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# Chromium (III) Biosorption of *Deinococcus radiodurans* and Its *Vitreoscilla* Haemoglobin (*vgb*) Gene-Transferred Recombinants

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**Abstract: Objectives:** This study used *Deinococcus radiodurans*, which is extremely resistant to oxidative damage, genotoxic chemicals, high levels of ionising and ultraviolet radiation and drying, and its *Vitreoscilla* haemoglobin (*vgb*) gene-cloned recombinant with the *vgb*$^{-}$ recombinant strain as a control. In addition to the conditions wherein bacteria have an optimum Cr (III) biosorption capacity, the contribution of the *vgb* gene to the biosorption ability of the bacteria has been investigated by providing the organism with a more oxygenic environment. **Methods:** Bacteria were produced and metal stock solution was prepared. To determine the Cr (III) removal capacities of wild and recombinant *D. radiodurans* strains, the residual metal concentration in aqueous media at the beginning and after biosorption was determined in Atomic Absorption Spectrophotometer. Some optimal conditions were created for the biosorption conditions to occur. **Conclusions:** The optimisation tests showed that Cr (III) reached the highest biosorption capacity within 15 minutes at a metal concentration of 2,000 ppm, 30 ℃, pH 5.0 and 150 rpm stirring speed in all the three bacteria. The *vgb* gene had no significant contribution to the biosorption capacity.

**Key words:** *Deinococcus radiodurans*, Chromium (III) Biosorption, *Vitreoscilla* Haemoglobine.

## 1. Introduction

### *1.1 Heavy Metals*

The “heavy metal” has become a commonly used term in recent years. It is usually defined as metals or semi-metals that are associated with contamination and potential toxicity or ecotoxicity. At present, there are many definitions of heavy metal depending on its density, atomic weight, chemical properties or toxicity. In medicine, heavy metals are defined as all toxic metals, regardless of the atomic weights of the element. Although more than 60 elements can be used as examples of heavy metals, the following are the most common and well-known heavy metal fields: Mercury (Hg), Manganese (Mn), Iron (Fe), Cobalt (Co), Nickel (Ni), Copper (Cu), Zinc (Zn), Cadmium (Cd), Arsenic (As), Chromium (Cr), Lead (Pb), Silver (Ag) and Selenium (Se) [1-3].

Many applications in nature cause metal pollution. The industrial applications that hold the most important place in this type of pollution are mining, garbage furnaces, the processing of radioactive metals, metal coating applications (coating of electronic equipment) and the use of paints, waste batteries, pesticides and exhaust gas [4-5].

Various chemical and physical methods are used to remove heavy metals from industrial wastewater and environmental water sources contaminated with heavy metals. However, because these methods are not economical and the purification level obtained is not sufficient, microorganisms that have an important potential in this field are used and preferred [6-9].

Although chromium can exist in many different forms, the most common and stable forms are trivalent Cr (III) and hexavalent Cr (VI). Chromium is

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naturally present in the form of $Cr_2O_3$ (trivalent (+3) form) in the environment [10]. Cr (III) is poorly soluble in groundwater and is firmly controlled by the soil. The insufficient solubility of Cr (III) minimises its toxic effect in the environment. However, Cr (VI) is highly soluble and combines with oxygen in the form of chromate ($CrO_4^{-2}$) or dichromate ($Cr_2O_7^{-2}$) ions. Its portability is also much higher than Cr (III). Cr (VI) is a considerably strong oxidant and will be reduced to Cr (III) in the presence of organic matter. This reduction is faster in acidic environments such as soils that contain acid. Cr (VI) can readily penetrate the prokaryotic and eukaryotic cell membranes [11]. It causes lung cancer, chromate ulcer, nasal septum perforation and kidney damage in humans [12].

Furthermore, it can interact with chromium (III) proteins and nucleic acids. Studies conducted on chromium chloride ($CrCl_3$) have shown that there is a delay in the onset of nucleic acid synthesis and, consequently, a decrease in nucleic acid content. Moreover, studies on potassium dichromate ($K_2Cr_2O_7$) show that there is a prolonged duration of cell division and a decrease in cell division. Potassium dichromate ($K_2Cr_2O_7$) has a great impact on DNA synthesis, likely exerting its effect by affecting DNA polymerase and interacting with double helix DNA. The hydrogen bonds between the bases and the negatively charged phosphate within them repel each other, keeping DNA in a stable form. Cr (III) in chromium chloride binds to negatively charged phosphate groups. Thus, negatively charged phosphates are neutralised; weak H bonds between bases in DNA are broken by increasing Cr (III); DNA loses its stable structure and its melting temperature decreases [11] (Figure 1).

1.1.1 Heavy Metal Biosorption

Biosorption is a promising alternative method for industrial waste removal primarily because of its low cost and high metal binding capacity. Biosorption can occur actively through metabolism or passively through some physical and chemical processes. In biosorption, the metal binding process takes place in two steps. The first step is the stoichiometric interaction between metal and reactive chemical groups in the cell wall; the second is the inorganic accumulation of increasing amounts of metal. The bacterial cell wall is the first component to come into contact with metal ions. As the type of metal sorption with dead or inactive cells is extracellular, chemical functional groups of the cell wall play an important role in biosorption. Bacterial cell walls contain several functional groups, including carboxyl, phosphonate, amine and hydroxyl groups [14].

Microorganisms are effectively utilized and are preferred for biosorption of heavy metals [6-9]. Various bacteria, fungi and algae are used for this purpose [15-22]. The biomolecules of these microorganisms ensure that both living and dead biomasses have a high metal affinity and thus offer a considerably high biosorption capacity.

### *1.2 Deinococcus Radiodurans*

*Deinococcus radiodurans* (Greek: deinos: strong, unusual and coccus: granular fruit) was first isolated from canned meat applied 4,000 gm gamma ray by Anderson et al. in 1956. Because of the similarity of its surface morphology to Micrococcus genus, the

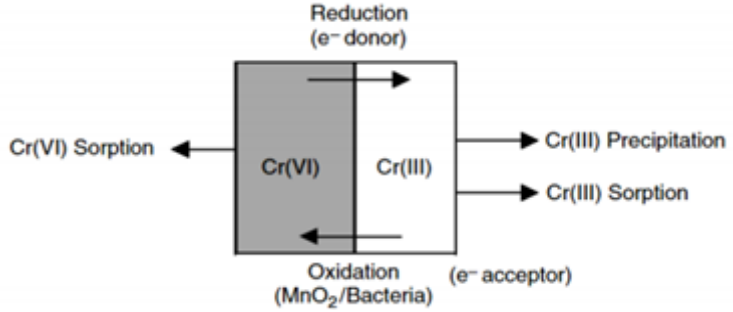


**Fig. 1 Cr (VI) and Cr (III) reactions [13].**

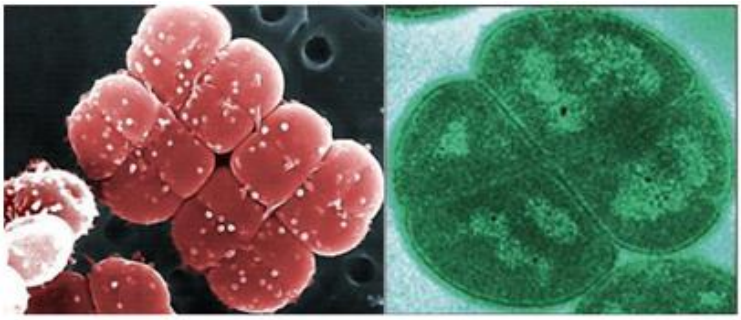

**Fig. 2** ***Deinococcus radiodurans*.**

bacteria were first classified as Micrococcus. Then, it was named as "Radiodurans" because of its resistance to radiation [23]. 16S rRNA analysis of *Micrococcus radiodurans* led to the reclassification of these species and their closest relatives. Thus, it was included in a new family, the Deinococcaceae, and renamed *Deinococcus radiodurans* [24].

*D. radiodurans* is a nonphotosynthetic, red-pigmented, nonsporulating, extremophile bacterium that have extreme resistance to ionizing radiation and numerous oxidizing agents. Although the chemical composition of the non-pathogenic *D. radiodurans* cell wall is similar to the Gram-negative, it is a Gram-positive and mesophilic organism with a thermal limitation above 39 ℃. This proteolytic bacterium, ranging in size from 0.5 to 3.5 μm, is in a spherical structure and present as single cells in the liquid culture. However, it is present as tetrates in solid and liquid media (Figure 2). In addition, *D. radiodurans* entered the Guinness World Records Book as the most resistant organism against radiation in the world [25].

*D. radiodurans* is conventionally grown at 32 ℃ in rich TGY medium 0.5% tryptone, 0.1% glucose, 0.15% yeast extract with aeration. The cell replication time is approximately 100 minutes under optimal conditions [26, 27]. *D. radiodurans* are resistant to genotoxic agents such as low humidity, UV-C rays, high amounts of reactive oxygen derivatives and mitomycin C as well as ionizing radiation [28-30]. *D. radiodurans* has become increasingly popular in comparative metabolic studies and bioremediation of areas contaminated with radioactive waste due to their exceptional resistance to conditions [27, 31].

### *1.3 Vitreoscilla Haemoglobine*

It was not until 1986 that haemoglobin was known to exist only in eukaryotes. It was later found in the Gram-negative filamentous bacteria *Vitreoscilla stercoraria*, a chemical organic nutrient from the *Beggiatoa* family. This bacterium is found where oxygen is limited, for example, in freshwater sediments and cow droppings. Thus, bacteria survive by expressing some kind of haemoglobin under hypoxic conditions. This haemoglobin increases respiration, oxygen intake and energy metabolism. When the bacteria obtained from *V. stercoraria* (Vitreoscilla hemoglobin) are expressed by transferring their haemoglobin to various bacteria that live in oxygen-restricted conditions, it improves the growth of bacteria, protein synthesis, metabolite production and resistance to stress [32, 33]. In addition, the heterologous expression of this gene (*vgb*) increases the production of different useful products in these hosts in the host. It also has a significant effect on reducing the effects of harmful compounds. Therefore, VHb can have a beneficial effect, particularly in biotechnological applications [34].

The structure of VHb (Figure 3) has a classical eight-helix (A–H) folding pattern with a high homology to eukaryotic haemoglobins. Vitreoscilla haemoglobin has two similar subunits with an average molecular weight of 15.775, and each subunit contains 146 amino acid residues and two B-type heme. Haemoglobin

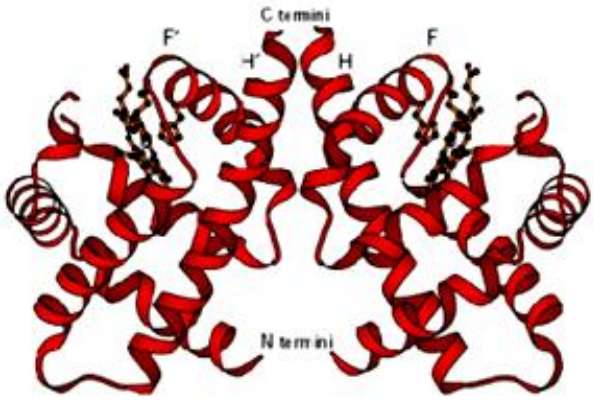


**Fig. 3 Dimensional structure of VHb.**

synthesis increases when the organism is present under hypoxic conditions. The amino acid sequence of this protein shows a structural homology to eukaryotic haemoglobins, but this haemoglobin is separated from the others in the N-terminal region, and a helix is absent [35].

## 2. Materials and Methods

The present study used *Deinococcus radiodurans* R1 (ATCC BAA-816) and its *vgb* gene-cloned recombinant with a $vgb^-$ recombinant strain as the control. The form containing puc8 plasmid from two recombinants of *Deinococcus radiodurans* is called Dr[pUC8], and the form containing *vgb* gene of the same plasmid is called Dr[pUC8:15] (Figure 4).

### *2.1 Production of Microorganisms*

Tryptone glucose yeast (TGY) agar and TGY broth medium were used for the production of *D. radiodurans*.

The long-term stocks of the bacteria used in the study were prepared in a TGY liquid medium containing 20% glycerol (v/v) and stored at −20 °C. However, among the bacteria used in each optimisation test, *D. radiodurans* and its recombinants were produced in a petri dish containing TGY agar and stored in a refrigerator at +4 ℃. The bacteria stored in the refrigerator were harvested under sterile conditions and planted in flasks containing TGY broth medium (20 ml/100 ml). Wild and recombinant bacteria grew

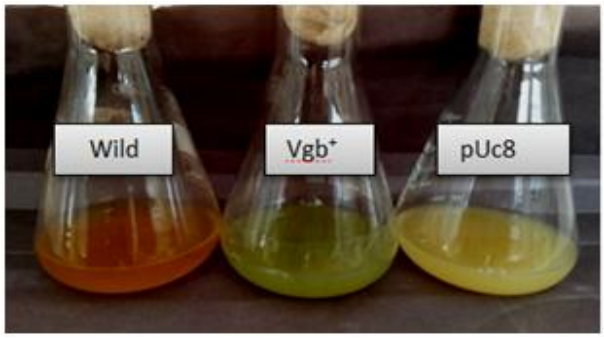


**Fig. 4 *Deinococcus radiodurans* and recombinants.**

at 32 ℃ with a stirring speed of 150 rpm. These stock cultures were obtained at the end of an overnight incubation and planted in 1ml/20ml flasks containing TGY broth medium and then used in the later stages of the experiment.

### *2.2 Preparation of Metal Solutions*

To determine the optimum Cr (III) biosorption conditions of *D. radiodurans* and its recombinants, a $CrCl_3$ stock of 50,000 ppm was prepared. To adjust the metal doses in the ranges of 250 ppm, 500 ppm, 1,000 ppm and 2,000 ppm for Cr (III), a metal sample from stock metal solutions and 1 ml of wild and recombinant strains were added to the TGY medium to provide the determined metal dose and subsequently incubated in the oven for the specified periods.

### *2.3 Metal Analysis*

To determine the Cr (III) removal capacities of wild and recombinant *D. radiodurans* strains, the residual metal concentration in aqueous media at the beginning and after biosorption was determined in air-acetylene flame in the Perkin ElmerAnalyst 800 Atomic Absorption Spectrophotometer.

### *2.4 Optimisation Studies*

Some optimal conditions must be formed for the biosorption conditions to be realised. Biosorption is affected by physicochemical factors such as metal ion type, biomass type and amount, temperature, solution pH and stirring speed. Biosorption parameters such as dose and duration, temperature, pH and shaking rate

were investigated to determine the optimal Cr (III) ion removal conditions with *D. radiodurans* and recombinants. Depending on the optimisation conditions, the incubated samples were centrifuged at 4,500 rpm, and the supernatant obtained was determined via atomic absorption spectroscopy (AAS) to determine the biosorption capacity.

2.4.1 Determination of Optimum Dose and Duration

In the biosorption tests, all three bacteria were treated at 32 ℃ for 30, 60, 90, 120 and 180 min at Cr doses of 250 ppm, 500 ppm, 1,000 ppm, 1,500 ppm and 2,000 ppm to determine the dose and duration that provided the best Cr (III) removal.

2.4.2 Determination of Optimum Temperature

After determining the best dose and duration biosorption capacities of wild and recombinant strains, the samples prepared at the determined dose and duration were incubated at 10 ℃, 20 ℃, 30 ℃ and 40 ℃ for temperature optimisation. The samples expired were measured in AAS, and their biosorption capacities were calculated.

2.4.3 Determination of Optimum pH

For optimisation, the pH of the samples was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Further, 0.1 MNaOH and 0.1 M HCI were used to adjust these pH values. In the study, the determined metal concentrations were added to the media prepared at different pH values and incubated after other optimum conditions were met. The finished samples were measured in Atomic Absorption Spectroscopy (AAS), and their biosorption capacities were calculated.

2.4.4 Determination of Optimum Stirring Speed

One of the factors affecting metal biosorption is the stirring speed in the environment in which the method takes place. The stirring speed with the best biosorption capacity of bacteria was tested at 50, 100, 150, 200 and 250 rpm. The determined metal concentrations were added to the media and incubated at different stirring speeds after other optimum conditions were met. The finished samples were measured in AAS, and their biosorption capacities were calculated.

## 3. Discussion and Results

The dose and duration with the best removal of *D. radiodurans* (wild), (*vgb*) and (pUC8) bacteria were found to be 2,000 ppm and 15 min (Table1).

**Table 1 Cr(III) biosorption of *D. radiodurans* wild and recombinant strains at certain dose and times. a. *D. radiodurans* (wild); b. *D. radiodurans* (vgb) and c. *D. radiodurans* (pUC8).**

| *D. radiodurans* | Time (min) | 250 ppm | 550 ppm | 1000 ppm | 1500 ppm | 2000 ppm |
|---|---|---|---|---|---|---|
| a. wild | 15 | 56.96 | 78.04 | 89.09 | 92.77 | 94.58 |
| | 30 | 57.12 | 77.94 | 88.95 | 92.21 | 94.55 |
| | 60 | 56.52 | 77.76 | 88.92 | 92.68 | 94.53 |
| | 90 | 56.12 | 77.58 | 88.82 | 92.63 | 94.46 |
| | 120 | 57.24 | 78.08 | 89.09 | 92.8 | 94.63 |
| | 180 | 57.6 | 78.28 | 89.23 | 92.83 | 94.65 |
| b. vgb | 15 | 57.16 | 78.04 | 89.06 | 92.72 | 94.59 |
| | 30 | 57.04 | 77.9 | 88.99 | 92.69 | 94.35 |
| | 60 | 56.64 | 77.8 | 88.95 | 92.65 | 94.51 |
| | 90 | 56.4 | 77.54 | 88.92 | 92.6 | 94.19 |
| | 120 | 57.36 | 78.18 | 89.11 | 92.78 | 94.63 |
| | 180 | 57.76 | 78.3 | 89.21 | 92.8 | 94.64 |
| c. pUC8 | 15 | 57.36 | 78 | 89.1 | 92.8 | 94.6 |
| | 30 | 59.69 | 77.94 | 89 | 92.71 | 94.55 |
| | 60 | 56.68 | 77.72 | 88.93 | 92.65 | 94.52 |
| | 90 | 56.04 | 77.66 | 88.88 | 92.64 | 94.51 |
| | 120 | 57.76 | 78.14 | 89.14 | 92.8 | 94.62 |
| | 180 | 57.6 | 78.3 | 89.2 | 92.82 | 94.67 |

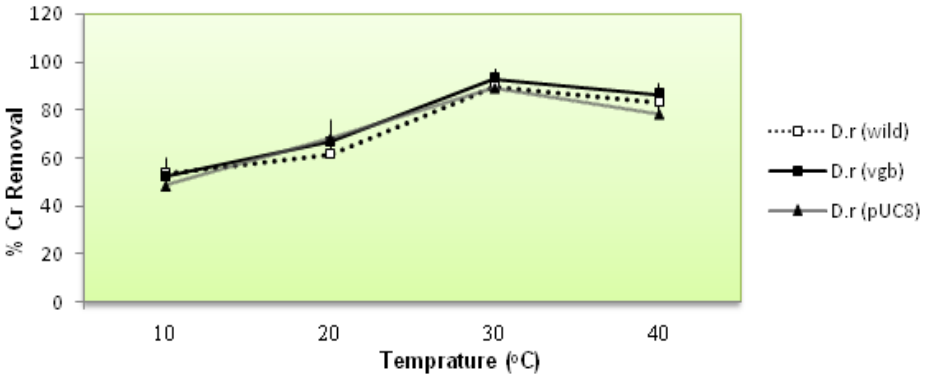


**Fig. 5 Cr (III) biosorption (%) D. radiodurans (wild) and recombinant strains at different temperatures.**

The examination of the Cr biosorption capacity of *D. radiodurans* (wild), (*vgb*) and (pUC8) at certain doses and durations revealed that the bacteria increased in the Cr biosorption ratio in parallel with the increase in dose and duration and gave similar results.

*3.1 Temperature Optimisation*

The temperature of the adsorption medium is important because of the energy-dependent mechanisms in metal biosorption with microbial cells [36]. The data obtained from the biosorption capacity of *D. radiodurans* (wild) and its recombinant bacteria at different temperatures are given in Figure 5 Accordingly, it was determined that the Cr biosorption capacity of *D. radiodurans* (wild) and its recombinant bacteria increased in parallel with the temperature increase up to 30 ℃ and started to decrease after 30 ℃. Therefore, the optimum temperature for all three bacteria has been recorded as 30 ℃.

Temperature is another factor that affects biosorption, and it is a significant parameter in reactions where biosorption occurs. Theoretically, biosorption decreases as temperature increases. The ions that bind to biomass in the early moments of their biosorption tend to be released back from the biomass because of the increased temperature [37, 38]. Zouboulis et al. showed that with bacteria species obtained from metal-contaminated soils, the metal uptake increased with the increase in temperature. The increase in the metal uptake capacity with temperature was explained by the increase of the metal-related attraction force on the biomass. However, metal sorption decreases because the cell surface is damaged by the excessive rise in temperature [39].

*3.2 pH Optimisation*

In metal-microorganism interactions, the pH value of the medium is an important parameter in biosorption processes because it significantly affects the form of the metal and the functional groups in the cell that have metal affinity [40, 41]. High protons and metal ions in low pH solutions compete to bind to active groups on the biosorbent, which reduces the potential for cell-metal interaction. However, as the pH value of the solution increases, the metal ions will be able to bind more to the active groups on the biosorbent with the decrease of protons. This explains why the biosorption capacity increases as the pH increases up to pH 5.0. In this study, the optimum pH value was found to be 5.0 (Figure 6).

Kiff and Little found that the biosorption of cadmium on *A. oryzae* increased with the increase in pH [42]. Further, Ross and Townsley showed that copper removal with *P. spinulosum* decreased at low pH values [43]. In addition, Zhang et al. stated that lead holding capacity was high above pH 6, but at higher pH values, lead started to precipitate by forming lead hydroxide and compounding with hydroxides in water [44].

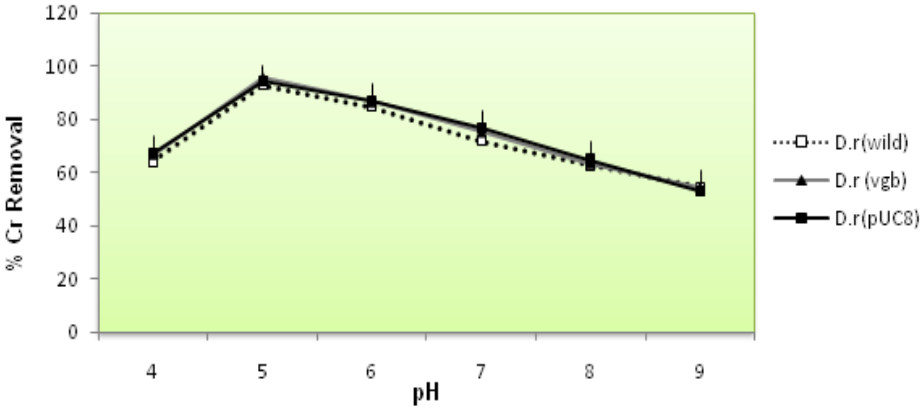


**Fig. 6 Cr (III) biosorption (%) *D. radiodurans* (wild) and recombinant strains at different pH values.**

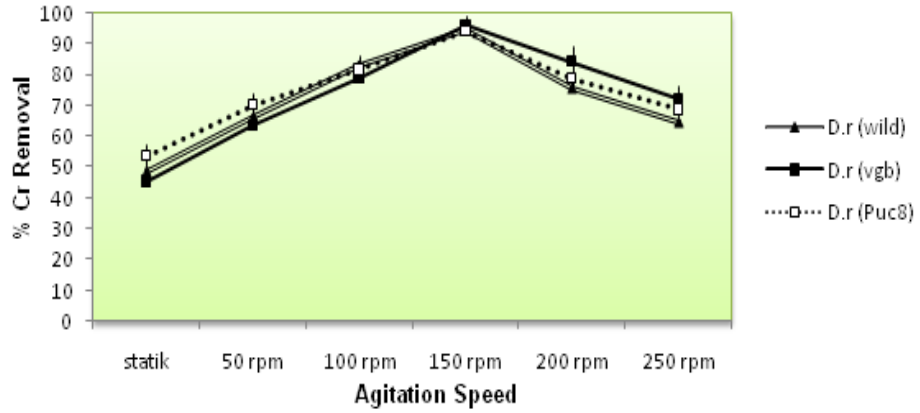


**Fig. 7 Cr (III) biosorption (%) *D. radiodurans* (wild) and recombinant strains at different stirring speeds.**

*3.3 Stirring Speed Optimisation*

To determine the effect of stirring speed on the biosorption capacity of *D. radiodurans* and its recombinants, biosorption capacities were determined by exposing the bacteria to different stirring speeds and other optimum conditions. The data obtained are given in Figure 7.

Accordingly, it was determined that the Cr biosorption capacity of *D. radiodurans* (wild) and its recombinant bacteria increased in parallel with the increase of the stirring speed up to 150 rpm and started to decrease after 150 rpm. Therefore, the optimum stirring speed for all three bacteria was found to be 150 rpm. The rate of the contact of the molecules in the solution, which is continuously stirred at high speed, to the surface of the adsorbent and the probability of penetrating its pores are higher [45]. Therefore, in the study, metal removal continued to increase up to a stirring speed of 150 rpm, but the adsorption of metals became difficult at high stirring speeds such as 200 and 250 rpm. There are different opinions in the existing literature with respect to the correlation of the biosorption properties of biomasses with metal tolerance [46-50]. Based on the data obtained herein, the highest biosorption capacity value is reached within 15 min at pH 5.0, 30 ℃ and 150 rpm. It is considered that the high biosorption capacity achieved in the determined optimum conditions is comparable to the literature. In addition, the advantages of the VHb/*vgb* system, such as its role in buffering the ambient oxygen and providing a better respiration, growth and reproduction ability to the old cells by transferring them to the membrane transferases in the advanced phases of the culture, could not be clearly seen in the recombinant bacteria that contained the gene.

## Acknowledgement

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## Author's Contribution

Elif Ozbey, carried out the experiment. Elif Ozbey, worked out almost all of the technical details, and performed the numerical calculations for the suggested experiment. Elif Ozbey, contributed to sample preparation and performed the calculations. Dilek Asma supervised the project and wrote the manuscript. Dilek Asma, devised the project, the main conceptual ideas and proof outline. Both Dilek Asma and Elif Ozbey authors contributed to the final version of the manuscript. Both researchers did the original drafting, review and editing.

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