https://dx.doi.org/10.22161/ijaers.6732 ISSN: 2349-6495(P) | 2456-1908(O)

Activity of two Exometabolites produced by Escherichia coli on the Synthesis of Pyocyanin

Ray Ravilly Alves Arruda[1], Bianca Teixeira Morais de Oliveira[1], Tarcísio Tárcio Corrêa Bonifácio[1], Vinícius Cavalcante Morais[1], Ian Porto Gurgel do Amaral[2], Ulrich Vasconcelos[1]

[1]Laboratório de Microbiologia Ambiental, Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa-PB, Brazil
[2]Laboratório de Biotecnologia de Organismos Aquáticos, Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa, Brazil

Abstract *— The secretion of metabolites with antimicrobial activity is one of the strategies employed by bacteria to respond to negative stimuli promoted during interspecies competition. In a long-term stationary phase. Pseudomonas aeruginosa and Escherichia coli can synthesize diffusible exometabolites whose action is to mutually inhibit the exposed cells, guaranteeing the balance of both populations in a certain site. The P. aeruginosa may have an advantage in that it produces pyocyanin. However, the excretion of indole and acetate by E. coli may reduce this advantage. This work aims to detect the influence of different concentrations of these two exometabolites on the synthesis of pyocyanin in two wild isolates of P. aeruginosa. After incubation under shaking for 72 h at 29°C, reduction of up to 50% of the concentration of pyocyanin in the presence of indole was observed. On the other hand, no change was observed in the production of the pigment with the acetate, alone or when in combination with concentrations of less than 0.5 mM indole. It reduced the inhibitory effect of the compound, reflecting an increase in pyocyanin production of more than 20%. The results contribute to help understanding the ecological mechanisms of competition between the two species.*

Keywords *— **Pseudomonas aeruginosa, Natural phenazine, Microbial antagonism, Indole, Anti-Quorum Sensing molecules.***

I. INTRODUCTION

The In natural environments, different bacterial species coexist, forming complex multicellular communities that collectively respond to stimuli from the environment they inhabit, resulting in the stability of their populations [1]. During a long-term stationary phase, competition for space and nutrients is a natural process that occurs in mixed microbial populations, where a given microorganism produces certain diffusible substances whose function is to inhibit the growth of a second microorganism. These substances can be of various natures, for example enzymes, organic acids or phenazine compounds, among them, pyocyanin [2].

Pyocyanin is a bright blue pigment, characteristic of the *P. aeruginosa* species. It is also the main pigment synthesized by fluorescent pseudomonads [3]. In addition, pyocyanin is known as one of the most important virulence factors of *P. aeruginosa*. In addition to the role of a signaling molecule in cell-dependent cell density phenomena [4], it has been reported to participate in events involving resistance to antibiotics [5], inflammatory processes [6] and competition with other microorganisms at a given site [7].

In aqueous media, the relationship between *P. aeruginosa* and *E. coli* reveals certain particularities during a long-term stationary phase. Both organisms can synthesize diffusible exometabolites whose action is to mutually inhibit the exposed cells, ensuring the balance of both populations at a given site [8]. In addition to pyocyanin, other important exometabolites have been identified in *P. aeruginosa*, such as proteases, hemolysins, rhamnolipids and pyoverdine, a green color pigment that also has a siderophore function [9].

In an attempt to overcome the pressures exerted by *P. aeruginosa*, as well as its exponent metabolic advantage, *E. coli* strains can release into the environment, for example, acetate and indole. The first is formed as a by-product in aerobiosis when the absorption rate of the primary carbon source is greater than its conversion to biomass and CO_2 [10]. On the other hand, indole is formed from the metabolism of tryptophan [11] and concentrations of 0.5 to 1 mM can regulate *E. coli* responses to stresses exerted by the environment, including competition with *P. aeruginosa* [12].

The microbial interspecies relationships are a subject with a number of aspects that can be explored. The

https://dx.doi.org/10.22161/ijaers.6732 ISSN: 2349-6495(P) | 2456-1908(O)

present work aimed to evaluate the influence of exogenous acetate and indole on the inhibition of the synthesis of pyocyanin in two wild strains of *P. aeruginosa*, submitted to direct contact with these metabolites.

II. MATERIAL AND METHODS

2.1 Microorganisms

Two isolates of *Pseudomonas aeruginosa*, TGC02 and TGC04, recovered from a petrol station in the city of Joao Pessoa, Brazil [13] were used. Both isolates exhibited pyocyanin by culturing at 30°C for 72 h in King A broth [14] and cetrimide agar [15].

2.2 Assay of exogenous indole and acetate activity on pyocyanin production

Recently cultured cells of TGC02 and TGC04 were suspended in 0.85% NaCl solution, standardizing the turbidity with tube # 1 of the MacFarland scale. Then, 5 mL of the suspension was transferred to flasks containing 50 mL of King A broth, to which had been added different concentrations of sodium acetate (0.25, 0.5 and 1.0 mM) and indole (1, 2 and 4 mM), totaling 16 conditions, including the control. The flasks were incubated under constant shaking at 150 rpm at 29±1°C for 72h [16]. The test was conducted in triplicate.

2.3 Extraction and quantification of pyocyanin

The assay was conducted according to methodology described by Oliveira et al. [17]. After the incubation period, 10 mL of the contents of the vials was transferred to 3 mL of chloroform. After vigorous vortexing and resting for 1h, 1.5 mL of the blue chloroform phase was acidified with 1 mL of 0.2M HCl, changing the color to red. After 1 h of rest, the concentration of pyocyanin was estimated by measuring the optical density of the acidified solution at $\lambda = 520$ nm (U2M chemistry), based on a standard curve prepared with 98% pure pyocyanin (Merck KGaA, Darmstadt, Germany) ($r = 0.9999$).

III. RESULTS

Both exometabolites promoted changes in the production of pyocyanin for the TGC02 and TGC04 isolates, especially indole. The results are shown in Figure 1. Pyocyanin concentrations are expressed as the mean of the three trials, with a standard deviation of ± 0.10.

In the absence of the inhibitors, the TGC02 and TGC04 isolates produced slightly more than 50 µg/mL pyocyanin. Indole alone, in the concentrations from 0.5mM and higher, was responsible for lower activity on the part of the *P. aeruginosa* isolates from the point of view of pyocyanin production, reducing the synthesis by about 40 and 50%, for TGC02 and TGC04, respectively. In contrast, the indole concentration of 0.25 mM did not promote reduction of the synthesis of pyocyanin for either of the *P. aeruginosa* isolates. This same result was observed under the conditions tested with the acetate, alone. Surprisingly, compared to the control, there was a 15% increase in the production of pyocyanin in the TGC02 isolate in the presence of 1 mM acetate. Under the other conditions, the concentration of pigment was not different from that observed in the control, with the increase of acetate in the medium.

When indole and acetate were associated, both TGC02 and TGC04 also exhibited a reduction in pyocyanin production, as the indole concentration increased. The concentration of the pigment obtained, however, was higher when compared to the results of samples only containing indole.

IV. DISCUSSION

There are two forms of microbial life in nature: planktonic and sessile. The second, more frequent, assures the formation of mixed communities with a high level of organization, whose maintenance in coexistence is guaranteed through several mechanisms, of which the nutrient concentration and chemical signalling stand out [18, 19].

Pseudomonas aeruginosa is an aerobic Gram-negative bacillus, a member of fluorescent pseudomonads [20], characterized by remarkable metabolic versatility, which gives it ubiquity, ensuring persistence in environments with different degrees of selective pressure [21, 22]. About 90-95% of all strains can produce pyocyanin [23], a pigment involved in the production of reactive oxygen species [24]. This is believed to constitute one of the main mechanisms that guarantees the advantage of *P. aeruginosa* against other bacteria [25].

https://dx.doi.org/10.22161/ijaers.6732 *ISSN: 2349-6495(P) | 2456-1908(O)*

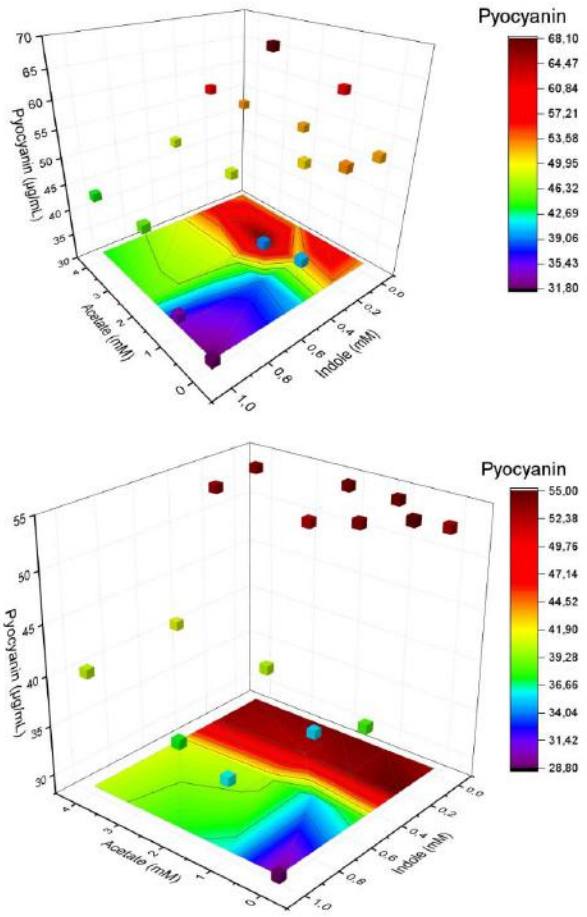

Fig. 1: Influence of indole and acetate on the production of pyocyanin by Pseudomonas aeruginosa TGC02 (A) and TGC04 (B). The colouring of the surface chart demonstrates the level of pyocyanin production: the nearly purple colour indicates a lower rate of production while that closer to red shows a higher production rate of the pigment.

It is known in the scientific community that in an aqueous media, *P. aeruginosa* and *E. coli* can interact [26]. This association sometimes represents disturbances especially to coliforms, although with a biostatic rather than a biocidal effect [2, 27]. In order avoid disturbance in the cells, *E. coli* releases secondary metabolites to keep itself viable in the environment, until it finds favourable conditions for regrowth [11, 28].

Among the exometabolites tested, indole proved to be more inhibitory to the production of pyocyanin by both TGC02 and TGC04 when compared to acetate, possibly due to its toxicity. On the other hand, it is known that acetate may also have a negative effect on organisms coexisting with *E. coli*. Concentrations of about 20 mM of acetate may slow growth or inhibit protein formation in competing organisms, however this concentration does not occur naturally [29].

It is emphasized that acetate reduced the inhibitory effect of indole on the TGC02 and TGC04 isolates when the two molecules were associated, especially under conditions where the indole concentration was 0.25 mM, as observed at acetate 2 mM and indole 0.5 mM, when TGC02 produced 68 µg/mL of pyocyanin, representing an increase of about 20% over the control. This apparent equilibrium suggests that acetate may have been used as an additional carbon source, based on a previous observation of the use of acetate by a *P. putida* strain in the presence of *E. coli* DOT-T1E [28].

E. coli excretes 10 to 30% of the carbon flux from glucose to an acetate in a glucose-containing medium, even when the culture is fully aerated [30]. Acetate can also be consumed by *E. coli* in terms of providing nutritional support to growth under stress conditions, and can be metabolized by two alternative routes: the first, by reversible Pta-AckA and the second, by irreversible, high-affinity acetyl-coA synthetase [31]. It is important to note that, because it also serves as an alternative source of carbon for the production of biomass and energy for *P. aeruginosa*, acetate is an exometabolite less damaging to the cell [32].

It is also important to remember that although there was a higher carbon input via acetate under some conditions, this did not promote significant differences in pyocyanin production when the indole concentration was equal to or greater than 0.5 mM, reinforcing the hypothesis that indole is a potentially more inhibitory molecule. Having information about the amount of indole and acetate produced when *E. coli* is disturbed in its environment can contribute to the elucidation of the population dynamics of the two species in mixed communities.

A previous study reported the reduction of pyocyanin production in three ATCC strains of *P. aeruginosa* when they were cultivated in a mixed culture with *E. coli*, attributing this reduction to the presence of exoproducts without naming them [8]. However, Chu et al. [11] studied the growth of *E. coli* in mixed culture, identifying indole as an anti-quorum sensing molecule for *P. aeruginosa*, which ensured the persistence of *E. coli* in the medium. In addition, Lee et al. [33] demonstrated that indole 1.0 mM inhibited 444 *P. aeruginosa* genes, including those regulating the production of the pyocyanin synthesis intermediates, such as *phz*C2, *phz*D2, *phz*E2 and *phz*F2, which are essential in the conversion of 5-methylphenazine-1-carboxylic acid betaine (PCA), to 1-hydroxy-5-methylphenazine, i.e., pyocyanin [23].

Pyocyanin biosynthesis is mediated by the quorum sensing system (QS) via the PQS system [4]. The QS is a

https://dx.doi.org/10.22161/ijaers.6732 *ISSN: 2349-6495(P) | 2456-1908(O)*

density-dependent cell-cell signaling mechanism, used by *P. aeruginosa* to guarantee, among other responses, the stability of its population in a given environment under pressures of different natures [34]. Pyocyanin was described as a physiological signal, assuming the role of regulator of quorum sensing sensors, controlling genes during the stationary phase of *P. aeruginosa* [35]. The participation of pyocyanin as the QS signalling molecule in *P. aeruginosa* was identified as an important factor during formation and stability of biofilms [36, 37]. It is also believed that pyocyanin is required as autoinducer of the expression of certain phenotypic characteristics in *P. aeruginosa*, among then, the biosynthesis of surfactants, thus favouring the degradation of hydrocarbons [38, 39].

Microbial interactions occur with the purpose of promoting the stability of populations in the environment, guaranteeing the recognition of substrates, as well as the transfer of genetic information, resulting in a diversity of phenotypes [40, 41]. In the microbial world, competition is a common and an expected event. However, some evidence suggests that interspecies interactions are weak, since they can be resolved by spatial separation [42]. For microbes, the balance between the populations brings many more advantages than the elimination of a particular population. This may reinforce and justify the reduction in the production of pyocyanin, but not more than by half.

V. CONCLUSION

Under the experimental conditions tested, the presence of indole promoted perturbations in the production of pyocyanin in TGC02 and TGC04 isolates. The presence of acetate contributed to equate this disturbance when the concentrations of exometabolites were lower than 0.5 mM.

ACKNOWLEDGEMENTS

The authors express their gratitude to CNPq, UFPB and CAPES for funding for this research. The isolates of *P. aeruginosa* are registered in the National System of Registration of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number AA1970F.

The English text of this paper has been revised by Sidney Pratt, Canadian, MAT (The Johns Hopkins University), RSAdip - TESL (Cambridge University).

REFERENCES

[1] Dohi M., Mougi A. (2018). A coexistence theory in microbial communities. Royal Soc Open Sci. 8, 180476. https://doi10.1098/rsos.180476.

[2] Vasconcelos U., Lima M.A.G.A., Calazans G.M.T. (2010). *Pseudomonas aeruginosa* associated with negative interactions on coliform bacteria growth. Can J Pure Appl Sci. 4(2): 1133-1139.

[3] Hassani H.H., Hasan H.M., Al-Saadi A., Ali A.M., Muhammad M.H. (2011). A comparative study on cytotoxicity and apoptotic activity of pyocyanin produced by wild type and mutant strains of *Pseudomonas aeruginosa*. Eur J Experiment Biol. 2(5): 1389-1394.

[4] Mangwani N., Kumari S., Das S. (2015). Involvement of quorum sensing genes in biofilm development and degradation of polycyclic aromatic hydrocarbons by a marine bacterium *Pseudomonas aeruginosa* N6P6. Appl Environ Biotechnol. 99(23): 10283–10297.

[5] Evans K.C., Benomar S., Camuy-Vélez L.A., Nasseri E.B., Wang X., Neuenswander B., Chandle J.R., Nature Jobs. (2018). Quorum-sensing control of antibiotic resistance stabilizes cooperation in *Chromobacterium violaceum*. ISME J. 12(5): 1263–1272.

[6] Patterson A.G., Jackson S.A., Taylor C., Evans G.B., Salmond G.P.C., Przybilski R., Staals R.H.J.,Fineran P.C. (2016). Quorum Sensing ccntrols adaptive immunity through the regulation of multiple CRISPR-Cas systems. Molecular Cell. 64(6): 1102–1108.

[7] Abisado R.G., Benomar S., Klaus J.R., Dandekar A.A., Chandler J.R. (2018). Bacterial quorum sensing and microbial community interactions. mBio. 9(3): e02331-17. DOI: 10.1128/mBio.02331-17.

[8] Kuznetsova M.V., Maslennikova I.L., Karpunina T.I., Nesterova L.Y., Demakov V.A. (2013). Interactions of *Pseudomonas aeruginosa* in predominant biofilm or planktonic forms of existence in mixed culture with *Escherichia coli in vitro*. Can J Microbiol 59(9): 604–610.

[9] Yin Y., Papavasiliou G., Zaborina O.Y., Alverdy J.C., Teymour F. (2017). *De novo* synthesis and functional analysis of polyphosphate-loaded poly(ethylene) glycol hydrogel nanoparticles targeting pyocyanin and pyoverdin production in *Pseudomonas aeruginosa* as a model intestinal pathogen. Ann Biomed Eng. 45(4): 1058-1068.

[10] Kleman G.L., Strohl W.R. (1994). Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. Appl Environ Microbiol. 60(11): 3952-3958.

[11] Chu W., Zere T.R., Weber M.M., Wood T.K., Whiteley M., Hidalgo-Romano B., Valenzuela E.J., McLean R.J.C. (2011). Indole production promotes *Escherichia coli* mixed-culture growth with *Pseudomonas aeruginosa* by inhibiting quorum signaling. Appl Environ Microbiol. 78(2): 411–419.

[12] Gaimster H., Cama J., Hernández-Ainsa S., Keyser U.F., Summers D.K. (2014). The indole pulse: A new perspective on indole signaling in *Escherichia coli*. PLoS One. 9(4): e93168. https://doi: 10.1371.

[13] Cavalcanti T.G., Souza A.F., Ferreira G.F., Dias D.S.B., Severino L.S., Morais J.P.S., Sousa K.A., Vasconcelos U. (2019). Use of agro-industrial waste in the removal of phenanthrene and pyrene by microbial consortia in soil. Waste Biomass Valor. 10(1): 205-214.

[14] King E.O., Ward M.K., Raney D.E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. J Lab Clin Med. 44(2): 30-307.

[15] Genhartdt P., Murray R.G.E., Wood W.A., Kieg N.R. (1994). Methods for general and molecular bacteriology (. 1st. ed.) Washington, WA: American Society for microbiology. 791p.

https://dx.doi.org/10.22161/ijaers.6732 *ISSN: 2349-6495(P) | 2456-1908(O)*

[16] El-Fouly M.Z., Sharaf A.M., Sahim A.A.M., El-Bialy H.A. (2015). Byosinthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. J Rad Res Appl Sci. 8(1): 36-48.

[17] Oliveira B.T.M., Barbosa P.S.Z., Cavalcanti T.G., Amaral I.P.G., Vasconcelos, U. (2019). Craft beer waste as substrate for pyocyanin synthesis. J Pharm Biol Sci. 14(1): 21-25.

[18] Tashiro Y., Yawata Y., Toyofuku M., Uchiyama H., Nomura N. (2013). Interspecies interaction between *Pseudomonas aeruginosa* and other microorganisms. Microbes Environ. 28(1): 13–24.

[19] Hibbing M.E., Fuqua C., Parsek M.R., Peterson S.B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol. 8(1): 15-25.

[20] Abdul-Hussein Z.R., Atia S.S. (2016). Antimicrobial effect of pyocyanin extracted from *Pseudomonas aeruginosa*. Eur J Experiment Biol. 6(6):3.

[21] Maia A.A., Cantisani M.L., Esposto E.M., Silva W.C.P., Rodrigues E.C.P., Rodrigues D.P., Lázaro N.S. (2009). Resistência antimicrobiana de *Pseudomonas aeruginosa* isolados de pescado e de cortes e de miúdos de frango. Ciência Tec Alim. 29(1): 114-119.

[22] Pirnay J.P., Matthijs S., Colak H., Chablain P., Bilocq F., Van Eldere J., de Vos D., Zizi M., Triest L., Cornelis P. (2005). Global *Pseudomonas aeruginosa* biodiversity as reflected in a Belgian river. Environ Microbiol. 7(7): 969-980.

[23] Mavrodi D.V., Bonsall R., Delaney S.M., Soule M.J., Phillips G., Thomashow L.S. (2001). Functional analysis of genes for biosynthesis of pyocianin and phenazine-1-carboxamine from *Pseudomonas aeruginosa* PA01. J Bacteriol. 183(21): 6454-6465.

[24] Muller M., Merrett N.D. (2014). Pyocyanin production by *Pseudomonas aeruginosa* confers resistance to ionic silver. Antimicrob Agent Chemother. 58(9): 5492-5499.

[25] Sales-Neto, J.M., Lima E.A., Cavalcante-Silva L.H.A., Vasconcelos, U., Rodrigues-Mascarenhas, S. (2019). Anti-inflammatory potential of pyocyanin in LPSstimulated murine macrophages. Immunopharmacol Immunotoxicol. https://doi.org/10.1080/08923973.2018.1555845

[26] Gonzales-Siles L., Sjoling, A. (2016). The different ecological niches of enterotoxigenic *Escherichia coli*. Environ Microbiol 18(3): 741–751.

[27] Viana A.A.G., Martins R.X., Ferreira G.F., Zenaide-Neto H., Amaral I.P.G., Vasconcelos, U. (2017). *Pseudomonas aeruginosa* and pyocyanin negatively act on the establishment of Enterobacteriacea biofilm on a ceramic surface. Int J Eng Res Appl.7(8): 23-30.

[28] Molina-Santiago C., Udaondo Z., Cordero B.F., Ramos J.L. (2017). Interspecies cross-talk between co-cultured *Pseudomonas putida* and *Escherichia coli*. Environ Microbiol Rep. 9(4): 441–448.

[29] Mey M., Lequeux G.J.., Beauprez J.J., Maertens J., Horen E.V., Soetaert W.K., Vanrolleghem P.A., Erick J., Vandamme E.J. (2007). Comparison of different strategies to reduce acetate formation in *Escherichia coli*. Biotechnol Prog. 23(5): 1053−1063.

[30] Farmer W.R., Liao J.C. (1997). Reduction of aerobic acetate production by *Escherichia coli*. Appl Environ Microbiol. 63(8): 3205-3210.

[31] Enjalbert B.. Millard P., Dinclaux M., Portais J.C., Létisse F. (2017). Acetate fluxes in *Escherichia coli* are determined by the thermodynamic control of the Pta-AckA pathway. Sci Rep. 7: 42135, https://doi: 10.1038/srep42135.

[32] Lopes S.P., Machado I.M., Pereira M.O. (2011). Role of planktonic and sessile extracellular metabolic by production *Pseudomonas aeruginosa* and *Escherichia coli* intra and interspecies relationships. J Ind Microbiol Biotechnol. 38(10): 133-140.

[33] Lee J.H., Lee J. (2010). Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev. 34(4): 426–444.

[34] Bruger E.L., Waters C.M. (2016). Bacterial quorum sensing stabilizes cooperation by optimizing growth strategies. Appl Environ Microbiol. 82(22): 6498-6506, 2016.

[35] Dietrich L.E.P., Price-Whelan A., Petersen A., Whiteley M., Newman D.K. (2006). The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Molec Microbiol. 61(5): 1308–1321.

[36] Skariyachan S., Sridhar V.S., Packirisamy S., Kumargowda S.T., Challapi S.B. (2018). Recent perspectives on the molecular basis of biofilm formation by *Pseudomonas aeruginosa*. Folia Microbiol. 63(4): 413–432.

[37] Khare E., Arora N.K. (2011). Dual activity of pyocyanin from *Pseudomonas aeruginosa* – antibiotic against phytopathogen and signal molecule for biofilm development by rhizobia. Can J Microbiol. 57(9): 708-713.

[38] Viana A.A.G., Oliveira B.T.M., Cavalcanti T.G., Sousa K.A., Mendonça E.A.M., Amaral I.P.G., Vasconcelos, U. (2018). Correlation between pyocyanin production and hydrocarbonoclastic activity in nine strains of *Pseudomonas aeruginosa*. Int J Adv Eng Res Sci. 5(7): 212-223.

[39] Das P., Ma L.Z. (2013). Pyocyanin pigment assisting biosurfactant-mediated hydrocarbon emulsification. Int Biodeterior Biodegrad. 85: 278-283.

[40] Braga R.M., Dourado M.N., Araújo W.L. (2016). Microbial interactions: ecology in a molecular perspective. Braz J Microbiol. 47(1): 86-98.

[41] Jimenez P.N., Koch G., Thompson J.A., Xavier K.B., Cool R.H., Quax W.J. (2012). The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev. 76(1): 46-65.

[42] Ghoul M., Mitri S. (2016). The ecology and evolution of microbial competition. Trends Microbiol. 24(10): 833-845.