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Assessment of Combined Effects of Selenium and Cadmium on Antioxidant Activity of Enzymes Produced by *Citrobacter freundii*

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Abstract

In this study, Citrobacter freundii (NRRL B-2643) bacteria were cultured in an LB medium with different cadmium (Cd) concentrations. To mitigate the deleterious impact of Cd, varying quantities of selenium (Se), renowned for its antioxidative power, were added to the cadmium-containing growth medium. Bacterial concentration, soluble protein, and activities of antioxidant enzymes (Glutathione peroxidase (GSH-Px), Glutathione reductase (GSH-Red), Superoxide dismutase (SOD), and Catalase (CAT) were determined by spectrophotometer. No significant microorganism growth was observed at 150 ppm and higher Cd concentrations. However, the bacterial growth was not affected up to 40 ppm Cd concentration. Bacteria were grown in media containing 0, 75, 100, and 125 ppm Cd, where the 0-ppm cadmium group served as control. The protein content of the microorganism grown in the medium containing 75, 100, and 125 ppm Cd decreased about 21, 40, and 62 percent, respectively, compared to the control. When 3.0 ppm selenium was added to the same growth medium, the percentage decrease in protein amount compared to the control was 12, 25, and 50, respectively. Compared to the control, an increase in the antioxidant enzyme activities in bacteria grown in cadmium-containing media was observed (p<0.05). With the addition of 1.0 and 3.0 ppm selenium to cadmium-containing media, a decrease was observed in the activities of antioxidant enzymes.

Keywords: Citrobacter freundii, selenium, cadmium, antioxidant enzymes, protein

INTRODUCTION

Bacteria are small single-celled microorganisms that are found nearly everywhere on Earth. They thrive in various environments, including the stomach, water, and soil. Certain species can withstand extremely high and low temperatures and pressures (Flemming &Wuertz 2019). *Citrobacter freundii* (*C. freundii*)is a facultative anaerobic gram-negative bacterium that belongs to the *Enterobacteriaceae* family. Not only does *C. freundii*live in soil, but it is also present in food, water, sewage, and the digestive tracts of both humans and animals (Wang *et al.* 2000). Although *C. freundii*is a bacterial pathogen, it also contributes significantly to the nitrogen cycle in the environment which converts nitrate to nitrite (Thompson *et al.* 2006).*C. freundii*was previously reported to have the ability to bioaccumulate metals such as copper (Sharma & Fulekar 2009).

Heavy metals are naturally occurring substances harmful to living organisms, even at low levels. Heavy metals like cadmium (Cd) have significant environmental and functional implications (Abd Elnabi *et al* 2023). It has been shown that heavy metals affect some metabolic enzymes, cell organelles, and organs in biological systems. Cd is one of the most dangerous heavy metals to living organisms, mainly due to its higher toxicity and severe extent of bioaccumulation. Cd presents a unique concern due to its notable mobility in soil environments. Unlike some heavy metals, Cd exhibits a relatively high degree of mobility within soils, facilitated by factors such as soil pH, organic matter content, and redox potential. This mobility renders Cd more hazardous even at relatively low soil concentrations, as it can readily leach into groundwater and accumulate in crops, posing environmental and human health risks (Zulfiqar *et al.*, 2023). In vitro tests with Cd reveal cytotoxic effects and DNA damage from free

radicals (Sall *et al.*, 2020). Arsenic and Cd are causing significant public health issues due to their high propensity for toxicity, carcinogenic tendency, and association with environmental contamination (Charkiewicz *et al.*, 2023). Due to the growing emissions of pollutants from industry and human activity, protection against the buildup and hazardous effects of heavy metals on humans, animals, and plants is a topic of contemporary attention (Feng *et al.*, 2013).

Substances such as ascorbic acid and selenium (Se), well known for their antioxidant qualities, have been used to reduce the impact of cadmium toxicity in bacteria (Ibrahim *et al.* 2021; Ibrahim *et al.* 2022). Se is identified mainly by its antioxidant function exerted via enzymes like glutathione peroxidase and thioredoxin reductase (Méplan & Hughes 2020). Se alleviated Cd and Pb toxicity by preventing oxidative stress in *Brassica napus L* (Wu *et al.* 2016). Se has been shown to play a helpful effect in reducing oxidative stress caused by heavy metals (Han *et al.* 2015). Numerous biological systems have demonstrated Se's critical function in antioxidant defense against heavy metal stress (Kumar et al., 2012; Lin et al., 2012; Malik et al., 2012). When taken in small amounts and integrated into proteins and enzymes, Se possesses antioxidant qualities that shield both people and animals from a variety of illnesses. Another well-known obstacle to its usage as a dietary supplement is its extraordinarily narrow range between dangerous and required amounts (Ramirez *et al.*, 2022). In research using cell culture and animal models, selenium, in any form, can lessen Cd-mediated toxicity in the liver, kidney, spleen, brain, or heart (Zwolak, 2020). Even though Se has been found to confer antioxidative function, exposure to levels higher than normal might also be toxic to the environment (Okonji *et al.*, 2021). Recently, the use of microorganisms-based bioremediation of heavy metals clean-up in freeing the environment of toxic heavy metals such as Cd happened to be a research focus and has gained more interest from researchers worldwide (Raklami *et al.*, 2022).

Oxidative stress causes by cadmium results in changes in the amount of total protein, elevated lipid peroxidation, and modifications to the antioxidant defense mechanism in the biological system (Ibrahim *et al.*, 2022). The antioxidant enzyme system is the most important defense against cell damage caused by oxidative stress. The defense system includes antioxidant enzymes such as Glutathione peroxidase (GSH-Px), Glutathione reductase (GSH-Red), Superoxide dismutase (SOD), Catalase (CAT), and non-enzymatic substances like glutathione (Shim & Kim 2013). Through the use of the antioxidant enzyme system, some species of bacteria, such as *Bacillussiamensis L.*, were found to reduce Cd toxicity in plants by reducing malondialdehyde (MDA) content and increasing CAT and SOD activities (Awan *et al.*, 2020). The increasing amount of Cd released into the environment necessitates the development of suitable mitigation strategies to lower soil Cd pollution. Microorganisms offer a sustainable and efficient way to clean up Cd-contaminated soil. Recent research points to microbial remediation as a dependable solution that can be used to remediate soil contaminated with Cd (Bing *et al.*, 2023).

The main aim of this work is to evaluate the effect of Cd combined with Se on the protein and antioxidant enzyme activity in *C. freundii*. Due to its capacity to proliferate quickly and easily and its similarities to other organisms, *C. freundii*(NRRL B-2643) was selected. To ascertain the impact of Cd on the activity of antioxidant enzymes and soluble protein, bacterial growth was conducted by adding 75, 100, and 125 ppm Cd to the LB medium. To mitigate the deleterious impact of Cd, 1.0 and 3.0 ppm Se, renowned for its antioxidative power, were added to the Cd-containing growth medium. The 75, 100, and 125 ppm Cd concentrations were selected because bacterial growth was unaffected by Cd up to 40 ppm and did not grow above 150 ppm. Additionally, a higher amount of Se is toxic. For that reason, 1.0 and 3.0 ppm Se concentrations were chosen.

MATERIALS AND METHODS

*C. freundii*was grown in an LB medium containing 2.5 g yeast extract, 2.5 g peptone, and 1.25 g NaCl per 250 mL. A stock solution of 1000 ppm of Cd was made from cadmium chloride ($CdCl_2$). A Selenium stock solution of 100 ppm was also prepared from selenium chloride ($SeCl_4$).

Experimental design

The following groups were created in the study;

1. Control: *C. freundii*was grown in a sterile LB medium with 0 ppm cadmium and 0 ppm selenium.

2. Cadmium group: The bacteria were grown by adding 75, 100, and 125 ppm cadmium concentration in the growth medium.

3. Selenium group: The bacteria were grown by adding 1.0 and 3.0 ppm selenium concentration to the 75, 100, and 125 ppm cadmium groups.

After injection, incubation was done in a shaking incubator (Selecta Rotabit) at 150 rpm for 18 hours at 37 °C. The concentration of bacteria was ascertained after the incubation time by measuring the absorbance at 600 nm using a spectrophotometer (UV -6300PC double beam model). The culture media was centrifuged at 10 °C for 10 minutes at 8000 rpm. After two rounds of double-distilled water washing, the bacterial precipitate was centrifuged again. The samples were sonicated ten times in the buffer employed in the procedures, for thirty seconds each time, to ascertain the samples' total protein and enzyme activity. The same centrifuge precipitated the cell debris, and the supernatant was used for analysis (Ibrahim *et al.*, 2022).

Total Protein Analysis

Total soluble protein was determined according to the modified Lowry method described by (Lu, 2020).

Determination of Enzymes Activity

kits from Solarbio Life Science were utilized for SOD (BC0170), CAT (BC0200), GR (BC1170), and GP (BC1190). 150 µL of each sample and 1 mL of the extraction reagent were combined and properly mixed in an ice bath in accordance with the manufacturer's instructions. The supernatant was obtained by centrifuging the mixture for 10 minutes at 4 °C and 8000 rpm. After precisely 90 µL of the mixture was collected using a glass cuvette, it was carefully combined with 180 µL of reagent III, 240 µL of reagent I, 6 µL of reagent II, and 30 µL of reagent V. The mixture was then mixed with 180 µL of distilled water and let to stand at 4°C for half an hour. The mixtures of each sample were tested for the activity of SOD, CAT, GR (GSH-Red), and GP (GSH-Px) using Spectrophotometer, as also used by Nafiu *et al* (2022).

Statistical Analysis

All measurements were taken in triplicate, and the mean ± standard deviation was computed. SPSS 10.0 for Windows was used to analyze variance (ANOVA) on the findings. P-values < 0.05 were used to signify statistical significance.

RESULTS

In this study, the amount of total soluble protein was found to be 26.12 ± 2.11 mg/g (control), and after the addition of 75, 100, and 125 ppm Cd to the LB medium, the concentrations were found to be 20.77 ± 1.14, 16.73 ± 1.05 and 9.97 ± 0.83 mg/g respectively (Figure 1), which shows significant decrease when compared with the control ($p<0.05$). Se was reported to have a protective role against Cd toxicity in bacteria (Araúz *et al.*, 2008). The amount of soluble protein after the addition of 1.0 ppm Se to the 75, 100, and 125 ppm Cd group was found to be 21.89 ± 1.16, 18.75 ± 1.11, and 11.34 ± 0.63 mg/g, respectively, indicating a significant increase in the amount of soluble protein in 100 and 125 ppm Cd groups ($p<0.05$) when compared to the same groups in the absence of Se. After adding 3.0 ppm Se to the 75, 100, and 125 ppm Cd group, the amount of soluble protein was found to be 23.04 ± 1.23, 19.66 ± 1.62, and 13.24 ± 0.76 mg/g respectively. This result shows a significant increase ($p<0.05$) in the concentration of soluble protein when compared to the same groups without Se addition (figure 1), showing the effect of Se in ameliorating Cd toxicity as previously reported by several researchers (Zwolak., 2020). According to our findings, compared to the control, the protein content of the microorganism cultivated in the media containing 75, 100, and 125 ppm Cd dropped by around 21, 40, and 62 percent, respectively. The same growth medium treated with 3.0 ppm Se showed a decrease in protein content of 12, 25, and 50% compared to the control.

In this study, the activity of CAT was seen to be 189.53 ± 9.72 U/mg protein (control), which significantly increased to 235.34 ± 11.23, 251.91 ± 12.06, and 266.33 ± 12.11 U/mg protein ($p<0.05$) after addition of 75, 100 and 125 ppm Cd to the LB medium respectively (Figure 2). However, addition of 1.0 and 3.0 ppm Se to the 75, 100 and 125 ppm Cd LB medium significantly lower the activity of CAT to 217.92 ± 12.11, 230.56 ± 9.88, 243.25 ± 11.66 and 195.65 ± 10.72, 214.19 ± 10.77, 209.12 ± 10.94 respectively when compared to the same group without Se ($p<0.05$) (Figure 2). Our work aligns with the work of Ibrahim *et al*. (2022), who also reported a significant increase in CAT activity after Cd addition and a significant decrease in CAT activity after adding an antioxidant (vitamin C) in the bacterial growth medium.

In this study, the activity of SOD was observed to be 87.43 ± 5.11 U/mg protein (control) which significantly increased to 125.72 ± 6.23, 140.17 ± 11.74, and 154.47 ± 13.77 U/mg protein ($p<0.05$) after addition of 75, 100 and 125 ppm Cd to the LB medium respectively (Figure 3).

However, the addition of 1.0 and 3.0 ppm Se to the 75, 100, and 125 ppm Cd LB medium significantly decreased the activity of SOD to 114.65 ± 5.31, 131.78 ± 6.77, 143.76 ± 12.88, and 101.84 ± 5.17, 124.43 ± 4.98, 133.41 ± 11.97 respectively when compared to the same group without Se ($p<0.05$) (Figure 3).

The present work shows the activity of GSH-Px to be 31.52 ± 1.42 U/mg protein (control), which significantly increased to 46.12 ± 3.59, 79.34 ± 3.74, and 113.58 ± 4.48 U/mg protein ($p<0.05$) after addition of 75, 100 and 125 ppm Cd to the LB medium respectively (Figure 4), when 1.0 and 3.0 ppm Se was added to the 75, 100 and 125 ppm Cd LB medium a significant decrease in the activity of GSH-Px to 39.96 ± 2.21, 70.75 ± 2.74, 101.44 ± 4.23 and 32.94 ± 1.01, 61.43 ± 2.13, 88.27 ± 3.98 respectively when compared to the same group without Se was observed ($p<0.05$) (Figure 4).

The present study shows the activity of GSH-Red to be 16.86 ± 1.87 U/mg protein (control) which significantly increased to 24.59 ± 1.11, 40.17 ± 3.94 and 59.16 ± 2.12 U/mg protein ($p<0.05$) after inclusion of 75, 100 and 125 ppm Cd to the LB medium respectively (Figure 5).

The addition of 1.0 and 3.0 ppm Se to the 75, 100, and 125 ppm Cd LB medium significantly decreased the activity of GSH-Red to 20.16 ± 1.72, 32.23 ± 1.24, 53.12 ± 2.71 and 17.91 ± 1.04, 28.65 ± 1.33, 47.72 ± 1.98 when compared to the same group without Se ($p<0.05$) (Figure 5).

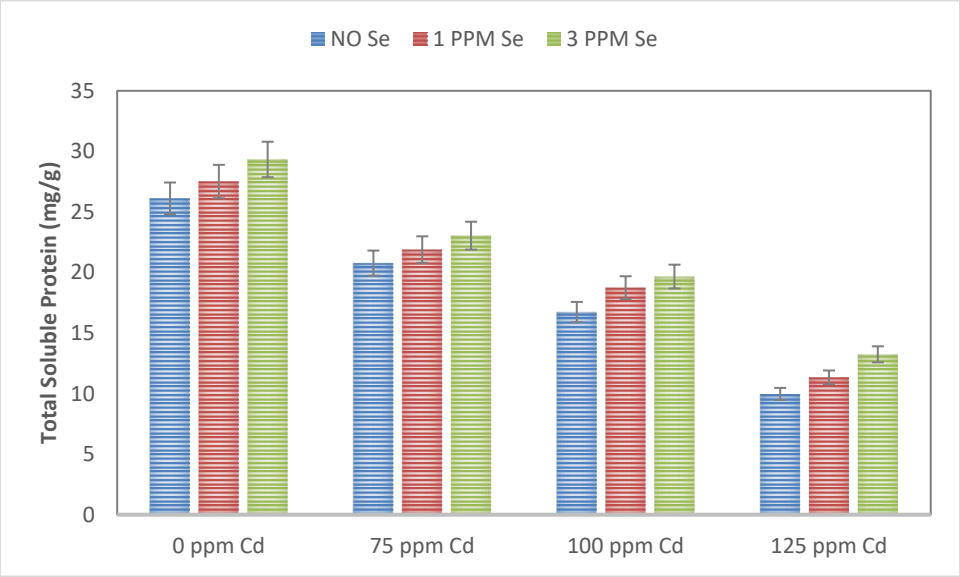

Figure 1: Combine Effect of Selenium and Cadmium on Total Soluble Protein in *C. freundii*.

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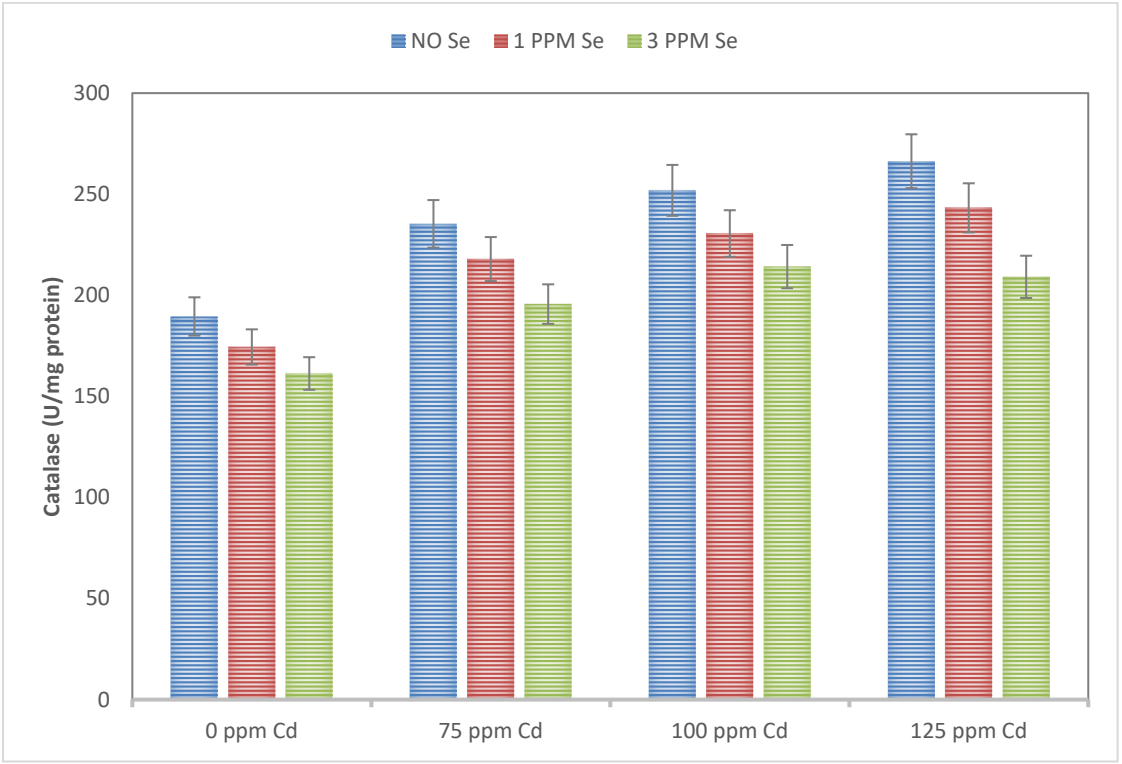

Figure 2: Combine Effect of Selenium and Cadmium on CAT Activity in *C. freundii*.

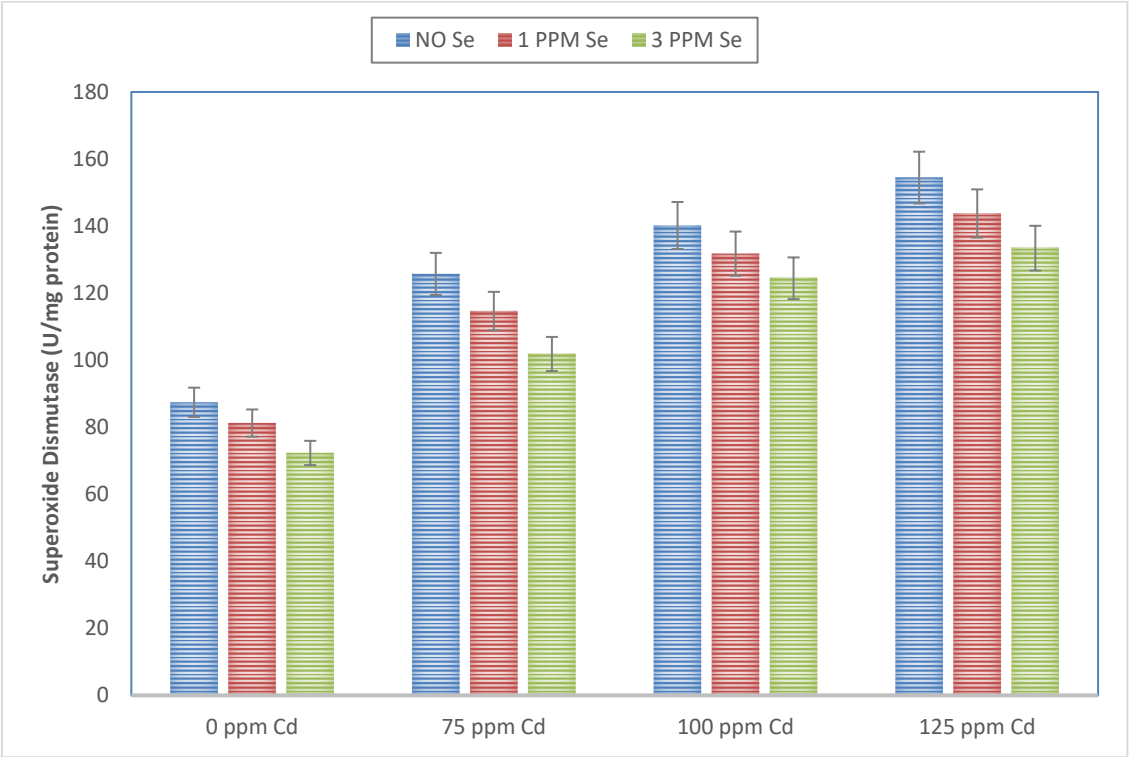

Figure 3. Combine effect of selenium and cadmium on SOD activity in *C. freundii*.

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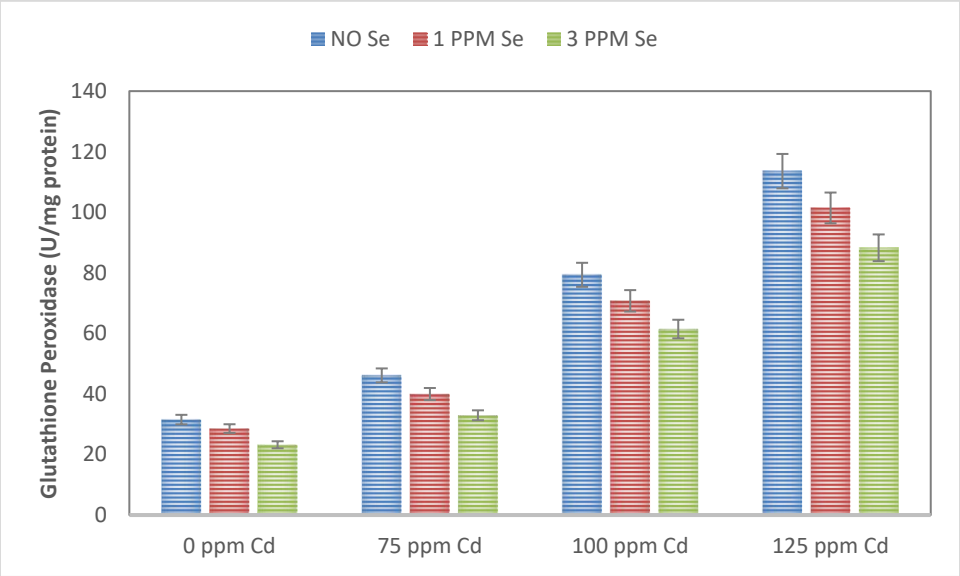

Figure 4. Combine effect of selenium and cadmium on GSH-Px activity in *C. freundii*.

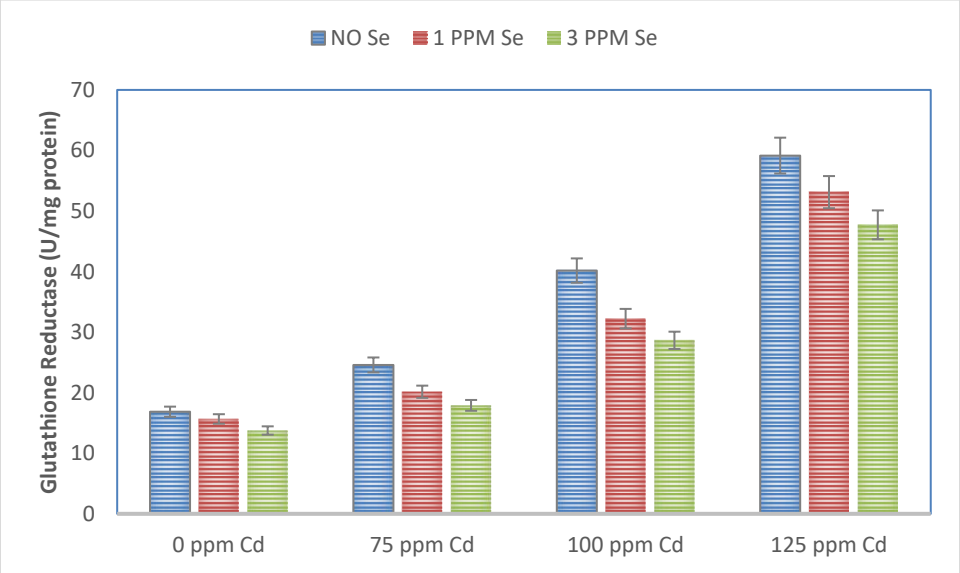

Figure 5. Combine effect of selenium and cadmium on GSH-Red activity in *C. freundii*.

DISCUSSION

The physiology, survival, and performance of organisms can be significantly impacted by heavy metals such as Cd, which are expressed through protein inhibition (Güner, 2010). Protein enables bacteria to respond to environmental changes, heavy metals stress, and nutrient availability (Njenga*et al.*, 2023). The present work shows a significant decrease in total soluble protein in *C. freundii* in the presence of cadmium (figure 1). However, the addition of 1.0 and 3.0 ppm selenium in the presence of Cd revealed a significant increase in the amount of soluble protein, showing the effect of Se in ameliorating Cd toxicity (figure 1). This work is in line with previous findings that indicate the antagonistic effect of Se against cadmium toxicity (Luo *et al.*, 2019). Previously, Ling *et al.* (2019) extensively studied in vivo and in vitro Cd-Se interactions in *Caenorhabditis elegans, revealing the* Se antagonistic effect of Cd toxicity via Cd detoxification stimulation.

Several studies have indicated the importance of antioxidant enzyme activity during oxidative stress (Birben *et al.*, 2012). Heavy metals such

as cadmium, lead, and mercury were reported to cause an increase in the activities of antioxidant enzymes (Mnkandla *et al* 2019). Antioxidants are substances that counteract ROS(reactive oxygen species) that harm living things. Research has demonstrated the critical function antioxidant enzymes play in defending cells from oxidative damage. Antioxidant enzymes like CAT, SOD, GSH-Px, and GSH-Red exert a key function in the antioxidant enzyme system, and changes in their activity due to increased ROS production gives the cell the ability to withstand the stress, thereby consequently leading to the neutralization of the ROS to nontoxic molecules (Adwas *et al.*, 2019; Kiran *et al* 2023). CAT is another important antioxidant enzyme that significantly reduces oxidative stress, which breaks down cellular hydrogen peroxide to produce oxygen and water (Nandi *et al.*, 2019). The activity of CAT was found to significantly increase in this study in the presence of Cd (figure 2). Our findings align with the work of Ibrahim *et al.* (2022), which also indicates an increase in CAT activity in *C. freundii* in the presence of Cd. Another earlier study also shows increased CAT activity in bacteria as a defense mechanism to counteract oxidative stress caused by Cd toxicity (Hadwan *et al.* 2024). However, the addition of Se (1.0 and 3.0 ppm) in the same growth medium shows a significant decrease in CAT activity, indicating the antioxidative effect of Se in combating Cd toxicity in the bacteria. Also, in line with our study, Akonaç and Boysan (2023) reported an increase in CAT activity in *Lupinus albus* L. after the addition Cd and a decrease in CAT activity after adding a low level of Se. They show the effect of Se in alleviating the toxic effect of Cd.

SOD is an enzyme that plays a pivotal role in the antioxidant system, which helps in breaking down oxygen molecules that are potentially toxic to cells. It catalyzes the conversion of superoxide into oxygen and hydrogen peroxide (Ying *et al.* 2018). In our study, SOD activity was seen to increase after Cd addition in *C. freundii* growth medium (figure 3). Our findings are consistent with earlier work that also revealed an increase in SOD activity in *Enterobacter cloacae* in the presence of Cd (Banerjee *et al.* 2015). Another previous work also showed a significant increase in SOD activity in the presence of Cd in *C. freundii* (Ibrahim *et al.*, 2022). Addition of 1.0 and 3.0 ppmSe in the same groups showed a significant decrease in SOD activity which further shows a relief in Cd toxicity by Se. Our work is also in line with the findings of Dzobo and Naik (2013), where they revealed a significant increase in SOD activity in rat liver in the presence of Cd and a significant decrease after Se addition. Akonaç and Boysan(2023) reported an increase in SOD activity in *Lupinus albus* L. in the presence of Cd and a decrease in SOD activity after the low-level addition of Se, which shows the ability of Se to alleviate the toxic effect of Cd.

GSH-Px is an antioxidant enzyme whose biochemical purpose is to convert lipid hydroperoxides to their alcohols and free H_2O_2 to water. In this study, the activity of GSH-Px was seen to significantly increase in the presence of Cd (figure 4), which is in line with previous studies also indicating an increase in GSH-Px activity in the presence of Cd (Pandey *et al.* 2013; Ibrahim *et al.* 2022). However, the addition of 1.0 and 3.0 ppm Se shows a significant decrease in the activity of GSH-Px (figure 4), indicating its antagonistic effect on Cd toxicity in the bacteria. GSH-Px is a selenocysteine (selenium-containing) compound class since it binds four Se atoms and gives glutathione peroxidase catalytic action. The GSH-Px co-substrate is glutathione, a popular antioxidant against ROS and free radicals for its various physiological roles in detoxifying xenobiotic compounds **(**Zoidis *et al.*, 2018**).** Ibrahim *et al.* (2021) reported a decrease in the concentration of reduced glutathione (GSH) and an increase in oxidized glutathione (GSSG) in the presence of Cd in the *C.freundii* culture medium, and when Se was added to the growth medium, an increase in GSH concentration together with a decrease in GSSG was observed. This finding indicates the bacterial ability to withstand the Cd toxicity by using GSH through the GSH-Px system and also explains the power of Se in ameliorating the heavy metal toxicity. These findings can also be correlated to our results in explaining the combined effect of Cd and Se in GSH-Px activity.

GSSG has to be transformed back to a reduced form of GSH to sustain free radical detoxification in the cell. When NADPH is present, GSSG is changed back to GSH in a reaction catalyzed by GSH-Red **(**Couto *et al.*, 2016**)**. The present study shows the GSH-Red activity significantly increased after Cd was added to the LB medium (figure 5). This work is also consistent with earlier findings that indicate increased GSH-Red activity in the presence of Cd (Cheng *et al.* 2016; Ibrahim *et al.* 2022). Addition of 1.0 and 3.0 ppm Se to the Cd-containing LB medium significantly decreased the activity of GSH-Red (figure 5). Se was also previously reported to antagonize Cd by

increasing the glutathione levels to minimize oxidative stress, alleviating Cd-induced damage (Ge *et al.*, 2021). Recent work also shows the ability of Se to reduce the harmful effects of Cd (Ozoani *et al.*, 2024). Another recent work also shows a protective role of Se against cadmium-induced nephrotoxicity (Jing *et al.*, 2021). Furthermore, it has been proven that Se upregulates the expression of Nrf2 (nuclear factor erythrocyte 2-related factor 2) dependent antioxidant enzymes, which enhances its protection against specific pathological changes (Schwarz et al., 2019).

CONCLUSION

This study investigated the combined effects of Se and Cd on the antioxidant activity of enzymes produced by *C. freundii*. The results showed that Se addition decreased antioxidant enzyme activity, while Cd exposure increased it. However, the presence of Se mitigated the negative effects of Cd on antioxidant activity. These findings suggest that Se plays a protective role against Cd-induced oxidative stress in *C. freundii*, highlighting the potential for Se supplementation to enhance bacterial antioxidant defenses in environments contaminated with Cd. This study might also suggest the importance of Se by increasing the bacterial tolerance to Cd, allowing it to survive and thrive in Cd contaminated environment. This research contributes to understanding the complex interactions between trace elements and their impact on microbial antioxidant systems, with implications for environmental and public health applications. As seen in the present work, the increase in antioxidant enzyme activity in the presence of Cd can serve as a biomarker for Cd exposure and toxicity in bacteria. The present work also indicates the ability of *C. freundii* to withstand Cd toxicity, which provides a tool for further research for its possibility as a potential bacterium for Cd bioaccumulation.

RECOMMENDATION

Further studies are recommended to investigate the molecular mechanisms by which selenium protects against cadmium-induced oxidative stress in *C. freundii*. Analyzing the expression of genes involved in antioxidant defenses to understand how selenium and cadmium affect gene regulation in *C. freundii* and performing proteomics analysis to identify changes in protein expression and modification in response to selenium and cadmium exposure is also recommended.

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