269.66% at Cr^{6+} concentrations of 20, 40 and 60 mg/L, respectively. When the carbon source was maltose (25 g/L) and the nitrogen source was trypsin (25 g/L), the best adsorption temperature and pH value weree 35 °C and 7.5, respectively. When the concentration of Cr^{6+} was 20mg/L, the adsorption rate was as high as 89.20%, which was 24.34% higher than that before optimization. Bacillus amyloliquefaciens has good adsorption potential for Cr^{6+}, which can provide excellent microbial resources for bioremediation or environmental pollution.

1 INTRODUCTION

With the rapid development of economic and industrial technology, heavy metal pollution gradually poses a serious threat to the natural environment and human health. As an essential raw material, chromium is widely used in metalworking, metallurgy, electroplating, leather processing, printing and dyeing industries, in which processes produce wastewater and waste containing hexavalent chromium (Ma, 2018. Brasili, 2020. Kazakis, 2017. Jones, 2019. Sukumar, 2014). Chromium in ecological environment mainly exists in hexavalent and trivalent forms, among which hexavalent chromium has high toxicity and mobility (Pellerin, 2000), which can cause skin allergy, dermatitis and chromium sores (Tumolo, 2020). Furthermore, it can cause nasal septum hemorrhage, erosion and even perforation (Lu, 2018); as well as diarrhea, decreased gastrointestinal function, gastrointestinal ulcer and even bronchial cancer (Sethuraman, 2010. Yuling, 2021).

Hexavalent chromium is considered one of the eight most harmful chemicals and one of the three metals most likely to cause cancer (Sethuraman, 2010). Therefore, it is very important and meaningful to treat hexavalent chromium in waste. Traditional treatment methods include electric repair, activated carbon adsorption, chemical precipitation, ion exchange method, membrane separation and other methods (Sukumar, 2014), but these methods have high cost, complex operation, easy to lead to secondary pollution and high treatment requirements. Biological method is characterized by wide source of adsorbent, high selectivity, no pollution and low cost, which can solve various problems existing in traditional

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physical and chemical methods and gradually become a hot spot in the field of environmental protection and heavy metal treatment (Tumolo, 2020). Biological methods are relative environmentally friendly methods, and they use biological cells and extracellular metabolites with heavy metals, through oxidation - reduction reaction, electrostatic adsorption, surface complexation and gravity of heavy metals and does not cause secondary pollution. Biological methods mainly include phytoremediation, biological flocculation method and microbial adsorption method (Xue-Nai, 2019. Valeria, 2020. Sasmita, 2014).

In this study, *Bacillus amyloliquefaciens* stored in the laboratory was mainly used as the research object to carry out the research on its adsorption characteristics and optimization of adsorption conditions for Cr^{6+}, so as to develop the potential of the bacteria as a microbial resource for bioremediation or environmental pollution control. Be advised that papers in a technically unsuitable form will be returned for retyping. After returned the manuscript must be appropriately modified.

2 MATERIALS AND METHODS

2.1 Microbial Strains and Medium

Bacillus amyloliquefaciens is a strain with strong stress resistance which was screened and preserved in laboratory (CGMCC 18719). The strain was stored on the LB solid ramp at 4℃ and activated before use. In the Cr(VI) chromium adsorption test, two kinds of media were used. The seed medium contained (per liter): 6 g beef extract, 12 g Glucose, 8 g NaCl,12 g peptone. The medium was adjusted to pH 6.8~7.2. 18 g agar powder was added to the solid medium on the above basis. The fermentation medium contained (per liter): 10 g Glucose, 8 g NaCl, 10 g peptone, 1.5 g K_2HPO_4, 1 g KH_2PO_4. The medium was adjusted to pH 6.8~7.2.

The Bacillus amyloliquefaciens stored on a solid inclined plane was inoculated into the seed culture medium by inoculation loop and incubated overnight in a constant temperature oscillating incubator at 37℃ and 150 rpm for 12 hours as the initial seed culture liquid.

2.2 Cr (VI) Resistance Test and Establish Standard Curve

Two groups of fermentation medium containing hexavalent chromium ion of 0, 20, 40, 60, 80 and 100 mg/L were prepared, respectively. One group was treated normally and the other group was added $CaCl_2$. Then, 3% (V/V) seed culture solution was inoculated into 100 mL seed medium and incubated overnight for 12 hours in a constant temperature oscillating incubator at 35℃ and 150 rpm. Finally, the absorbance of the bacterial solution was measured at the wavelength of 600 nm, and the tolerance curve of *Bacillus amyloliquefaciens* to hexavalent chromium ions was plotted.

Appropriate amount of 1 g / L chromium ion mother liquor (Precisely weigh 0.282 9 g K2Cr2O7 into a 100ml volumetric flask and fix the volume) and fermentation culture were added to 50 ml volumetric flasks to make the concentration of heavy metal chromium ion in the culture medium 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mg / L respectively. Then 0.6mL (1+1) hydrochloric acid solution and 1.0 mL diphenylcarbazide solution were added successively, immediately mixed and the volume was fixed to the scale line with deionized water. After standing reaction for 9 min, the absorbance was measured at 540 nm wavelength with the solution without chromium ion mother liquor as the control. The standard curve reflecting the relationship between chromium ion concentration and absorbance value was drawn (Hadia-E, 2018).

2.3 Effect of the Adsorption Conditions on the Adsorption Effect

Batch experiments were carried out in 250mL conical flasks containing 100 mL medium with appropriate volume of chromium ion mother liquor. Then, the bacterial solution was inoculated into the medium with 3%(V/V) of the inoculation amount and placed in a constant temperature oscillating incubator culture at 150 rpm. After overnight culture for 12 h, the samples were extracted and centrifuged at 5 000 rpm for 10 min. After the reaction with dibenzoyl hydrazine to form purplish red complex, spectrophotometer was used to determine the absorbance value at 540nm, and the residual concentration of Cr (VI) in the supernatant was analyzed. The adsorption rate (β) of Cr (VI) is calculated according to the following formula:

$$\beta = (C_0 - C_e) \div C_0 \quad (1)$$

where C_0 and C_e are the initial and residual Cr (VI) concentrations, respectively.

In order to optimize the Cr(VI) adsorption efficiency of selected strains, the effects of Carbon sources (sucrose, fructose, lactose, maltose, glucose), nitrogen sources (ammonium citrate, soybean peptone, tryptone, peptone), temperature

(30, 32, 35, 38, 40℃) were studied, pH (6.0, 6.5, 7.0, 7.5, 8.0) and initial Cr(VI) concentration (10, 20, 30, 40, 50mg/L) were investigated.. Each set of experiments was in triplicate, and the average value was taken for further analysis.

3 RESULTS AND ANALYSIS

3.1 Effects of Chromium and Calcium on the Growth of the Strain

This study verified whether Ca^{2+} could improve the tolerance of *Bacillus amyloliquefaciens* to Cr^{6+}. As can be seen from Figure 1, with the increase of Cr^{6+} concentration, the tolerance of the strain to Cr^{6+} increased significantly with the addition of calcium chloride. When calcium chloride was present, the tolerance to Cr^{6+} increased by 21.26%, 76.21% and 239.66% at 20, 40 and 60mg/L of Cr^{6+}, respectively.

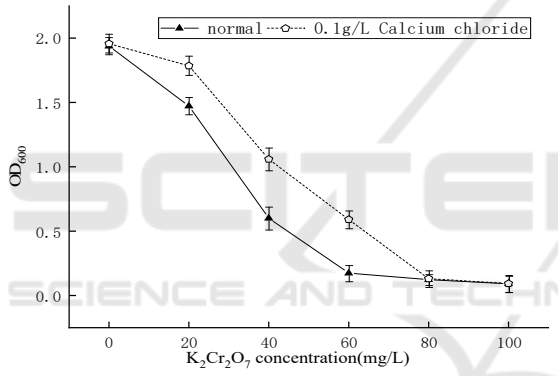

Figure 1: Tolerance curve of Bacillus amyloliquefaciens to Cr^{6+}(conditions: temperature = 35℃, pH=7, agitation rate = 150 rpm, contact time = 12 h).

In acidic environment, Cr^{6+} reacts with dibenzodiazide solution to form a purplish red complex with a maximum absorption wavelength of 540 nm. Therefore, the absorbance value at 540 nm was used as the abscissa and the concentration of chromium ions as the ordinate to draw the working curve. The linear regression equation y = 2.562 1x-0.053 and the correlation coefficient R^2 = 0.994 were obtained. The linear relationship was good as well as the experimental stability.

3.2 Effect of Carbon Source on Adsorption Effect

It can be seen from Figure 2 that when the carbon source was maltose, the adsorption effect was better. The concentration of residual Cr^{6+} in the medium was 4.78 mg/L, the bacteria weight was 0.56 g and the adsorption rate was as high as 76.08%. When the carbon source was fructose, it had the worst adsorption effect. The concentration of residual Cr^{6+} was 12.52 mg/L, the bacteria weight was 0.24 g and the adsorption rate was only 37.40%. With the increase of carbon source concentration, the adsorption rate reached 88.94% when maltose concentration was 25 g/L, which increased by 9.48%. It can be clearly seen from Figure 2 that, with the increase of concentration and adsorption rate, the bacterial weight also increased. Therefore, it can be inferred that the increase of carbon source concentration provides sufficient carbon source for the growth of bacterial strains, leading to the increase of the number of bacterial strains in the medium, thus enhancing the adsorption effect.

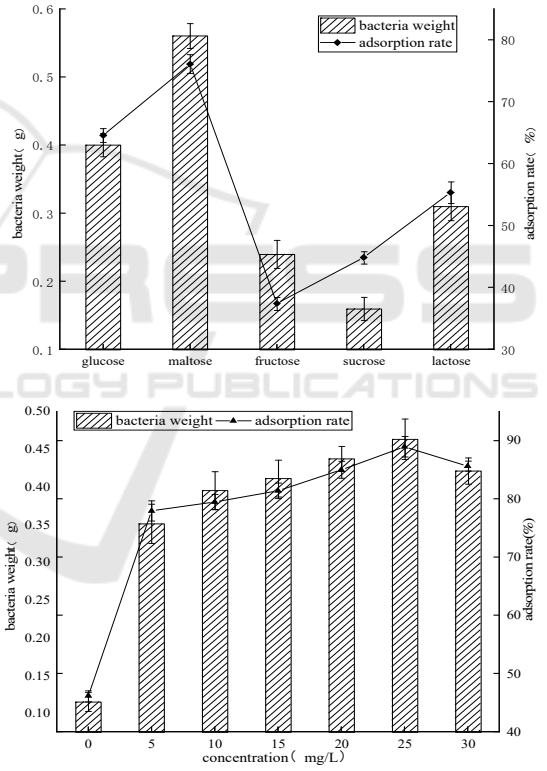

Figure 2: Effect of carbon sources on the adsorption effect (conditions: temperature = 35℃, pH=7, agitation rate = 150 rpm, contact time = 12 h).

3.3 Effect of Nitrogen Source on Adsorption Effect

As can be seen from Figure 3, when the nitrogen source was peptone, the adsorption effect was the best, the concentration of residual Cr^{6+} in the medium was 3.48 mg/L, the bacteria weight was

0.99 g and the adsorption rate was as high as 82.67%. When the nitrogen source was ammonium citrate, the concentration of residual Cr^{6+} was 19.35 mg/L, the bacteria weight was 0.07 g, and the adsorption rate was only 3.24%. The cells may have died and the dead cells still had certain adsorption capacity for heavy metals. With the increase of nitrogen source concentration, the adsorption rate reached 88.43% when the concentration of peptone was 25 g/L, which increased by 18.57%.

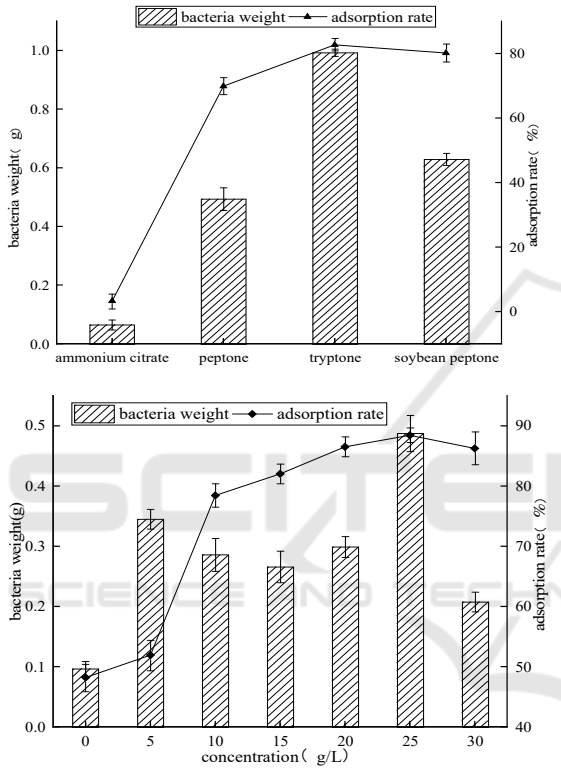

Figure 3: Effect of the nitrogen source on the adsorption effect (conditions: temperature = 35℃, pH=7, agitation rate = 150 rpm, contact time = 12 h, carbon source = 25 g/L maltose).

3.4 Effect of Temperature on Adsorption Effect

The life activities of microorganisms cannot be separated from the help of enzymes and enzyme activity is closely related to temperature18. The adsorption of Cr(VI) was studied at 20 mg/L initial Cr(VI) concentration, pH=7 and 150 rpm and 5 different temperatures (30, 32, 35, 38, 40℃). As can be seen from Figure 4, when the temperature was 35℃, the activity of enzymes related to adsorption was strong and the effect was the best. The concentration of residual Cr^{6+} in the medium was only 4.06 mg/L, the bacteria weight was 0.64 g and the adsorption rate was as high as 79.72%. At 30 ℃, the enzyme activity was weak and the effect was the worst. The concentration of residual Cr^{6+} was 11.61 mg/L, the bacteria weight was 0.47 g and the adsorption rate was only 41.93%.

Extreme temperature will have adverse effects on the growth of bacteria and chrome reductase, and the growth and development of bacteria will be inhibited at low temperature. At higher temperature, the conformation of ribosome will change to some extent, which will lead to the change of membrane structure and decrease or even inactivation of chromium reductase.

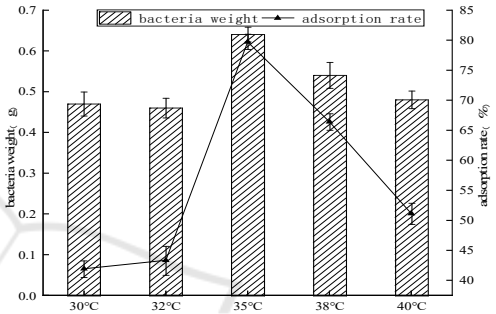

Figure 4: Effect of the temperature on the adsorption effect (conditions: agitation rate = 150 rpm, pH=7, contact time = 12 h, carbon source = 25 g/L maltose, nitrogen source =25 g/L tryptone).

3.5 Effect of pH on Adsorption Effect

In the adsorption process, the pH of the medium can affect the ionized state of the major functional groups responsible for metal ion binding, such as carboxyl, amino, and phosphorylation. At low pH, these groups retain their protons, which reduces the possibility of binding with other positively charged ions. On the other hand, at higher pH, the carboxyl groups become deprotonated and negatively charged, which helps to attract positively charged metal ions.As can be seen from Figure 5, when pH=7.5, the effect was the best, the concentration of residual Cr^{6+} in the medium was 3.83 mg/L, the bacterial weight was 0.51 g and the adsorption rate was 80.87%. When pH=8, the effect was the worst, the bacteria weight was 0.44 g, the residual Cr^{6+} concentration was 9.49 mg/L and the adsorption rate was only 52.56%.

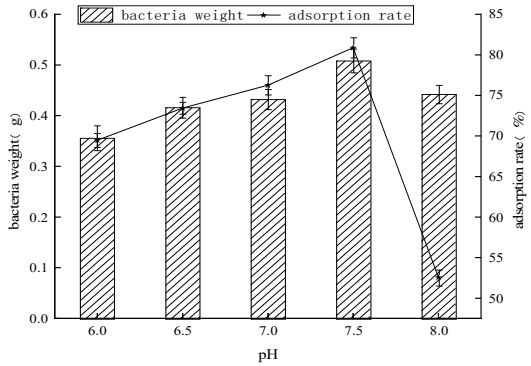

Figure 5: Effect of the pH on the adsorption effect (conditions: temperature = 35℃, agitation rate = 150 rpm, contact time = 12 h, carbon source = 25 g/L maltose, nitrogen source =25 g/L tryptone).

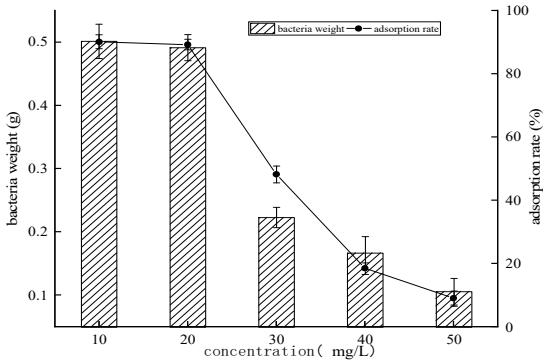

Figure 6: Effect of the initial Cr6+ concentration on the adsorption effect (conditions: temperature = 35℃, pH=7.5, agitation rate = 150 rpm, contact time = 12 h, carbon source = 25 g/L maltose, nitrogen source =25 g/L tryptone).

3.6 Effect of Initial Concentration of Cr^{6+} on Adsorption Effect

Five different initial metal ion concentrations (10, 20, 30, 40 and 50 mg/L) and 3% inoculation were used to determine the biosorption of chromium by the strain. The effect of the initial concentration of chromium on the adsorption is shown in Figure 6. It can be clearly seen that, with the increase of concentration, the adsorption effect decreased gradually, which may be related to the saturation degree of the adsorption site of the bacteria4. When the initial concentration was 10 mg/L and 20 mg/L, the adsorption sites on the cell wall of the bacteria had basically reached the saturation state, so the effect was good. The bacteria weight was 0.501 g and 0.491 g, respectively and the adsorption rate was 90.10% and 89.20%. When the concentration of Cr^{6+} was 20 mg/L, the adsorption rate of the optimized medium increased from 64.56% to 89.20%, which was 24.64% higher than that before the optimization. When the initial concentration was 50 mg/L, metal ions had a great toxic effect on cells18 and inhibited the adsorption effect and the adsorption rate was only 9.01%.

4 DISCUSSION

The mechanisms of chromium ion removal by microorganisms mainly include membrane surface adsorption, transformation, intracellular absorption, intracellular transformation and extracellular transformation. Chromium ions can be fixed on the binding sites provided by polysaccharides, proteins and lipids on the surface of the cell membrane by the chemical binding ability of functional groups on the surface of the cell membrane, electrostatic force and the electrostatic attraction of cations on the surface of the cell membrane(Hadia-E, 2018. Sasmita, 2014. Ramírez-Díaz, 2008.Gunnar, 2018). Most of these chromium ions enter the cell through a unique mechanism and a small part may be reduced by membrane-bound reductase mediated action and extracellular polysaccharide complexation with the involvement of membrane surface functional groups(Tang, 2020).

Since chromium ions exist in the form of CrO_4^{2-}, a regular tetrahedral structure similar to SO_4^{2-} in spatial conformation, they can enter the cell channel through SO_4^{2-}. Then, soluble reductase (NADH, NemA, NAPH, etc.) and reducing agents (ascorbic acid, glutathione, etc.) in the cytoplasm were reduced to Cr^{3+} with lower toxicity(Karthik, 2017). In the environment of ferric reducing bacteria and sulfate reducing bacteria, the extracellular products (ferrous ions, hydrogen sulfide, etc.) that can be anaerobic metabolized by bacteria are reduced without ATP consumption(Long, 2021).

The adsorption tests in this study were all carried out in an environment containing only chromium ions, while the actual chromium pollution treatment often contains metal ions such as Cu^{2+}, Mg^{2+}, Pb^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+} and some oxygen anions (sulfate ion, nitrate ion, etc.), which may affect the adsorption. HANG found that 5 mg/L Cu^{2+} promoted the adsorption of Cr6+ to Bacillus sp. CrB-B1, and the adsorption rate increased to 92.21%. When Cd^{2+} concentration was 5 mg/L, the adsorption rate decreased by 25.03%(Tang, 2020). Oxygen anions such as SO_4^{2-} and NO_3^- have little effect on the adsorption of Cr6+. LUO showed that

Ca^{2+} and SO_4^{2-} promoted the reduction of chromium by 52.5% and 55.9%, respectively(Luo, 2020). In addition, studies have shown that some microorganisms can also remove other ions (Pb^{2+}, NO^{3-}, etc.) in the environment in the process of chromium ion adsorption(Zhong, 2017. Yu, 2016. An, 2020). XU isolated a strain of Serseria marcescens from tannery wastewater, which can remove carcinogenic o-dichlorobenzene while absorbing hexavalent chromium(Xu, 2018). The co-removal of chromium ions and other ions as well as the co-removal of chromium ions and organic matter further provides a theoretical basis for the application of microorganisms in the practical heavy metal pollution treatment, which is of great significance to the remediation of chromium pollution by microorganisms. In practical application, the influence of the external environment on the growth of microorganisms and the adsorption effect should be considered comprehensively. Therefore, while excavating the adsorption potential of microorganisms for heavy metal ions, we should also pay attention to their adaptability to the complex and changing environment.

5 CONCLUSIONS

In this experiment, the adsorption capacity of Cr^{6+} of a strain of *Bacillus amyloliquefaciens* with strong stress resistance preserved in the laboratory was studied. At the same time, it was also verified that Ca^{2+} improved the tolerance of the strain to metals. The results showed that when the concentration of Cr^{6+} was 20 mg/L, the temperature was 35℃, the pH value was 7.5, the carbon source was maltose (25 g/L) and the nitrogen source was tryptone (25 g/L), the adsorption rate of Cr^{6+} was 89.20%, which was 24.34% higher than that before optimization. When calcium chloride (0.1 g/L) was added to the culture medium, the tolerance was increased by 21.26%, 76.21% and 239.66% when the concentration of Cr^{6+} was 20, 40 and 60 mg/L, respectively. In the Cr^{6+} adsorption test, it was found that the cell content may have a certain relationship with the adsorption effect. Within the limited concentration range, the cell content has a negative correlation with the residual Cr^{6+} concentration, that is, the cell content has a positive correlation with the adsorption effect, and the more cells, the better the adsorption effect.

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