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Araştırma Makalesi / Research Article

Effect of Cadmium and Vitamin C on *Citrobacter Freundii's* Antioxidant Enzymes and Stress Markers

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Abstract

In this study, *Citrobacter freundii* (NRRL B-2643) bacteria were grown in LB medium containing varying concentrations of cadmium (Cd). In order to reduce the negative effect of Cd, different concentrations of vitamin C, known for its antioxidant properties, were added to the Cd-containing growth medium. Bacterial concentration, soluble protein and activities of antioxidant enzymes (Glutathione peroxidase (GSH-Px), Glutathione reductase (GSH-Rd), Superoxide dismutase (SOD), Catalase (CAT) and peroxidase (POD)) were determined by spectrophotometer. In addition, reduced and oxidized glutathione (GSH and GSSG), 4-hydroxyneoneal (4-HNE), malondialdehyde (MDA) amounts were determined by HPLC. No significant microorganism growth was observed at 150 ppm and higher Cd concentrations. Bacteria production was not affected up to 40 ppm Cd concentration. Bacteria were grown in media containing 0, 75, 100 and 125 ppm Cd. The protein content of the microorganism grown in the medium containing 75, 100 and 125 ppm Cd decreased about 24, 44 and 62 percent, respectively, comparisons to the control. When 50 ppm of vitamin C was added to the same growth medium, the percentage decrease in protein amount compared to the control was found to be 10, 31 and 50, respectively. An increase was observed in the antioxidant enzymes activities and stress markers in bacteria grown in cadmium-containing media compared to the control ($p<0.05$). With the addition of 25, 50 and 75 ppm vitamin C to cadmium-containing media, a decrease was observed in the activities of antioxidant enzymes and the amounts of stress markers.

Keywords
Antioxidant Enzymes; *Citrobacter freundii;* Cadmium; Stress Markers

Kadmiyum ve C Vitamininin *Citrobacter Freundii*'nin Antioksidan Enzimleri ve Stres Belirteçleri Üzerine Etkisi

Öz

Bu çalışmada *Citrobacter freundii* (NRRL B-2643) değişik konsantrasyonlarda kadmiyum içeren LB besi yerinde üretildi. Kadmiyumun (Cd) oluşturduğu olumsuz etkiyi azaltmak için, kadmiyum içeren besi yerine antioksidan özelliği ile bilinen değişik konsantrasyonlarda C vitamini katılarak da bakteri çoğaltıldı. Çoğaltılan bakteri konsantrasyonu, protein miktarı ve antioksidan enzimlerin (Glutatyon peroksidaz (GSH-Px), Glutatyon redüktaz (GSH-Rd), Süperoksit dismutaz (SOD), Katalaz (CAT) ve peroksidaz (POD)) aktiviteleri spektofotometre ile belirlendi. Ayrıca redükte ve okside glutatyon (GSH ve GSSG), 4-hidroksineoneal (4-HNE) ve malondialdehit (MDA) miktarları ise HPLC ile tayin edildi. 40 ppm kadmiyum konsantrasyonuna kadar bakteri üretiminin etkilenmediği 150 ppm ve daha yüksek Cd konsantrasyonlarında de ise anlamlı mikroorganizma üremesi gözlenemedi. Bu nedenle 0, 75, 100 ve 125 ppm Cd içeren besi yerlerinde bakteri üretimi gerçekleştirildi. 75, 100 ve 125 ppm Cd içeren besi yerinde üretilen mikroorganizmaların protein miktarı kontrole göre sırasıyla yüzde 24, 44 ve 62 oranında azalmıştır. Aynı besi ortamına 50 ppm C vitamini eklendiğinde ise kontrole göre protein miktarındaki yüzde azalma sırasıyla 10, 31 ve 50 olarak bulunmuştur. Kadmiyum içeren besi ortamında üretilen bakterilerdeki antioksidan enzimlerin aktiviteleri ve stres biyomarkerları kontrole göre artış,

Anahtar kelimeler
Antioksidan Enzimler; *Citrobacter freundii;* Kadmiyum; Sitres Belirteçleri

göstermiştir (p<0.05). Kadmiyum içeren besi ortamına 25, 50 ve 75 ppm C vitamini katılmasıyla birlikte antioksidan enzimlerin aktiviteleri ve stres belirteçlerinin miktarlarında azalma olduğu gözlenmiştir.

1. Introduction

C. freundii is a member of the *Enterobacteriaceae* family which is gram-negative bacterium (O'Hara *et al*. 1997), that is a soil micro-organism, may also be seen in other places such as foods, intestinal tracts and sanitation (Wang *et al*. 2000). Even though *C. freundii* is a bacterial pathogen, also plays a big part in the environment's nitrogen cycle, which is responsible for environmental reduction of nitrate to nitrite (Puchenkova 1996). Heavy metals are toxic to living things even at low concentrations (Banfalvi 2011). Cadmium is a heavy metal that has a substantial environmental and functional effect (Paschal *et al*. 2000). In biological systems, cellular organs and components such as cell membranes, mitochondria, lysosomes, endoplasmic reticulum, nuclei, certain metabolic enzymes, detoxification and cell damage repair have been documented to be impaired by heavy metals. Metal ions interact with components in cells, including DNA and nuclear proteins, causing damage to DNA and then altering conformation (Beyersmann and Hartwig 2008). Cadmium induces cytotoxic effects in an *in vitro* experiment at concentrations 0.1 to 10 mM and the free radical damage to DNA (Al-Ghafari *et al*. 2019). Proteins, which are the building blocks of tissues and cells, have an important role in the growth and development of cells and tissues. It is important to keep the amount of protein constant in order for the living thing to continue its normal functioning, and changes in the total amount of protein may cause some disruptions (Shacter 2000). Oxidative stress caused by cadmium in biological systems causes an increase in lipid peroxidation and changes in the antioxidant defence system (Manca *et al*. 1991, Jemai *et al*. 2007). The most important defence mechanism against oxidative stress-induced cell damage is exhibited by the antioxidant enzyme system. These antioxidant enzymes, together with their by-products, are highly important proteins involved in the catalytic conversion of ROS into non-toxic stable molecules (Sáez *et al*. 2017). This defence system includes antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GSH-Rd), as well as nonenzymatic antioxidant glutathione (GSH) (Taysi 2005). It has been reported that the change in antioxidant enzyme activities is important during oxidative stress (Adwas *et al*. 2019). In addition to determining antioxidant enzyme levels, malondialdehyde (MDA) and 4-hydroxyneoneal (4-HNE) levels, which are formed as a result of lipid peroxidation, also serve as good markers in determining cellular damage caused by ROS depending on stress conditions (Gaweł *et al*. 2004, Schaur *et al*. 2015). Vitamin C has a role in tissue repair, protein formation, inactivation of toxic metals and protection of other vitamins (such as A and E), DNA from the harmful effects of oxidation (Hamza 2017).

Citrobacter freundii (NRRL B-2643) was preferred for its ability to reproduce easily and rapidly and to have many common features with other living things. The aim of this study is to investigate effect of cadmium together with vitamin C to counteract on the protein, antioxidant enzymes and stress markers.

2. Materials and Methods

2.1. Material

Citrobacter freundii (NRRL B-2643) grown in LB medium (10.0 g peptone, 5.0 g yeast extract, 10.0 g NaCl per liter) was used. A stock solution of 1000 ppm cadmium was prepared from cadmium chloride ($CdCl_2$). The 500 ppm Vitamin C stock solution was freshly prepared every time and used. Microorganism production was carried out in 250 mL flasks containing 50 mL broth. The following groups were studied;

Control: *C. freundii* was added to sterile LB medium. Cadmium and vitamin C concentrations are given as 0 ppm.

Cadmium group: The microorganism was produced by adding different amounts of cadmium stock solution to the control, according to the desired medium concentration (75, 100 and 125 ppm) of cadmium.

Vitamin C group: The microorganism was reproduced by adding the required amount of vitamin C stock solution to the cadmium group according to the desired medium concentration (25, 50 and 75 ppm) of vitamin C.

After inoculation, it was incubated at 37 °C with 150 rpm, for 18 hours in an orbital shaker (Selecta Rotabit). At the end of the incubation period, the concentration of bacteria was determined by reading the absorbance at 600 nm with UV-Visible spectrophotometer (CHEBIOS s.r.l.).

Then growth medium centrifuged at 8000 rpm, at 10 °C for 10 minutes, (Nüve NF 800 R) the precipitated bacteria were washed twice with distilled water, and centrifuged again and used in further processing. In order to determine the total protein and enzyme activities in the cell, it was sonicated ten times in an ice water bath for thirty seconds in the buffer used in the methods. Cell debris was precipitated in the same centrifuge and conditions, and the supernatant was used in the necessary analysis.

2.2. Protein analysis

Total soluble protein analysis was performed according to the Lowry method (Lowry *et al*. 1951)

2.3. Determination of Glutathione Peroxidase activity

GSH-Px enzyme activity was measured by monitoring the change in absorbance at 340 nm during the oxidation of NADPH to $NADP^+$ (Paglia and Valetine 1967). GSH-Px enzyme activity (ε_{340}: 6220 M/cm) was calculated as the amount of NADPH consumed by 1.0 mg protein in one minute and the specific activity of the enzyme was given as U/mg protein.

2.4 Determination of Glutathione reductase activity

Glutathione reductase catalyses the reduction of GSSG to GSH by NADPH. Enzyme activity is determined by the difference in absorbance of NADPH oxidized during the reaction at 37 °C at a wavelength of 340 nm (Beutler 1984).

2.5 Determination of superoxide dismutase activity

Superoxide dismutase activity was performed according to the method developed by Marklund and Marklund (1974). The principle of the experiment is based on the inhibition of autoxidation of pyrogallol by the SOD enzyme. One unit of SOD activity was determined as the amount of protein that inhibited pyrogallol autoxidation by 50%.

2.6 Determination of Catalase activity

The activity of catalase enzymes was determined according to the Aebi (1984) method which is based on spectrophotometric measurement of the conversion of hydrogen peroxide to water by catalase at 240 nm. Catalase activity was defined as the amount of hydrogen peroxide neutralized by one milligram of protein per minute.

2.7 Determination of peroxidase activity

Peroxidase activity determination was made according to the method of Kumar and Khan (1982). One unit (U) was defined as 0.1 unit change in absorbance per minute per mg protein.

2.8 Determination of 4-HNE

A certain weight of microorganism was fragmented by sonication in ethanol. Analysis were performed using methanol-acetonitrile-water mixture (33:63:4 v/v) as mobile phase on ODS-2 column (25 cm, 4.6 mm ID, 5 µm) in HPLC (Ligor *et al*. 2015).

2.9 Determination of GSH, GSSG and MDA

A certain weight of microorganism was fragmented by sonication in ice-water. Analyses were performed using a Utisil-XB-C-8 (25 cm, 4.6 mm ID, 5 µm) column in HPLC (Ibrahim *et al*. 2017, Karatas *et al*. 2002).

2.10. Statistical Analysis

All measurements were triplicated and mean ± standard deviation was determined. The results were subjected to Variance Analysis by SPSS 10.0 for Windows. The level of statistical significance was expressed as $p < 0.05$.

3. Results and Discussion

In order to determine the effect of cadmium on soluble protein, antioxidant enzymes activity and stress markers in the cell, microorganism production was carried out by adding 75, 100 and 125 ppm cadmium to LB medium. In addition, in order to observe the combined effect of cadmium and vitamin C, which is known for its antioxidant properties, microorganisms were produced by adding 25, 50 and 75 ppm vitamin C to the media containing the same concentration of cadmium. The obtained results were compared with the control (LB medium) values (Figure 1-11). As seen in Figure 1, the amount of soluble protein in microorganisms produced by adding control, 75, 100 and 125 ppm Cd was found to be 23.775±1.25, 18.12±1.13, 13.26±0.92 and 8.99±0.62 mg g^{-1} dw, respectively.

Heavy metals such as cadmium cause metabolic, biological, and physiological modifications that are also expressed via protein inhibition (Güner 2010). Sahiti *et al*. (2020) reported that vitamin C reduces heavy metal accumulation in tissues. Due to these properties of vitamin C, when 25, 50 and 75 ppm vitamin C was added to LB medium containing 100 ppm cadmium to reduce the negative effect of cadmium, the amount of soluble protein was found to be 14.48±0.64, 16.41±0.57 and 17.55±0.50 mg g^{-1} dw, respectively. From these results, it is seen that the amount of protein decreased due to the increasing concentration of cadmium ($p<0.05$), while the amount of vitamin C added to the medium increases the amount of protein depending on the concentration. 50 and 75 ppm vitamin C added to the growth medium led to significant change in the total amount of soluble protein in bacteria ($p<0.05$). Cells exposed to heavy metals show mutation-like changes in the DNA structure, and decreases in the amount of RNA, soluble protein and sugar **(**Yerli *et al*. 2020).

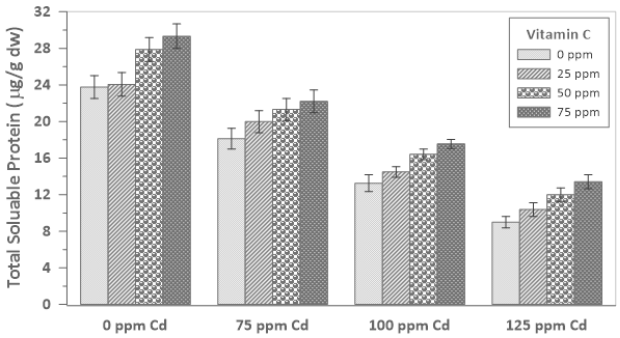

Figure 1. Combined effect of cadmium and vitamin C on the total soluble protein in *C. freundii*.

There may be metal tolerance mechanisms in bacteria such as precipitation of metal salts, alteration of membrane permeability, cell wall immobilization, production of chelating agents and biochemical conversion of metal ions **(**Pandey *et al*. 2013). In addition, antioxidant enzymes have important roles in metal tolerance. Heavy metals create oxidative stress, leading to the formation of reactive oxygen species (ROS). Normally, the amount of ROS remains low due to the activities of antioxidant enzymes such as superoxide dismutase, catalase, lipoxygenase and glutathione peroxidase. Expressions of these enzymes are thought to increase under metal stress conditions to detoxify reactive oxygen species (Choudhary *et al*. 2007).

GSH-Px is an enzyme that helps relieve stress through hydrogen peroxide removal in the presence of reduced glutathione. Current findings show a significant increase in GSH-Px activity with an increase in cadmium concentration compared to control. The addition of 75, 100 and 125 ppm cadmium significantly increased the GSH-Px activity from 1.96±0.18 (control) to 3.33±0.25, 6.71±0.37 and 10.08±0.60 U/mg protein respectively ($p<0.05$) (Figure 2). This shows 1.7, 3.4 and 5.1 times increase in GSH-Px activity at 75, 100 and 125 ppm cadmium respectively when compared with control. In the study by Lenártová *et al*. (1998), it was reported that mercury increased the GSH-Px activity in *Streptococcus bovis*. This finding is also in line with previous work that showed an increase in GSH-Px activity in the presence of cadmium (Pandey *et al*. 2013).

When 0, 25, 50 and 75 ppm vitamin C was added to the medium containing 100 ppm cadmium, the GSH-Px activity was found to be 6.71±0.37, 5.63±0.34, 4.71±0.32 and 4.06±0.26 U/mg protein, respectively.

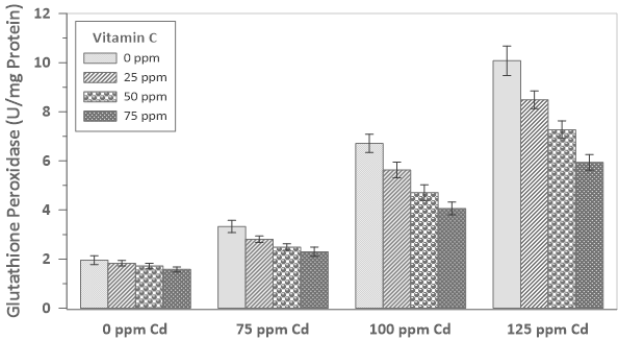

Figure 2. Combined effect of cadmium and vitamin C on the GSH-Px activity in *C. freundii*.

GSH-Red is the enzyme that catalyse the conversion of oxidized form of glutathione to its reduced form. This reaction is very important in maintenance of the glutathione level which is also important in oxidative stress conversion. In this study, the addition of cadmium significantly increases the activity of GSH-Red when compared with control. As seen in Figure 3, GSH-Red activity as a result of 0 (control), 75, 100 and 125 ppm cadmium additions are 0.62±0.06, 1.46±0.10, 2.87±0.18 and 4,16±0.20 U/mg protein, respectively. GSH-Rd activity also increases depending on cadmium concentration ($p<0.05$). This result is also consistent with previous findings pointing to an increase in GSH-Red activity in the presence of cadmium (Cheng *et al*. 2016). In a similar study conducted by Corticeiro *et al*. (2006) on *Rhizobium leguminosarum*, it was reported that both GSH-Red and GSH-Px activities increased in the presence of cadmium, consistent with the current study. When 0, 25, 50 and 75 ppm vitamin C was added to the medium containing 75 ppm cadmium, the GSH-Rd activity was found to be 0.62±0, 1.46±0.10, 1.11±0.08, 0.95±0.07 and 0.86±0.07 U/mg protein, respectively ($p<0.05$) (Figure 3).

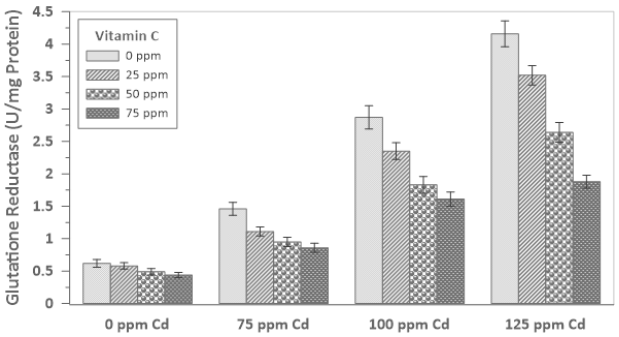

Figure 3. Combined effect of cadmium and vitamin C on the GSH-Rd activity in *C. freundii*.

Addition of 0, 75, 100 and 125 ppm cadmium to the growth medium, the SOD activity was found to be 3.46±0.28, 6.65±0.39, 10.23±0.75 and 16.68±1.12 U/mg protein, respectively. SOD activity also increases depending on the cadmium concentration ($p<0.05$). (Figure 4). When 25, 50 and 75 ppm vitamin C were added to the LB medium containing 75 ppm cadmium, the SOD activity values were found to be 5.53±0.35, 4.77±0.37 and 4.22±0.35 U/mg protein, respectively.

Under stress conditions, microorganisms can develop self-protection mechanisms such as accumulation of suitable substances and increase of antioxidant enzymes. SOD is an antioxidant enzyme that functions by converting a highly toxic superoxide radical to oxygen and less toxic hydrogen peroxide (Franklin *et al*. 2013). Lenártová *et al*. (1998) explained the removal of toxic oxygen species by an increase in SOD activity.

CAT is an important antioxidant enzyme that contributes to the antioxidant enzymes system through the detoxification of H_2O_2 to oxygen and water. After adding 0, 75, 100 and 125 ppm cadmium to the LB medium, the CAT activity of the microorganism was found to be 7.34±0.51, 12.36±0.90, 17.77±1.05 and 27.82±1.28 U/mg protein, respectively (Figure 5). It was observed that CAT activity in the presence of 75, 100 and 125 ppm cadmium was increased by 68, 142 and 279 percent, respectively, compared to the control ($p<0.05$).

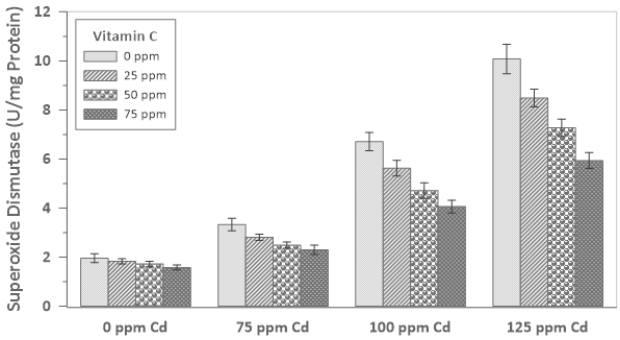

Figure 4. Combined effect of cadmium and vitamin C on the SOD activity in *C. freundii*.

In the study conducted by Banerjee *et al*. (2015) with *Enterobacter cloacae*, it was reported that cadmium caused an increase in CAT and SOD activities. When 25, 50 and 75 ppm vitamin C was added to the medium containing 125 ppm cadmium, the CAT activity was found as 21.59±1.24, 17.29±0.97 and 14.32±0.92 U/mg protein, respectively.

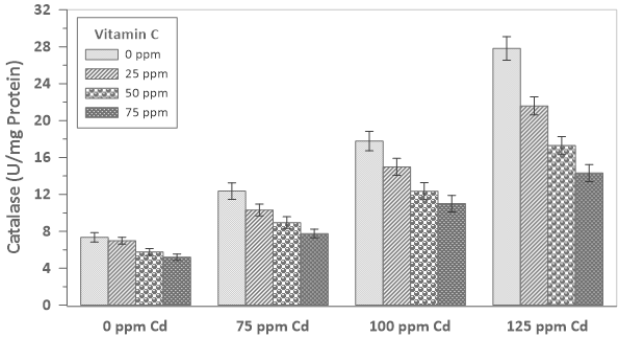

Figure 5. Combined effect of cadmium and vitamin C on the CAT activity in *C. freundii.*

Peroxidase (POD) is an oxidoreductase that catalyses the reaction between compounds that tend to donate hydrogen atoms and the H_2O_2 compound that has these atoms in the acceptor state (Vlasova 2018). While the POD activity in the control was 1.67±0.12 U/mg protein, the POD activities of the microorganisms in the medium containing 75, 100 and 125 ppm cadmium were found to be 4.52±0.34, 7.65±0.47 and 11.59±0.78 U/mg protein, respectively (Figure 6).

Results showed that the increase in POD activity of bacteria grown in LB media containing 75, 100 and 125 ppm cadmium compared to the control was 2.74, 4.58 and 6.94 times, respectively ($p<0.05$).

In a study by Hussein and Joo (2013) with two different bacterial species, *Basillus subtilis* and *Pseudomonas putida*, it was reported that heavy metals cause a significant increase in POD activity.

POD activity values of microorganisms produced by adding 25, 50 and 75 ppm vitamin C to the medium containing 125 ppm cadmium were found as 9.19±0.55, 7.63±0.50 and 6.42±0.45 U/mg protein, respectively. Depending on the increased of cadmium concentration, an increase was observed in the activity of antioxidant enzymes that protect the cell against oxidative stress. Our findings are consistent with the results of the study by Pandey *et al*. (2013). In addition, vitamin C, known for its antioxidant properties, was added to the cadmium-containing nutrient LB medium, a decrease in antioxidant enzyme activity was observed depend on the vitamin C concentration.

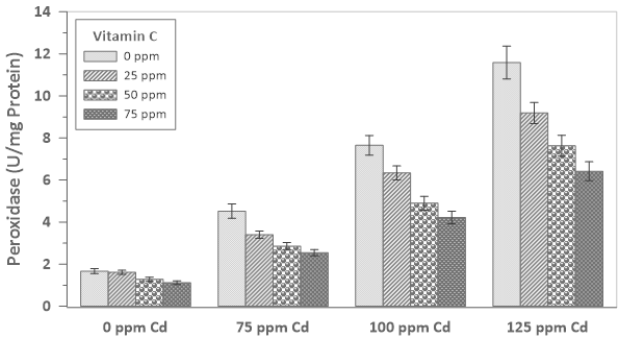

Figure 6. Combined effect of cadmium and vitamin C on the POD activity in *C. freundii.*

Cadmium causes oxidative stress by increasing the production of reactive oxygen species (ROS) in metabolism. Oxidative stress, on the other hand, causes changes in the activities of antioxidant enzymes and an increase in lipid peroxidation (Kumar *et al*. 2019).

Oxidative stress was created in the microorganism by adding cadmium at different concentrations to the medium. The effects of both cadmium and cadmium + vitamin C on stress markers (GSH, GSSG, MDA and 4-HNE) were investigated by adding different concentrations of vitamin C to the cadmium-containing medium.

Glutathione is a tripeptide antioxidant that prevents damage to cell components by free radicals,

peroxides, lipid peroxides and heavy metals (Smirnova and Oktyabrsky 2005). The reduced (GSH) and oxidized (GSSG) forms of glutathione amounts are important indicators of cell and organism health, with cellular redox status. The GSH and GSSG are in equilibrium in the cell, and the disruption of this balance against GSH causes negative effects in the cell, and the GSH/GSSG ratio is also known as a stress marker (Cnubben *et al*. 2001).

Compared to the control, the percentage decrease in the amount of GSH of the microorganism in the nutrient medium containing 75, 100 and 125 ppm cadmium was found as 67, 72 and 76, while the increase in the amount of GSSG was found to be 190, 214 and 303. As seen in Figures 7 and 8, as the cadmium concentration increased, the amount of GSH decreased while the amount of GSSG increased ($p<0.05$). In addition, depending on the concentration of vitamin C added to the cadmium-containing growth medium, it increased the amount of GSH and decreased the amount of GSSG. In cases where oxidative stress is low, the level of GSH increases as a result of adaptation mechanisms. But; In cases where oxidative stress is high, GSH level decreases due to weakened adaptation mechanisms and increased GSSG formation (Zhang *et al*. 2005). As a result of the increase in ROS production due to the increased concentration of cadmium added to the nutrient medium, the GSH/GSSG ratio decreased, while the GSH/GSSG ratio increased as a result of the addition of vitamin C, which has antioxidant properties (Figure 9).

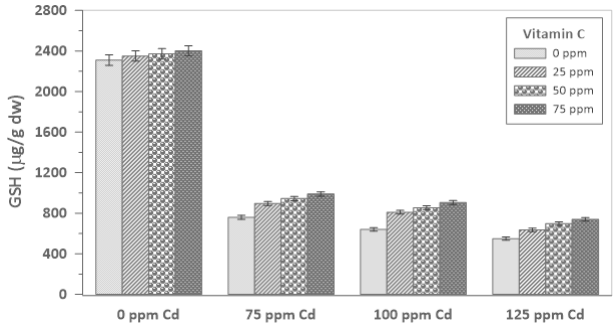

Figure 7. Combined effect of cadmium and vitamin C on the level of GSH in *C. freundii.*

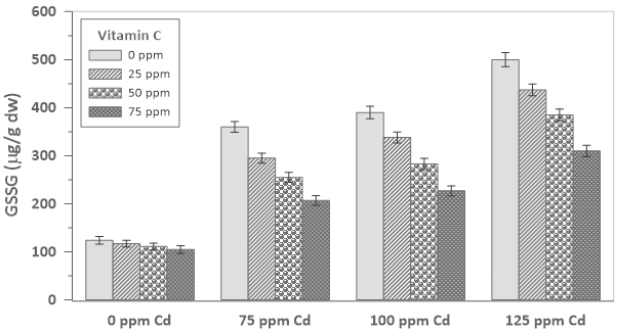

Figure 8. Combined effect of cadmium and vitamin C on the level of GSSG in *C. freundii.*

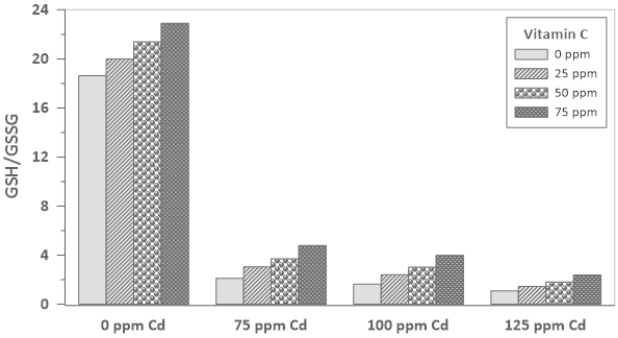

Figure 9. Combined effect of cadmium and vitamin C on the GSH/GSSG ratio in *C. freundii*

Free radicals cause lipid peroxidation by affecting unsaturated fatty acids in cell membranes. Lipid peroxides decompose rapidly to form reactive carbon compounds. Among these compounds, MDA and 4-HNE are an indicator of lipid peroxidation and are widely used important reactive carbon compounds (Gaweł *et al*. 2004).

The amounts of MDA and 4-HNE in the control were 2.9±0.12 and 2.44±0.06 µg/g dw, respectively, when 100 ppm cadmium was added to the LB medium, values of these parameters were found to be 14.5±1.0 and 10.2±0.44 µg/g dw, respectively. In addition, when 75 ppm vitamin C was added to the medium containing 100 ppm cadmium, these parameters were determined to be 8.10±0.56 and 7.5±0.25 µg/g dw, respectively (Figure 10 and 11). The results obtained for GSH, GSSG, GSH/GSSG and MDA are consistent with the results of the study by Kireçci (2017).

25, 50 and 75 ppm Vitamin C added to the cadmium-containing growth medium changed the activities of antioxidant enzymes (GSH-Px, GSH-Rd, SOD, CAT,

POD) and the amounts of stress parameters (MDA, 4-HNE, GSSG) ($p< 0.05$).

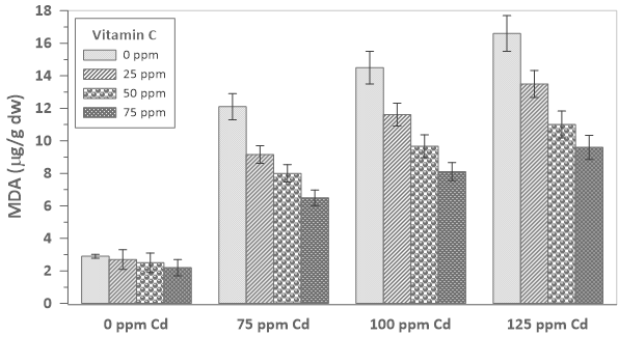

Figure 10. Combined effect of cadmium and vitamin C on the level of MDA in *C. freundii.*

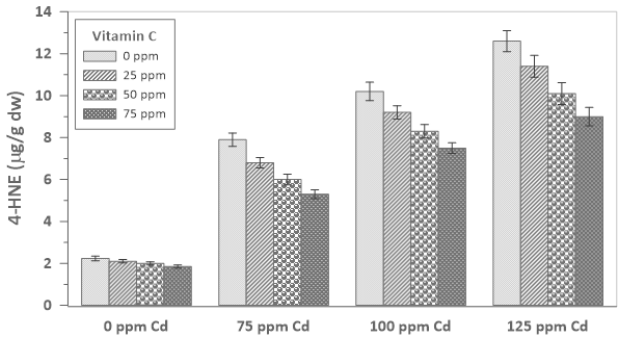

Figure 11. Combined effect of cadmium and vitamin C on the level of 4-HNE in *C. freundii.*

4. Conclusions

Antioxidants are compounds that neutralize reactive oxygen species that are biologically toxic. It is seen that antioxidant enzymes play an important role in the fight against oxidative stress and protect the cell. As a result, it is seen that cadmium added to the medium increases the toxic effect and ROS formation, reducing the protein and GSH amount of the microorganism, and increasing the amount of antioxidant enzyme activities and stress biomarkers.

It can be said from these results that vitamin C added as an antioxidant to the cadmium-containing nutrient medium reduces the negative effects of cadmium on bacteria, increasing the amount of protein and GSH, while decreasing the amount of antioxidant enzyme activities and stress biomarkers**.** In addition, antioxidant enzymes can also be used for biological monitoring of heavy metal pollutions.

5. Conflicts of Interest

The authors declare no conflict of interest.

6. References

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