

# Effect of Ca<sup>2+</sup> on *P. aeruginosa* and *B. cereus* metabolites

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## Research

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# Abstract

**Background:** The effects of  $\text{Ca}^{+2}$  on virulence and some parameters were tried to be analyzed in this study. *P. aeruginosa* and *B. cereus* were used as Gram (-) bacteria as a control and Gram (+) bacteria as a control. Both bacteria are soil bacteria.

**Results:** Amylase; *P. aeruginosa* is 102% increase in 0.1 M  $\text{Ca}^{+2}$  concentrations and *B. cereus* caused an increase in 123%. Biofilm *P. aeruginosa*; 200% increase in 0.1 M  $\text{Ca}^{+2}$  concentrations. *B. cereus* caused a 252% increase in the presence of 0.15 M  $\text{Ca}^{+2}$ . Las B; in accordance with *B. cereus*, Las B activity decreased 1.7 times more than with *P. aeruginosa*.  $\text{H}_2\text{O}_2$ ; *P. aeruginosa* increased by 60% resistance. *B. cereus* caused only a 3% increase. Protease in *P. aeruginosa* increase of 220%. *B. cereus* caused a 548% increase. Pyorubin, a pigment, caused an increase in the presence of  $\text{Ca}^{+2}$ . However, these increases are not seen as significant increases.

**Conclusion:** In this study; the effect of  $\text{Ca}^{+2}$  on protease, amylase, LasB elastolytic assay,  $\text{H}_2\text{O}_2$ , pyorubin and biofilm was investigated in a 24 hour time. In the study we have conducted here, the effect of  $\text{Ca}^{+2}$  meat on the production of some secondary metabolites on Gram (-) *P. aeruginosa* and Gram (+) *B. cereus* was investigated for the first time. As a result, although there are many different related studies, they are not comparative as in ours. Therefore, our study has progressed in a different direction and it is a first in its field.

## Introduction

$\alpha$ -amylase (E.C.3.2.1.1; 1,4- $\alpha$ -D-glucan-glucanohydrolase) is a hydrolytic enzyme that hydrolyzes  $\alpha$ -1,4-glycosidic linkages in starch and forms products such as glucose or maltose. This enzyme is one of the most important biotechnological products used in various industrial processes such as food, paper, textile and detergent [1].

$\alpha$ -amylase can be isolated from plants, animals or microorganisms. It is found in many bacteria (*Bacillus* spp. *B. amyloliquefaciens*, *Bacillus subtilis*, *B. cereus*, *B. amyloloquefaciens*, *B. amloloquefaciens*, *B. megaterium*, *B. licheniformis*, *Lactobacillus*, *Escherichia*, *Proteus*, *Clostridium* and *Pseudomonas* sp.) and fungi (*A. niger*, *Penicillum*, *Sefalosporium*, *Neurospora* and *Rhizopus*). For this reason, microorganisms in particular, are used more in the production of this enzyme. However, *Bacillus* species are more widely used in commercial protease production [2–7].

*P. aeruginosa* is a metabolically versatile Gram (-) pathogenic bacterium that has adapted to many environments associated with terrestrial, aquatic, animal, human and plants. In addition, *Pseudomonas* species are bacterial groups with many scientific and technological importances. It is a metabolically versatile and powerful organism that can use many simple or complex organic compounds.

*Pseudomonas* has a fairly large genome in its genes that contains many different virulence factors. In this way, it has the ability to adapt to almost any environment. As a result of processes such as phase

variation or adaptive mutations to changing environmental conditions, rapid change of the *P. aeruginosa* genotype is possible in producing morphologically different phenotypic variants. *Pseudomonas* sp. is capable of producing many extracellular enzymes such as lipase and amylase. Proteases secreted, play an important role in pathogenesis during acute infections. Apart from this, *P. aeruginosa* can produce LasB elastase and LasA staphylolytic protease secretion [8–14]

Metalloproteinase elastase A, which belongs to *Pseudomonas*, has been reported to break down the elastin and increase the substrate range of elastase B. Elastase B and alkaline protease specifically destroy host defense proteins. This is very important in virulence [15]. Elastase has three active amino acids. These are the catalytic triads that work together; aspartate, histidine and serine. Elastase, *E. coli* and other Gram (-) bacteria outer membrane elastase, also have the property of breaking down *Shigella* virulence factors, which can be done by carboxy of small and hydrophobic amino acids such as glycine, alanine and valine [16].

The structures we call biofilms are actually a group of microorganisms attached to a surface and covered with an exopolysaccharide matrix. It is most commonly created by *P. aeruginos*. The presence of chemotaxis, motility, surface adhesions and surfactants towards the surface are factors affecting biofilm formation [17]. Biofilm, in phytopathogenic microorganisms and animal pathogens, adaptation promotes survival. Cells in biofilm are said to be more resistant to oxidative stress than free cells [18].

*Bacillus cereus* can be found widely in soil and plants. Bacteria possessing psychrotrophic, spore, Gram (+), flagella, aerobic, and peritric flagels are aerobic. Optimum breeding is usually 30 °C. *B. cereus* has lecithinase, gelatinase, amylase and protease activity. It can reduce nitrate and is resistant to polymyxin. Many strains can also breed in 7.5% salt. *Cereus* takes its name from cereal, which means grain [19].

Studies suggest that cellular  $\text{Ca}^{+2}$  in a host can be an environmental clue for opportunistic bacteria and can trigger their virulence. As already known,  $\text{Ca}^{+2}$  in prokaryotes has roles in many physiological events such as spore formation, motility, cell differentiation, transport and virulence [20].

In the study we have conducted here, the effect of  $\text{Ca}^{+2}$ 's meat on the production of some secondary metabolites on Gram (-) *P. aeruginosa* and Gram (+) *B. cereus* was investigated for the first time.

## Materials And Methods

### Reagents

All chemicals were of the highest purity available commercially.

### Microorganism

*P. aeruginosa* (ATCC 27853) and *B. cereus* (ATCC 10876) obtained from the ATCC and used this study.

# Enzyme production

Microorganisms were grown and cultivated as follows: 3 ml of 24 h bacterial inoculum ( $OD_{600} = 0.3-0.4$ ) was inoculated into 5 ml growth media and agitated at the rate of static for 24 h at 37 °C. Crude extracellular enzyme solutions were prepared by removing the cells by centrifugation at 13,500 rpm and room temperature for 5 min. Supernatant harvested was assayed for proteolytic activity [21].

## Amylase activity assays

The amount of the reducing sugars released by the action of amylases on starch was currently performed at 37 °C and pH 7 phosphate buffer for 15 min and the increase in the glucose was determined by antron method. The reaction mixture contained 0.5 ml starch (2% in 0.01 M phosphate buffer) and the 0.5 ml enzyme solution in a final volume of 1 ml. One unit of amylase was defined as the amount of enzyme, required to produce reducing sugars equivalent to 1  $\mu\text{mol}$  glucose/min at 37 °C and at pH 7.0 [7, 21].

## Pyorubin

Pyorubin, a pigment, caused an increase in the presence of  $\text{Ca}^{+2}$ . However, these increases are not seen as significant increases. Subsequent concentrations, the highest in the presence of 0.1 M  $\text{Ca}^{+2}$ , also began to decline. The value of 88.21 was obtained. As a result,  $\text{Ca}^{+2}$  had no effect on the increase or decrease of pyorubin. As a result, it increased the production of 0.1 M  $\text{Ca}^{+2}$  pyorubin, which has the highest effect, by only 102% (Fig. 6). Why are there no *B. cereus* values in this chart? Because of pyorubin is a metabolite specific to *Pseudomonas aeruginosa*. Therefore it is not shown on the chart.

## Assay of protease activity

Protease activity was measured by some modification of the reaction mixture consisted of 1.0 ml enzyme solution preincubated at 37 °C for 10 min. The reaction was started by the addition of 1.0 ml casein 6.5 mg/ml (in 0.05 M phosphate buffer pH 7.0). The reaction mixture was then incubated in the incubator at 37 °C for 10 min shaker and terminated by the addition of 1 ml 10% trichloroacetic acid (TCA). A vortex mixer was used. This mixture was further incubated at 37 °C for 20 min, followed by centrifugation at 13,500 rpm for 10 min. The supernatant was harvested. To 300  $\mu\text{l}$  supernatant, 750  $\mu\text{l}$  of 0.5 M  $\text{Na}_2\text{CO}_3$  and 150  $\mu\text{l}$  folin ciocalteau reagent: water (1:3 v/v) was added to yield a blue color. The colored mixture was incubated in an incubator at 37 °C for 20 min. Absorbance was read at  $OD_{660 \text{ nm}}$ . The amount of tyrosine was determined from the tyrosine standard curve [4, 21, 22].

## Biofilm assay

Biofilm generated by each strain of *P. aeruginosa* were evaluated using the following crystal violet assay [10]. The biofilm forming ability isolates were tested using glass tube with little modifications. The overnight cutlers of *P. aeruginosa* isolates were inoculated in Nutrient broth supplemented with  $\text{Ca}^{+2}$  (0; 0.05; 0.1 and 0.15 M) for 24 hours at 37 °C. After incubation, removing the planktonic bacteria, the wells were carefully rinsed three times-distilled water and then stained with 0.1% of crystal violet (10 minutes, at room temperature) and washed with distilled water (three-times). After air drying, ethanol (95%) was added and incubated for 15 min to remove the bound crystal violet. The absorbance was measured spectrophotometrically at  $\text{OD}_{570}$  nm, for quantification of biofilm biomass [10–11, 23–25].

## LasB elastolytic assay

The test was performed with some modifications. 1 ml supernatan, 1 ml reaction mixture [1 mg/ml ECR in 200 mM Trizma-base buffrer (pH 8.8)] and then incubated with shaking at 37 °C for 30 min. Then reaction stopped 0.5 ml 100 mM EDTA added. Insoluble ECR was removed by centrifugation (6000 rpm and room temperature 5 min), and the absorption of the supernatant was measured at  $\text{OD}_{495}$  nm. Activity was expressed as change in  $\text{OD}_{495}$  g/protein [13, 16, 26].

## Results

In our study, various physiological parameters of *B. cereus* and *P. aeruginosa* were investigated in solid media, and 24-hour incubation at 37 °C was evaluated.

The effects of  $\text{Ca}^{+2}$  on virulence and some parameters were tried to be analyzed in this study. *P. aeruginosa* and *B. cereus* were used as Gram (-) bacteria as a control and Gram (+) bacteria as a control. Both bacteria are soil bacteria. The concentration was left at 0.15 M because the bacteria lost its ability to reproduce in the above concentrations. A severe inhibitory effect was observed. For this reason, as they were found to be the most suitable concentrations as a result of optimization studies, 0.05, 0.1 and 0.15 M  $\text{Ca}^{+2}$  were used. Why did we work with a high level of  $\text{Ca}^{+2}$ ? We preferred it because it has the highest values we can reach with optimization studies.

Why did *P. aeruginosa* compare with *B. cereus*? a) Both are soil bacteria. b) *P. aeruginosa* Gram (-) and *B. cereus* Gram (+). Therefore, it gives the opportunity to compare. c) *P. aeruginosa* sporeless and *B. cereus* sporulated bacteria. d) Pathogenicity levels are different. e) We used our work in this preference.

## Amilaz

Amylase activity, in the presence of  $\text{Ca}^{+2}$ , *B. cereus*, achieved a maximum increase of up to 1.2 times. The highest increase was in the presence of 0.15 M  $\text{Ca}^{+2}$  with 25.32 U/ml. For *P. aeruginosa*, an increase of 1.6 times more amylase activity was observed on average compared to *B. cereus*. When *P. aeruginosa* was evaluated in itself, no significant increase was observed in the presence of  $\text{Ca}^{+2}$ . The highest P.

*aeruginosa* amylase activity was observed in the presence of 0.1 M Ca with 32.13 U/ml. Although *P. aeruginosa* is more advantageous on the graph, in its production it caused a 102% increase in 0.1 M Ca<sup>+2</sup> concentrations. However, this ratio in *B. cereus* caused an increase in 123% amylase production in the presence of 0.15 M Ca<sup>+2</sup>. All of these values are calculated according to the controls (Fig. 1).

## Biofilm

As was expected, biofilm formation in *B. cereus* was low. The highest biofilm formation occurred in OD<sub>570</sub> with 0.284 in the presence of 0.1 M Ca<sup>+2</sup> for *B. cereus*. According to the control, an increase of up to 2.5 times was observed in the presence of Ca<sup>+2</sup>. In *P. aeruginosa*, a higher biofilm formation occurred, as was expected. Unlike *B. cereus*, in the presence of 0.1 M Ca<sup>+2</sup>, a higher biofilm formation was observed in OD<sub>570</sub> with 0.735. It appears to be the most suitable concentration for biofilm formation for *P. aeruginosa*. The difference was two times more than in the control and there was approximately three times more biofilm formation than in *B. cereus*. Although *P. aeruginosa* is advantageous, a 200% increase in 0.1 M Ca<sup>+2</sup> concentrations was observed compared to the control. However, this ratio in *B. cereus* caused a 252% increase in the presence of 0.15 M Ca<sup>+2</sup> compared to the control (Fig. 2).

## LasB elastolytic assay (ECR)

The addition of Ca<sup>+2</sup> in both bacteria decreased ECR activity. There was a decrease of 7.14 times in *B. cereus* and 4.8 times in *P. aeruginosa*. It occurred mostly in *B. cereus*. The greatest decrease in both bacteria occurred in the presence of 0.15 M Ca<sup>+2</sup>. In accordance with *B. cereus*, ECR activity decreased 1.7 times more than with *P. aeruginosa*. The graphic here shows a harmonious decrease (Fig. 3).

## H<sub>2</sub>O<sub>2</sub>

In *B. cereus*, the highest sensitivity was observed in the presence of 0.15 M Ca<sup>+2</sup> with 33 mm. H<sub>2</sub>O<sub>2</sub> resistance only increased compared to the control in the presence of 0.05 M Ca<sup>+2</sup>. This was observed with a 7 mm zone decrease. While *P. aeruginosa* was 35.3 mm in the highest zone control, the highest resistance was realized in the presence of 0.1 M Ca<sup>+2</sup>, just like in *B. cereus*. This was observed with a zone diameter of 14 mm. Resistance increase in both bacteria was observed in the presence of 0.05 M Ca<sup>+2</sup>. An increase in sensitivity started to occur above these concentrations. While Ca<sup>+2</sup> increase in *B. cereus* causes a sensitivity increase compared to the control, in *P. aeruginosa*, resistance increased continuously compared to the control. H<sub>2</sub>O<sub>2</sub> resistance in *P. aeruginosa* caused an increase in resistance in all three concentrations compared to the control (Fig. 4).

*P. aeruginosa* increased by 60% resistance at a concentration of 0.05 M Ca<sup>+2</sup>. However, this ratio in *B. cereus* caused only a 3% increase in the presence of 0.05 M Ca<sup>+2</sup>. These values are calculated according

to the controls.

## 3.5 | Protease

The presence of  $\text{Ca}^{+2}$  in both bacteria caused an increase in protease activity. This increase caused enzyme activity in *B. cereus* in the presence of 0.05 M  $\text{Ca}^{+2}$  up to 0.746 U/ml and up to 5.6 times according to the control. Therefore, this appears to be the most appropriate concentration in protease activity for *B. cereus*. In *P. aeruginosa*, only a 2.3-fold increase in the presence of  $\text{Ca}^{+2}$  was 1.635 U/ml in the presence of 0.1 M  $\text{Ca}^{+2}$ . Consequently, this appears to be the most appropriate concentration in protease activity for *P. aeruginosa*. However, the concentration of 0.15 M  $\text{Ca}^{+2}$  caused a decrease in both bacteria. In general, the presence of  $\text{Ca}^{+2}$  increased protease activity. Although *P. aeruginosa* is advantageous when the graph is examined, it caused an increase of 0.1%  $\text{Ca}^{+2}$  concentrations by 220%. However, this ratio in *B. cereus* caused a 548% increase in the presence of 0.05 M  $\text{Ca}^{+2}$ . These values are calculated according to the controls (Fig. 5).

## Pyorubin

Pyorubin, a pigment, caused an increase in the presence of  $\text{Ca}^{+2}$ . However, these increases are not seen as significant increases. Subsequent concentrations, the highest in the presence of 0.1 M  $\text{Ca}^{+2}$ , also began to decline. The value of 88.21 was obtained. As a result,  $\text{Ca}^{+2}$  had no effect on the increase or decrease of pyorubin. As a result, it increased the production of 0.1 M  $\text{Ca}^{+2}$  pyorubin, which has the highest effect, by only 102% (Fig. 6). Why are there no *B. cereus* values in this chart? Because of pyorubin is a metabolite specific to *Pseudomonas aeruginosa*. Therefore it is not shown on the chart.

## Conclusion

The determination of reducing sugars was generally carried out by the antrone method. The concentration of the glucose was determined at  $\text{OD}_{620}$  nm spectrophotometrically. It is mainly used in the assay of  $\alpha$ -amylase activity [2]. In some studies, it has been observed that calcium ion influences the biofilm structure in *P. aeruginosa* cultures. They stated that by adding calcium it was 10 to 20 times thicker than the formation of non-added calcium biofilm [27, 28]. The extracellular elastase (LasB) and LasA amounts, which are the products of the Type II secretory pathway, increase in the presence of additional calcium. In addition, the amount of extracellular protease increased with the addition of  $\text{Ca}^{+2}$  [28]. Another study showed a 20-fold increase in biofilm thickness after 24 hours in the presence of  $\text{Ca}^{+2}$  (1.0 and 10.0 mM  $\text{CaCl}_2$ ). In this study, by growing the culture with 10 mM  $\text{CaCl}_2$ , approximately two to three times more biofilm structures were observed compared to the non- $\text{Ca}^{+2}$  medium [28]. Some studies have stated that the amount of protease and pyocyanin increase with the addition of  $\text{Ca}^{+2}$ . The addition of calcium has been shown to increase the biofilm structure in *Vibrio cholerae*. Although, in

*Pectobacterium carotovorum*, it has been found to increase the activity of the type III secretion system and the expression of effector proteins, as well as the modulation of hydrolytic enzymes (such as polygalacturonase and pectate lyase), which are considered to be important in virulence. Calcium also plays a role in *X. fastidiosa* infection. In addition, the presence of  $\text{Ca}^{+2}$  is said to increase biofilm formation, cell binding and motility *in vitro*. These results show that the role of  $\text{Ca}^{+2}$  in biofilm formation is important [27, 28]. It is also stated in Prokaryotes that the presence of  $\text{Ca}^{+2}$  regulates and increases bacterial gene expression. Many prokaryotes, including *Escherichia coli*, *Propionibacterium acnes*, *Streptococcus pneumoniae*, *Bacillus subtilis* and *Cyanobacteria*, have also been shown to maintain intracellular  $\text{Ca}^{+2}$  levels at micromolar levels, producing in response to environmental and physiological conditions. This suggests that  $\text{Ca}^{+2}$  play an important role in prokaryotic physiology and virulence. Apart from this, it is stated that  $\text{Ca}^{+2}$  increases biofilm formation in *P. aeruginosa* and protease, and pyocyanin virulence factors induce biosynthesis [20, 29]. In addition, it has been stated in a study that the presence of  $\text{Ca}^{+2}$  in the environment increases the expression of genes that cause proteolysis and stress response [20]. In another study, the presence of  $\text{Ca}^{+2}$  has been shown to increase the virulence of *Pseudomonas aeruginosa* and the thickness of the biofilm structure. It also shows that  $\text{Ca}^{+2}$ , *X. fastidiosa*'s biofilm formation, has the ability to cling to the cell surface and play a role in the regulation of the movement of twitches [27, 28]. However, when  $\text{Ca}^{+2}$  were added, intense protein bands of AprA, LasB, and PrpL were observed. The chemical analysis showed an increased production of pyocyanin with  $\text{Ca}^{+2}$  additions in *P. aeruginosa* FRD1 [28]. Chemical analysis shows that pyocyanin production increases with the addition of  $\text{Ca}^{+2}$  [28]. We did not find any study on the effect of  $\text{Ca}^{+2}$  on pyoverdine. It is stated that protease enzymes need some metal ions in order to maintain their stability and maintain their active form. According to this study; it has been stated that  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$  and  $\text{Mn}^{+2}$  ions increase the protease activity [20]. In our study, it is a rare study because it is studied with high molarity  $\text{Ca}^{+2}$ , which is not found in the literature. As a result, although there are many different related studies, they are not comparative as in ours. Therefore, our study has progressed in a different direction and it is a first in its field.

## Declarations

## Authors' contributions

HK and CCK designed this study. HK and ZBK performed most experiments. Authors analyzed the data, wrote the manuscript, read and approved the final manuscript.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Acknowledgements

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## Figures

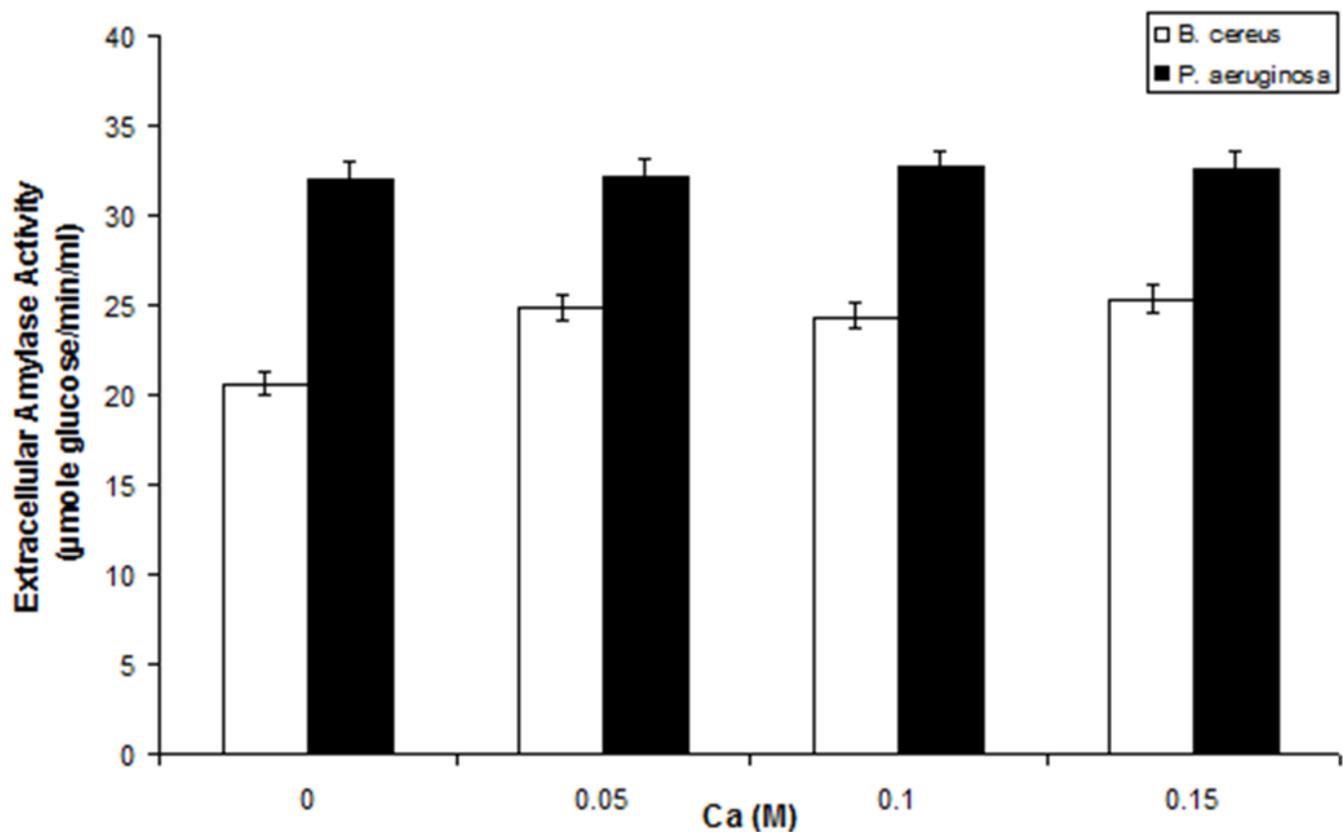


Figure 1

Amylase unit activity of *B. cereus* (●) and *P. aeruginosa* (●), grown in NB medium under static conditions at 37 °C.

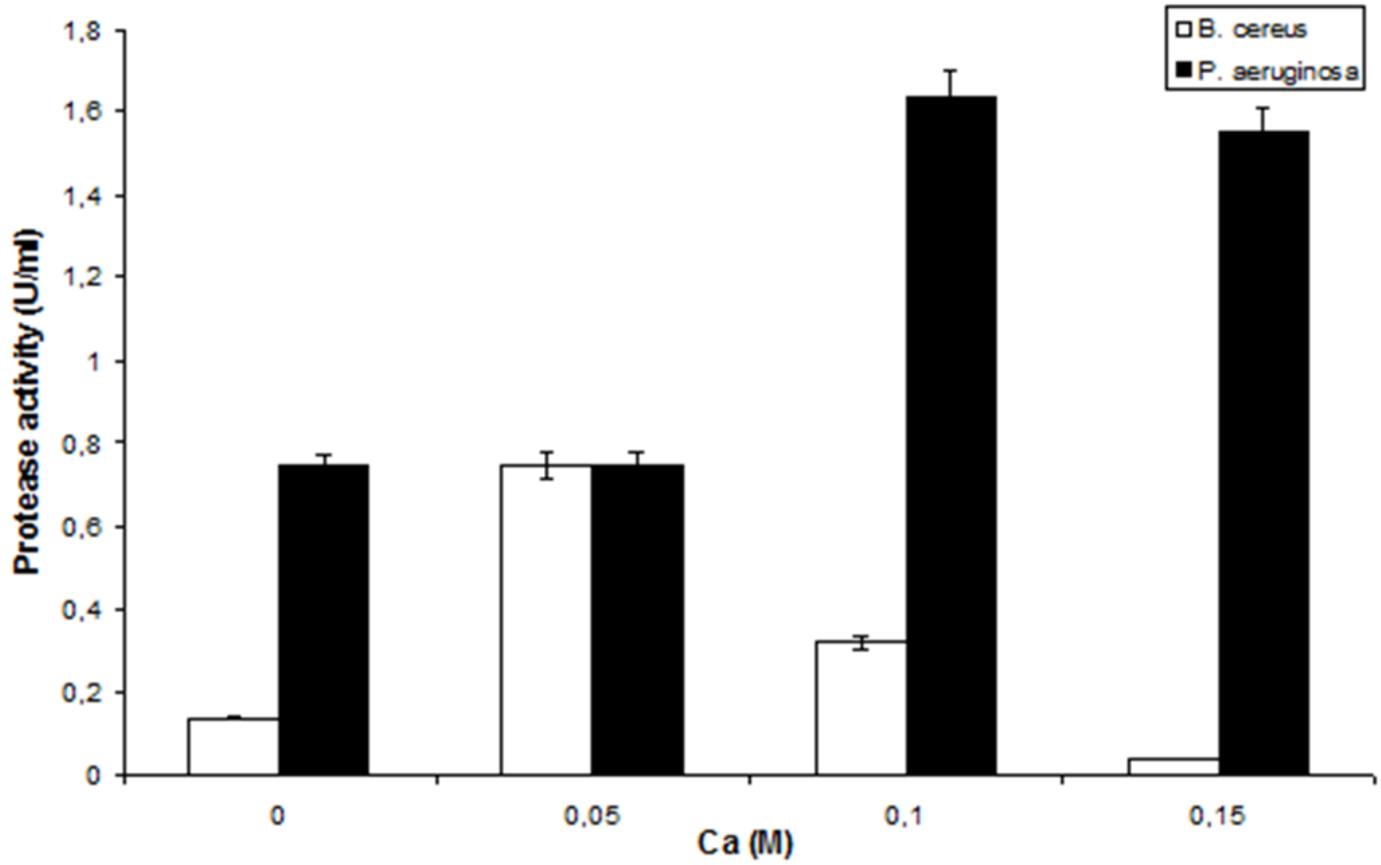


Figure 2

Biofilm levels of *B. cereus* (●) and *P. aeruginosa* (●), grown in NB medium under static conditions at 37 °C.

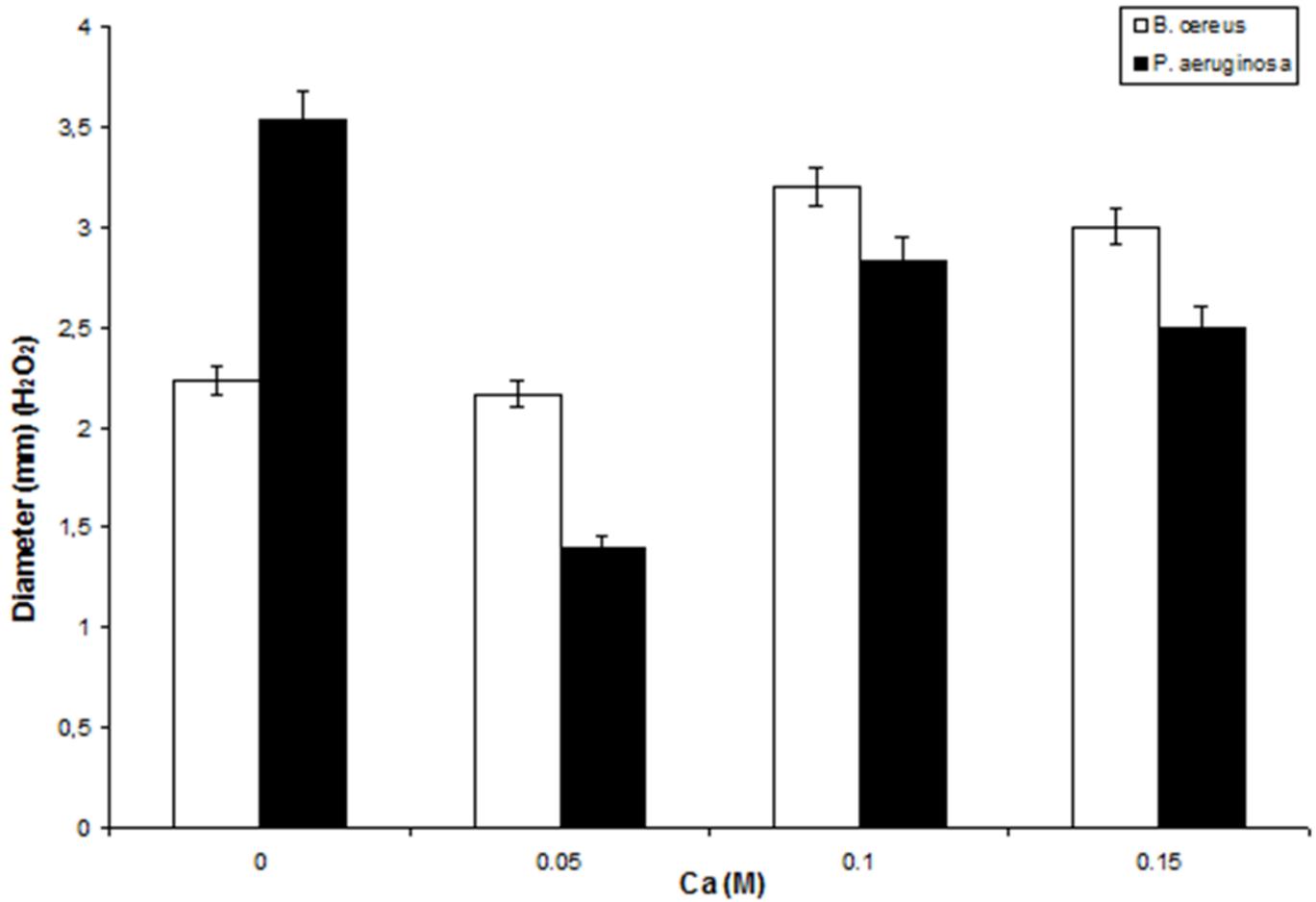


Figure 3

Las B activity of *B. cereus* (●) and *P. aeruginosa* (●), grown in NB medium under static conditions at 37 °C.

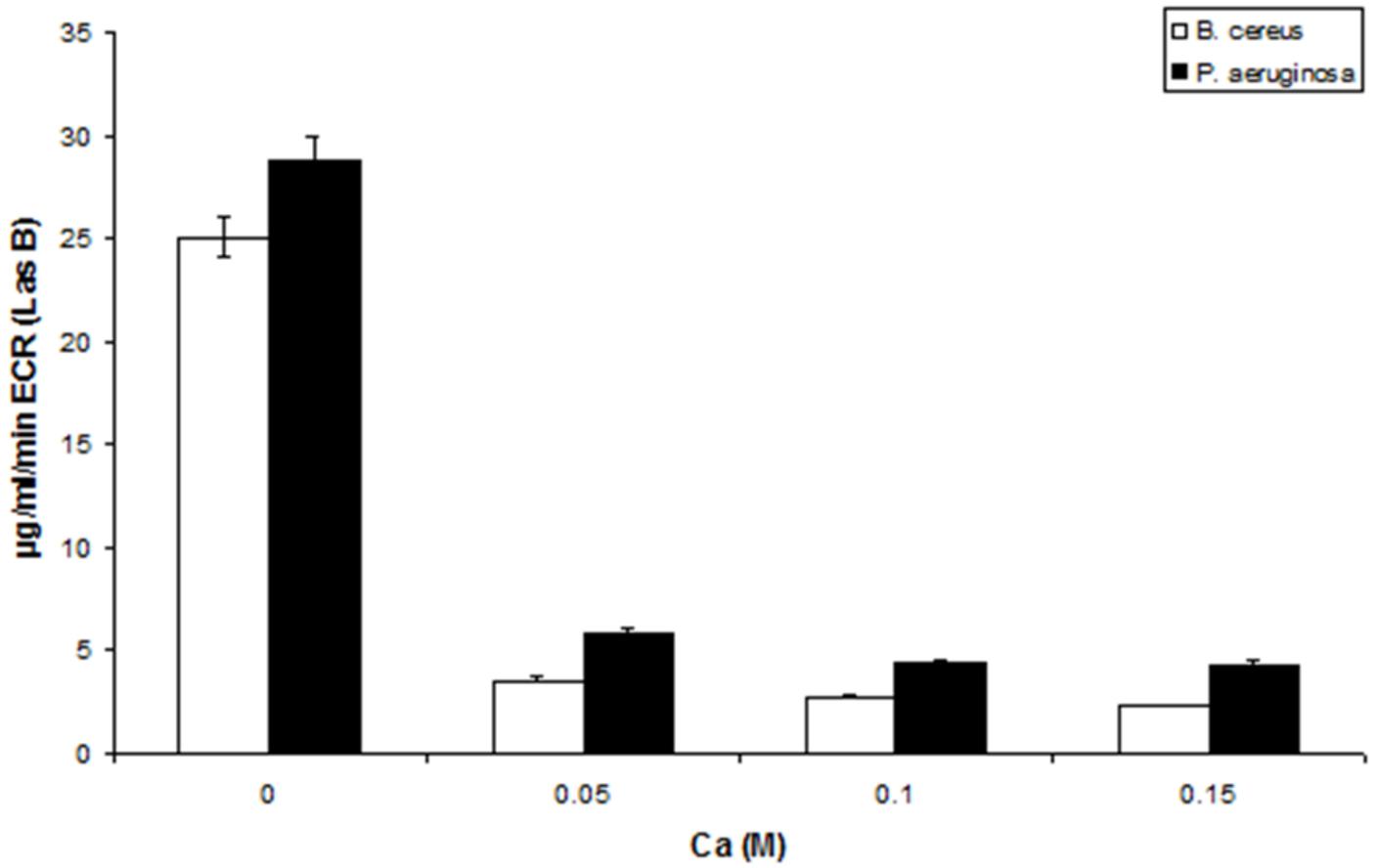


Figure 4

H<sub>2</sub>O<sub>2</sub> diameter of *B. cereus* (●) and *P. aeruginosa* (●), grown in NB medium under static conditions at 37 °C.

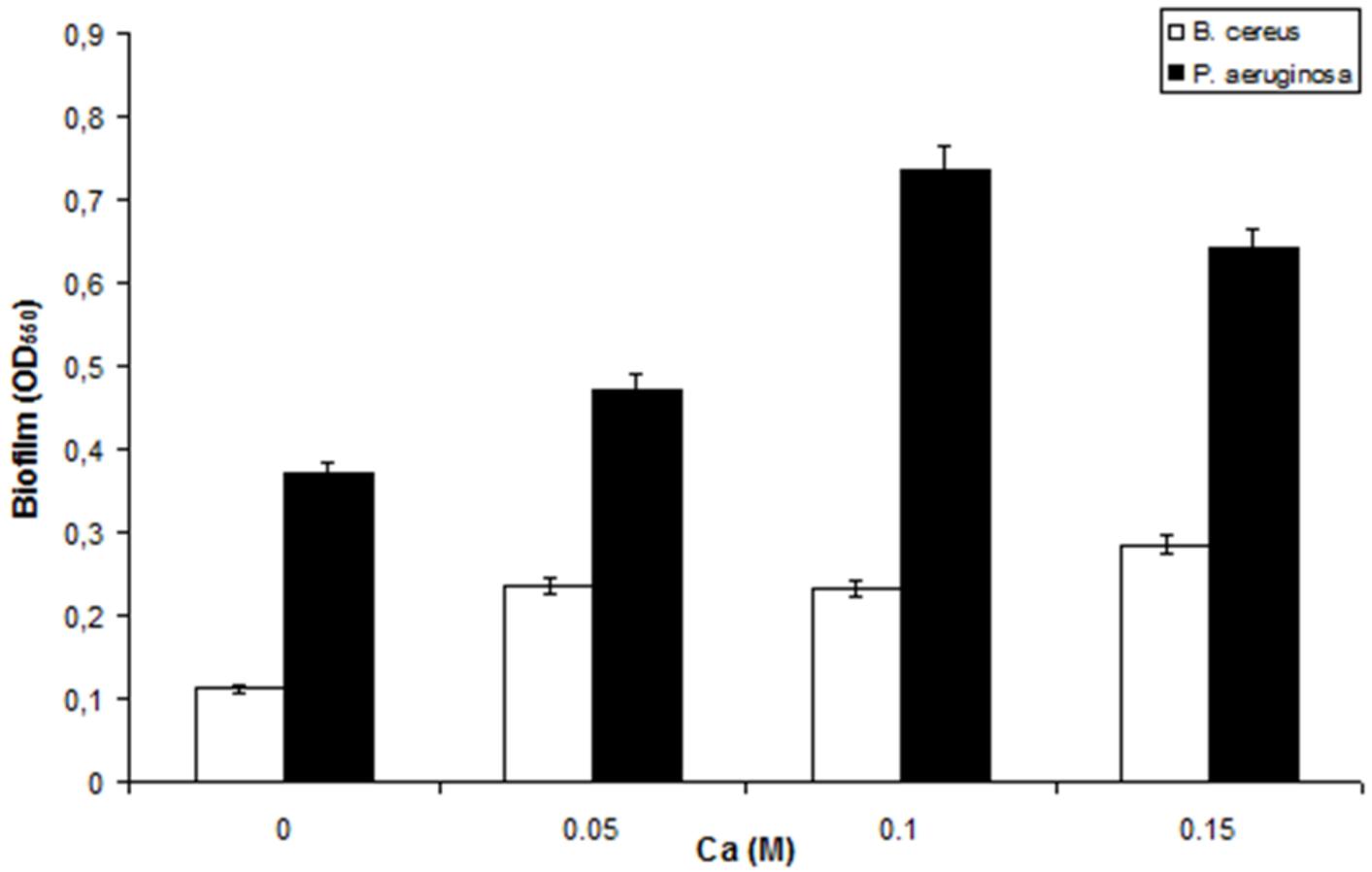


Figure 5

Protease activity of *B. cereus* (●) and *P. aeruginosa* (●), grown in NB medium under static conditions at 37 °C.

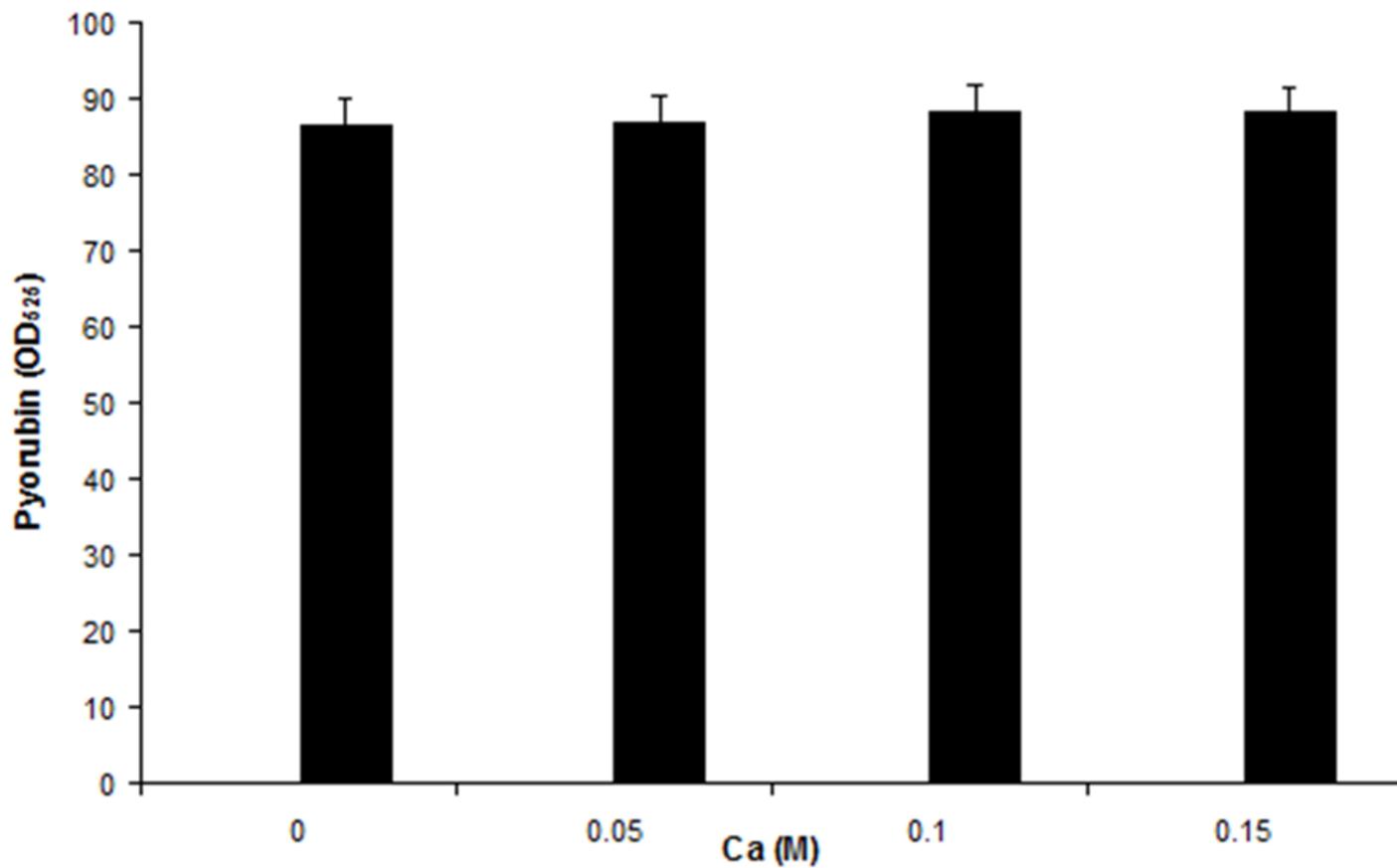


Figure 6

Pyorubin production of *P. aeruginosa* (●), grown in NB medium under static conditions at 37 °C.