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# Pyocin QDD1: A highly thermostable bacteriocin produced by *Pseudomonas aeruginosa* QDD1 for the biocontrol of foodborne pathogens *Staphylococcus aureus* and *Bacillus cereus*

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ABSTRACT

This study was designed to explore alternative antibacterial bioresource products, bacteriocins produced by local strain of *Pseudomonas aeruginosa* QDD1. This bacteriocin (termed as Pyocin) has high inhibitory activity against Gram-positive bacteria including *S. aureus*, *B. cereus*, *B. thuringiensis* and *B. subtilis*. QDD1 Pyocin production was constitutive, auto-regulated and reached an activity level of 110 AU/mL. It affects 40 % of sensitive cells starting from the 1 st hour. Pyocin QDD1 activity was not affected by Proteinase K, α-amylase, β-mercaptoethanol and 8 M urea. It displayed broad pH stability (pH 3–9) and significant thermostability (121 °C). Considering that the spores produced by *Bacillus cereus* are highly resistant to cooking temperatures and cause food poisoning upon germination in gastrointestinal tract, Pyocin QDD1's ability to withstand high temperatures can help control *Bacillus cereus* populations in contaminated food products. Thus, Pyocin QDD1 can be considered as an important bioresource product of industrial applications.

## 1. Introduction

According to the World Health Organization, there are an estimated 600 million cases of foodborne illnesses worldwide every year, out of which 420,000 leads to death (Lee and Yoon, 2021; World Health Organization, 2015). More than 30 % of these are caused by bacteria (Lee and Yoon, 2021), mainly *Bacillus cereus* and *Staphylococcus aureus* (York-Moore et al., 2017).

*Bacillus cereus* is an endospore forming bacteria that are commonly found to contaminate carbohydrate rich food like rice, pasta and noodles, meat and milk products, soups and vegetables (Berthold-Pluta et al., 2019; Rodrigo et al., 2021). Their spores are resistant to heat, ultra-violet light, gastric acid, freezing and desiccation (Tewari and Abdullah, 2015) and can easily survive on food products for more than 48 weeks without any loss of viability (Rodrigo et al., 2021). Consumption of food contaminated by these spores can result in severe food poisoning and diarrhoea as the spores germinate under the favourable conditions of the human small intestine and produce a number of pore-forming enterotoxins (Berthold-Pluta et al., 2019). Occasionally, food contaminated by *B. cereus* that was left in the open for an extended period of time can cause the emetic form of the disease, where in the bacterium releases the heat and acid resistant toxin – cereulide, into the food (Rajkovic et al., 2008). Exposure to cereulide can cause a loss of mitochondrial membrane potential and reduce oxidative phosphorylation, ultimately leading to cell death (Alonzo et al., 2015).

Unlike *B. cereus*, *Staphylococcus aureus* does not form endospores. However, it is a desiccation tolerant microbe and can survive stressful environments, with the ability to grow in a wide range of pH conditions (pH 4.2–9.3), temperatures (7 °C – 48.5 °C) and salinities (up to 15 % NaCl) (Kadariya et al., 2014). *S. aureus* has been found to produce more than 20 enterotoxins, more than half of which have proven emetic in the human body (Hennekinne et al., 2012). All these enterotoxins have superantigenic activity with the ability to non-specifically activate T-cell receptors. This could potentially result in lethal toxic shock syndrome due to the release of massive amounts of pro-inflammatory cytokines and chemokines (Hennekinne et al., 2012). Additionally, there have been increasing reports of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from food in recent years (da Silva et al., 2020; Dougeraki et al., 2017). With the emergence of antibiotic resistant bacteria, it is critical to find alternative antibacterial compounds with comparable activity to prevent foodborne illnesses (Benítez-Chao et al., 2021; Soltani et al., 2021).

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Bacteriocins are ribosomally synthesized proteins or peptides produced by bacteria that typically inhibit the growth of closely related species (Zimina et al., 2020). Bacteriocins offer several advantages over antibiotics. For instance, their mechanisms of action are generally different from antibiotics, which makes it more potent against antibiotic resistant strains. Most bacteriocins reported in literature have low oral toxicity and are amenable to bioengineering to improve their stability and function (Cotter et al., 2013). Over the past decade, several bacteriocins have been identified with a broad-spectrum of activity, however only one called Nisin has entered the commercial market for use as a food preservative (de Arauz et al., 2009; Gharsallaoui et al., 2016). While Nisin has been useful in inhibiting several Gram-positive food pathogens like *Clostridium botulinum*, *Bacillus cereus*, *Listeria monocytogenes* and *Staphyloccocus aureus* (Le Lay et al., 2016; Jensen et al., 2020; Martinez et al., 2016), it is limited in its application due to its low stability at neutral and alkaline pH conditions (Rollema et al., 1995). Additionally, a few Nisin-resistant strains have been recently reported which makes it imperative to find new bacteriocins to prevent food spoilage (Draper et al., 2015).

The Gram-negative bacterium, *Pseudomonas aeruginosa* is a good source to identify new antimicrobials as more than 90 % of strains produce at least one bacteriocin (Michel-Briand and Baysse, 2002). The bacteriocins of *Pseudomonas aeruginosa* are called Pyocins and are classified as soluble type (S) or particle/tailocin type (R and F). S-type Pyocins are made up of 2 sub-units: an endonuclease domain with DNase activity and an inhibitor domain that confers the producer strain, immunity to its own Pyocin. The S-type Pyocins resemble the colicins from *Escherichia coli* in their translocation domains and are typically sensitive to heat and protease treatments (Ghequire and De Mot, 2014; Michel-Briand and Baysse, 2002; Scholl, 2017). The R-type Pyocins are high molecular weight particles that resemble the non-flexible and contractile tails of the *Myoviridae* bacteriophages. They kill the cell by creating pores within the cell envelope, resulting in a loss of membrane potential. The F-type Pyocins are high molecular weight particles that resemble the flexible and non-contractile rod like structures of the *Siphoviridae* bacteriophages. While they are typically bactericidal like the S and R type Pyocins, their exact mechanism of action has not been well characterized. Both the R and F type Pyocins are typically resistant to heat and protease treatments (Ghequire and De Mot, 2014; Michel-Briand and Baysse, 2002; Scholl, 2017).

In this study, the antibacterial potential of *Pseudomonas aeruginosa* QDD1 was investigated against foodborne pathogens *Bacillus cereus* and *Staphylococcus aureus*. Upon identifying a potential Pyocin, its mode of action, kinetics of production during the growth cycle, and its sensitivity to different enzymes, chemicals, changing pH and temperature conditions was determined.

## 2. Materials and methods

### 2.1. Micro-organisms and culture conditions

In this study, a local strain of *Pseudomonsa aeruginosa QDD1* previously isolated by Attar et al. (2017) from oil-contaminated soils in Qatar was used for the bacteriocin production. Several indicator strains were used to evaluate the bacteriocin activity. These include reference strains of *Bacillus cereus (strain A* and *strain B), Bacillus thuringiensis (Bt) kurstaki HD1 cryB* (Stahly et al., 1978)*, Bacillus thuringiensis israelensis H14* (Lahkim-Tsror et al., 1983)*, Bacillus thuringiensis israelensis 4Q7* (Jeong et al., 2014)*, Bacillus thuringiensis* strains isolated from Qatar soil QBT 422*,* QBT 625 and QBT 56 (Nair et al., 2018)*,* reference strains of *Staphylococcus aureus, Agrobacterium tumefaciens C58* and *Escherichia coli* Top 10. For long term storage, the bacterial strains were maintained at -80 °C in Luria-Bertani (LB) broth supplemented with 30 % glycerol (*v/v*) and were routinely streaked on 1.5 % LB agar at 30 °C to obtain fresh, viable and pure cells before any experiment. *Agrobacterium tumefaciens* was maintained on yeast extract mannitol agar (YMA) with congo red. LB was prepared by adding Tryptone-water (10 g) (Merck: 110859.0500; Composition: 10 g/L peptone from caesin +5 g/L NaCl), Yeast extract (5 g) + NaCl (5 g) to 1000 mL of deionized water. 15 g of agar was added to the broth for 1.5 % LB agar plates and 0.7 g of agar for 0.7 % LB soft agar plates. The mixture was sterilized by autoclaving at 121 °C for 15 min. Tryptic soy agar (TSA) was prepared using HiMedia: Lot 0000047521 (pancreatic digest of casein (17 g), peptic digest of soyabean meal (3 g), D(+)-Glucose (2.5 g), NaCl (5 g), $K_2HPO_4$ (2.5 g)) + 15 g agar in 1000 mL of deionized water, adjusted to pH 7.3.

### 2.2. Determination of anti-microbial activity of Pseudomonas aeruginosa QDD1

The agar spot-overlay method (Paik et al., 1997) was initially used to determine *Pseudomonas aeruginosa QDD1's* anti-bacterial activity. Briefly, *QDD1* was streaked on 1.5 % LB agar and incubated at 30 °C for 24 h to obtain fresh, isolated colonies. A single colony was then picked and transferred using a sterile toothpick onto another 1.5 % LB agar plate and grown at 30 °C for 12 h. The plates were then overlaid with 4 mL of warm 0.7 % LB agar premixed with $10^7$ log phase cells of the indicator strain. To allow the bacteriocin diffusion into the agar, plates were kept for 24-h at 4 °C. Finally, the plates were placed at 30 °C for 12 h to allow for the growth of the indicator strain after which a zone of clearance was checked around each colony of QDD1. The diameter of this clearance zone was measured. A clear zone around the QDD1 colony indicates that the indicator strain cannot grow in the presence of QDD1 and is potentially inhibited by a bacteriocin produced by QDD1. The indicator strains used in the agar-overlay experiment were *Staphylococcus aureus, Bacillus cereus (strain A* and *strain B), Bacillus thuringiensis (Bt) kurstaki HD1 cryB, Bt. israelensis 4Q7* and *Qatari Bacillus thuringiensis* (QBT) 56.

### 2.3. Effect of temperature and media composition on bacteriocin activity in solid medium

*Pseudomonas aeruginosa QDD1* was streaked on 1.5 % LB agar and grown at 30 °C for 24 h to obtain fresh, isolated colonies. A single colony was then picked and transferred using a sterile toothpick onto 1.5 % Luria-Bertani (LB) agar or Tryptic soy agar (TSA) at varying dilutions: 0.2×, 0.4×, 0.5×, 0.6×, 0.8× and 1× LB/TSA. The plates were then incubated at either 30 °C or 37 °C for 12 h after which they were overlaid with 4 mL of 0.7 % LB agar pre-mixed with $10^7$ log phase cells of the indicator strain *Bacillus thuringiensis* QBT 56. This was followed by a 24-h incubation of the plates at 4 °C after which the indicator strain was allowed to grow for 13 h at 30 °C. Zones of clearance were checked around each colony of QDD1 and its diameter measured. The experiment was duplicated.

### 2.4. Kinetics of bacteriocin production

To detect bacteriocin activity during the growth cycle, *Pseudomonas aeruginosa QDD1* was first streaked on 1.5 % LB agar and incubated at 30 °C for 20 h to obtain fresh, isolated colonies after which a single colony was inoculated in 3 mL of LB broth in a 15 mL falcon tube. This first culture was grown for 2 h in a shaking incubator at 200 rpm, 30 °C. A total of 100 µL of this first culture was then transferred to 3 mL of fresh LB broth and grown for 18 h at 200 rpm, 37 °C. The $OD_{600}$ of this second culture was checked the following day using a UV–Vis spectrophotometer and a part of it was used to inoculate 50 mL of fresh LB broth (0.4× and 1×) in a 250 mL Erlenmeyer flask at a starting OD = 0.05. This third culture was then grown for 48 h in a shaking incubator at 180 rpm, 37 °C. Culture samples were periodically taken every few hours and centrifuged at 14,000 rpm for 20 mins or 5500 rpm for 50 mins and the supernatant was filter sterilized using a 0.2 µm filter (Acrodisc, 40PN4612). The optical density of the growing culture was measured every few hours at absorbance of 600 nm. The cell-free supernatant was

then tested against an indicator strain using the well diffusion method (Kamoun et al., 2005). Briefly, 2.5 % LB agar was overlaid with 4 mL of 0.7 % LB agar pre-mixed with $10^7$ log phase cells of the indicator strain *Bacillus thuringiensis* QBT 56. Wells were then punched in the agar using the end of a sterilized glass pipette after which 180 µL of the filter sterilized supernatant was added into the well. 2×, 5×, 7.5×, 10×, 15× and 20× dilutions of the supernatant was made and added to different wells to see which dilution gives a zone of inhibition. Autoclaved deionized water was used as a control. The plates were then incubated at 4 °C for 4 h to allow for bacteriocin diffusion through the wells after which they were incubated at 30 °C for 12 h to allow for indicator strain growth. After 12 h, zones of inhibitions were noted and the activity unit per mL was calculated by taking the reciprocal of the highest dilution that produced a detectable zone of inhibition divided by the volume of supernatant added into the well (Choeisoongnern et al., 2019). The experiment was replicated twice.

### 2.5. Inhibitory spectrum of the bacteriocin

The antibacterial activity of QDD1 was tested against several Gram-positive and Gram-negative bacteria using spot-on-lawn assay (Batdorj et al., 2006; Hockett and Baltrus, 2017). Briefly, 4 mL of 0.7 % soft agar was mixed with $10^7$ log phase cells of the indicator strain and overlaid over a 1.5 % LB agar plate. YMA agar was used for *Agrobacterium tumefaciens* instead of LB. After cooling, 20 µL of the filter-sterilized supernatant containing 110 AU/mL of the bacteriocin was spotted onto the agar. The plate was then incubated at 4 °C for 2 h after which it was placed at 30 °C for 12 h. The following day, zones of clearances were noted. The indicator strains used include *Bacillus cereus (strain A* and *strain B), Bacillus thuringiensis (Bt) kurstaki HD1 cryB, Bt. israelensis H14, Bt. israelensis 4Q7, Qatari Bacillus thuringiensis* QBT 422, QBT 625, QBT 56, *Staphylococcus aureus, Agrobacterium tumefaciens* and *Escherichia coli* Top 10.

### 2.6. Mode of action of the bacteriocin

To determine the mode of action of the bacteriocin, the cell-free supernatant containing 200 AU of bacteriocin was mixed with $10^4$ log phase cells of QBT 56 in a 10 mM phosphate buffer at pH 7.0. The mixture was gently shaken at 25 °C for 14 h. Samples were periodically removed at hourly intervals to determine the residual viable cell count. Serial dilutions of the samples were plated on LB agar and the colony forming units (CFU) were counted after incubation at 30 °C for 12 h. Triplicate measurements were taken.

### 2.7. Sensitivity of the bacteriocin to heat, pH, enzymes and chemicals

The thermostability of the bacteriocin was studied by exposing the cell-free supernatant containing 75 AU/mL of bacteriocin to varying temperatures (30, 50, 70, 100 °C) for 30 min, (−20 °C) for 20 h, (4 °C) for 2 months and was autoclaved at (121 °C, 15 psi) for 15 min. Similarly, the pH stability of the bacteriocin was studied by incubating 75 AU/mL of bacteriocin in different buffers (66 % *v*/v) at 4 °C for 6 h. The buffers included 10 mM Phosphate buffer (pH 7.0), 50 mM Citrate buffer (pH 3, 4, 5, 6) and 50 mM Tris-HCl buffer (pH 8, 9). The residual activity of the bacteriocin was checked against QBT 56 using well-diffusion assay, as previously described in Section 2.4. The sensitivity of the bacteriocin to enzymes and chemicals was determined by treating the supernatant containing the bacteriocin to Proteinase K (1 mg/mL; Sigma-Aldrich: P6556), α-amylase (1 mg/mL; Sigma-Aldrich: 10065), 8 M urea, 1 % Tween-80 and 1 % β-mercaptoethanol for 4 h at 37 °C. The residual activity of the bacteriocin was then checked against QBT 56 using spot-on-lawn assay, as previously described in Section 2.5. The untreated bacteriocin containing supernatant, the enzyme/chemical preparations and buffer solutions were used as controls. Experiments were repeated twice.

### 2.8. Statistical analysis

The effect of temperature and media composition on antimicrobial activity of *Pseudomonas aeruginosa QDD1* were compared by presenting means of bacterial zone of inhibition at different condition. The kinetics of Pyocin QDD1 Production by *Pseudomonas aeruginosa* QDD1 was presented by calculating growth curve and bacteriocin production (mean ± SD) at different time-points. Mode of action of bacteriocin was determined by calculating cell death in percent (%) at different time intervals after mixing bacteriocin with bacterial culture.

## 3. Results

### 3.1. Determination of anti-bacterial activity and inhibitory spectrum of Pyocin QDD1

The anti-microbial potential of *Pseudomonas aeruginosa strain* QDD1 was determined against the Gram-positive pathogen *Staphylococcus aureus*, using soft-agar-overlay method. *P. aeruginosa* QDD1 produced a clear 25-mm zone of inhibition against *S. aureus* indicating a significant antagonistic activity by a bacteriocin. After determining when the Pyocin was produced in liquid medium (further detailed in Section 3.3), the cell-free supernatant containing the Pyocin was spotted onto agar plates containing other Gram-positive and Gram-negative bacteria as indicator strains. The crude extract containing Pyocin QDD1 inhibited the growth of several Gram-positive bacteria including *Bacillus subtilis,* several strains of *Bacillus thuringiensis* and the foodborne pathogen *Bacillus cereus.* However, Pyocin QDD1 had no effect on Gram-negative bacteria like *Agrobacterium tumefaciens* and *Escherichia coli* (Fig. 1 and Table 1).

As a control, the crude extract of Pyocin was spotted onto a plate containing *Pseudomonas aeruginosa* QDD1 and no inhibition zone was seen as expected. The bacterial strains that were found to be sensitive to

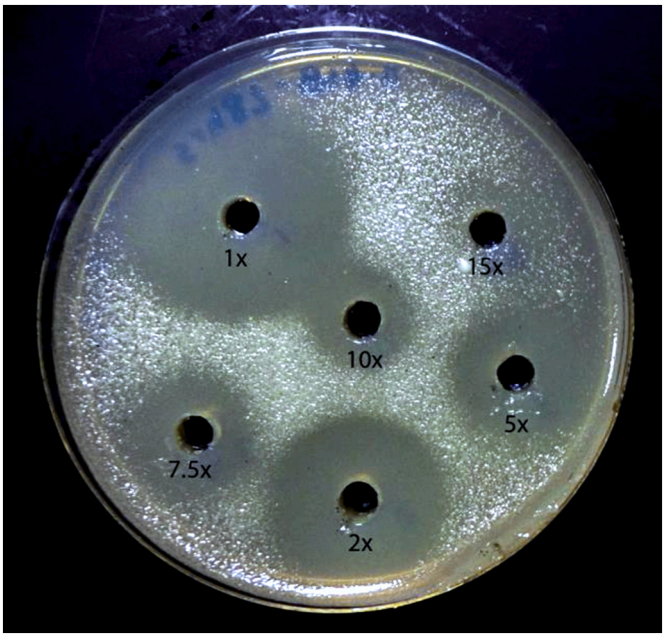


**Fig. 1.** Antimicrobial activity of Pyocin QDD1 against *Bacillus thuringiensis* QBT 56 as determined by well-diffusion assay. Different dilutions (1, 2, 5, 7.5, 10 and 15×) of a cell-free supernatant from a 11 h culture of *Pseudomonas aeruginosa* QDD1 was added into the wells of an LB agar plate overlaid with $10^7$ log-phase QBT 56 indicator cells. After 12 h, zones of inhibitions were noted and the activity unit per mL was calculated by taking the reciprocal of the highest dilution that produced a detectable zone of inhibition divided by the volume of supernatant added. In this image, the highest dilution factor that produced a visible zone of inhibition is 10×.

**Table 1**
Antimicrobial spectrum of Pyocin QDD1 as determined by spot-on-lawn assay.

| Indicator strain | Bacteriocin activity[a] |
|---|---|
| Gram-positive strains | |
| *Staphylococcus aureus* | + |
| *Bacillus cereus strain A* | + |
| *Bacillus cereus strain B* | + |
| *Bacillus subtilis* | + |
| *B. thuringiensis* subsp. *Kurstaki* HD1 cryB | + |
| *B. thuringiensis* subsp. *Israelensis* H14 | + |
| *B. thuringiensis* subsp. *Israelensis* 4Q7 | + |
| *B. thuringiensis* QBT 422 | + |
| *B. thuringiensis* QBT 625 | + |
| *B. thuringiensis* QBT 56 | + |
| Gram-negative strains | |
| *Agrobacterium tumefaciens* | − |
| *Escherichia coli Top 10* | − |

[a] Cell-free supernatant from a 35 h liquid culture of *Pseudomonas aeruginosa QDD1* containing Pyocin QDD1 was spotted on a lawn of indicator cells. The presence or absence of an inhibition zone was noted after 12 h of growth of the indicator strain. (+) indicates the presence of an inhibition zone, i.e., the indicator strain is sensitive to Pyocin QDD1. (−) indicates the absence of an inhibition zone, i.e., the indicator strain is insensitive to Pyocin QDD1.

Pyocin QDD1 will hereby be referred to indicator strains in the following sections.

### 3.2. Effect of temperature and media composition on Pyocin QDD1 production on solid medium

To determine the optimal culture conditions for maximum Pyocin production, *Pseudomonas aeruginosa* QDD1 was grown and tested at different temperatures (30, 37 °C), media compositions (LB, TSB) and media dilutions against indicator strain *B. thuringiensis* QBT 56 on solid medium, using agar-overlay assay. *Pseudomonas aeruginosa* QDD1 produced the lowest amount of Pyocin on 1× TSB agar at 30 °C, as evidenced by the 14-mm zone of inhibition (Table 2). On the other hand, Pyocin production increased almost 2.5× more on 0.2/0.4× LB agar at 37 °C, as evidenced by the 40-mm zone of inhibition against QBT 56, which was highest amount obtained in this experiment. In general, higher and more optimal growth temperature, nutritionally diluted media and simpler media (LB) increased Pyocin production.

### 3.3. Kinetics of Pyocin QDD1 production by Pseudomonas aeruginosa QDD1

In order to determine the time-point of Pyocin production during *Pseudomonas aeruginosa* QDD1 growth, cell-free supernatants were taken from a growing liquid culture of *Pseudomonas aeruginosa* QDD1 every few hours and was tested against a lawn of *B. thuringiesnsis* QBT 56 cells. Bacteriocin production form this strain was estimated using well-diffusion method and activity unit (AU) was calculated from the highest dilution that was able to produce a zone of inhibition against indicator strain - QBT 56. The time course of bacteriocin production and bacterial growth is shown in Fig. 2. Since maximum Pyocin production on solid medium occurred at 37 °C, 0.4× LB (Section 3.2), the same condition was adopted during liquid culture. To ensure that reduced nutrient level would not limit bacteriocin production in liquid medium over extended periods of time, this experiment was repeated in 1× LB medium.

In liquid mediuum, maximum Pyocin production occurred at 1× LB condition, contrasting the previous experiment on solid medium where 0.4× LB condition gave maximum Pyocin production. 110 ± 10 AU/mL of Pyocin activity was achieved in 1× LB broth (Fig. 2A) while only 55 ± 10 AU/mL of Pyocin activity was achieved in 0.4× LB broth (Fig. 2B).

In both cases, Pyocin production started during early exponential level and increased quickly up to stationary phase after which Pyocin production levelled off. However, during the transition from stationary phase to death phase, Pyocin production peaked, almost doubling and then levelled off to a constant value. This suggests that Pyocin QDD1 follows both primary and secondary metabolite kinetics. In 0.4× LB, Pyocin production started after 3 h while in 1× LB, bacteriocin production started after 6 h. Similarly, maximum Pyocin production was achieved by incubating for 24 h in 0.4× LB while maximum bacteriocin production was achieved by 28 h in 1× LB.

**Table 2**
Effect of temperature, medium composition on antimicrobial activity of *Pseudomonas aeruginosa QDD1* Pyocin against *Bacillus thuringiensis* QBT 56 on solid medium.

| Temperature | Type of media[a] | Diameter of zone of inhibition (mm)[b] ± 1 mm | | | | |
|---|---|---|---|---|---|---|
| | | Dilution level of media | | | | |
| | | 0.2× | 0.4× | 0.6× | 0.8× | 1× |
| 30 °C | LB | 30 | 28 | 26 | 20 | 16 |
| | TSA | 23 | 22 | 19 | 16 | 14 |
| 37 °C | LB | 40 | 40 | 38 | 33 | 30 |
| | TSA | 29 | 28 | 27 | 26 | 26 |

[a] LB – Luria Bertani agar, TSA – Tryptic Soy agar.
[b] The diameter of the zone of inhibition produced by *Pseudomonas aeruginosa QDD1* Pyocin on solid agar was measured at different temperatures and media composition. The indicator strain used was *Bacillus thuringiensis* QBT 56. The biggest zone of inhibition at 40 mm was seen on 0.2/0.4× diluted LB agar at 37 °C and the smallest zone of inhibition at 14 mm was seen on 1× TSA at 30 °C.

### 3.4. Mode of action of Pyocin QDD1

In order to determine whether Pyocin QDD1 had a bactericidal or bacteriostatic effect on the indicator strain, the cell-free supernatant from a logarithmic culture of *Pseudomonas aeruginosa* QDD1 was mixed with a *Bacillus thuringiensis* QBT 56 and the remaining viable cell count was determined using CFU/mL measurements every few hours. QBT 56 was selected as the indicator strain because it is closely related to the food pathogen *Bacillus cereus* and also exhibited the highest sensitivity to Pyocin QDD1 in solid medium (Table 1).

The addition of 200 AU/mL of Pyocin QDD1 to ~$10^4$ log phase cells of QBT 56 caused a rapid decrease in the number of QBT 56 cells within the first 5 min. Within 1 h, 40 % of all cell viability was lost, indicating an initial bactericidal mode of action. This was then followed by a very gradual decrease (~10 %) in the number of QBT 56 cells over the next 13 h. No significant change in the number of cells was observed after 13 h, indicating a later bacteriostatic mode of action.Thus, Pyocin QDD1 seems to exhibits an initial bactericidal mode of action followed by a bacteriostatic mode of action against QBT 56 (Fig. 3).

### 3.5. Effect of heat, pH, and enzymes on the anti-bacterial activity of Pyocin QDD1

To determine the biochemical nature of Pyocin QDD1, the cell-free supernatant containing the Pyocin was exposed to varying temperature and pH conditions. Additionally, it was treated with the protease Proteinase K to check for its proteinaceous nature, α-amylase to check for the presence of any carbohydrate moiety, 1 % β-mercaptoethanol to check for the presence of disulfide bonds, 1 % of the surfactant Tween-80 and 8 M Urea, a dissociating agent that can unfold proteins.

Interestingly, Pyocin QDD1 was found to be resistant to all the enzymes and chemicals that it was treated with and retained its antibacterial activity against the indicator strain QBT 56 (Table 3). Additionally, it was found to be extremely stable at pH 3–9 and at all the temperatures (−20 °C to 121 °C) that it was incubated at (Table 4). It retained full activity even after autoclaving it for 15 min at 121 °C, 15 psi.

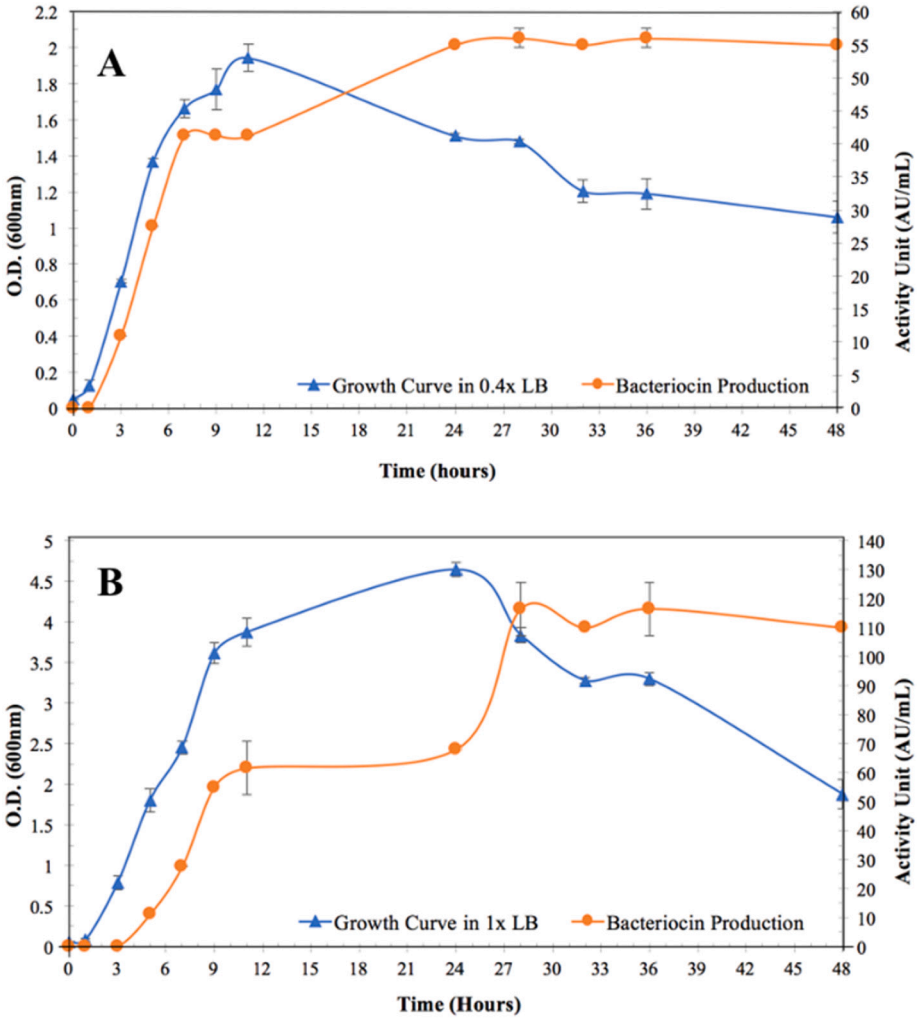


**Fig. 2.** Kinetics of Pyocin QDD1 production during the growth of *Pseudomonas aeruginosa QDD1* in (A) 0.4× LB, (B) 1× LB at 37 °C. Growth of *P. aeruginosa QDD1* (▲) was measured at an optical density (OD) = 600 nm at 1, 3, 5, 7, 9, 11, 24, 28, 32, 36 and 48 h. Bacteriocin production (●) was determined by taking cell-free supernatants periodically and testing it against *Bacillus thuringiensis* QBT 56 using well diffusion assay. Concentration of active Pyocin QDD1 is expressed as AU/mL. The results are presented as mean ± standard deviation from duplicate experiments.

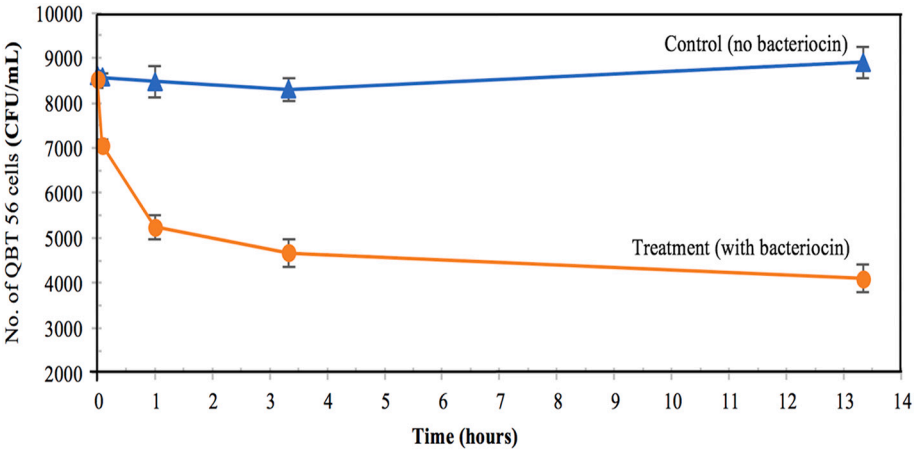


**Fig. 3.** Determination of mode of action of Pyocin QDD1. The cell-free supernatant of a *Pseudomonas aeruginosa QDD1* culture containing 200 AU of Pyocin QDD1 was mixed with ~$10^4$ log phase *Bacillus thuringiensis* QBT 56 cells in 10 mM phosphate buffer at 25 °C. The survival of the indicator strain QBT 56 with time was measured using CFU/mL counts in the absence (▲) and presence (●) of Pyocin QDD1. A bactericidal mode of action was seen at 5 mins and 1 h while a bacteriostatic mode of action was seen at 3 h and 13 h. The results are presented as mean ± standard deviation from triplicate measurements.

## 4. Discussion

In this study, we describe a novel pH and thermostable bacteriocin called Pyocin QDD1 that was produced by *Pseudomonas aeruginosa* QDD1, a strain previously shown by Attar et al. (2017) to have good hydrocarbon degrading potential. Pyocin QDD1 was found to have broad pH stability from pH 3–9, which makes it ideal for food applications as the current and only bacteriocin in the market, Nisin, operates

**Table 3**
Effect of enzymes and chemicals on activity of Pyocin QDD1.

| Treatment[a] | Bacteriocin activity[b] |
|---|---|
| Proteinase K (1 mg/mL) | + |
| α-amylase (1 mg/mL) | + |
| 1 % β-mercaptoethanol | + |
| 1 % Tween 80 | + |
| 8 M Urea | + |

[a] Cell-free supernatant from a 35 h liquid culture of *Pseudomonas aeruginosa QDD1* containing Pyocin QDD1 was treated with different enzymes and chemicals at 37 °C, 5 h after which it was tested against *B. thuringiensis* QBT 56 using spot-on-lawn assay. Untreated cell-free supernatant containing Pyocin QDD1 was used as positive control and the respective enzymes/chemicals as negative control.

[b] (+): Pyocin QDD1 is not affected by treatment and retains activity against *B. thuringiensis* QBT 56; (−): Pyocin QDD1 is affected by treatment and loses activity against *B. thuringiensis* QBT 56.

**Table 4**
Stability of QDD1Pyocin at different temperature and pH.

| Treatment[a] | Duration | Residual bacteriocin activity |
|---|---|---|
| Temperature | | |
| −20 °C | 20 h | 100 % |
| 4 °C | 2 months | 100 % |
| 30 °C – 100 °C | 30 mins | 100 % |
| 121 °C, 15 psi | 15 mins | 100 % |
| pH | | |
| 3.0–9.0, 4 °C | 5 h | 100 % |

[a] Cell-free supernatant from a 35 h liquid culture of *Pseudomonas aeruginosa QDD1* containing 75 AU/mL of Pyocin QDD1 was incubated at different temperatures and in buffers with varying pH after which it was tested against *Bacillus thuringiensis* QBT 56 using well-diffusion assay. Untreated Pyocin QDD1 was used as positive control and respective buffers as negative control.

optimally only at acidic pH conditions (Rollema et al., 1995). Additionally, Pyocin QDD1 was found to be thermostable from −20 °C to 121 °C, and was resistant to autoclaving conditions. This makes it ideal for use as a potential food preservative as food is subjected to several different temperatures during the entire manufacturing processes – from low temperatures during storage to high temperatures during cooking. The endospores of *Bacillus cereus,* a pathogen responsible for thousands of foodborne related outbreaks each year, is resistant to high cooking temperatures (Rodrigo et al., 2021). Thus, Pyocin QDD1's ability to inhibit *Bacillus cereus* growth makes it an attractive candidate for its use as a bio-control agent. Pyocin QDD1 was also found to be resistant to protease degradation. Protease resistance is often seen in circular bacteriocins or those with unusual amino acids (Gabrielsen et al., 2014; Lu et al., 2020; Nishie et al., 2012). Thermal stability and resistance to proteases is generally a characteristic that is seen in the tailocin/particle type Pyocins of *Pseudomonas aeruginosa*, specifically the R and F type Pyocins but not the S-type Pyocins (Michel-Briand and Baysse, 2002; Ghequire and De Mot, 2014; Scholl, 2017). A similar stability to Pyocin QDD1 in terms of resistance to autoclave temperature, enzymes and retention of bioactivity in a broad range of pH was previously reported in Pyocin SA189, a bacteriocin produced by the clinical isolate *Pseudomonas aeruginosa* SA189. However, its structure and type has not been reported yet (Naz et al., 2015). It is worth mentioning that in contrast to the high molecular weight and stable R and F type Pyocins produced by *P. aeruginosa*, a few strains of the *Pseudomonas* genus also produce colicin M like bacteriocins called microcins. Microcins are low molecular weight peptides less than 10 kDa in size that have high thermal and pH stability, and are resistant to protease degradation (Ghequire and De Mot, 2014; Telhig et al., 2020). Thus, it would be interesting to find out in future studies whether Pyocin QDD1 fits into any of the more common bacteriocin types produced by *Pseudomonas aeruginosa* like the R, F and S type Pyocins or the lesser common types like the microcins, lectin-like bacteriocins or any other types (Ghequire et al., 2018).

The vast majority of Pyocins reported to date appear to be produced in response to stressful conditions like DNA damage and oxidative stress (Chang et al., 2005; Ghequire and De Mot, 2014); In laboratory conditions, fluoroquinolone antibiotics, hydrogen peroxide and mutagenic agents like UV and mitomycin C are often used to activate the SOS response in *Pseudomonas aeruginosa* which in turn induces the production of Pyocins (Chang et al., 2005; Ghequire and De Mot, 2014). A few exceptions have been reported in literature. For instance, upon induction by fluoroquinolone antibiotics, strains deficient in XerC tyrosine recombinase produced Pyocins in an SOS independent manner (Baggett et al., 2021). On the other hand, *Pseudomonas aeruginosa* SA189 produced its Pyocin SA189 during exponential phase without any inducing agent in the culture medium (Naz et al., 2015). Expression of most Pyocins is generally heterogeneous across a cell population, with only a few cells producing Pyocins at any given time. This is due to the costly nature associated with Pyocin production as release of most Pyocins typically requires a cell to self-lyse and die (Penterman et al., 2014). However, in contrast to the general trend seen in the production of most Pyocins that have so far been reported, Pyocin QDD1 was constitutively produced at high levels without the use of any inducing agent. The production of Pyocin QDD1 by *Pseudomonas aeruginosa* QDD1 started at early logarithmic phase and increased steadily up to early stationary phase after which it levelled off. However, during the transition of stationary phase to death phase, there was a sudden surge in Pyocin QDD1 production, almost doubling in activity, after which it levelled off again by mid-death phase.

In other species, generally bacteriocin production start during the logarithmic phase and stop by stationary phase. It is not typical to observe a re-surge in bacteriocin production from the transition from stationary to death phase, after production had already stopped by stationary phase. One possibility could be that as low levels of cell lysis start towards the end of stationary phase, quorum sensing molecules are released that communicate the decreasing cell viability to neighbouring cells (Chang et al., 2005). This in turn starts the production of two genes under the same operon. Gene A produces a highly stable activator of bacteriocin production at higher quantities while gene B produces an unstable repressor of bacteriocin production at lower quantities. The activator has a lower threshold amount than the repressor and its sudden release during cell lysis reaches the threshold amount necessary to induce bacteriocin production, thus explaining the surge in bacteriocin production. However, as more cells lyse, there is more release of the repressor which then reaches its threshold level and begins to suppress bacteriocin production. An alternative hypothesis could be that *P. aeruginosa* is not actually re-starting its bacteriocin production but instead releasing molecules during cell-lysis that partially cleave the Pyocin to make it biologically more active. For instance, Pyocin QDD1 may contain a leader region that needs to be cleaved for it to be more biologically active, similar to those found in many lantibiotics (Gabrielsen et al., 2014; McAuliffe et al., 2001). Alternatively, it may be bound to an immunity protein that prevents it from exhibiting its full toxicity effects as seen in most S-type Pyocins (Michel-Briand and Baysse, 2002; Ghequire and De Mot, 2014). Interestingly, a colicin M-type bacteriocin produced by *Pseudomonas aeruginosa* JJ692 called PaeM exhibited 70-fold higher enzymatic activity upon deletion of its N-terminal domain (Barreteau et al., 2012).

It was interesting to note the differing levels of Pyocin QDD1 production in solid and liquid medium. On solid agar, a media lacking nutrients (0.4× LB) enhanced bacteriocin production while in liquid medium, a media enriched with nutrients (1× LB) enhanced bacteriocin production. While 0.4× LB broth resulted in faster bacteriocin production, the net bacteriocin production over extended periods of time were half of that of 1× LB, thus suggesting that the production of Pyocin QDD1 is directly proportional to the number of cells which consequently depends on the level of nutrients present in the broth. Thus, it is likely

that Pyocin QDD1 is a primary metabolite.

Pyocin QDD1 seemed to exhibit both a bactericidal and bacteriostatic mode of action against the indicator strain *Bacillus thuringiensis* QBT 56. Specifically, an initial bactericidal effect caused a 40 % decrease in cell viability in QBT 56 by the 1st hour. However, only 10 % of cells died within the next 13 h, indicating a majorly bacteriostatic effect by Pyocin QDD1. This sudden appearance of a bacteriostatic effect might be due to the resistance gained by QBT 56 cells towards the Pyocin. DNA damage and antibiotic induced cell lysis has been previously shown to generate persister cells and population wide tolerance to antimicrobials (Podlesek et al., 2016; Podlesek and Žgur Bertok, 2020); The initial bactericidal mode of action by QDD1 might have caused many cells to lyse, releasing their nutrients and molecules that would signal nearby cells of this rapid loss in cell-viability of its species. QBT 56 might have then responded to these signalling molecules by transcribing a new set of genes that entailed it protection from the bacteriocin.

It is also possible that QBT 56 cells in an environment lacking nutrients (phosphate buffer) becomes insensitive to Pyocin QDD1. Bacterial absorption of the bacteriocin is affected by the surrounding environment. For instance, organic solvents, detergents and ions can modify specific receptors that are targeted by the bacteriocin. Cells that were starved of for carbon and nitrogen became insensitive to Pyocin AP41 (Sano and Kageyama, 1981). Interestingly, a colicin M type bacteriocin produced by *Pseudomonas aeruginosa* JJ692 called PaeM was found to be bacteriostatic towards other *Pseudomonas aeruginosa* strains by degrading lipid II intermediates involved in cell wall peptidoglycan synthesis in the periplasm and thus arresting cell division. It had no cytotoxic effect against *Escherichia coli* strains. However, a truncated version of this toxin containing only its C-terminal half was able to kill *E. coli* cells, indicating that maturation of the bacteriocin might be required to convert it from bacteriostatic to bactericidal (Barreteau et al., 2012).

While Pyocin QDD1 did not inhibit Gram-negative bacteria like *Escherichia coli* and *Agrobacterium tumefaciens*, it had good inhibitory activity against several gram-positive bacteria like *Bacillus thuringiensis*, *Bacillus subtilis*, the foodborne pathogen *Bacillus cereus* and *Staphylococcus aureus*, making it a promising antimicrobial agent for food preservation and human health. Future work should be also on the characterization and composition of Pyocin QDD1, and the main contributor(s) of its properties and functions.

## 5. Conclusion

This study presents a thermostable and pH stable bacteriocin produced by *Pseudomonas aeruginosa* QDD1 with a promising inhibitory activity against the foodborne pathogens *Staphylococcus aureus* and *Bacillus cereus*. Production of this bacteriocin was autoregulated and occurred during exponential phase and during the transition from stationary to death phase. It displayed both bactericidal and bacteriostatic modes of action of against the indicator strain *Bacillus thuringiensis* QBT 56. Future work will focus on the safety aspects of this bacteriocin, its inhibitory activity and mode of action on a higher variety of Gram-positive and Gram-negative strains.

## CRediT authorship contribution statement

MD., and S.J.: Conceptualization; MD., and S.J.: Methodology; MD., K.N., Z.U.and S.J.: Validation and analysis of results; S.J: Resources provided; MD., Z.U., and S.J.: Writing and reviewing; S.J.: Supervision.

## Declaration of competing interest

Then authors declare that there is no conflict of interest regarding this manuscript submitted for publication in Bioresource Technology Reports.

Then authors also declare that this manuscript has neither been published elsewhere, nor has been simultaneously submitted elsewhere for publication.

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