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Influence of Environmental Factors on the Production of Violacein Synthesized By *Janthinobacterium lividum*

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ABSTRACT

Violacein is a purple pigment extracted from bacteria, which has been reported with antimicrobial, anticancer, antioxidant and insecticide activities. Due to its biotechnological potential, studies have focused on the ability to synthesize this pigment from Chromobacterium violaceum, *however* Janthinobacterium lividum *also synthesizes it. Therefore the aim of this research was to determine the optimum growth conditions of* J. lividum *for increasing violacein production. The pigment production was evaluated under different environmental growth conditions: stirring speed (150rpm/200rpm), light conditions (light/dark) and temperature (4 °C/30 °C).The optimal culture conditions were used to evaluate different carbon sources: tryptone and nutrient broth supplemented with glucose or glycerol (1% w/v). The cultures were centrifuged at 16000xg by 20 minutes, and the liquid-liquid extraction of violacein was made with ethyl acetate. The sample was concentrated by rotavaporation, and the crude extract fractions were recovered by using HPLC. The best culture conditions were 150 rpm, without light, and refrigeration (4 °C). The culture medium favoring violacein production was the nutrient broth supplemented with 1% w/v glucose. The HPLC chromatogram showed the presence of two compounds that correspond to violacein and deoxyviolacein*.

Keywords: *Deoxyviolacein, HPLC,* Janthinobacterium lividum*, Pigment production, Violacein, Violet pigment.*

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I. INTRODUCTION

Currently, numerous researches are made, looking for natural sources of antibacterial, antifungal, antiviral and generally against diseases either on human or animals and plants [1,2]; secondary metabolites produced by microorganisms have proven to be resources with great biotechnological potential, being highly effective, safe, with fewer adverse effects than chemical growth conditions and also environmentally friendly [3-5].

Violacein is a purple pigment product of the secondary metabolism of bacteria, whose biological activities have been widely reported, including antimicrobial activity (antibacterial, antiviral, antiprotozoal and antifungal) [3,4,6-14] anticancer activity [15-19] antioxidant activity [20] and recent studies also show insecticidal activity [5]. Due to its biotechnological potential, numerous studies have been done indicating that *Chromobacterium violaceum* and *Janthinobacterium lividum* are primarily responsible for its synthesis [3-5,9,21,22].

Violacein has an indole derivative structure [8,15,17,18,21] characterized as C20-H13-N3-O3 [4,22,23]. The pigment is insoluble in water but soluble in alcohols (methanol, ethanol) and acetone.

The performance of the violacein biosynthesis pathway can be affected by different factors involved in the process [6,24], hence the nutritional requirements needed to increase the production of this metabolite have been widely studied [24-26], indicating the importance of controlling growth nutritional and environmental factors for the production of the pigment [22,27-30].

Violacein biosynthesis is regulated by a quorum-sensing system [21,31], which is a form of intercellular communication and population density-dependent gene regulation found in Gram-negative bacteria [23]. Despite the growing amount of related research, most of the knowledge on violacein is derived from studies carried out on the pigment produced by *Chromobacterium violaceum* [22] therefore, it is imperative to characterize the best conditions to produce violacein from *J. lividum*.

Janthinobacterium lividum is a Gram-negative, motile, strictly aerobic bacterium [32], commonly isolated from the microbiota of soils and water of rivers, lakes and springs [22]. It was isolated by Shivaji from the soils of maritime Antarctic [33], and Brucker and collaborators report that *J. lividum* can be found on salamander skin, because it is likely to be a mutualistic partner with salamanders and other amphibians [34].

Recent studies have focused on evaluating the activity of *J. lividum* [10-12,33-35], but it has not been defined a protocol indicating the optimum production conditions of violacein by this bacterium. Furthermore, different methodologies are found in literature [4,29,36,37], which is why it is essential to identify variations that occur in pigment production by altering growth conditions either environmental or nutritional for *J. lividum*. The aim of this research was to determine the optimal growth conditions of *Janthinobacterium lividum* for violacein production.

II. EXPERIMENTAL WORK

2.1 Microorganism:

The bacterial strain *Janthinobacterium lividum* was provided by Dr. Vance Vredenburg, ecologist from the Biology Department at San Francisco State University. It was isolated after culture by the streak plate method in tryptone agar (1% w/v) and nutrient agar media supplemented with glycerol (1% w/v) incubated at 24 °C for 24 h. *J. lividum* was maintained frozen at -4 °C in tryptone broth (1% w/v) and nutrient broth containing glycerol (25% w/v).

Bacterial inoculums were made inoculating colonies from culture plates of *J. lividum* in 90 mL of nutrient broth with glycerol (1% w/v) and incubated at room temperature (23 °C) at 150 rpm for 24 h. The production of crude violacein was carried out using a series of 125 mL Erlenmeyer flasks containing 30 mL of culture medium. Bacterial inoculums (10% v/v) were then added into the growth mediums followed by incubation in a rotatory shaker at the specific environmental conditions according to each experiment. Five different replicates were conducted for each growth condition and as a control; one flask with initial culture medium was used in all experiments.

Pigment concentration was determined by the collection every 24 h of an aliquot of 1 mL and its absorbance measured at a wavelength of 575 nm [4,29] for up to 5 days. The values of OD presented in figures were obtain after a mathematical treatment of dilutions and the measures.

2.2 Evaluation of environmental conditions:

Several experiments were carried out in nutrient broth supplemented with glycerol (1% w/v). Three factors were studied by conducting 8 experiments with the fermentation conditions shown in Table 1.

Table 1. 2^3 Factorial design for violacein production. The coded values corresponding for light conditions: - (Without light), + (Light).

Experiment	Factor		
	Agitation (rpm)	Temperature (°C)	Light
A	150	4	+
B	150	4	-
C	150	30	+
D	150	30	-
E	200	4	+
F	200	4	-
G	200	30	+
H	200	30	-

Evaluation of carbon sources for violacein production: The optimum fermentation conditions for violacein production were used to grow the cultures in the appropriate medium to evaluate different carbon sources: Tryptone broth (1% w/v) and nutrient broth (OXOID) were used and were supplemented with glucose (1% w/v) or glycerol (1% w/v) and as control culture medium without additional carbon sources.

2.3 Pigment extraction:

After the incubation period, violet pigment was extracted from cell suspension of *J. lividum* using the procedure adapted from a method described by Blosser and Gray [23] as follows: 40 mL aliquots of culture medium were transferred in 50 mL Falcon tubes and centrifuged at 16,000 *x g* for 20 min. Cell-free supernatants and bacterial cultures (pellet) were separated and the extraction was carried out according to Matz et al. [37] and Brucker et al. [34] respectively.

Extraction was performed from the supernatant, evaluated as organic solvents ethyl acetate (EtOAc) [34] and ethanol (EtOH) [38] in 1:1 v/v by a separating funnel. It was shaken and formed two phases; the aqueous (lower) phase was recovered and washed successively collecting the organic (top) phase containing the pigment. For pellet extraction [37], this was resuspended in 400 uL of medium using vortexing and then the cells were lysed with 400 uL of sodium dodecyl sulfate (SDS) 10% v/v undergoing vortexing. After 5 minutes incubation at room temperature, extraction was performed using ethyl acetate and ethanol 1:1 v/v and centrifuged again at 16000xg for 10 minutes to get the upper phase containing violacein. In both cases ethyl acetate was used as organic solvent to obtain violacein crude extracts from each different growth condition. Each extract was concentrated in a rotaevaporator under low pressure at 40 °C and were kept frozen until further analysis.

2.4 HPLC analysis:

Samples from each treatment were purified by Shimadzu - Prominence HPLC-(High-Performance Liquid Chromatography), following the protocol proposed by Brucker et al. [34]: the crude extract was dissolved in 2 mL of EtOAc and filtered using 0.22 micron PTFE filters; DAD detector was programmed to record absorbance between 200 and 600nm. A column of reverse phase C18 (150 mm × 4.6 mm × 5 um) brand Restek, with flow of 1 mL/min was used and 20 mL of sample was injected into the machine. The initial eluant was 10% v/v acetonitrile/water (acidified with 0.1% w/v acetic acid) for 2 minutes, then a linear gradient to 100 % v/v acetonitrile acidified for 18 minutes, and finally the solvent was eluted by 3 minutes.

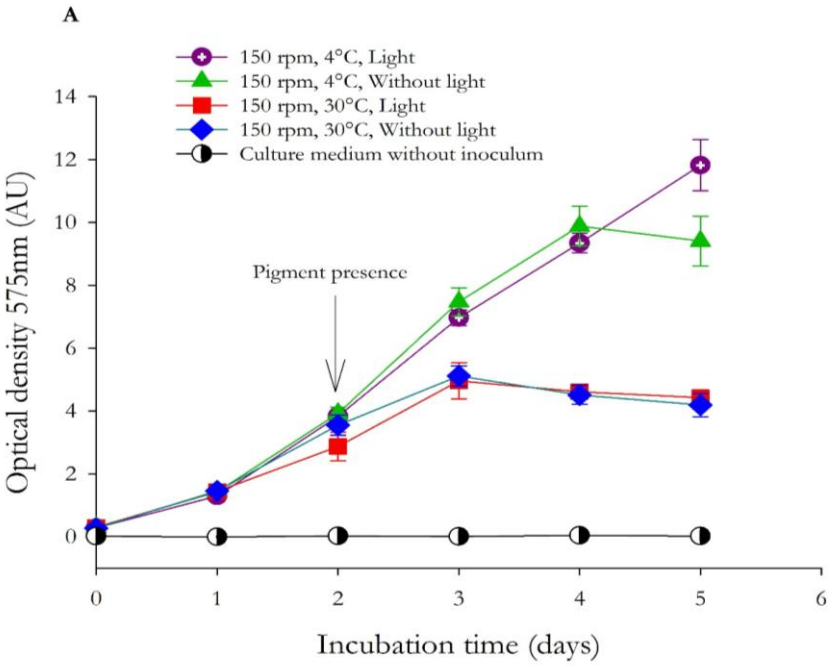
2.5 Statistical analysis:

Results were expressed as mean ± SD. All data collected from the experimental design were analyzed for variance using one-way analysis of variance (ANOVA) followed by Tukey's and Duncan post hoc test with statistics *software* IBM SPSS Statistics version 19. Statistical significance was established at $P < 0.05$.

III. RESULTS AND DISCUSSION

3.1 Evaluation of environmental conditions:

The results obtained for the 2^3 factorial design are presented in Figure 1. The beginning of cell pigmentation was observed on day 2 of the culture, in the growth conditions that induced violacein production (Fig. 1 A.B).



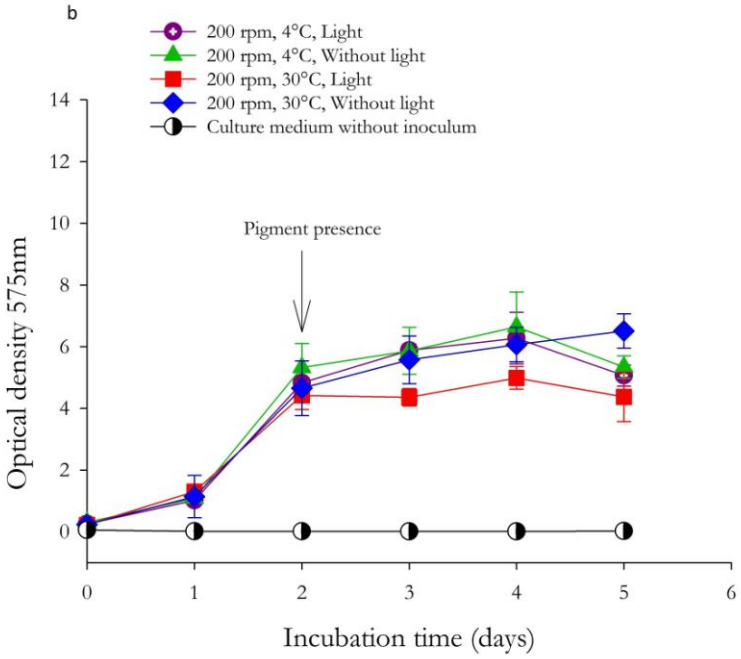


Figure 1. Effect of different environmental conditions **A.** 150 rpm, **B.** 200 rpm on the growth and violacein production (OD575nm) by *J. lividum* versus time. Results are reported as mean values ± standard deviation (SD) of experiments performed in quintuple. Arrows indicate commencement of pigment formation in each of the growth conditions.

Pigmentation was observed in growth conditions of 4 °C (Fig. 1), while cultures grown at 30 °C showed no color in any case. The optical density of 9 and 11 obtained in the treatments performed at 150 rpm, 4 °C with and without light respectively, were the highest concentrations of the pigment presented (Fig. 1A); while growing at 200 rpm, 4 °C in light and dark conditions, the optical density was 4 AU (Fig. 1B).

According to the analysis of variance for the growth conditions evaluated at 150 rpm, it was found that there are differences between treatments (ANOVA, $p = 0.001$), therefore, post hoc tests for comparison of means were performed and two treatment groups were statistically significant, which led to the conclusion that the most suitable growth conditions for the pigment production were at 150 rpm, 4 °C either with or without light. Additionally, in the comparison of incubation time, significant difference was found, indicating that the incubation up to 5 days will allow to obtain more pigment.

Moreover, in the case of the treatments evaluated at 200 rpm, no significant difference between these (ANOVA, $p = 0.529$) were found, although two of them induced the production of pigment (4 °C) and the other two inhibited this process (30 °C).

Different criteria were consider for choosing the most appropriate growth condition: Increased pigment production, evidenced by the significant differences that occur in the absorbance measurements; increased speed inducing the synthesis of violacein, defined by generating pigmentation treatment in the shortest time and uniformity in replicates, assessed by the standard deviation of the data.

Since the presence or absence of light showed no significant difference, the criteria described above allowed to select 150 rpm, 4 °C as the optimal growth conditions for violacein production by *J. lividum*, using them in subsequent tests assessing different nutritional conditions.

Growth at 4 °C, and the lack of pigmentation at 30 °C confirmed previous reports [33,38,39] who isolated psychrotrophic strains *Janthinobacterium* sp. growing at temperatures between 2 °C and 28 °C, but above 30 °C showed no growth. Additionally, Lu et al. [38] reported that the production of violacein is favorable at lower temperatures, which can be explained by the results of recent researches [35] suggesting that violacein can have cryoprotectant properties. According to their experiments, the bacteria that produce the pigment were more resistant to freeze-thaw cycles than non-pigmented, indicating that these pigments may play a significant role in the maintenance of the membrane fluidity, protecting them from freezing damage and cold adaptation.

The results indicate that pigment production was enhanced under growth at 150 rpm, 4 °C in both, light and without light. Although no significant difference was found between light conditions, Ahmad et al. [26] reported

that violacein may be susceptible to light, because the absorption of light in the UV and visible range leads to excitation of electrons in the molecule, increasing their reactivity and inducing chemical reactions such as photo-oxidation.

These results agree with previous reports [22,30], who obtained higher violacein production by growing bacteria at low agitation speeds. They explain that this phenomenon is possible due to the formation of cell aggregates under these conditions, which also let bacteria adhere to surfaces and develop biofilms, inducing different metabolic pathways that increase violacein production, while higher agitation speed, affects the aggregation of bacterial cells, thus the pigment biosynthesis process might be affected.

According to previous researches, high pigment concentration varies significantly with temperature changes [38,40], resulting favored by low temperatures [38], which confirms the psychrotrophic characteristics showed by the strain worked in this investigation.

3.2 Carbon sources for violacein production:

Liquid cultures obtained under growth conditions with different carbon sources were centrifuged, and the pigment was observed in both pellet and supernatant, whereby violacein extraction was performed from both, according to the methodology described.

When the extracts were concentrated by rotavaporation, the mass of each extract obtained from the growth conditions under different nutritional conditions was measured. We were able to recover pigment from each of the fractions, both the supernatant and the pellet (Fig. 2) finding significant differences on pigment production from nutrient medium supplemented with glucose.

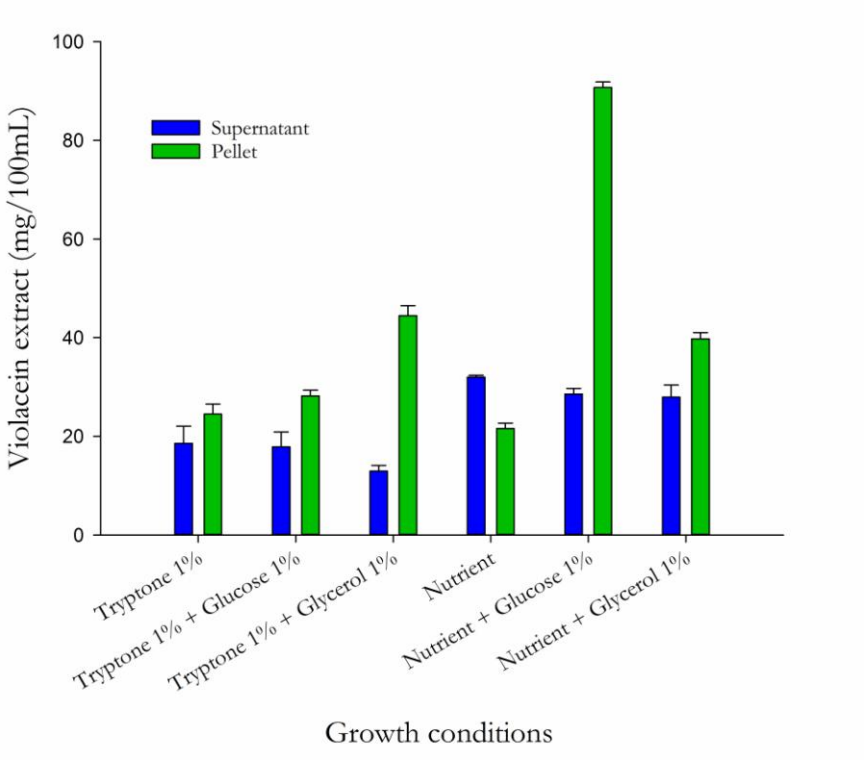


Figure 2. Concentration of violacein crude extract produced by *J. lividum* in pellet and supernatant from growth conditions under different nutritional culture conditions.

Although the pigment was observed in both fractions, from the pellet was recovered the highest concentration of violacein crude extract (Fig. 2). The quantification of the extract mass under each growing condition was similar to data obtained in initial tests in which the pigment concentration was measured by optical density, confirming that the nutrient broth supplemented with glucose induces greater pigment amount.

Pantanella et al. [22] and Lu et al. [38], indicated that simple culture media containing tryptophan, favors pigment production, because it appears to be the only precursor molecule in violacein biosynthesis, suggesting that violacein production could be associated with tryptophan regulation, that at high concentrations is toxic for bacteria [24,41], which explains *J. lividum* capacity to grow and produce pigment both tryptone medium and nutrient medium as they have high amino acid content.

Additionally it was reported that violacein production is dependent on the carbon source and the concentration in the culture medium [41] furthermore, that violacein biosynthesis is dependent on the metabolism of carbohydrates [38,41]. It is also reported that violacein production is stimulated by glycerol but is reduced in the presence of an easily metabolized carbon source such as glucose or fructose [22].
It is known as well, that for some microorganisms, carbon sources with rapid metabolisms result in an increase in cellular mass and a decrease of secondary metabolic production, however the use of carbon sources of slower consumption results in a decrease of cellular growth and an increase in secondary metabolic production [29] which let us understand the ability of *J. lividum* to synthesize pigment using glycerol as carbon source.
Similarly, it is reported that carbon sources of easy consumption, such as glucose or fructose, may affect violacein biosynthesis process, since they stimulate biomass growth and thus reduce secondary metabolic production [21,29].
Ahmad et al. [26] suggests the existence of genetic repression mechanisms during the production of violacein by *C. violaceum* under high concentration of glucose, which can be associated with the observed in this research with tryptone medium, where could be some interaction between the carbon compounds, leading into repression systems. However, these phenomena do not match the results obtained with nutrient medium supplemented with both glucose and glycerol, since in both cases pigment was observed.
Although pigment was recovered from the two fractions (Figure 2), the higher concentration of violacein crude extract was recovered from the pellet, which is consistent with previous reports indicating that the pigment is produced intracellularly, therefore it is retained within the cells after centrifugation [37,38]. The results of extract mass quantification in each growth condition was similar to data obtained in initial tests in which pigment concentration was measured by optical density, confirming that nutrient broth supplemented with glucose induces the pigment production.

3.3 Identification of chemical structure of the violet pigment produced by *J. lividum*

Compounds produced by *J. lividum* that were retained in the solvent, and therefore are part of the violacein crude extract, were fractionated by HPLC, obtaining two peaks at 575nm, around 16.1 minutes and 17.8 minutes (Fig. 3A).

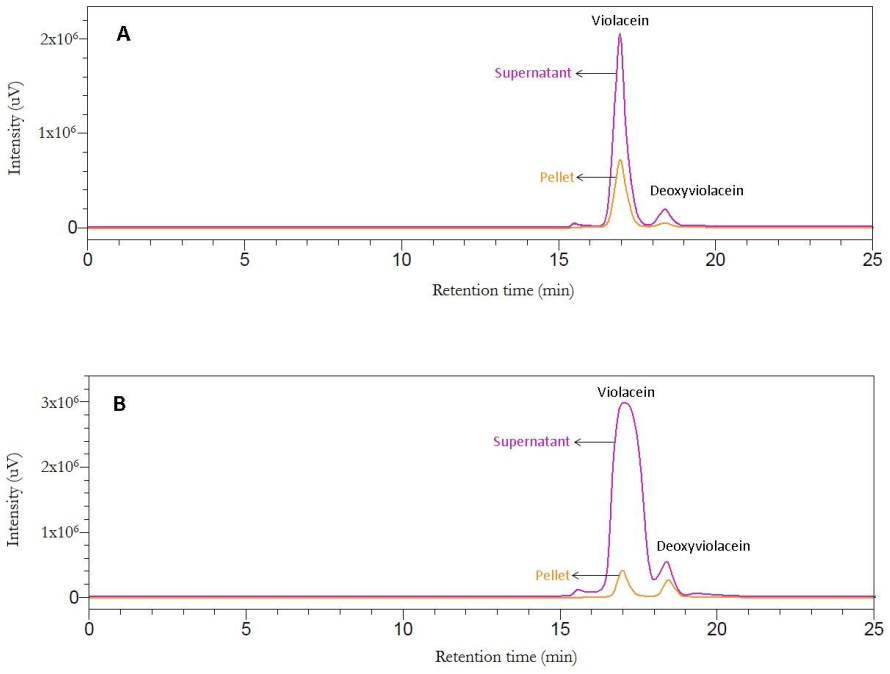


Figure 3. HPLC chromatograms (575nm) of crude extract *J. lividum* cultures under different nutritional growth conditions. **A.** Tryptone 1% w/v + Glucose 1% w/v. **B.** Nutrient + Glucose 1% w/v.

According to the size of the peak obtained, it can be qualitatively estimated from the chromatograms (Fig. 3), that the concentration of violacein produced was higher than the concentration of deoxyviolacein. Additionally, it was observed that the pigment concentration of the supernatant obtained was greater than the concentration obtained from the pellet.

Finally, qualitative estimation of the pigment concentration, allowed to confirm that the treatment carried out using nutrient broth supplemented with glucose, induced the major pigment production (Fig. 3b).

Several authors have reported that the violacein crude extract usually is found as a mixture of violacein and deoxyviolacein [21,29,32,38]. Particularly, Rodrigues et al. [42] reported that, by separating the mixture of these compounds, it can be obtain two completely separate fractions: the first peak containing violacein and subsequently deoxyviolacein [42,43].

According to the size of the peak obtained from the chromatograms (Figure 3) the concentration of violacein produced, can be qualitatively estimated, finding that the concentration of violacein was higher than deoxyviolacein concentration; Rodrigues et al. [42] argues that these pigments display a broad range of interesting biological properties but their production is rarely distinguished due to the lack of suitable analytical methods.

Finally, it was observed that the pigment concentration obtained from the supernatant was higher than the obtained from the pellet, which is opposite to the measures made by optical density and the extract mass. Several factors can affect the absorbance measurement of the culture, mainly the presence of biomass affects optical density and thus the measurement, however, in the present study, culture centrifugation was not conducted, since the pigment is retained in the two fractions: extracting the pigment either from pellet or the supernatant would be mistake in the quantification.

IV. CONCLUSION

According to the criteria for evaluating growth conditions, it was established that the optimum for violacein production by *J. lividum* are 150 rpm, 4 °C; and the most adequate culture media were nutrient broth supplemented with glucose or glycerol. Since the pigment exhibits several biological activities, this study allows the standardization of growth conditions, increasing its commercially interesting uses, especially for industrial applications in cosmetics, medicines and fabrics.

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