IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 14, Issue 1 Ser. IV (Jan – Feb 2019), PP 21-25
www.Iosrjournals.Org

Craft beer waste as substrate for pyocyanin synthesis

Bianca Teixeira Morais de Oliveira[1], Patrik Saul Zarpellon Barbosa[1], Thiago Gonçalves Cavalcanti[1], Ian Porto Gurgel do Amaral[2], Ulrich Vasconcelos[1]
[1](*Laboratório de Microbiologia Ambiental/ Universidade Federal da Paraíba – Campus I, João Pessoa, Brazil*
[2](*Laboratório de Biotecnologia de Organismos Aquáticos/ UFPB, João Pessoa, Brazil*
Corresponding author: Ulrich Vasconcelos

Abstract: *Pyocyanin is a fluorescent phenazine pigment of a bright blue color, synthesized exclusively by about 95% of Pseudomonas aeruginosa strains. Laboratory acquisition of the pigment is achieved by employing simple protocols. The aim of this work was to increase the production of pyocyanin by adding different concentrations of craft beer waste (w/v) to King A broth, incubated by shaking at 150 rpm, at 29±1ºC for 96 hours. Compared to the control group, a positive correlation was observed between the production of pyocyanin and the percentage of malt bagasse added. This contributed to the increase of pigment concentration up to approximately 70%, over the control, with values ranging from 21 to 58 µg/ml. The craft beer waste proved to be an environmentally friendly option as an adjuvant in the process of pyocyanin production in a synthetic medium.*

Keywords: *Blue pigments, Natural phenazines; Natural pigments, Pseudomonas aeruginosa, Waste reuse*.

Date of Submission: 30-01-2019 Date of acceptance:16-02-2019

I. Introduction

Natural pigments are secondary, low molecular weight metabolites synthesized by eukaryotes and prokaryotes [1]. A large number of bacteria produce pigments of the most diverse stains, assigning different functions to the organism, for example, photosynthesis [2], protection of the cell against ultraviolet radiation [3], iron uptake [4] and promotion of antibiosis [5], among others.

P. aeruginosa is a Gram-negative rod member of the fluorescent pseudomonads group, easy to cultivate in the laboratory. The bacterium exhibits an extremely versatile metabolism and can synthesize at least 6 different pigments, among them pyocyanin, a water soluble phenazine compound with a bright blue color, produced exclusively by 90-95% of the strains [6]. The main function of this pigment is related to its participation in reactions involving the production of reactive oxygen species [7]. Additionally, pyocyanin is involved in iron metabolism as well as in the extracellular electron transport by the bacterium [8-9].

Implicitly, most of the studies involving pyocyanin treat synthesis as a mechanism of response to environmental stresses [10], focusing on medical science topics such as multiresistance [11], antimicrobial activity [12], quorum sensing [13] and immunological changes or inflammatory responses [14]. However, the application of the pigment can also be investigated in the context of the production of tensoactive molecules [15], bioremediation [16], the ecological interactions of *P. aeruginosa* with other organisms [5, 17] and in the generation of bioelectricity [18].

Some investigations may require certain amounts of phenazine, however, the average cost is high, about US$ 30 for 1 mg of pyocyanin. The literature provides protocols for synthesis and purification of pyocyanin on a laboratory scale [19]. Based on the metabolism of *P. aeruginosa*, given the ease of its manipulation in the laboratory, pyocyanin production can be increased by using processing residues as supplementary carbon sources, minimizing costs. Thus, the aim of this work was to stimulate the synthesis of pyocyanin by adding different concentrations of waste from craft beer production to the King A broth.

II. Material and Methods

Microorganisms

Four strains of *Pseudomonas aeruginosa*, recovered from soils of gas stations in city of João Pessoa, Paraíba, Brazil [20] were used: two pyocyanin-producing isolates (TGC02 and TGC04) and two non-pyocyanin-producing strains (TGC03 and RX10).

Tests for pyocyanin production

The strains from fresh culture on nutrient agar were suspended in 0.9% NaCl, standardizing the turbidity by tube #1 on the McFarland Scale. A volume of 5 mL was transferred into flasks containing 100 mL

of King A broth [21], with neutral pH, supplemented with 0.25, 0.5, 1 and 2% (w/v) of malt bagasse. Incubation was carried out under agitation at 150 rpm, at 29±1 °C for 96 hours.

Malt bagasse as a supplement to the King A broth

The malt was provided by a craft brewing industry. The material was washed with hot water, dried and then comminuted. The total nitrogen content was 1.45 mg/kg, as determined by the Kjeldahl method [22].

Extraction and estimation of pyocyanin

The assays were adapted from the methodology described by literature [23], using a mixture of 10 ml of the medium containing the pigment and 3 ml of chloroform. After vigorous vortexing (Warmnest VX-28), it was allowed to stand for 2 hours.

After this time, a 1.5 mL aliquot of chloroform (blue phase) was acidified with 1 mL of 0.2 mol/L HCl, changing the color to pink. The estimate of the concentration of pyocyanin in µg/ml was determined by measuring the optical density of the pink solution at λ = 520 nm (U2M Chemistry), based on a standard curve prepared with 98% pure pyocyanin (Merck KGaA, Darmstadt, Germany) (r = 0.9999).

Statistical analysis

The correlation between the malt bagasse concentration and the amount of pyocyanin produced, compared to the control groups, was verified. Significance was established at $p < 0.05$, using the free IBM®SPSS® Statistics version 21 program.

III. Results

Pyocyanin-producing TGC02 and TGC04 exhibited the pigment under all conditions tested (Fig. 1). In the absence of the malt bagasse, mean yield about 17 µg/mL was observed and the concentration of pyocyanin was increased proportionally to the percentage of malt bagasse added to the medium. The best condition being obtained when this substrate was 2% (w/v), as summarized in Table 1.

Table 1. Concentration of pyocyanin (µg/mL)

Strains	Percentage of malt bagasse added to King A broth				
	0.00	**0.25**	**0.50**	**1.00**	**2.00**
TGC02	17.8±0.1	23.0±0.5	35.3±0.3	32.6±0,2	58.2±0,1
TGC04	16.9±0.1	21.5±0.3	22.2±0.5	27.1±0.9	58.3±0.1
TGC03	0.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
RX10	0.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

A high correlation was observed between the production of pyocyanin with the concentration of malt bagasse added to the culture medium, in both strains, TGC02 and TGC04, 87.6 and 81.8%, respectively. On the other hand, the addition of malt bagasse did not stimulate pigment production in the non-producing strains, TGC03 and RX10.

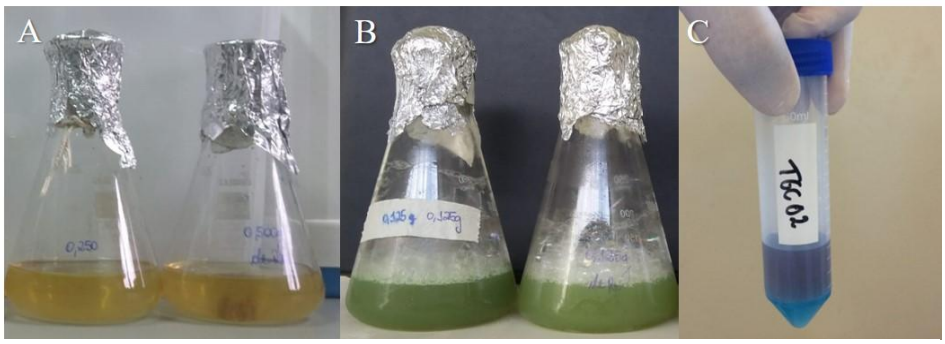

Figure 1. King A broth before (A) and after incubation (B) and extracted pyocyanin (C)

The TGC02 strain produced between approximately 18 and 58 µg/ml of pyocyanin, representing an increase from 22.6 to 69.4%, compared to the control in King A broth, i.e., without malt bagasse supplementation. In the concentrations 0.5 and 1% of the residue, there was no statistical difference in the values of the obtained pigment.

Similar results were observed with TGC04, for which concentrations between 0.25 and 1% of malt bagasse were used. The strain produced between 17 and 58 µg/ml of pyocyanin, representing an increase between 21.4 and 71%, when compared to the control group.

IV. Discussion

The expression of pyocyanin is one of the most important phenotypes of *P. aeruginosa*, particularly due to it is unique to this species [24]. The pigment participates in a number of biological processes that have not yet been completely elucidated, thus stimulating research involving different scientific approaches in the fields of biotechnology, engineering, environmental sciences and medical sciences.

The production of pyocyanin in traditional media used in the routine of a microbiology laboratory is based on the energy state of *P. aeruginosa*, which is reduced under conditions of low nutrient concentration, resulting in a decrease in the growth rate and increase the concentration of pigment. Nutritional scarcity, especially related to the PO_4^{-3} and Ca^{+2} ions, forces the pyocyanin-producing strains to exhibit the pigment, which diffuses in the medium [25].

This hypothesis, which was raised almost 4 decades ago, supports the observations of the performance of *P. aeruginosa* in environments with highly selective pressures, such as stabilization ponds [26], hospitals [11] and soils contaminated by oil hydrocarbons [27]. Although organic matter is widely available in these environments, the selective pressure exerted on the microbiota drive forces the bacterium to gather mechanisms that guarantee its persistence [28], with pyocyanin being a crucial element in the regulation of these responses [29].

The *in vitro* production of pyocyanin occurs at least 48 hours after the onset of growth, when specific conditions of temperature and agitation are encountered, i.e., 30°C and 150 rpm [30]. In addition, the culture medium also influences this result. In mineral broth and GSNB, for example, the pigment was observed to be diffused in both media, after 96h of incubation at 37°C [31]. This production usually starts at the beginning of the stationary phase, an event dependent on the generation time of the strains. *P. aeruginosa* under these growing conditions tends to have a generation time ranging from 3 to 6 hours [32], which justifies the appearance of the blue color between 48 and 72 hours after the start of the incubation.

Data on the production and quantification of pyocyanin obtained from *P. aeruginosa* are rare. Most data estimate the pigment concentration in the context of some bioprocess that depends on this data, with results varying between 9 and 42 μg/mL [31, 33]. To the best of our knowledge the literature reports that in an aqueous medium, under specific incubation conditions, which include the use of the King A medium or modification thereof, the pyocyanin is synthesized during the end of the log phase and the beginning of the stationary phase, being able to reach up to 80 μg/ml. The primary source of carbon and nitrogen are the most important factors on yield [23, 34], although the presence of glycerol is essential, a condition known since the first quarter of the 20th century [35].

A previous study attained a maximum production of pyocyanin, approximately 26 μg/ml, after 72h of incubation [30]. This concentration was obtained when the main source of nitrogen was peptone (13 g/L), at neutral pH and 30°C. Other sources of the element, such as urea, potassium nitrate and sodium nitrate, promoted lower yields of pyocyanin, 17, 15 and 11 μg/ml, under the same incubation conditions.

On the other hand, the addition of malt bagasse did not stimulate the synthesis of pyocyanin in the non-producing strains, TGC03 and RX10, indicating that these strains do not present the genes responsible for the conversion of the intermediate phenazines to pyocyanin [13, 36].

Based on the results of Gharieb et al. [37], which reported the production of approximately 65 μg/mL of the pigment within 120h in a medium containing 1% peptone, representing a 55% increase in the concentration of pyocyanin between 72 and 120h of incubation, the strains TGC02 and TGC04 could possibly produce more pyocyanin if the incubation time were increased. Compared with the pyocyanin values achieved by other *P. aeruginosa* strains grown in medium containing 1% peptone [30, 38], the results obtained with the TGC02 and TGC04 strains were more promising, since in the presence of malt bagasse, from 0.5 and 1%, respectively, both strains exhibited higher pigment concentrations. The difference was most significant when double the percentage of peptone was applied. Compared to the study by Devenath et al. [39], we found that even using the smallest percentage of malt bagasse, we achieved more than twice the amount of pyocyanin.

Both TGC02 and TGC04 in an earlier study by our group produced respectively 38 and 17% more pyocyanin in King A broth [16]. It is important to clarify that the amount of pyocyanin may be variable for the same strain, submitted to the same incubation conditions, on different occasions. This may be related to the possible stresses to which the strains are exposed when kept in the laboratory. Media supplementation with malt bagasse, on the other hand, stimulated increased pigment production by strains, indicating that optimization of pyocyanin production can be planned using non-conventional substrates, often intended for disposal or burning. The reuse of these can have a significant impact on the reduction of costs, as well as on the re-signification of wastes, especially the craft brewing industry.

V. Conclusion

Malt bagasse, serving as a supplement to the traditional medium of *P. aeruginosa* production of pyocyanin, promoted a significant increase in the production of pyocyanin in two wild strains. The increase was proportional to the concentration of added malt bagasse. These results suggest that other industrial residues of plant origin could be tested, as well, with experimental planning trials to identify the best conditions for obtaining higher concentrations than those achieved in this study.

Acknowledgements

The authors would like to thank the Brazilian National Council for Scientific and Technological Development (CNPq) and the Federal University of Paraíba (UFPB) for the financial supporting of this research.

This work has registration nº AA1970F in the National System of Genetic Heritage Management and Associated Traditional Knowledge.

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

References

[1]. Chadni Z, Rahaman MH, Jerin I, Hoque KMF, Reza MA. 2017. Extraction and optimization of red pigment production as secondary metabolites from *Talaromyces verruculosus* and its potential use in textile industries. *Micology*, **8**(*1*): 48-57.
[2]. Nowicka B, Kruk J. 2016. Powered by light: phototrophy and photosynthesis in prokariotes and its evolution. *Microbiological Research*, **186-187**: 99-118. DOI: 10.1016/j.micres.2016.04.001.
[3]. Moeller R, Horneck G, Facius R, Stackebrandt, E. 2005. Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. *FEMS Microbiology and Ecology*, **51**(*2*): 231-236.
[4]. Moradali MF, Ghods S, Rehm BHA. 2017. *Pseudomonas aeruginosa* lifestyle: A paradigm for adaptation, survival, and persistence. *Frontiers in Cellular and Infection Microbiology*, **7**: 1-29. DOI.: 10.3389/fcimb.2017.00039.
[5]. Viana AAG, Martins RX, Ferreira GF, Zenaide-Neto H, Amaral IPG, Vasconcelos U. 2017. *Pseudomonas aeruginosa* and pyocyanin negatively act on the establishment of Enterobacteriaceae biofilm on a ceramic surface. *International Journal of Engineering Research and Applications*, **7**(*8*): 23-30.
[6]. Mavrodi DV, Bonsall R, Delaney SM, Soule MJ, Phillips G, Thomashow LS. 2001. Functional analysis of genes for biosynthesis of pyocianin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PA01. *Journal of Bacteriology*, **183**(*21*): 6454-6465.
[7]. Muller M, Merrett ND. 2014. Pyocyanin production by *Pseudomonas aeruginosa* confers resistance to ionic silver. *Antimicrobial Agents and Chemothererapy*, **58**(*9*): 5492-5499.
[8]. Jayaseelan S, Ramaswamy D, Dharmaraj S. 2014. Pyocyanin: production, applications, challenges and new insights. *World Journal of Microbiology and Biotechnology*, **30**(*4*): 1159- 1168.
[9]. Abdul-Hussein ZR, Atia SS. 2016. Antimicrobial effect of pyocyanin extracted from *Pseudomonas aeruginosa. European Journal of Experimental Biology*, **6**(*1*): 1-4.
[10]. Bahari S, Zeighami H, Mirshahabi H, Roudashti S, Haghi F. 2017. Inhibition of *Pseudomonas aeruginosa* quorum sensing by subinhibitory concentrations of curcumin with gentamicin and azithromycin. *Journal of Global Antimicrobial Resistence*, **10**(*1*): 21-28.
[11]. Kerr KG, Snelling AM. 2009. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *Journal of Hospital Infection*, **73**(*4*): 338-344.
[12]. Martins VV, Macareno AC, Gradella DG, Stehling EG. 2014. Antagonism between clinical and environmental isolates of *Pseudomonas aeruginosa* against coliforms. *Water Science and Technology: Water Supply*, **14**(*1*): 99-106.
[13]. Dietrich LEP, Price-Whelan A, Petersen A, Whiteley M, Newman DK. 2006. The phenazine pyocyanin is a terminal signaling factor in the quorum sensing network of *Pseudomonas aeruginosa. Molecular Microbiology*, **61**(*5*): 1308-1321.
[14]. Sales-Neto JM, Lima EA, Cavalcante-Silva LHA, Vasconcelos U, Rodrigues-Mascarenhas S. Anti-inflammatory potential of pyocyanin in LPS stimulated murine macrophages. 2019. *Immunopharmacology and Immunotoxicology*, **9**: 1-7. DOI.:10.1080/08923973.2018.15558452019.
[15]. Das P, Ma LZ. 2013. Pyocyanin pigment assisting biosurfactant-mediated hydrocarbon emulsification. *International Biodeterioration and Biodegradation*, **85**: 278-283.
[16]. Viana A, Oliveira B, Cavalcanti T, Sousa K, Mendonça E, Amaral I, Vasconcelos U. 2018. Correlation between pyocyanin production and hydrocarbonoclastic activity in nine strains of *Pseudomonas aeruginosa*. *International Journal of Advance Engineering Research and Science*, **5**(*7*): 212-223.
[17]. Kerr JR, Taylor GW, Rutman A, Hoiby N, Cole PJ, Wilson R. 1999. *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *Journal of Clinical Pathology*, **52**(*5*): 385–387.
[18]. [18] Bosire EM, and Rosenbaum M. 2017. Electrochemical potential influences phenazine production, electron transfer and consequently electric current generation by *Pseudomonas aeruginosa. Frontiers in Microbiol*, **8**: 1-11. DOI: 10.3389/fmicb.2017.00892.
[19]. Cheluvappa R. Standardized chemical synthesis of *Pseudomonas aeruginosa* pyocyanin. 2014. *MethodsX*, **1**(*1*): 67-73.
[20]. Cavalcanti TG, Souza AF, Ferreira GF, Dias DSB, Severino LS, Morais JPS, Sousa KA, Vasconcelos U. 2019. Use of agro-industrial waste in the removal of phenanthrene and pyrene by microbial consortia in soil. *Waste and Biomass Valorization*, **10**(*1*): 205-214.
[21]. King EO, Ward MK, Raney DE. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine*, **44**(*2*): 301-307.
[22]. USEPA. Method 351.2. Revision 2.0. Washington: USA, 1993.
[23]. Hassani HH, Hasan HM, Al-Saadi A, Ali AM, Muhammad MH. 2012. A comparative study on cytotoxicity and apoptotic activity of pyocyanin produced by wild type and mutant strains of *Pseudomonas aeruginosa*. *European Journal of Experimental Biology*, **2**(*5*): 1389-1394.
[24]. Bellin DL, Sakhtah H, Rosenstein JK, Levine PM, Thimot J, and Emmets K. 2014. Integrated circuit-based electrochemical sensor for spatially resolved detection of redox-active metabolites in biofilms. *Nature Communications*, DOI: 10.1038/ncomms4256.

[25]. Whooley MA, McLoughlin AJ. 1982. The regulation of pyocyanin production in *Pseudomonas aeruginosa. European Journal of Applied Microbiology and Biotechnology*, **15**(*1*): 161-166.
[26]. Vasconcelos U, Lima MAGA, Calazans GMT. 2010. *Pseudomonas aeruginosa* associated with negative interactions on coliform bacteria growth. *Canadian Journal of Pure and Applied Science*, **4**(*2*): 1133-1139.
[27]. Mittal A, Singh P. 2009. Isolation of hydrocarbon degrading bacteria from contaminated with crude oil spills. *Indian Journal of Experimental Biology*, **47**(*9*): 760-765.
[28]. Deng H. 2012. A review of diversity-stability relationship of soil microbial community: What do we not know? *Journal of Environmental Science*, **24**(*6*): 1027-1035.
[29]. Samrot AV, Azeemullah A, Sujhita R, Azharudeen S, Samanvitha S, Sneha S. 2016. Characterization of Acyl Homoserine Lactone of pigment producing *Pseudomonas aeruginosa* SU-3. *Der Pharma Chemica*. **8**(*9*):74-79.
[30]. Agrawal AH, Chauhan PB. 2016. Effect of cultivation media components on pyocyanin production and its application in antimicrobial property. *International Journal of Current Advances Research*, **5**(*4*): 829-833.
[31]. El-Fouly MZ, Sharaf AM, Sahim AAM, and El-Bialy HA. 2015. Byosinthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. *Journal of Radiation Research and Applied Science*, **8**(*1*): 36-48.
[32]. Tamagnini LM, Gonzales RD. 1997. Bacteriological stability and growth kinetics of *Pseudomonas aeruginosa* in bottled water. *Journal of Applied Microbiology*, **83**(*1*): 91-94.
[33]. Barakat R, Goubet I, Manon S, Berges T, Rosenfeld R. 2014. Unsuspected pyocyanin effect in yeast under anaerobiosis. *Microbiology Open*. **3**(*1*): 1-4.
[34]. El-Shouny WA, Al-Baidani ARH, Hamza WT. 2011. Antimicrobial activity of pyocyanin produced by *Pseudomonas aeruginosa* isolated from surgical wound-infections. *International Journal of Pharmacy and Medical Science*, **1**(*1*): 1-7.
[35]. Hays EE, Wells IC, Katzman PA, Cain CK, Jacobs FA, Thayer SA, Doisy EA, Gaby WL, Roberts EC, Muir RD, Carroll CJ, Jones LR, Wade NJ. 1945. Antibiotic substances produced by *Pseudomonas aeruginosa*. *The Journal of Biological Chemistry*, **159**(*3*): 725-750.
[36]. Huang J, Xu Y, Zhang H, Li Y, Huang X, Ren B, Zhang X. 2009. Temperature-dependent expression of *phzM* and its regulatory genes *lasI* and *ptsP* in rhizosphere isolate *Pseudomonas* sp. strain M18. *Applied and Environmental Microbiology*, **75**(*20*): 6568-6580.
[37]. Gharieb MM, El-Sheekh MM, El-Sabbagh SM, Hamza WT. 2013. Efficacy of pyocyanin produced by *Pseudomonas aeruginosa* as a topical treatment of infected skin of rabbits. *Indian Journal of Biotechnology*, **7**(*5*): 184-193.
[38]. Das S, Das P. 2015. Effects of cultivation media components on biosurfactant and pigment production from *Pseudomonas aeruginosa* PAO1. *Brazilian Journal of Chemical Engineering*, **32**(*2*): 317-324.
[39]. Devnath P, Uddin MK, Ahmed F, Hossain MT, Manchur MA. 2017. Extraction, purification and characterization of pyocyanin produced by *Pseudomonas aeruginosa* and evaluation for its antimicrobial activity. *International Research Journal of Biological Sciences*, **6**(*5*): 1-7.

IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) is UGC approved Journal with Sl. No. 5012, Journal no. 49063.

Bianca Teixeira Morais de Oliveira " Craft beer waste as substrate for pyocyanin synthesis" IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 14.1 (2019): 21-25.