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Electrochemical Detection of *Pseudomonas aeruginosa* in Polymicrobial Environments

MASTER'S DEGREE FINAL PROJECT

Supervised by Dr. Edgar D. Goluch

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Abstract. A major cause of death among hospitalized patients results from infections acquired in the hospital setting, with *Pseudomonas aeruginosa* being one of the most prevalent hospital-acquired bacterial pathogens. This bacterium produces a unique, redox-active molecule known as pyocyanin. Because pyocyanin can be detected using electrochemistry, it serves as a useful biomarker for identification and detection of this opportunistic pathogen. While previous research has electrochemically detected *P. aeruginosa*'s production of pyocyanin in complex media environments, this study addresses the need to understand how *P. aeruginosa* behaves when co-cultured with other bacterial pathogens by electrochemically monitoring *P. aeruginosa's* production of pyocyanin in polymicrobial samples. Liquid cultures of the most common clinically-relevant bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*) were grown overnight at 37 °C in lysogeny broth or trypticase soy broth growth media. From these stock cultures, different polymicrobial combinations were tested. Production of pyocyanin was monitored every hour for the first 24 hours and then every 6 hours afterwards for a total of 3 days. The results from this study demonstrate that *P. aeruginosa* produces pyocyanin at similar rates, regardless if other bacterial pathogens are present, aiding in development of this sensing platform for clinical point-of-care diagnostics.

Introduction

An infection is an invasion of microorganisms not naturally found in the human body. Bacteria, viruses, and parasites are among the various infectious agents that can cause disease in humans. While the vast majority of microorganisms do not successfully infect healthy individuals, humans with immunocompromised systems are at a higher risk for disease and death. Microbial infections contribute to more deaths worldwide than any other single cause. According to experts, it is estimated that the annual cost of medical care for treating infectious diseases in the United States alone is approximately $120 billion [1].

Infections acquired in healthcare facilities during medical treatment, also referred to as

nosocomial infections, impact 1 in 25 hospital patients [2]. The most common infection sites include surgical wounds (28.1% of all infections), pneumonia (21.8%), gastrointestinal infections (17.1%), urinary tract infections (12.9%), and primary bloodstream infections (9.9%) [2]. Wounds are often colonized by multiple microorganisms and when not treated in a timely manner, patients can experience increased trauma, leading to poorer patient outcomes, and as a direct result, higher treatment costs [3].

Pseudomonas aeruginosa, *staphylococcus aureus*, coagulase-negative *staphylococci*, *Escherichia coli*, *Enterobacter* spp., and *Enterococcus* spp., are the primary pathogens responsible for delayed healing and infection in acute and chronic wounds, particularly in surgical wounds [4]. An analysis of 108 post-surgical wounds concluded that on average, a patient stayed an additional 10.2 days in the hospital due to wound infection and that the associated hospital cost was $3,937 per infected patient [5].

Pseudomonas aeruginosa, one of the most common opportunistic pathogens in nosocomial infections, is a rod-shaped aerobic gram-negative bacterium measuring 0.5-0.8 μm wide and 1.5-3.0 μm long. *Pseudomonas* can be found in soil, water, and vegetation as well as isolated from the skin and throat of a healthy individual. Because *P. aeruginosa* can additionally be found in medical environments, such as on sinks, taps, and respiratory equipment [6], it poses a specific risk to hospitalized patients whose immune systems may not be strong enough to fight off infection. *P. aeruginosa* is frequently linked to patients with cystic fibrosis [7], severe burn victims [8], and immunocompromised hosts such as patients with AIDS [9]. This pathogen is responsible for 30% of deaths related to pneumonia and septicemia and 38% of deaths related to ventilator-associated pneumonia in intubated patients [10-12].

P. aeruginosa synthesizes different aromatic, tricyclic compounds known as phenazines. Figure 1 shows the phenazine biosynthesis pathway for *P. aeruginosa*. Derivatives of phenaxine-1-carboxylic acid (PCA) include phenazine-1-carboxamide, 1-hydroxy-phenazine, and pyocyanin [13].

Pyocyanin is a blue redox-active secondary metabolite produced by *P. aeruginosa*. It is recovered in large quantities in sputum from patients with cystic fibrosis who are infected by this opportunistic pathogen. Pyocyanin is a water-soluble pigment, acting as both a virulence factor and a quorum sensing

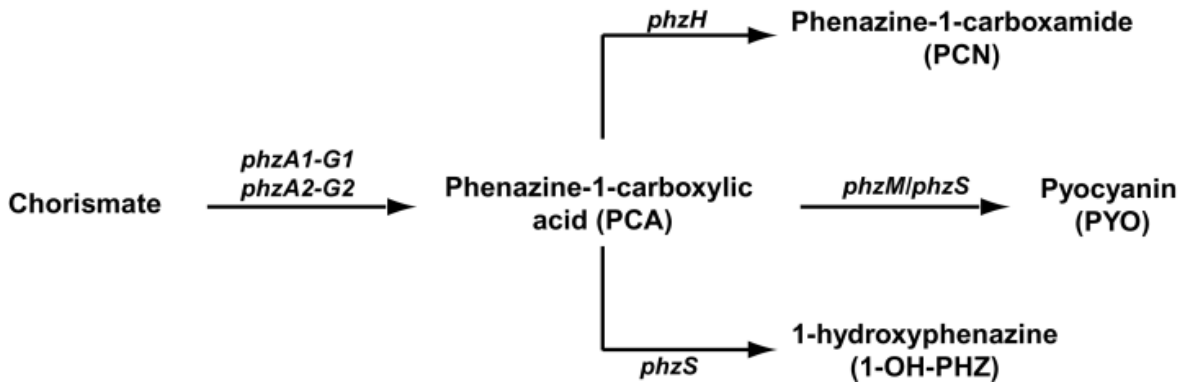

Figure 1. Phenazine biosynthesis in *P. aeruginosa* [13].

molecule during wound colonization [14]. Because of its redox-active activity, pyocyanin be detected using electrochemistry [15-16]. It is a useful biomarker for *P. aeruginosa* identification and detection because no other bacterium is known to produce this molecule.

In a study performed with 676 surgery patients with signs of wound infections, it was observed that the common pathogens were *Staphylococcus aureus* (28.2%), *Pseudomonas aeruginosa* (25.2%), *Escherichia coli* (7.1%), *Staphylococcus epidermidis* (7.1%), and *Enterococcus faecalis* (5.6%) [17].

Infections caused by gram-negative bacteria are relevant because they are highly efficient at acquiring genes that code for mechanisms of antibiotic drug resistance [18]

Previous work in this area has shown that electrochemical sensors can be used to rapidly detect *P. aeruginosa*'s production of pyocyanin in complex media environments [15-16]. However, no previous work has been done to investigate how this opportunistic pathogen behaves when it is cultured alongside other bacteria in the same media environment.

The aim of this project is to investigate whether *P. aeruginosa*'s production of pyocyanin changes when it is cultured alongside other bacterial pathogens. This research project will lead to a better understanding of pyocyanin production rates by *P. aeruginosa* in polymicrobial samples. In addition, the data from this study will help elucidate the value of measuring pyocyanin concentrations in point-of-care applications, leading to improved patient care and reduced hospital expenditures.

Materials and methods

Materials

Pseudomonas aeruginosa (PA14), *Staphylococcus aureus* (RN4220), *Staphylococcus epidermidis* (RP62A), *Enterococcus faecalis* (ATCC 29212) and *Escherichia coli* (DH5α m-cherry) were grown overnight at 37 °C and 150 rpm, in both lysogeny broth (LB) (BD 244620) and trypticase soy broth (TSB) (BD 211768) growth media.

Electrochemical measurements were done using disposable, screen-printed electrode sensors (Zensor TE100) containing carbon working and counter electrodes and a Ag/AgCl reference (Figure 2). Electrochemical measurements were controlled and recorded with a potentiostat (CHI842C, CH Instruments).

Cell cultures

Stock cell cultures were prepared in 3 mL of LB and TSB growth media. From these stock cultures, different polymicrobial combinations (SI) were inoculated into fresh media, starting with a single species co-cultured with *P. aeruginosa*. Combinations of two bacterial pathogens were tested with *P. aeruginosa*, including a final sample that contained all five bacterial species. For each test, 100 µL of sample was placed onto a sensor and scanned from -0.5 to 0.2 V using square-wave voltammetry at a frequency of 15 Hz

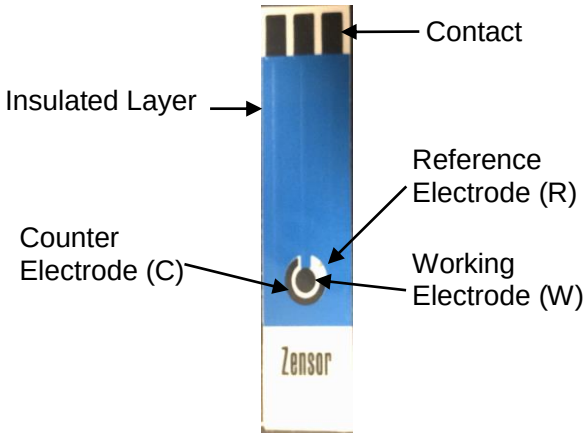

Figure 2. Zensor TE100.

and an amplitude voltage of 0.05 V to detect *P. aeruginosa*'s production of pyocyanin. Measurements were recorded every hour for the first 24 hours and then every 6 hours for 2 days; resulting in 3 days of measurements. The data were analyzed using OriginPro 9.1 (OriginLab) using baseline-subtraction to determine the peak current that is observed due to pyocyanin oxidation. It is expected that pyocyanin oxidizes at -0.25 V vs. a Ag/AgCl reference electrode [19]**.** These peak currents can be correlated to the concentration of pyocyanin in the sample using a calibration curve made from known pyocyanin standards [SI1]

To correlate pyocyanin concentrations to cell densities, cell counts were performed using a hemocytometer (Hausser Scientific Modell 3500) for each sample at 6-hour intervals.

Results and discussion

Liquid cultures of *P. aeruginosa* combined with different bacterial pathogens were grown in either LB or TSB media to investigate whether *P. aeruginosa*'s production of pyocyanin changes when it is cultured alongside other bacterial pathogens. Figure 3. shows the concentration of pyocyanin over time for *P. aeruginosa* cultured alongside other bacteria in (a, c) LB or (b, d) TSB growth media. A control sample with *P. aeruginosa* alone was also tested in each medium. From the data presented in Figure 3. (a, c) a significantly higher concentration of pyocyanin is observed in the LB media vs. TSB media, which can partially be attributed to the lower cell densities that are observed between both media (e, f). From the data, we also observe that when *P. aeruginosa* is cultured alongside other

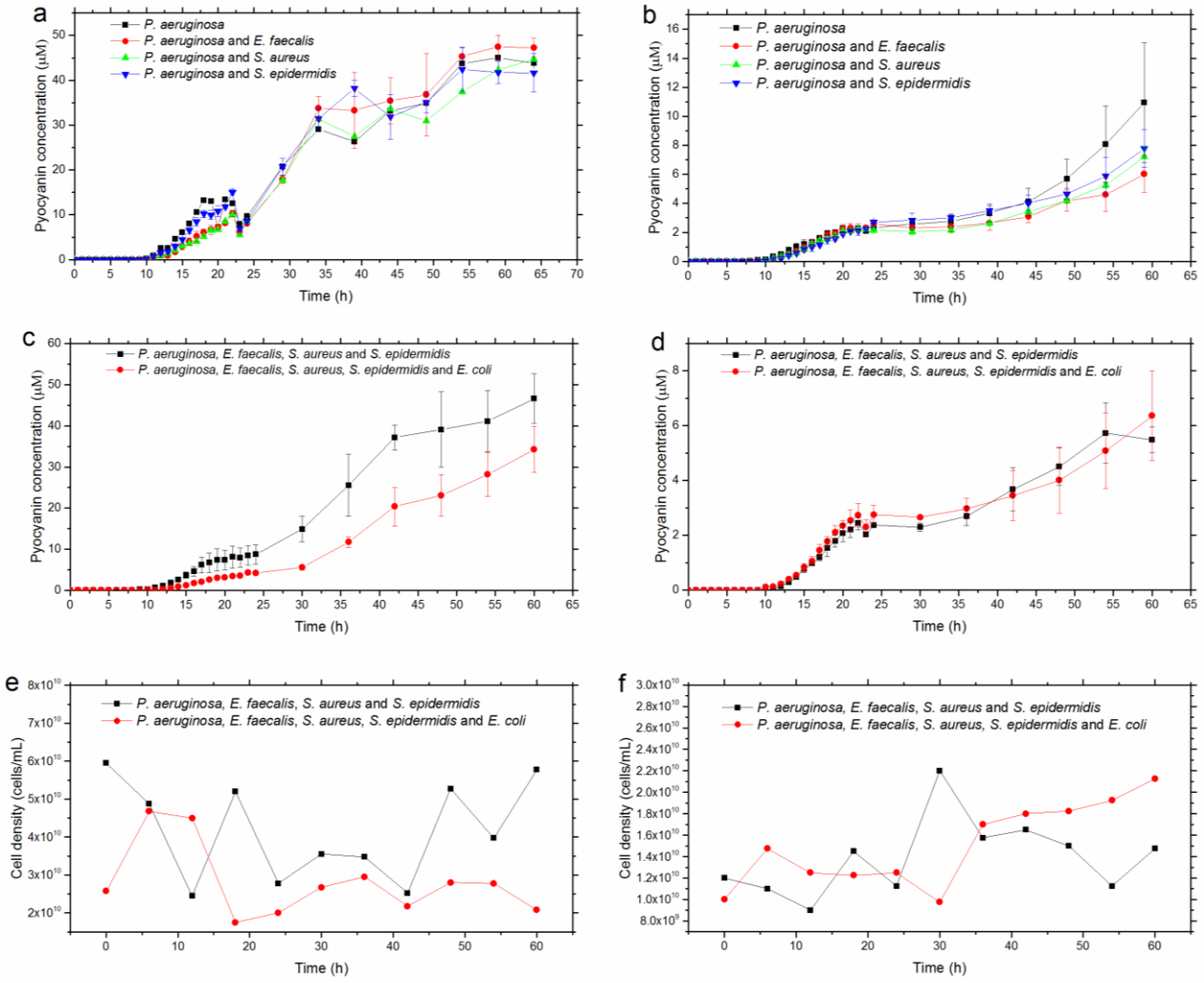

Figure 3. Monitoring pyocyanin concentration over time of different polymicrobial co-cultures for 3 days in (a,c) LB or (b,d) TSB media. Bacterial cell density measurements were taken over time in (e) LB and (f) TSB.

bacteria, the increase of pyocyanin during the first 24 hours follows a sigmoidal curve, which corresponds to the lag phase and exponential phase of bacterial growth. After that time, the curves have different structures for each media. In LB media (Figure 3. a and c), the pyocyanin concentration increases at a faster rate than when the bacteria are cultured in TSB media (Figure 3. b and d), which increases more gradually. This may be due to the different components found in each medium, where LB is a nutrient-rich medium that allows rapid and robust growth of specific bacteria, whereas TSB is a general-purpose medium with enough nutrients to allow the growth of a wide variety of microorganisms. With a higher nutrient base medium, the bacteria have to compete against each other to grow and therefore, *P. aeruginosa* will produce pyocyanin in higher concentration in LB.

Based on the cell counts (Figure 3. e and f), the total concentration of bacteria does not increase over time. This can be attributed to the pyocyanin produced by *P. aeruginosa*, as it has been shown that pyocyanin is able to kill mammalian and bacterial cells through the generation of reactive oxygen intermediates [20]. Therefore, the overall uniform cell densities that are observed throughout the experiment may be due to pyocyanin disrupting the growth of other pathogens in the media, resulting only in *P. aeruginosa* growth. It is observed that in TSB media (Figure 3. f), the total concentration of bacteria is lower than in LB media (Figure 3. e).

Differences between pyocyanin concentrations in the same media (Figure 3. c) may be due to the difference in initial bacterial concentration (Figure 3. e). This indicates that the initial bacterial concentration of *P. aeruginosa* plays a role in pyocyanin production rates, which is in agreement with previous research [15].

Overall, the relative production rate of pyocyanin is not affected by the bacterial pathogens in the media but is influenced by the type of media in which the bacteria grows.

Conclusions

Pseudomonas aeruginosa's production of pyocyanin was electrochemically monitored in polymicrobial samples containing different bacterial pathogens and media types. This study shows that *P. aeruginosa* produces pyocyanin at similar rates, regardless if other bacterial pathogens are present. Although the total concentration of bacteria does not increase over time, the initial concentration

may influence the concentration of pyocyanin produced by *P. aeruginosa*.

This data provides useful information supporting the use of electrochemical sensors as a point-of-care diagnostic for *P. aeruginosa* in hospital-associated infections that often contain multiple bacterial pathogens.

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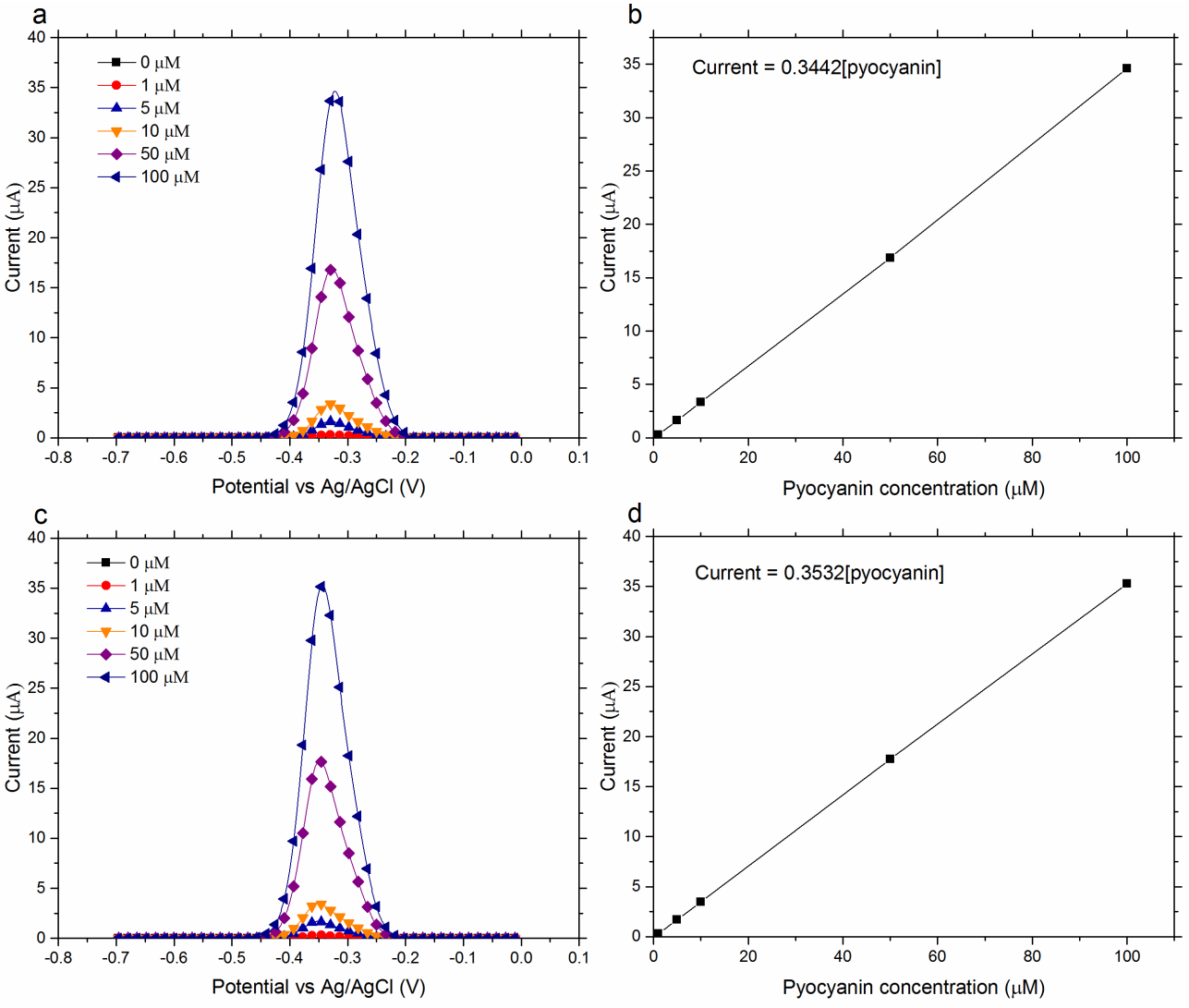
Supplementary information

The bacterial combinations used were:

1. *P. aeruginosa*
2. *P. aeruginosa* and *E. faecalis*
3. *P. aeruginosa* and *S. aureus*
4. *P. aeruginosa* and *S. epidermidis*
5. *P. aeruginosa, E. faecalis, S. aureus* and *S. epidermidis*
6. *P. aeruginosa, E. faecalis, S. aureus, S. epidermidis* and *E. coli*



SI1. Pyocyanin calibration curves in (a) LB and (c) TSB and linear dynamic range of the sensors from 0 to 100 µM in (b) LB and (d) TSB.