Use of ultraviolet-light mutagenesis to generate a mutant with elevated cadmium resistance, *B. agri* C15 CdR

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Abstract. Cadmium (Cd) is toxic to living organisms; however, bacteria are adaptable to severe conditions, including Cd contamination. Bacteria can develop in the natural environment, as they generate resistant strains that can be used to remove Cd, but getting such adaptive strains usually takes a long time. In this study, ultraviolet (UV) light mutagenesis was used to accelerate the strain-resistant ability of *Brevibacillus agri* C15 to generate CdR mutant with high resistance to Cd. Its maximum tolerable concentration (MTC) to Cd was 15.00 mM. The UV light mutagenesis process resulted in the generation of the mutant *B. agri* C15 CdR (MTC: 20.00 mM Cd). Morphological and biochemical tests showed that there were no major differences between *B. agri* C15 CdR and *B. agri* C15. This study provides a basis for developing microbial Cd resistance and facilitating the application of Cd remediation.

Keywords. Cadmium, *Brevibacillus*, bioremediation, ultraviolet radiation, mutant, maximum tolerable concentration.

1. Introduction

Bioremediation is the process of transfer, forming contaminants into less harmful substances and, depending on the type of pollutant, and different approaches can be employed. One approach is the development of bacterial resistance to the pollutant with the aim of enhancing remediation efficiency. At this moment, engineering strategies are being investigated to increase further the Cd resistance of microbes, such as cloning the gene for metallothioneins, over-expressing metal-binding proteins, such as poly-histidine [1] or poly-cysteines [2]. Chemical mutagenesis approaches have been widely applied to develop bacterial detoxification systems for Cd, such as *Lactobacillus plantarum* ATCC 8014 and *Enterobacter cloacae* TU [3]. An alternative approach to increase bacterial resistant ability is the use of ultraviolet (UV) light mutagenesis. The advantages of using UV light are its simplicity, safety, and cost-effectiveness; there is also no need to establish a genetic system for the target organism, which can be useful when using it to create a mutant from a newly isolated strain. Ultraviolet (UVC) causes direct DNA damage due to the induction of the formation of DNA lesions.
(photoproducts), most notably pyrimidine dimers, which block DNA replication, and RNA transcription [4]. Cd-resistant bacteria are often used as one of the remediation technologies for Cd. The main aims of this study were to generate a mutant with elevated resistance to Cd using UV-light mutagenesis. The objectives of this study were to induce mutations in wild type B. agri C15, a mutant screen, and to determine the effects on their specific growth rate and maximum amount of biomass formed.

2. Materials and Methods

2.1. Strain

*Brevibacillus agri* C15 was obtained from our previous study [4] (Jebril et al., 2020).

2.2. Mutagenesis of *B. agri* C15 and screening of mutant Cd

The *B. agri* C15 was mutagenized using UV light, according to [5] with some modifications. To obtain large quantities of *B. agri* C15, which were exposed to Cd and needed for the mutagenesis assay, the specific growth rate was first determined in 500 mL. Three thoroughly mixed 50 mL batch cultures from the late exponential phase were inoculated into 500 mL of EBS/pyruvate in Erlenmeyer flasks (2000 mL), in triplicate and with a nominal concentration of 10 mM Cd. This concentration of Cd was chosen as the possible concentration for achieving high biomass of the cells under Cd stress. The cultures were grown at 37 °C in a rotary shaking incubator at 100 rpm, and the amount of biomass was estimated as described in our previous study [4]. The effect of Cd (10 mM) on the growth of *B. agri* C15 was evaluated for the comparison of the specific growth rate values. These experiments were carried out with control in 50 mL and 500 mL of EBS without Cd. After determining the specific growth rate of *B. agri* C15 in 500 mL of batch cultures supplemented with 10 mM Cd, 50 mL culture of *B. agri* C15 was grown in a new fresh 500 mL of EBS /pyruvate in an Erlenmeyer flask (2000 mL) in triplicate, with a nominal concentration of 10 mM Cd, and incubated shaking at 100 rpm, 37 °C. The amount of biomass was determined, and from the late exponential phase, the cells were cooled in ice for an hour, centrifuged, and washed with and suspended in 0.5 EBS (two folds of diluted EBS with sterilized ddH2O, without pyruvate). The cell suspension was snap-frozen in liquid nitrogen. The counting of colony-forming units (CFUs) was used to estimate the survival of frozen cells using the serially diluting bacterial suspensions up to 1X106 CFU/mL. Each dilution was then spread on the nutrient agar plates in triplicate and incubated at 37 °C for 16 h. A CFU was determined from three sequential dilutions, and the mean was taken from all three. During the actual experiment, UV exposure was optimized by determining a survival time of *B. agri* C15 under UV exposure, a 90% drop of CFU required to increase the chance of mutant isolation. Five mL of cell suspensions of 500-1000 CFU/mL was transferred into empty plates in triplicate and exposed to a UV light lamp of 340 nm, with a distance of 50 cm from the plate to the light. The plates were irradiated for the set times (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60 mins) using a digital timer. For each UV-exposure time point, three plates were randomly removed. For determination, the number of dead CFUs, which were killed by incubation rather than by the UV light, 500-1000 CFU/mL was transferred into empty plates in triplicate, covered with aluminium foil, and incubated on the bench at constant room temperature (22 °C). At the same time, three plates were collected as before. The removed plates were recovered on ice in the dark for 10 mins, then, 100 µL of the cell from each suspension was spread directly on nutrient agar, in triplicate. All nutrient agar plates were incubated at 37 °C for 16 h, and CFU was determined, plotted as survivors vs exposure times. The lethal time for 50% mortality (*LT*50) was estimated from the survival curve and the time, which kills 90% of the total CFU, was determined as 20 mins. Therefore, for mutation experiments, 5 mL of cell suspension having 500-1000 CFU/mL were irradiated for 20 mins, in triplicate. The exposed cells were recovered, and then screened for mutants with elevated resistance to Cd. 100 µL of exposed, recovered cells of *B. agri* C15 were
inoculated into 50 mL of EBS/ pyruvate in Erlenmeyer flasks (250 mL), in triplicate and with different nominal concentrations of Cd. The concentrations were below MTC of *B. agri* C15 (7 mM), equal (15 mM), and above (18, 20, 22, and 24 mM). The control of EBS broth without Cd was used to determine whether there was growth at each concentration of the screening process. The batch flasks were incubated at 37 °C for 72 h. The isolation of mutants was done in terms of MTC to Cd [6]. 100 µL from each batch culture was transferred and spread onto EBS agar/ pyruvate and with the same nominal concentrations of Cd that were used in the exposed batch cultures. After growing, the colonies of the mutants were purified and the mutant amongst several mutants, which had the highest MTC to Cd, was selected and coded as the mutant CdR. The selected mutant was further screened for its MTC to Cd. The nominal concentrations of Cd in the broth were MTC and 0.1 MTC of the selected mutant (increases of 2 µM). MTC value obtained was tested up to three separate occasions, followed by the confirmation on EBS agar.

2.3. Characteristics of *B. agri* C15 and *B. agri* C15 CdR

Several experiments were performed to evaluate whether the mutant had lost some of its physical characterisations identified in *B. agri* C15 due to UV mutagenesis.

2.4. Identification analyses and optimising growth conditions

The morphological test, including Gram stain and biochemical tests (IMVIC) was performed, in triplicate, with control. Optimal temperature, carbon, and nitrogen sources were also studied for the mutant *B. agri* C15 CdR, according to the previous protocol described for the wild type [4]. Different pH (4.0, 4.6, 5.8, 6.4, 7.7, 8.8, and 9.0) in EBS/pyruvate was adjusted to obtain the optimum environmental conditions of these strains. The pH (4.0 and 4.6) was adjusted using acetic acid and sodium acetate. K2HPO4 and KH2PO4 were also used to acquire pH between 5.0 and 8.5, while pH 8.6 and 9.0, HCl, Trise-base were used. According to the ratio of each solution, 2 mL was added in a Falcon tube (50 mL) containing 20 mL of EBS/pyruvate. The bacterial culture was inoculated in triplicate and incubated at the optimal temperature (37 °C) for 72 h. Turbid growth was observed, and the results were recorded.

2.5. Measurement of core kinetic parameters

The growth curve, specific growth rate, and pyruvate utilised with its respective values of specific growth yields were studied for *B. agri* C15 and *B. agri* C15 CdR. The experiments were carried out by inoculating 1 mL of overnight culture into 50 mL of EBS/pyruvate in Erlenmeyer-flasks (250 mL), in triplicate, incubated at 37 °C and shaken at 100 rpm. 1 mL of growth culture was collected at interval times, and the dry biomass was measured; the culture was then centrifuged, and the supernatants were kept in Eppendorf tubes and stored at -20 °C until being analysed for pyruvate concentration using a pyruvate kit assay as described in our previous study [4].

2.6. Inhibition effects of cadmium on the specific growth rate and maximum amount of biomass formed

The effect of Cd on specific growth rate and the maximum amount of biomass formed *B. agri* C15 and *B. agri* C15 CdR were investigated under different concentrations of Cd according to the method used by [7]. One mL of the culture was inoculated into 50 mL of EBS/pyruvate in Erlenmeyer-flasks (250 mL), in triplicate, and with different nominal concentrations of Cd: 2.5, 5, 7.5, 9, 10, 11, 12.5, 15, 16, 17.5, and 20 (mM), while the control was without Cd. Then, flasks were incubated at 37 °C for 72 h shaken at 100 rpm. The samples were collected at intervals, the amount of biomass formed was determined, and the growth curve was plotted, followed by the determination of the specific growth rate and the maximum amount of biomass formed.
2.7. Statistical analysis

Statistical analysis for these data was performed. All values were expressed as the mean and standard error of the mean (SEM). The bacterial growth curves were fitted with nonlinear regression (global curve fitting, four parameters) of the mean data utilizing the SigmaPlot (version 13). The specific growth rates on the bacterial growth curves were subjected to the Student’s t-test.

3. Results

3.1. UV-mutagenesis of B. agri C15 and screening of mutant CdR

It was essential to keep the cells of B. agri C15 in EBS/pyruvate completely frozen at -20°C for three weeks until the UV-mutagenesis was complete. Frozen cell beads have been known to be stable against freezing damage. It is not expected that the variable number of frozen cells will be affected for three months at -20°C [8]. To obtain large quantities of the cells, the specific growth rate of B. agri C15, grown in a 500 mL of batch culture, was determined with a nominal concentration of 10 mM Cd. This experiment was carried out along with growth in a 50 mL batch culture with 10 mM Cd for the comparison in the specific growth rate between both volumes of cultures. The growth of B. agri C15 in 50 mL and 500 mL without Cd was studied. The specific growth rate of B. agri C15 grown under the effect of Cd in 500 mL was significantly low (µ = 0.11 ± 0.07 h⁻¹, Figure 1D, p<0.05) compared to growth in 500 mL without Cd, which showed a higher specific growth rate (0.19 ± 0.1 h⁻¹, Figure 1C, p<0.05). Similarly, specific growth rates were decreased from 0.12 ± 0.01h⁻¹ (Figure 1A, p<0.05) when grown in 50 mL of batch culture without Cd to 0.11 ± 0.03 h⁻¹ (Figure 1D, p<0.05) when grown in 50 mL of batch culture under the effect of Cd. However, there were no significant differences observed between specific growth rates when grown under Cd effects in 50 or 500 mL (Figure 1B, D, p<0.05).

After the determination of the specific growth rate during growth at 500 mL of EBS under Cd effects, the growth of B. agri C15 in EBS broth (500 mL) was performed; the cells were harvested and suspended in 0.5 EBS without pyruvate to make the cell suspension form as beads to keep the cells of frozen at -20°C until the UV-mutagenesis was complete. After forming the frozen cells as beads, CFU/mL of B. agri C15 in these beads was estimated at 1×10⁶ CFU/mL. The length of UV light
exposure that would kill 90% of cells (as CFU) to increase the chance of mutant isolation was
determined at a fixed distance. Controls for CFU death just by incubation at room temperature were
performed; 90% of B. agri C15 cells were killed after 20 mins of UV exposure (Figure 2). Thus, this
was chosen as the length of exposure to induce the mutagenesis. The 50% mortality (LT$_{50}$), which was
the time for 50% of B. agri C15 CFU to die, was 5.92 mins.

![Figure 2. The lethal time curve for B. agri C15 after UV light exposure. 5 mL of 500–1000 CFU/mL
in empty plates in triplicate were exposed to UV light (340 nm, brand Pisces, watt 13) at a 50 cm
distance. The control plates were incubated on the bench at a constant room temperature of 22 °C
(data not shown, used to get the final amounts of CFU death). The error bars represent the standard
error of the three plates. The lethal time for 50% mortality (LT$_{50}$) after UV light exposure was
estimated to be at 5.92 mins.](image)

UV-mutagenesis of B. agri C15 was done for 20 mins, followed by the growth of exposed cells in
batch cultures, with nominal concentrations of Cd: 7, 15, 18, 20, 22, and 24 mM Cd. The selection of
putative mutants on EBS/pyruvate agar supplemented with the same concentrations of Cd was
performed. The mutant selection was based on the growth of exposed cells at a given concentration of
Cd in the plates. This showed no growth on plates with > 20 mM Cd, with few colonies at 15, 18, and
20 mM Cd. Among these, the most resistant colony to Cd was selected and considered a Cd-resistant
mutant strain due to its ability to grow at 20 mM Cd and was termed as ‘B. agri C15 Cd$^R$’. The MTC
of this mutant was 20.00 mM Cd, giving a higher MTC value than the wild type (15.00 mM).

3.2. Characteristics of B. agri C15 and B. agri C15 Cd$^R$

Several experiments were carried out to compare the different characteristics between B. agri C15 and
B. agri C15 Cd$^R$.

3.2.1. Identification analyses and optimising growth conditions

Optimal growth conditions were studied to investigate whether B. agri C15 Cd$^R$ had major phenotypic
changes other than B. agri C15. Gram-stained smears of the B. agri C15 and the B. agri C15 Cd$^R$
showed no key morphological changes. Both remained Gram-stain-positive (Figure 3A, B). Basic
chemotaxonomic characters (IMVIC test) showed the same results for B. agri C15 Cd$^R$, as reported
for B. agri C15 earlier in our previous study [4]. This data and the morphological data gave a
preliminary indication of no major phenotypic changes and that B. agri C15 Cd$^R$ was a bona fide
mutant, not a contaminate. Amounts of biomass formed by both strains under various conditions are
compared in Table 1. The statistical analysis of the biomass values showed the significant biomass
obtained at 37 °C, using pyruvate and ammonia as carbon and nitrogen sources, respectively. These
biomasses were not significantly different between the two strains ($p<0.05$). The growth at a different range of pH showed that both strains could grow within a range of 4.00 to 7.70, with no significant differences between them.

**Figure 3.** Morphological characterisation using Gram stain of (A) *B. agri* C15, (B) *B. agri* C15 Cd$^\text{R}$, (C) Bacillus subtilis (Gram-stain-positive control), (D) C. metallidurans DSM 2839$^\text{T}$ (Gram-stain-negative control) and (E) uninoculated slide. Stains were performed simultaneously in triplicate on three separately grown nutrient agar plates. Gram-stain-positive: purple to the blue colour; Gram-stain-negative: orange to brown colour; Bismarck brown

**Table 1.** The growth conditions of the wild type *B. agri* C15 and the mutant *B. agri* C15 Cd$^\text{R}$. ± represents the standard errors of the mean ($n = 3$, batch experiments). The biomass values for the growth conditions of each strain were subjected to a t-test to analyse the difference within each growth condition, and *show a significant difference in growth values resulting from different temperature, carbon source or nitrogen source, respectively within the strain. **No biomass was obtained.

| Growth conditions | Growth conditions values of: |
|-------------------|-----------------------------|
|                   | *B. agri* C15 | *B. agri* C15 Cd$^\text{R}$ |
| **Temperature**   |               |                             |
| 4 °C              | **           | **                         |
3.2.2. Measurement of core kinetic parameters

Specific growth rates for *B. agri* C15 and *B. agri* C15 CdR are 0.12 ± 0.07 h⁻¹ and 0.11 ± 0.02h⁻¹, respectively, with no statistically significant differences (Figure 4A, B, respectively). Mutant *B. agri* C15 CdR formed a specific growth yield higher than the wild type *B. agri* C15 (Figure 4C).

![Graphs](image)

**Figure 4.** The amount of dry biomass formed (■) and pyruvate utilisation (◊) of (A) *B. agri* C15 and (B) *B. agri* C15 CdR, and their respective (C) Yₚ of (■) *B. agri* C15 and (□) *B. agri* C15 CdR. The specific growth rate (●) is shown. The error bars indicate the standard error of the mean of three batch cultures.

3.3. Inhibition effects of cadmium on the specific growth rate and maximum amount of biomass formed
The effects of Cd on the specific growth rate and maximum amounts of biomass formed \( B. \text{agri} \ C15 \) and \( B. \text{agri} \ C15 \text{Cd}^R \) were compared. Both strains showed a Cd-dependent fall in a specific growth rate, and the significant rate was for \( B. \text{agri} \ C15 \) (Figure 5A). The maximum amount of biomass under different concentrations of Cd showed that the lethal doses for \( B. \text{agri} \ C15 \) and \( B. \text{agri} \ C15 \text{Cd}^R \) were 15.80 and 20.00 mM Cd, respectively (Figure 5B).

![Graph showing specific growth rate and maximum amount of biomass formed with Cd concentration](Figure 5)

**Figure 5.** The inhibition effects of Cd on (A) specific growth rate and (B) maximum amounts of biomass formed of (○) \( B. \text{agri} \ C15 \) and (●) \( B. \text{agri} \ C15 \text{Cd}^R \). The error bars indicate the standard error of the mean of three batch cultures at each Cd concentration. *Significant difference between strains at each Cd concentration.

4. Discussion

In this study, a distinct approach was carried out to achieve increased resistance in the \( B. \text{agri} \ C15 \). Isolate \( B. \text{agri} \ C15 \), which has an intrinsic ability to resist Cd (MTC: 15.00 mM), was used to increase its natural resistance to Cd. [9] found that growing a bacterium with Cd induces a mutant with elevated resistance to Cd. Using this process, several mutants were successfully generated from the wild type \( B. \text{agri} \ C15 \) by UV light mutagenesis; one of these mutants \( B. \text{agri} \ C15 \text{Cd}^R \) was able to grow in the highest Cd concentration (20.00 mM) and was chosen. The generation of Cd-resistant mutants in the growth medium can be affected by the concentration of Cd, the steps to isolate the mutants and the increase of the Cd concentrations in the medium during each step. Exposing cells, cultivating in the medium (with a high Cd concentration), and spreading exposed cells on agar plates containing Cd may have increased the resistance of the \( B. \text{agri} \ C15 \) by an MTC value of 0.25 fold. However, if the process was used repeatedly with increasing Cd concentrations in the low fold [10], it could generate a mutant with higher MTC value (eightfold). Mutant \( B. \text{agri} \ C15 \text{Cd}^R \) had improved resistance to Cd, tolerant to 20.00 mM, and showed no key morphological or biochemical characteristics compared to wild type \( B. \text{agri} \ C15 \). Mutant \( B. \text{agri} \ C15 \text{Cd}^R \) had higher biomass than the wild type and exhibited slower growth when grown with Cd than wild type \( B. \text{agri} \ C15 \). The inhibition of the bacteria’s growth led to a diminished specific growth yield; these results are similar to the results of the study performed by [11] on the Cd- mutant \( P. \text{aeruginosa} \) G-1. After three days of Cd exposure, it was found that wild type \( B. \text{agri} \ C15 \) was less likely to respond to Cd compared to mutant \( B. \text{agri} \ C15 \text{Cd}^R \). Also, the inhibition appeared Cd-dependent, and both strains showed similar responses in specific growth rate and biomass under different Cd concentrations. Still, mutant \( B. \text{agri} \ C15 \text{Cd}^R \) had less specific growth rates and biomass with addition Cd concentrations (15-20 mM). Few
studies have investigated Cd effects on mutants. The mutants of Escherichia coli were found to be less responsive to a concentration of 6 mM Cd $\text{Cd}^{10}$. [7] found that the mutants of E. coli had slower growth rates when exposed to a tolerant Cd concentration of 0.7 mM. These results, together with this study, suggest that Cd concentrations influence mutant growth.

5. Conclusion

The B. agri C15 CdR mutant was generated from isolated B. agri C15 by UV light mutagenesis, and this mutant showed enhanced resistance to Cd and had a MTC value of 20.00 mM to Cd, which was higher than the MTC of the wild type (15 mM). Thus, it is concluded that this mutant could be suitable for the development of a Cd-bioremediation process; therefore, further studies are needed to investigate this possibility.

6. References

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