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Exposure To Pyocyanin Promotes Cellular Changes In *Candida* spp.

Tarcísio Tarcio Corrêa Bonifácio, Ray Ravilly Alves Arruda, Bianca Teixeira Morais de Oliveira, Jonas Emanuel Guimarães da Silva, and Ulrich Vasconcelos*.

LAMA- Laboratório de Microbiologia Ambiental, CBIOTEC, Rua Ipê Amarelo, UFPB, Campus I, CEP – 58051-900, João Pessoa-PB, Brazil.

ABSTRACT

This study evaluated the cellular changes promoted by pyocyanin exposure among *Candida albicans* ATCC 76485, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 13803 and *C. krusei* ATCC 6258. The Minimum Inhibitory (MIC) and Minimum Fungicide concentrations (MFC) of pyocyanin were identified in the strains. Then tests were carried out to determine the action of the pyocyanin on the cell wall, plasmatic membrane, cell viability and in the formation of biofilm. The pyocyanin MIC was 600 µg/mL for all strains tested, while the MFC was 1200 µg/mL for *C. albicans* ATCC 76485, *C. parapsilosis* ATCC 22019 and > 1200 µg/mL for *C. tropicalis* ATCC 13803 and *C. krusei* ATCC 6258. There was damage to the cell wall, without compromising the plasmatic membrane. In addition, a significant reduction in cell viability was observed after 24 hours of exposure to subinhibitory and inhibitory concentrations of pyocyanin, revealing greater sensitivity of *C. krusei* ATCC 6258. Pyocyanin altered yeast adhesion, while showing no activity in mature biofilm. The findings suggest that pyocyanin is more active against planktonic forms of *Candida* spp.

Keywords: pyocyanin, bioactive pigments, antifungal agents, biofilm.

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Corresponding author

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INTRODUCTION

Pyocyanin is a bright blue nitrogen-containing aromatic compound synthesized exclusively by 95% of *Pseudomonas aeruginosa* strains.[1] The pigment participates in the reduction of Fe^{3+}, essential for its growth.[2] Pyocyanin is attributed the ability to generate reactive oxygen species (ROS) in other organisms.[3] In addition, ROS formation is described as the main mechanism involved in the antifungal properties of pyocyanin.[4]

The genus *Candida* (phylum Ascomycota) is composed of about 200 species, some of which are of interest due to the nature of an opportunistic pathogen, in particular *C. albicans*, *C. parapsilosis, C. tropicalis* and *C. krusei*.[5] It is known that *Candida* ssp. are susceptible to pyocyanin and the mechanisms are most efficiently explored using C*. albicans*. In this species, pyocyanin acts to inhibit the development of hyphae[6], in addition to interfering with the production of cyclic adenosine monophosphate (cAMP), resulting in severe damage to irreversible adhesion, cell aggregation and biofilm formation, as well as increasing exposure of the cell to pyocyanin.[7]

The search for biodegradable bioactive natural compounds is an important research tool for the design of new antimicrobials.[8] On the other hand, natural bioactive compounds with a history of success in past therapeutic use and currently in disuse can be reviewed and tested for new applications in the context of antimicrobials, for which pyocyanin has great potential. [9-10]

There are some studies which have focused on molecular mechanisms and blockage of cellular signalling related to pyocyanin activity in certain microbes, including yeasts.[11-12] To our knowledge, no studies have been published on the identification of possible targets of pyocyanin, located in the cellular framework, since an injured cell provides pigment entry routes, intracellular damage, disturbance of biofilm formation and perhaps changes in vegetative cell morphology. In view of this, this work assessed the exposure of four strains of *Candida* spp. to pyocyanin, evaluating the effect of this exposure on the integrity of the cell wall and its plasmatic membrane, on cell viability and on the formation of biofilms.

MATERIAL AND METODS

Microorganisms

Four strains of *Candida* spp.: *C. albicans* ATCC 76485, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 13803 and *C. krusei* ATCC 6258 were used in this study.

Obtaining and quantifying pyocyanin

In order to obtain pyocyanin in sufficient amounts, *P. aeruginosa* TGC04 was used, collected from hydrocarbon-contaminated soil in the Metropolitan Region of João Pessoa, Brazil[13] and registered with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (# AA1970F). To stimulate pigment production, 1 ml of suspension TGC04 from fresh culture was added to flasks containing 200 ml of Pseudomonas P broth.[14] The suspension was prepared in 0.85% NaCl, with standard turbidity using the No. 1 tube on the MacFarland scale. The incubation took place for 72 hours under agitation of 150 rpm at 29±1°C.[15]

The pigment was extracted from a 30 ml sample of the growth medium, containing the developed *P. aeruginosa* TGC04 cells and the dissolved pigment. After centrifugation at 10,000 rpm for 15 minutes, the supernatant was transferred to a separating funnel and mixed with chloroform in a ratio of 2:1. After 1 minute of vigorous agitation and a rest for 10 minutes, the chloroform phase (blue color) was removed and mixed with 20 mL of 0.2 mol/L HCl (red color). After another 10 minutes, the solution was neutralized with a Tris-Base buffer solution (blue color). After 1h, the concentration of pyocyanin was determined by measuring the optical density of the neutralized solution (690 nm).[16]

Determination of the Minimum Inhibitory Concentration (MIC) of pyocyanin

MIC was determined by microdilution using adaptation of protocol M27-A3.[17] Briefly, each well on each microdilution plate was injected with a double concentration of 100 µL of 2% Sabouraud Dextrose Broth

(SDB). Then, 100 µL of the pyocyanin solution was added distributed serially in a ratio 1:2 in order to obtain concentrations ranging from 1200 to 18.75 µg/mL. Afterwards, 10 µL of *Candida* spp. suspensions (10^6 cells/mL) were added to the each well. Two controls were tested: one, the broth and the other, the action of nystatin in concentrations from 36 and 9.2 µg/mL.[18] The plates were sealed and incubated at 35±2°C for 48 hours. MIC was defined as the lowest concentration able to inhibit fungal growth, observed by visual inspection.[19]

Determination of the Minimum Fungicidal Concentration (MFC) of pyocyanin

MFC was determined by the pour plate technique.[20] After determining the MIC, the content of the wells was homogenized by pipetting. Then, 10 µl from each well was transferred to Petri dishes containing Sabouraud-Dextrose agar 2% (SDA). The plates were incubated at 35±2°C for the 48 hours. MFC was defined as the lowest concentration of pyocyanin that inhibited the growth of any yeast colony. Control testing on the medium and growth of *Candida* spp. in the absence of pyocyanin was carried out

***In vitro* test of the action of pyocyanin on the cell wall**

A sorbitol test was carried out using the microdilution method.[21] Two rows on each microdilution plate had its wells filled with 100 µL of SDB or the same volume of SDB supplemented with sorbitol 0.8 mol/L. Then, pyocyanin was added in concentrations equivalent to the known MIC and MFC values together with 10 µL of the *Candida* spp suspension. (10^6 cells/mL). The microdilution plates were incubated at 35±2°C for 48 hours. The action of pyocyanin on the cell wall was detected when the MIC or MFC values increased, since sorbitol acts as an osmotic protector. Sterility controls were performed in wells with and without sorbitol in the absence of pyocyanin.

***In vitro* assay of the action of pyocyanin on the plasmatic membrane**

The protocol was followed as previously described, substituting the sorbitol with a 400 µg/mL ergosterol supplemented to the SDB.[22] The microdilution plates were incubated at 35±2°C for 48 hours and the action of pyocyanin on the plasmatic membrane was detected when the known values of MIC or MFC increased. Sterility controls were performed in wells with and without ergosterol in the absence of pyocyanin.

Pyocyanin cytotoxicity assay

The colorimetric method with 3-(4,5-dimethylthiaxolone-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used.[23] The wells of the microdilution plates were filled with 200 µl of YPD2% broth and 100 µl of pyocyanin solutions corresponding to the MIC and MIC÷2 values. Then, 10 µl of the suspension of each *Candida* spp. (turbidity standardized with tube No. 0.5 on the McFarland scale) was injected into each well. After 48 h of incubation at 35±2°C, the yeast growth was interrupted. The microdilution plates were subjected to centrifugation for 5 min (1500 rpm at 4°C) and the supernatant was carefully aspirated and discarded and the cells were rinsed with sterile 0.85% NaCl. This procedure was repeated three times. Then, 200 µL of MTT (0.5 mg/mL) was added to the wells and the microdilution plates were incubated at 35°C for 3h. Afterwards, the supernatant was aspirated from the wells, and 200 µL of the extraction reagent added (SDS 10 g, dimethylformamide 50 mL and sterile distilled water 50 mL, pH 4.7, adjusted with acetic acid solution). The microdilution plates were again incubated at 35°C for 15 minutes and then shaken for 5 minutes to complete the dissolution of the formazan crystals. Absorbance was measured at 570 nm. The positive and negative controls of the test were inoculation of *Candida* spp. in YPD2% (positive) and 32 µg/mL of nystatin added to the broth (negative).

Action of pyocyanin in disturbing the formation of the biofilm of *Candida* spp.

Two conditions were evaluated: interference in adhesion and the action of pyocyanin on the mature biofilm. In the first test, wells in the microdilution plates were filled with 100 µl of YPD2% and 100 µl of inoculum (10^6 cells/mL). After incubation for 90 minutes (75 rpm at 35°C±2°C), pyocyanin was added at the known concentrations of MIC and MIC÷2 and incubated again under the same conditions.[24] Then, the liquid phase was discarded and the wells were washed 3-5 times with distilled water, to remove any deposited planktonic cells. After these were dried, 200 µL of 1% violet crystal was transferred to the wells and after 20 minutes the dye was

discarded and the excess was removed with distilled water. After a second drying, 200 µL of P.A. ethanol was added and 30 minutes later the absorbance at 570 nm of the content in the wells was measured.[25]

The test of pyocyanin activity in the mature biofilm started as described above, with the incubation period extended to 24h. Afterwards, the pyocyanin in the known concentrations of MIC and MIC÷2 was added and the microdilution plates incubated for another 24 hours. Then the violet crystal test was conducted as described previously. The test control considered yeast growth as YPD2%.

Statistical analysis

All tests were performed in triplicate. The tests for determining MIC, MFC and pyocyanin activity on the cell wall and plasmatic membrane were expressed by the geometric mean of the results. In the cytotoxicity and biofilm assays, results were expressed as mean±standard error. Additionally, Dunnett's analysis was used to determine significant differences, when p <0.05.

RESULTS

Antimicrobial activity and action of pyocyanin on cell wall and plasmatic membrane

Pyocyanin activity was observed against yeast strains (Table 1). The MIC was similar for all, 600 µg/mL. The lethal concentration of pyocyanin was only possible to determine for *C. albicans* ATCC 76485 and *C. parapsilosis* ATCC 22019, since the limit test concentration was 1200 µg/m. This suggests the CFM for *C. tropicalis* ATCC 13803 and *C. krusei* ATCC 6258 should be higher than this concentration. In addition, MIC increased in the sorbitol test and remained the same in the ergosterol test, indicating that pyocyanin promoted cell wall damage.

Table 1: MIC, MFC and action of pyocyanin in the cell wall and plasmatic membrane of *Candida* spp.

Tests	Strains			
	C. albicans ATCC 76485	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 13803	*C. krusei* ATCC 6258
MIC (µg/mL)	600	600	600	600
MFC (µg/mL)	1200	1200	>1200	>1200
NYS (µg/mL)	>36	>36	>36	>36
SORB (0.8 mol/L)*	>1200	>1200	>1200	>1200
SORB (0.0 mol/L)*	600	600	600	600
ERG (400 µg/mL)*	600	600	600	600
ERG (0 µg/mL)*	600	600	600	600
Control (SDB)	+	+	+	+
Control (YSP2%)	+	+	+	+

* considered MIC = 600 µg/mL, (+) growth; MIC – Minimum Inhibitory Concentration, MFC – Minimum Fungicide Concentration, NYS – nystatin, SORB – Sorbitol, ERG – ergosterol,

Pyocyanin cytotoxicity

Important metabolic reductions were observed in the four strains tested (Fig. 1). Compared to the control group (untreated), the cell viability percentages were more significant when exposed to 600 µg/mL (MIC) than 300 µg/mL of pyocyanin. In the subinhibitory concentration, the cell viability percentages were 49.73 and 55.29% for *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, respectively. However, *C. albicans* ATCC 76485 and *C. tropicalis* ATCC 13803 did not show significant low viability for this treatment. On the other hand, in the inhibitory concentration of pyocyanin, the values were significantly reduced, as follows: 13.62 (*C. tropicalis* ATCC 13803), 10.83 (*C. parapsilosis* ATCC 22019), 7.69 (*C. albicans* ATCC 76485) and 2.19% (*C. krusei* ATCC 6258). In addition, there was no significant positive or negative change in cell viability percentages, compared to the control group.

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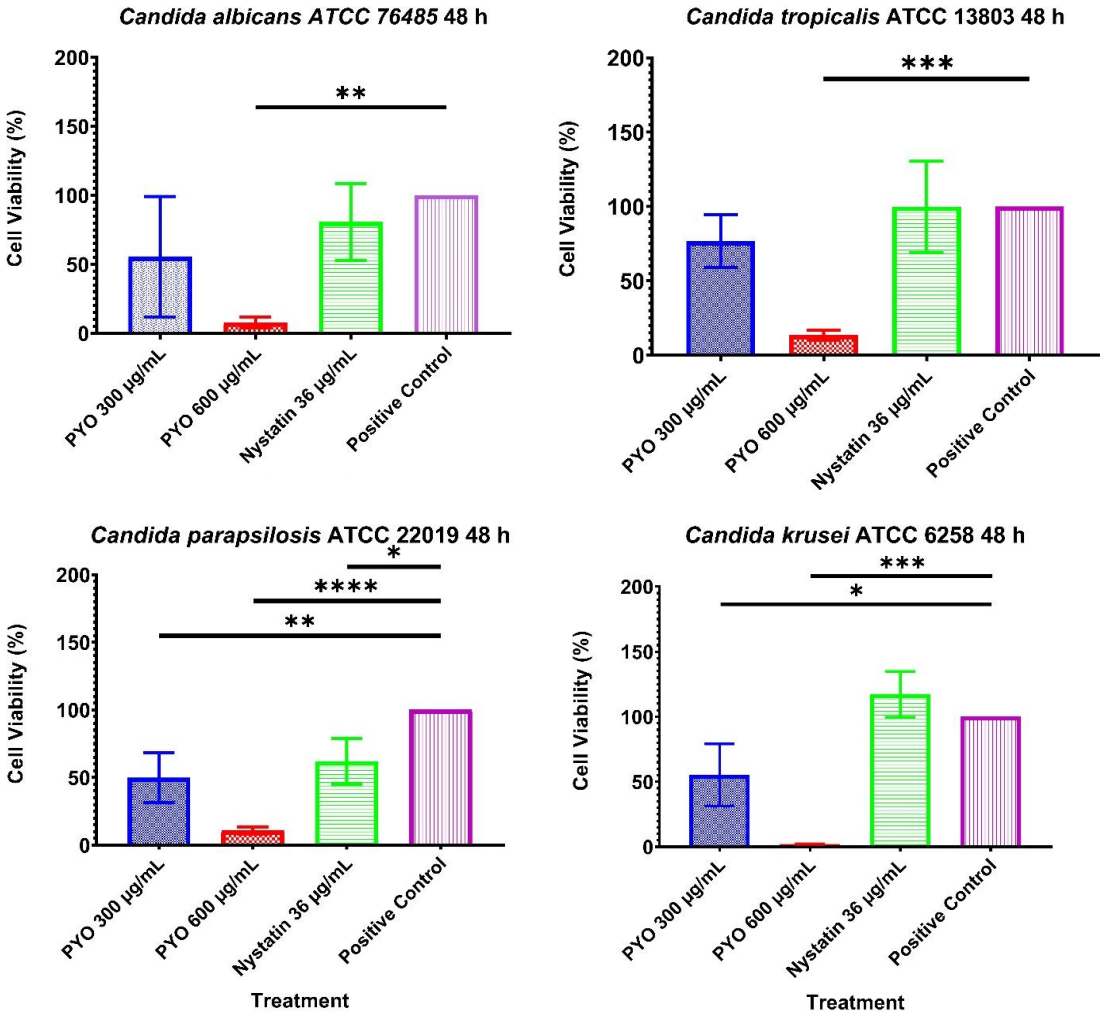
Fig. 1: Cellular percentage viability (48h) of *Candida* spp. exposed to pyocyanin (PYO). Significant differences in control are indicated with the asterisk (* p <0.05, ** p <0.01, *** p <0.005 and **** p <0.005).



Action of pyocyanin in the biofilm disturbance of *Candida* spp. Strains

There was a reduction in adherence only in *C. tropicalis* ATCC 13803. Interestingly, in the other strains, a significant increase in absorbance values was observed, compared to control (untreated), with no differences between the two treatments, except for the condition of exposure to 600 µg/mL of pyocyanin for *C. krusei* ATCC 6258. On the other hand, there was no disturbance of the biofilm when already established for all *Candida* spp. tested after 24 hours of exposure to pyocyanin (Fig. 2).

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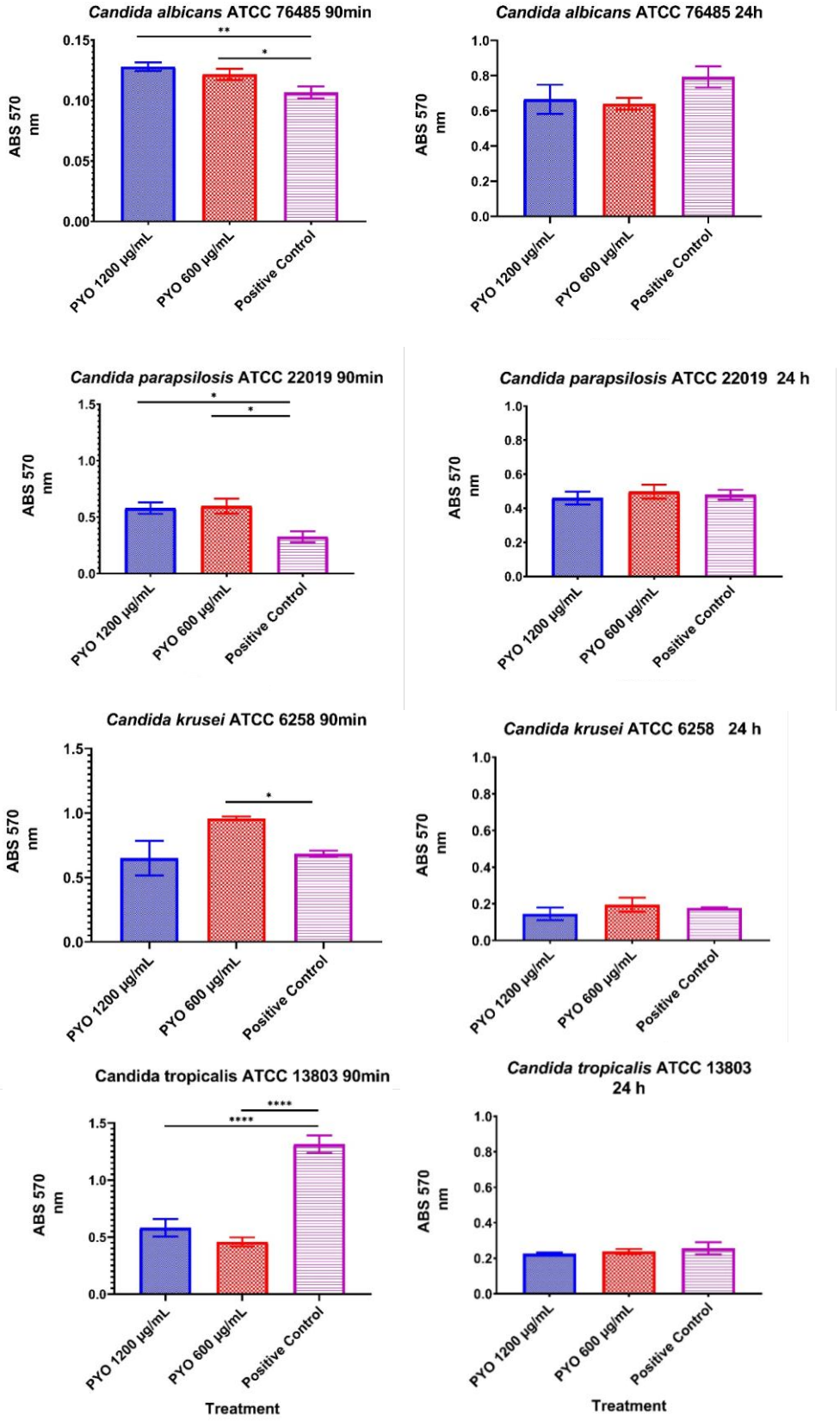
Fig. 2: Action of pyocyanin (PYO) on adhesion and mature biofilm of *Candida* sp. Significant differences in control are indicated with an asterisk (* p <0.05, ** p <0.01 and **** p <0.005)



DISCUSSION

It is known that microbial cells and their metabolites can interact and define the complexity of interspecific relationships, such as the relationship between fungi and bacteria.[26] Studies involving the role of secondary microbial metabolites with bioactive properties help to understand how these molecules can be determinants in the establishment and development of pathogens in different environments.[27] However, the cellular events of these interactions, such as morphological changes and the formation of biofilms, action on motility and especially disturbances of the cell wall and plasma membrane have been little explored.

Opportunistic lung conditions are often associated with *P. aeruginosa*.[28] In some cases, *Candida* spp. may be involved and both species can coexist in these patients.[29] Metabolic versatility, as well as different virulence factors, confer greater advantages to *P. aeruginosa* over other organisms, assuming that pyocyanin is one of the greatest strategies of the bacterium.[30] Data on the activity of pyocyanin against *Candida* sp. are available, however the information is limited to *in vitro* tests and the majority against *Candida albicans*.[15-16, 31] At this point, the present study aimed to evaluate cell events and phenotypic changes promoted by pyocyanin, in addition to identifying targets of the pigment in the yeast cell.

A bioactive antimicrobial potential is revealed when a lower MIC value is obtained. In this study, the MIC of 600 µg/mL attributed a moderate activity of pyocyanin against the strains of *Candida* spp.[32-34]; the distinct characteristics of the strains of certain species, however, can produce different responses. Previous studies have observed very high MIC values of pyocyanin against *C. albicans*, ranging between 5 and 150 g/mL.[31, 35] In addition, a MIC 100 times greater of pyocyanin was obtained in these studies when investigating the action of pyocyanin against *Aspergillus* spp., a mould member of the filamentous group.[36]

In order to detect cell damage in *Candida* spp., two initial targets were tested. The first one was the cell wall and the second, the plasmatic membrane. The fungal cell wall represents a dynamic structure that protects protoplasts from external osmotic shocks and controls cell growth; as well, it serves to provide form and to participate in interactions with the environment.[37] In this study, the sorbitol test indicated that pyocyanin promoted damage to the cell wall of *Candida* spp. Sorbitol acts as an osmotic stabilizer and an increase of the MIC indicates damage to the cell wall.[21] In addition, the fact that damage to the cell wall occurs in concentrations of pyocyanin above 1,200 µg/mL may indicate that higher pigment concentrations is needed to overcome the osmotic protection provided by sorbitol. These results may be compared to observations of an eight-fold increase in MIC in a study of trimethoxflavone activity against *C. albicans* ATCC 76645[38] and geraniol against *Candida albicans* ATCC 60193.[39]

A common target for many fungicidal or fungistatic substances is the plasmatic membrane. Ergosterol is an essential and the most abundant lipid component of the *Candida* spp. For this reason, the ergosterol test is a widely used methodology to infer if any damage has been done after exposure to antifungals.[22] However, even though pyocyanin easily crosses the plasmatic membrane of some eukaryotic cells[40], the results indicated that ergosterol was not a target of pyocyanin.

Additionally, exposure to pyocyanin was shown to be cytotoxic and promoted a significant reduction of viable cells in yeast cell viability. MTT is converted by dehydrogenases of fungal cells into formazan blue, making the test a way to indirectly measure the mitochondrial respiration of fungi.[41] Thus, the results suggest the activity of pyocyanin at the mitochondrial level. This highlights one of the ways in which pyocyanin acts on cells sensitive to pigment, i.e., it harms the respiratory chain, disrupting the active transport mechanism across the membrane, leading to a reduction in the oxygen supply to cells, as well as promotes the accumulation of hydrogen peroxide and superoxide.[42]

Biofilm formation is considered one of the most important virulence factors in *Candida* spp.[43] Only *C. tropicalis* ATCC 13803 had significant negative changes in terms of adherence, which is consistent with studies on the activity of phenazines, a large class of redox-active secondary metabolites produced by many bacteria, to which pyocyanin is part. These changes possibly act in blocking the formation of filaments in *Candida* spp.[44-45] On the other hand, the acceleration in the formation of biofilm, as well as the non-interference in mature biofilm by the other strains, suggests that pyocyanin is more active for the planktonic form of yeasts. The mechanisms by which *Candida* spp. avoids chemical stresses are not yet fully elucidated. However, four reasons can be hypothesized: 1. chemical stress causes the death of susceptible cells, however those that seem to fail

in the electron transport chain, adhere and multiply.[46]; 2. the growth rate of yeasts, combined with a high cell density, favours the emergence of pre-formed cell aggregates.[47]; 3. the contact of pyocyanin occurred after 90 minutes of incubation, sufficient time for the installation of a yeast community protected by EPS. When these cells are confined, they persist, compared to planktonic cells which are thousand times more susceptible.[48] and 4. the stability provided by the organization in biofilm increases resistance to different molecules, including toxic compounds.[25]

In the available literature the maximum concentration of pyocyanin naturally obtained was 245.39 µg/mL.[49] It was approximately four times lower than the MFC detected in our study. Under certain culture conditions, however, high concentrations may be obtained. [50] This was the reason why the TGC04 isolate was chosen for this study.

In a natural environment, the concentration of pyocyanin does not reach high values for two reasons. The first is that laboratory conditions do not match the environment, considering that in these studies, many variables are eliminated. The second reason refers to the fact that some microbes may exhibit exometabolites with antimicrobial activity [51]. These compounds are only synthesized in order to inhibit and not to kill potential competitors for nutrients and space. The interactions between species in a given system, including negative relationships, guarantee the balance of these populations, preventing any of them from standing out, otherwise the entire balance would collapse.[52]

Since this study did not evaluate the intimate contact between *P. aeruginosa* and *Candida* spp. strains, it can be presumed the interaction between both species is of the antagonistic type. However, planktonic forms are more susceptible to pyocyanin than the sessile form. Future studies can investigate the molecular events involved in this phenomenon.

CONCLUSION

In the experimental conditions employed in this study, pyocyanin showed moderate activity against *Candida* spp. strains, promoting damage to the cell wall and reduced viability of the planktonic cells, especially in *C. krusei* ATCC 6258. Mature biofilm was robust and not affected by pyocyanin; the adherence stage, however, is worth further investigation of the antibiofilm effect.

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