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Characterization of *Chromobacterium violaceum* isolated from Paca River, Pernambuco, Brazil

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ABSTRACT

Chromobacterium violaceum is a facultative anaerobic, motile, gram-negative bacillus, inhabitant of soil and water, and the most strains produce a violet pigment, violacein. Studies were carried out a strain of *Chromobacterium violaceum*, UCP 1489 isolated from Paca river of Pernambuco, evaluating the growth in different culture media, the biochemical characteristics, the susceptibility to the antimicrobian, and the biosurfactant production. The isolates was demonstrated exuberant growth on Luria Bertani medium, supplemented with glucose, negative for the following tests: the urea, manitol, mannose, lactose, indol, and lysine; and positive for gelatinase, glucose, motility, catalase, sucrose, oxidase and fructose. The strain showed resistant to cephalothin, imipenem, chloramphenicol, ampicillin and amicacin. The biosurfactant production by *Chromobacterium violaceum*, UCP 1489, showed reduction of superficial tension of water from 71 mNm-1 to 26mN/m-1, indicating high biotechnological potential for producing bioactive agent.

Key words: *Chromobacterium violaceum*, Paca river, antibiotical resistence, biosurfactant production

RESUMO

Chromobacterium violaceum é um anaeróbio facultativo, móvel, bacilo gram-negativo, habita solos e águas, e a maioria das espécies produzem um pigmento violeta. Estudos foram conduzidos com o isolado *Chromobacterium violaceum*, UCP 1489 isolado do rio Paca, Pernmabuco; sendo avaliado o crescimento em diferentes meios de cultura, as características bioquímicas, a susceptibilidadeaos antimicrobianos e a produção de biossurfactante. O isolado demonstrou crescimento exuberante no meio Luria Bertani suplementado com glicose, negativo para os seguintes testes: uréia, manitol, manose, lactose, indol e lisina; e positivo para gelatinase, glicose, motilidade, catalase, sacarose, oxidase e frutose. A amostra apresentou resistência para: cefalotina, imipenem, cloranfenicol, ampicilina e amicacina. A produção de biossurfactante pelo *Chromobacterium violaceum* UCP 1489, apresentou uma redução da tensão superficial da água de 71 mNm-1 para 26mN/m-1, indicando alto potencial biotecnológico para a produção de agentes bioativos.

Key words: *Chromobacterium violaceum*, rio Paca, resistência antibiótica, produção de biossurfactante.

1 INTRODUCTION

Chromobacterium violaceum is a Gram-negative bacterium, abundant in a variety of ecosystems in tropical and subtropical regions, including soil, water and borders of the Negro River, a major component of the Amazon Region. As a free-living microorganism, *C. violaceum* is exposed to a series of variable conditions

such as different sources and abundance of nutrients, changes in temperature and pH, toxic compounds and UV rays (CALDAS, 1990; BOONE, CASTENHOLZ, 2001; HUNGRIA et al., 2004).

In general, have focused on the most notable product of the bacterium, the violacein, a purple pigment first isolated in 1944 (STRONG, 1944), which has already been introduced as a therapeutic compound for dermatological purposes (CALDAS et al., 1978). Violacein also exhibits antimicrobial activity against the important tropical pathogens *Mycobacterium tuberculosis* (SOUZA et al., 1999), *Trypanosoma cruzi* (DURÁN et al., 1994; RETTORI, 2000; DESSAUX et al., 2004), *Leishmania* sp. (LEON et al, 2001), antiviral (DURÁN et al., 2001), anticancer activity (UEDA et al., 1994; MELO et al, 2000, DIAS JR. et al., 2002), and is reported to have other bactericidal activity (CALDAS, 1990; LICHSTEIN & VAN DE SAND, 1945, 1946; DURÁN et al., 1983; DURÁN, 1990). Otherwise, *C. violaceum* was first described by Bergonzini, (1881 in Johnson et al, 1971). The first recognized human cases occurred in Malaysia in 1927 and reported by Johnson et al., (1971) and Lesslar, (1953) in the 1950s. Other countries and regions where infection with *C. violaceum* has been recognized include Argentina, Brazil, India, Malaysia, Senegal, Singapore, Thailand, Vietnam, and Northern Australia. In the past, it is likely that *C. violaceum* was isolated but not recognized as a pathogen (SCHATTENBERG, HARRIS, 1941; SNEATH et al., 1953). The bacteria are the largest responsible for the production of these compounds. These microorganisms have been isolated of the soil, sea water, sediments, and polluted areas (ABU-RUWAIDA et al., 1991; MAKKAR AND CAMEOTRA et al., 2000).

Surfactants are amphipathic molecules have both clearly defined hydrophilic and hydrophobic groups. The formation of a molecular film, orderly in the interfaces, it reduces the tension interfacial and superficial being responsible for the only properties of the surfactants. These moieties partition preferentially at the interface between fluid phases with different degrees of polarity, such as oil-water or air-water interfaces (HEALY et al. 1996). These compounds have the property of reducing surface and interfacial tension in both aqueous solutions and hydrocarbon mixtures, and thereby have potential applications in the recovery of oil, and the pharmaceutical and food.

The aim of this work was to isolate, identification, semi-defined medium for the growth, and biochemical characterization of the new strain compared with standard strain of *C. violaceum.* The biotechnological potential of biosurfactant production of the both strains were investigate using some selected oils (corn oil, soy oil and canola oil) as a carbon source.

2 MATERIAL AND METHODS

2.1 Microorganism:

Collect and isolation

A water sample was collected in Paca, river located in the city of Camaragibe, Pernambuco. The water was collected and kept in bottle amber and submitted to the isolation and identification of microorganisms. Technique of the multiple pipes was used, being carried through the presumptive assay, using 10 mL of the sample and inoculated in Petri dishes containing nutritive agar, and incubated at 37 ^{o}C, for 24 hours for counting of the colonies. The microorganisms isolated from contaminated water produced violet colonies, characterized as *Chromobacterium violaceum*. The strain was deposited in the Bank of cultures of the Nucleus of Researches in Environmental Sciences of UNICAP/PE, Brazil, maintained in the Luria Bertani solid medium (SAMBROOK et al., 1989) at 5^{o}C.

*Growth and Biochemical Characterization***:**

The strain was transferred for Petri dishes for testing different culture media: Brain Heart Infusion (BHI) [Peptone of gelatin – 10,5g/L; brain heart infusion g/L; peptone of meat – 11g/L; dextrose – 2g/L; sodium chloride – 5g/L; phosphate dibasic of sodium – 2,5g/L], Luria Bertani (LB)

[Triptone – 10g/L; sodium chloride – 5g/L; yeast extract – 5g/L; agar – 15g/L], LB with glucose [Triptone – 10g/L; sodium chloride – 5g/L; yeast extract – 5g/L; glucose – 5g/L; agar – 15g/L], Müeller Hinton agar [infusion of meat – 2g/L; casein hydrolyzed – 17,5g/L; starch – 1,5g/L; agar – 15g/L], King A agar [peptone – 20g/L; magnesium chloride – 1,4g/L; sulfato of ammonium – 10g/L; agar – 15g/L], Mac Conkey ágar (MC) [peptone – 19g/L; lactose – 10g/L; sais biliares – 1g/L; sodium chloride – 5g/L; crystal violet – 0,001g/L; red neutro – 0,03g/L; agar – 15g/L], M9 agar [NH_4Cl – 1g/L, Na_2HPO_4 – 6g/L; K_2HPO_4 – 3g/L; NaCl – 0,5g/L; glycerol ou asparagine – 5g/L; magnesium sulfate 1M – 1mL; calcium chloride 0,01M – 10mL; agar – 15g/L] Cled agar [peptone – 4g/l; meat extract – 3g/L; tryptone – 4,0g/L; lactose – 10g/L; l-cystine – 1,128 g/L; blue of bromothimol – 0,02g/L; agar – 15g/L], BTB Lactose agar [meat extract – 5g/L; peptone – 10g/L; lactose – 10g/L; blue of bromothimol – 0,008g/L; agar – 15g/L] and incubated for 48 hours at 30°C. For characterization of the strain, tests of fermentative activity were accomplished (glucose, mannose, fructose, sucrose, lactose and manitol), and urea, indol, lysine, catalyses, gelatinize and oxidize tests disk CECON, and motility test (BALOWS et al., 1991; KONEMAN et al., 2001).

Antimicrobial Activity

The antimicrobial tests was accomplished according to methodology described by Kirby-Bauer. Initially the isolate was grown in LB solid medium for 48 hours to 30°C. The isolated was transferred for test tubes containing 5mL of the nutritious broth (TSB), and incubated for 2 hours at 30°C. After this period brackets of the nutritious broth were removed, sowed in the medium plates containing Müeller Hinton ágar, with the aid of sterile swab. Afterwards, disks of 6 millimeters of diameter of the antibiotics (CECON) amicacin, ampiciclina, aztreonam, cefalotina, ciprofloxacina, cloranfenicol, gentamicina, imipenem, nitrofurantoina, and tetraciclina, were deposited in the surface of the medium in a halfway. The plates were incubated to the temperature of 30°C for 18 hours and the reading of the halos formed around the disks was accomplished with a halometer, expressed in sensible or resistant. All tests were compared with *C. violaceum* standard strain.

Biosurfactant production:

The strains *C. violaceum* UCP 1489 and was maintained and transferred to Luria Bertani (LB) solid medium, at 5°C. The biosurfactant production was carried out in Erlenmeyer flasks of 250mL capacity containing 50mL of LB liquid Luria Bertani medium [triptona - (10g/L); yeast extract - (5g/L); NaCl - (5g/L); glucose - (5g/L). Glucose (5g/L), added of corn oil, soy oil and canola oil, maintained in orbital shaker 150 rpm, at 30°C, in different periods of time (24hours, 48 hours and 72 hours]. The production of the biosurfactant was determined by superficial tension using automatic tensiometer (Sigma 70-KSVLTD/Finland) according Kuyukina, (2001).

3 RESULTS AND DISCUSSIONS

The analysis of the obtained results showed that the isolated of *C. violaceum*, UCP 1489, presented growth in the following used medium: Luria Bertani (LB) without glucose and LB with glucose, Müeller Hinton, M9, MacConkey (MC), BHI (Brain Heart Infusion), as well as in the introduction of new medium as: Cled agar, King A and BTB Lactose. It was still observed, that the lineage of *C. violaceum*, UCP 1489, grew in all of the culture means used (Table 1). Among the tested means, the LB medium with glucose stood out as middle of larger growth for the lineage UCP 1489, being confirmed by all of the lineages of *C. violaceum* used by Antunes et al, (2006). It was still observed, that the isolated of *C. violaceum*, UCP 1489, grew in all of the culture medium used (Table 1). The isolated studied, UCP 1489, presented white and violet colonies in King A and BTB lactose medium (Table 2). According to Trabulsi et al., (1999), Myers et al., (1992) and Sorenson et al, (1985), not all stains are pigmented, and some strains may produce

pigmented and nonpigmented colonies on the same plate (DAUPHINAIS, ROBBEN, 1968; OGNIBENE, THOMAS, 1970; VICTORICA et al, 1983). Creczynski-Pasa and Antonio, (2004), tell that the results of the good growth of *C. violaceum* in the different culture medium are leaning for the literature and they consider the microorganism as a bacterium no demanding. The authors say that *C. violaceum* is able to live in both aerobic and anaerobic conditions. In aerobic conditions, *C. violaceum* is able to grow in a minimal medium with simple sugars, such as glucose, fructose, galactose, and ribose; Embden-Meyerhoff, tricarboxylic acid and glyoxylate cycles are used.

Like this, the obtained results confirm the easiness of cultivating *C. violaceum* in Luria Bertani medium with glucose, and was confirmed using the standard strain (CRECZYNSKI-PASA AND ANTONIO, 2004).

Table 1. . Growth of *Chromobacterium violaceum*, UCP 1489 compared with *C. violaceum* IUCP1471 (standard), on different culture media.

MEIOS DE CULTURA	UCP 1489	UCP 1471
KING A	+*	+*
CLED ÁGAR	++	++
LB	++	++
LB + GLUCOSE	++++	++++
M9 + GLUCOSE	+	+
BTB LACTOSE	++*	++
MAC CONKEY	++	++
MUELLER HINTON	++	++
BHI	++	++

(-) absence of growth
(+) 10 UFC/mL
(++) 70-100UFC/mL
(++++) >200 UFC/mL
∗ colonies of violet and white color

The biochemical tests realized with the isolated of *C. violaceum*, UCP 1489, are presented in the table 3, being confirmed in the literature by several authors. (BAILEY AND BARON, 1994; RETORI, 2000; DURÁN et al., 2001; KONEMAN et al., 2001).

Table 2. Result of the biochemical tests accomplished with the isolated of *Chromobacterium violaceum*, UCP 1489 compared with *C. violaceum* IUCP 1471 (standard),.

TESTS	UCP 1489	UCP 1471
GELATINASE	+	+
URÉIA	-	-
GLUCOSE	+	-
MANITOL	-	-
MANOSE	-	+
MOTILIDADE	+	+
CATALASE	+	+
LACTOSE	-	-
SUCROSE	+	+
INDOL	-	-
OXIDASE	+	+
LISINA	-	-
FRUTOSE	+	+

(+) Positive
(-) Negative

The antimicrobial therapy demonstrated that besides presenting resistance to the ampicillin and cephalothin, this isolated also presented resistance to imipenem, to chloramphenicol and and amicacin (Table 3). The largest sensibility halos were observed for ciprofloxacin, with superior diameters to 30 mm. According Sorenson et al., (1985); Kaufman et al., (1986); Suarez et al., (1986); Georghiou et al, (1989); Ponte e Jenkins, (1982) ; Hassan et al, (1993), *C. violaceum* should be considered resistant to penicillin, amoxicillin, amoxicillin-clavulanate, ampicillin, cephalothin, cefamandole, and vancomycin. Once again, the obtained results are confirmed by the literature; where the resistance is observe by *C. violaceum* to cephalothin and ampicillin (Tabela 3).

Although many authors repeat the statement that C. violaceum is always susceptible to chloramphenicol, tetracycline, and the aminoglycosides (Yo et al, 1999), corroborating with the obtained results by Antunes et al., (2006), with 12 of the studied strains, except for the isolated UCP 1489,

the chloramphenicol, tetracycline, and the aminoglycosides (Table 3).

Os resultados obtidos sugerem que o local de origem do isolado e as condições ambientais apresentam uma relação direta com a susceptibilidade aos antimicrobianos.

Table 3. Result of the antimicrobial activity with the isolated of *Chromobacterium violaceum*, UCP 1489 compared with *C. violaceum* IUCP1471 (standard).

ANTIBIOTICS	UCP1489	UCP 1471
CEFALOTINA	R	R
IMIPENEM	R	S
GENTAMICIN	S	S
CHLORAMPHENICOL	R	S
AMPICILLIN	R	R
TETRACYCLIN	S	S
AMICACIN	R	S
NITROFURANTOIN	S	S
AZTREONAM	S	S
CIPROFLOXAXIN	S	S

R - Resistente
S - Sensível

The superficial tensions of the strain UCP 1489 showed significant reductions of superficial tension between 26.6 and 26 mN/m respectively to soy oil, glucose, corn oil and canola substrates, during 24 hours. The biosurfactant produced by *Chromobacterium violaceum* UCP 1489 show the best results of reduction of superficial tension of water from 71 mN m-1 to 26mN/m, and indicated high biotechnological potential for producing biosurfactants agent.

Table 4. Biosurfactant production by *Chromobacterium violaceum*, (UCP 1489) using different substrates and the superficial tensions of each condition for fermentation.

C.violaceum UCP 1489	24 HOURS	48 HOURS	72 HOURS
SOY OIL	26.00	26.31	27.86
CORN OIL	26.16	27.05	27.32
CANOLA OIL	26.58	27.04	27.13
GLUCOSE	26.07	27.15	27.61

Table 5. Biosurfactant production by *Chromobacterium violaceum*, ATCC 12472 (standard) using different substrates and the superficial tensions of each condition for fermentation.

C.violaceum UCP 1471	24 HOURS	48 HOURS	72 HOURS
SOY OIL	30.46	35.13	32.39
CORN OIL	34.21	35.89	32.99
CANOLA OIL	33.14	43.45	32.18
GLUCOSE	34.31	38.57	36.94

4 CONCLUSIONS

The isolate strain from Paca river is confirmed to be *Chromobacterium violaceum* and shown similar characteristics with *C. violaceum* UCP1471, and is the first description of isolation of *C. violaceum* in Pernambuco. The new strain demonstrated different profile of antimicrobial resistence, evidencing genetic variability. *C. violaceum* 1489 showed higher ability of biosurfactant production and distinguished from the standard strain UCP 1471, and besides being a good surfactant, has attractive properties as an emulsifier, two characteristics that are not easily found together in other kinds of biosurfactants.

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