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Research Article

Production of carotenoid sarcinaxanthin by *Kocuria palustris* isolated from Northeastern Brazil Caatinga soil and their antioxidant and photoprotective activities ☆

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ABSTRACT

Background: This study aimed to produce carotenoids of two bacterial strains obtained and isolated from Caatinga soil in Northeastern Brazil and to evaluate their antioxidant and photoprotective activities. The morphological identification of bacteria was performed by Gram staining and molecularly confirmed through the *16S rRNA* gene. The production of carotenoids was performed on two 2^3 factorial designs to analyze the influence of independent variables (temperature range, luminosity, agitation, spiral presence, and bacterial isolate type) for maximum carotenoid yield. The selected condition has been transferred to a bioreactor (10L). The identification of carotenoids was performed by liquid chromatography (HPLC) and mass spectrometry (LC-MS). Antioxidant activity was determined by inhibiting the β-carotene/linoleic acid system and the effectiveness as sunscreen was measured through its sun protection factor (SPF).

Results: The results revealed that the isolates FT-7.22 and FT-5.12 were identified as *Kocuria palustris;* producers of a rare C50 carotenoid sarcinaxanthin. This is the first report on the production of carotenoids by this species from the Caatinga Domain. The pigment that was obtained from the Tryptic Soy Broth (TSB) medium in the best conditions of the factorial designs (increased agitation, aeration, and light exposure) exhibited a significant increase in the carotenoid production. The isolated FT-7.22 reached a higher sarcinaxanthin concentration (112,480 μg/L), and it exhibited promising antioxidant (76.53 ± 0.09%) and photoprotective activities (SPF = 9.36 ± 0.52).

Conclusions: This study demonstrated the ability of *K. palustris* to produce carotenoid sarcinaxanthin with antioxidant and photoprotective activities so that it can be applied in cosmetic formulations.

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1. Introduction

Carotenoids belong to a group of natural pigments responsible for giving the yellow, orange, and red colors. They are found in fruits, vegetables, flowers, fishes, birds, and microorganisms [1].

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Different structures of carotenoids allow their usage in food and feed industries (as dyes and nutritional supplements), presenting excellent capacity for singlet oxygen-scavenging, UV protection, provitamin A properties, and anticancer activities, apart from being anti-inflammative and antidiabetic [2,3]. This biological role strengthens the immune system, reducing the risk of degenerative diseases, and also improves skin texture and gap-junction communication [4,5].

Carotenoids can be produced in more than 700 different known structures [6]. More than 95% carotenoids are formed by a chain of 40 carbons; lesser numbers are formed from 30 carbon atoms; and even fewer are formed by 50 carbon atoms [7]. However, to date, β-carotene and astaxanthin (C40) make up the largest market share of carotenoids, worth about USD 2500/kg [8,9]. Medical research has studied only a limited number of carotenoid species, such as lutein and lycopene [10].

Little investigation has been conducted on the C50 carotenoids, which are known to have several conjugated double bonds, with at least one hydroxyl group. These characteristics contribute to strong antioxidant and photoprotective properties [7,11]. One such C50 carotenoid is sarcinaxanthin, which has been patented as an effective sunscreen [12].

While carotenoids produced from fruit and vegetables have been well investigated, other important carotenoid sources, such as microorganisms, have received far lesser attention. Bacterial carotenoid synthesis is a viable alternative for carotenoid production. Some examples of good carotenoid-producing species identified are: *Staphylococcus aureus* [13], *Flavobacterium* sp. [14], *Paracoccus carotinifaciens* [15], *Xanthophyllomyces dendrorhous*, *Agrobacterium aurantiacum* [16], and *Micrococcus luteus* [11]. The bacterial pigments are generally stable when exposed to UV light. Industrially, they have been already used as absorbers and free radical scavengers, suggesting their use in conjunction with bacterial pigments; especially the carotenoids [14].

Despite challenges related to the scaling up of pigment production from bacteria, the present technology has the potential to overcome these challenges for various applications [14]. Studies using statistical methods for the optimization of carotenoid production have made it possible to raise productivity – by modifying physical and cultural factors such as temperature, light, and aeration, to augment the concentrations of pigments produced [17,18]. Furthermore, another strategy is to use bioreactors for large-scale production.

The soil of Caatinga is a very peculiar habitat, with its own characteristic temperatures which range between 20°C and 30°C that reach up to 60°C in drought periods [19,20,21]. This region has a significant microbial biodiversity, with numerous microorganisms that produce natural molecules with excellent biological activities and high biotechnological potential. For example, microorganisms can accumulate carotenoids in response to various environmental stresses, such as oxidative stress caused by high solar incidence [22]. *Actinomycetes* that grow under these extreme conditions include some carotenoid-producing species, such as some representatives of the Micrococcaceae family [23,24,25].

However, bioprospecting research activities for biotechnological applications are still quite limited in this domain [23,24,25,26]. Little is known about the bacteria that produce carotenoids in the Caatinga, as only 1% of them (including pigmented and nonpigmented) have been described, stimulating interesting discoveries of new natural sources of carotenoids and their applications Within this context, we investigated two yellow-colored bacterial isolates from the Caatinga Domain, to characterize and optimize the production of carotenoids. In addition, the biological activities of the carotenoids produced were analyzed.

2. Materials and methods

2.1. Isolation

The isolates FT-5.12 and FT-7.22 were collected from the Private Natural Heritage Reserve (RPPN) Tamanduá Farm, situated in the municipality of Santa Terezinha, (coordinates 7° 2″ 20″S and 37° 26″ 43″W) in the Mesoregion of the state of Paraiba backlands. The samples were collected aseptically in plastic bags from the 0–20 cm layer of the soil, transported at 4°C, and kept in a refrigerator till processing. Enrichment was carried according to Soares et al. [27]. Finally, the selected colonies were purified by depletion and preserved in a 20% glycerol solution at −20°C. Each experiment was carried out in triplicate.

2.2. Bacterial identification

The morphological identification was carried out using Gram-staining [28] and molecular identification by sequencing of the *16S rRNA* gene. For molecular identification, PCR was performed using the forward (5′-3′-AGAGTTTGATCCTGGCTCAG) and reverse (5′-3′-AAGGAGGTGATCCAGCC) primers, under specified conditions for amplification [29]. The amplicons were visualized in agarose gel electrophoresis. The amplified products were purified with a PureLink purification kit (Invitrogen, USA) and sequenced (Applied Biosystems 3500 Series Genetic Analyzers, California USA).

The sequences were analyzed using Pregap4 and Gap4 tools in the software, STADEN 1.6, deposited to the GenBank under the accession numbers KX990291 (FT-5.12) and KX990292 (FT-7.22), and submitted to a similarity search in the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST) [30,31] and Ribosomal Database Project (RDP). Bacterial identification was assumed reliable when the query sequence exhibited a similarity >97% for the *16S rRNA* gene sequence [32].

2.3. Culture conditions

Purified cultures of FT-7.22 and FT-5.12 were transferred into 250 mL Erlenmeyer flasks containing 50 mL TSB medium (17.0 g casein, 3.0 g soy, 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g dextrose to 1000 mL distilled water). Preinoculum cells were grown with agitation at 30°C (150 rpm) in an incubator shaker (TECNAL TE-420, São Paulo Brazil) for 24 h. Initial kinetic growth was determined with a standardized optical density measurement of cell suspension (OD, 600 nm) of 0.1.

2.4. Production of carotenoid

The production of the carotenoid was performed in a broth (TSB) medium. Factorial designs were performed to evaluate the effect of light (L), incubation temperature (T), bacterial isolate type (Kpa), increased oxygen transfer rate through aeration increments in the presence or absence of plastic spiral (S), and concomitant with agitation (A). Coded levels and actual values of the variables investigated in this study are summarized in Table 1. Each statistically designed experiment comprised a total of eight assays, using a factorial design 2^3.

The "Experimental Design (DOE)" module of the program, Statistica 8.0 (Soft) was used to analyze the responses, to determine the effect of the independent variables and the interactions among them with the following responses: total production of carotenoids, specific production of carotenoids, and dry weight biomass after 48 h; thus optimizing the production of carotenoids by bacterial isolates.

Table 1
Independent variable levels used in factorial design assays.

Level (coded)	Independent Variables				
	Luminosity (L)	Temperature (T) (°C)	Agitation (A) (rpm)	Aeration (S)	Bacterial isolated type (Kpa)
−1	Light	30	180	− spiral	FT-7.22
+1	Dark	40	250	+ spiral	FT-5.12

2.5. Pigment production in bioreactor

After determining the best experimental conditions, a benchtop bioreactor (New Brunswick Scientific Bioflo 110, Edison New Jersey) was used for scaling up the pigment bioproduction in a 10 L culture in the TSB medium.

2.6. Determination of biomass

To determine the biomass dry weight (g/L), the culture was centrifuged at 14.000 rpm at 15°C for 7 min. The decanted cells were washed with distilled water and centrifuged again to remove the supernatant for quantitation of the remaining substrate. The resulting cell pellet was dried in a lyophilizer (Terroni Enterprise II, São Paulo Brazil) at 60°C for 48 h.

2.7. Carotenoids extraction and quantitation

The carotenoids were extracted from the dried biomass in accordance with the method described by Arulselvi et al. [33], with the following modifications: vortexing (Phoenix/AP 56, São Paulo Brazil) for 30 s and 2 min of ultrasonic bath (Unique/USC 800, São Paulo Brazil). Subsequently, they were centrifuged at 14.000 rpm for 5 min (HERMLE Z383K, Wehingen Germany). The extract obtained was concentrated in a rota evaporator (IKA® RV 2, São Paulo Brazil) and the rich carotenoid material was subjected to a saponification process [34] with the following modifications: using dichloromethane and methanolic KOH (30%) in a ratio of 1:1, vortexing (Phoenix/AP 56, São Paulo Brazil) for one hour in a dark room, adding 1 mL NaCl (5%); and centrifuging at 12.000 rpm for 5 min (HERMLE Z383K, Wehingen Germany), after which the supernatant was transferred to another tube.

The total carotenoid concentration was determined spectrophotometrically and calculated according to the formula provided [35]. The absorbance of the extract was measured at maximum wavelength obtained in a spectrophotometer at 439 nm (A) (Bioespectro SP-220, Paraná Brazil), the volume of the solvent used in the extraction (y), and using an absorption coefficient $A^{1\%}_{1cm} = 2500$ [36], according to [Equations 1, 2 and 3]. The total carotenoid content was expressed as μg/L and specific carotenoids as μg/g.

$$\mu g = \frac{A.y(\mathrm{mL}).10^6}{A^{1\%}_{1\mathrm{cm}}.100} \quad \text{[Equation1]}$$

where A = maximum absorbance, y = the volume of solvent used in the extraction, and $A^{1\%}_{1\mathrm{cm}}$ = the absorption coefficient, which is 2500.

$$\textit{Specific carotenoids}(\mu g/\mathrm{g}) = \frac{\mu g}{\textit{Sample weight}(\mathrm{g})} \quad \text{[Equation2]}$$

$$\textit{Total carotenoid } \mu g/\mathrm{L} = \textit{Dry weight biomass}(\mathrm{g/L}).\ \textit{specific carotenoids } (\mu g/\mathrm{g}) \quad \text{[Equation3]}$$

2.8. HPLC carotenoid analysis

The carotenoids were identified using high pressure liquid chromatographic (HPLC) separation with a reversed-phase column (C18 4.6 × 150 mm) (Sunfire Waters, Rio de Janeiro Brazil), an oven temperature of 30°C, with a mobile phase consisting of acetonitrile/methanol/ethyl acetate (10:50:40), eluted at a flow rate of 0.6 mL/min and monitored with a PDA detector (2998 Waters, Rio de Janeiro Brazil); and scanning at 200–600 nm, with a detection wavelength of λ = 450 nm. Beta-carotene was used as the external standard (Sigma, São Paulo Brazil).

2.9. LC–MS carotenoid analysis

To identify the carotenoid peaks confirmed by ultra performance liquid chromatography (UPLC) (Acquity UPLC H-Clas), it is coupled to a single quadrupole mass spectrometer SQ Detector 2 (Waters, Rio de Janeiro Brazil) and equipped with an ESI source. Chromatographic separation was performed in a reversed-phase column C18 (2.1 × 100 mm) (Acquity UPLC HSS C18 Waters, Rio de Janeiro Brazil), with 1.8 μm particle size, an oven temperature of 40°C, and an autoinjector at 10°C. The mobile phases consisted of an acetonitrile solution containing 0.1% formic acid (eluent A), a methanolic solution containing 0.1% formic acid (eluent B), and an ethyl acetate solution containing 0.1% of a formic acid (eluent C), with a flow rate of 0.37 mL/min and injection of 10 μL carotenoid extract. The carotenoids were eluted isocratically (10% A, 50% B, and 40% C) for 5 min.

The operation was performed in a positive-ion mode, with ESI needle voltage set at 3.5 kV, cone voltage 50 V, and ESI capillary temperature at 350°C, with a source gas flow rate of 650 L/h. Data were acquired in SIR (Selected Ion Recording) mode, searching for masses at 705 Da, 1029 Da, and 887 Da corresponding to sarcinaxanthin carotenoid, sarcinaxanthin diglucoside, and sarcinaxanthin monoglucoside, respectively, based on the sarcinaxanthin carotenoid characteristics and molecular weight data, described by Netzer et al. [11].

2.10. Kinetic parameters

Profiles of cell growth over time allowed us to determine the maximum speed of the microbial growth (μmax). Additionally, the following parameters were determined from the supernatants of the cultures in the bioreactor: glucose and nitrogen conversion factors in carotenoids and cells. The glucose concentration was determined by HPLC and free amino nitrogen was determined by the ninhydrin method according to Abernathy et al. [37]. The relationship between carotenoid production and cell growth, along with the yield in both was also estimated, based on the methodology of Aksu and Eren [38].

2.11. Antioxidant activity in vitro

The $^1O_2$ scavenging capacity was measured by monitoring the effect of the carotenoid extract on the oxidation (discoloration) of β-carotene, induced by the products of linoleic acid oxidative degradation, according to the methods of Rufino et al. [39] and Duarte-Almeida et al. [40]. The extracts were diluted to concentrations from 0.56 to 18 mg/mL. Approximately 50 μL of the samples were added to 1 mL of the β-carotene emulsion/linoleic acid, and subsequently, 200 μL of this mixture was placed on a microplate. Measurements were made for time point zero (t0), immediately at 470 nm; then, the samples were incubated for 120 min at 50°C, and measurement at this time was read again (t120). All analyses were performed in triplicate with BHT (butylated hydroxytoluene) (Sigma, São Paulo Brazil) as standard. The calculation of inhibition was performed with the described [Equation 4].

$$\%\ inhibition = \left[\frac{(abs\ system\text{-}abs\ sample)}{abs\ system}\right] x100 \qquad \text{[Equation4]}$$

where Abs system = Absorbance of β-carotene control (t0–t120) – White Absorbance (t0–t120), and Abs sample = Absorbance of the sample (t0–t120) – White Absorbance (t0–t120).

2.12. Photoprotective activity in vitro

The effectiveness of a sunscreen is measured by its sun protection factor (SPF), which is defined as a function of UVB radiation that causes erythema. Methanolic solutions in concentrations of 100 mg/L were prepared in triplicate with the extracted pigment. The absorbance was measured in a spectrophotometer at wavelengths between 290 and 320 nm. The values obtained were used to calculate the SPF according to the method of Velasco et al. [41], using the described [Equation 5].

$$FPS = FC. \sum_{290}^{320} EE\ (\lambda).\ I\ (\lambda).\ Abs\ (\lambda) \qquad \text{[Equation5]}$$

where CF = correction factor (=10); EE (λ) = erythematogenic effect radiation wavelength (λ); I (λ) = intensity of the sunlight at a wavelength (λ); and Abs (λ) = sample absorbance at wavelength (λ).

3. Results and discussion

3.1. Description of the isolates and 16S rRNA sequence analysis

The FT-7.22 and FT-5.12 showed yellowish colonies, and grew well in the TSB medium, both on agar plates and in liquid broth cultures. Strains stained Gram-positive appeared as cocci, and the colonies were observed to be arranged in packets. Cells were nonmotile.

The *16S rRNA* of the two isolates was sequenced and identified as *K. palustris*. The sequences exhibited 99% similarity with known deposited sequences of this species. Our results corroborate the description and identification of this species as denoted by Kovács et al. [42]. It is a representative of the family *Micrococcaceae*, order *Actinomycetales*; and is encountered in niches, such as soil samples, clinical samples, fermented foods, oral and skin flora [43]. Although *Kocuria* can be isolated from various sources, the size of this genus is relatively small, suggesting that each species of *Kocuria* is highly adapted to its ecological niche [44].

Microorganisms producing pigments are common in soil. The yellow color suggests a specialized microbiota of protective biomolecules against ultraviolet radiation because, this tonality is part of the carotenoid class, which have structures that determine and contribute to the biological property of photoprotection, according to Kampe et al. [45] and Kavamura et al. [46]. *Kocuria* genus contains several species with potential carotenoid production, such as *K. flava* [47], which produces yellow carotenoid, besides *K. carniphila* and *K. polaris* β-carotene producers [48]. Thus, *K. palustris* presence in the soil at the Tamanduá Farm (Caatinga) indicates a new source of bacteria that can produce natural molecules, such as carotenoids, with potential for biotechnological applications.

3.2. Effect of temperature incubation, luminosity, and bacteria Kocuria palustris FT-5.12 and FT-7.22 on carotenoid production

The first factorial design 2^3 including effects of temperature incubation (T), luminosity (L) and bacterial isolates type (Kpa) on carotenoid production (Table 2) denoted significant differences at 0.05 levels ($p < 0.05$) for all factors analyzed (Fig. 1a).

The assays at 30°C denoted a carotenoid production (on average 1718 μg/L) 1.3 times higher than at 40°C (in average at 1327 μg/L), with an estimated effect of −391. Similar data were described for yeast *Rhodotorula mucilaginosa* [49] and bacterium yellow pigment producer [50,51]. The second largest influence was the luminosity (negative effect), where light exposure increased carotenoid production by 4.9%, with an estimated effect of −73. The positive relation of light exposure on carotenoid production possibly occurs because of the carotenoids' photoprotection properties [52]. In addition, the isolate FT-5.12 presented a positive effect on carotenoid production, with an average carotenoid production of 1550 μg/L. This represents a significant increase of 3.6% in total carotenoid production than that of the FT-7.22 isolate.

Considering the regression coefficient values, it is possible to obtain [Equation 6], to represent the linear model that describes the effect of the variables considered in the total carotenoid production. The correlation coefficient (R^2) obtained was 0.99 (close to 1), indicating that the models were 99% accurate. These results indicate reliability between the experimental values and the values predicted by the model.

Table 2
Factorial design 2^3 with coded values and experimental responses obtained after 48-h of production of *Kocuria palustris* for total carotenoid, specific carotenoid, and dry weight biomass.

Essay	Independents Variables			Response		
	Luminosity (L)	Temperature (T) (°C)	Bacterial isolated type (Kpa)	Total carotenoids (μg/L)	Specific carotenoids (μg/g)	Dry weight biomass (g/L)
1	−1 (Light)	−1 (30)	−1 (FT-7.22)	1752.00	489.01	3.58
2	+1 (Dark)	−1 (30)	−1 (FT-7.22)	1632.00	454.14	3.59
3	−1 (Light)	+1 (40)	−1 (FT-7.22)	1356.00	447.29	3.03
4	+ 1(Dark)	+1 (40)	−1 (FT-7.22)	1240.00	407.34	3.04
5	−1 (Light)	−1 (30)	+1 (FT-5.12)	1760.00	53.82	32.70
6	+1 (Dark)	−1 (30)	+1 (FT-5.12)	1728.00	52.66	32.81
7	−1 (Light)	+1 (40)	+1 (FT-5.12)	1368.00	49.55	27.60
8	+1 (Dark)	+1 (40)	+1 (FT-5.12)	1344.00	47.95	28.03

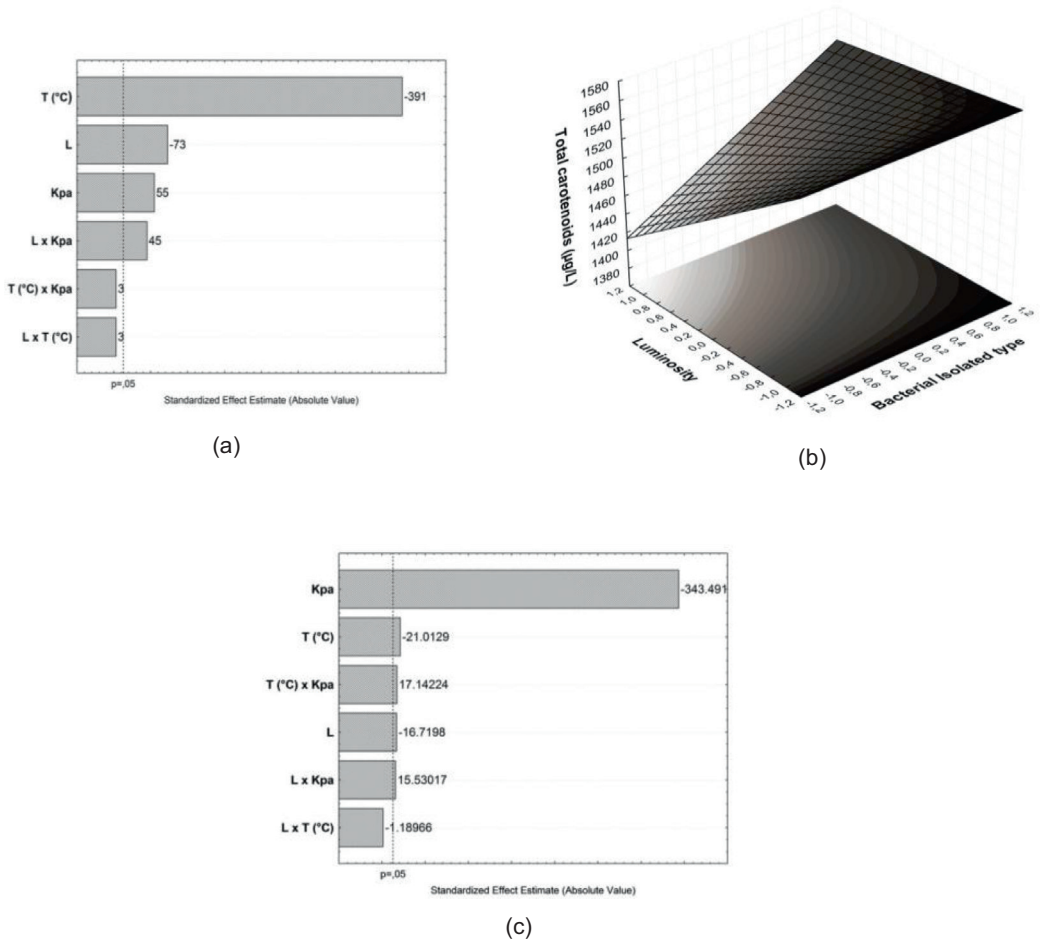

Fig. 1. Factorial design 2^3 with the effect of factors of luminosity (L), temperature (T), and bacterial isolated type on carotenoid production (Kpa). (a) Pareto diagram with the effects of these variables on total carotenoid production (μg/L). (b) Response surface with the influence of luminosity and bacterial isolated type on total carotenoid production in the factorial design 2^3. (c) Pareto diagram with the effects on specific carotenoid production (μg/g).

Total carotenoid production ($\mu g/L$)

$$= 1522.500\text{-}36.500\ L\text{-}195.500\ T + 27.500\ Kpa + 1.500L\ T + 22.500L\ Kpa + 1.5\ T\ Kpa \quad \text{[Equation6]}$$

where L is the luminosity, *T* is the temperature, and Kpa is the bacterial isolate type.

The model allowed us to generate a superficial response analysis of the influences of the variables on carotenoid production. As given in Fig. 1b, the cultivation of bacterial FT-5.12 with exposure to light resulted in increased carotenoid production.

A more precise analysis of pigment production, however, revealed a different situation. Upon examining specific carotenoid production, we observed that in terms of pigment produced by biomass unity (μg/g), FT-7.22 isolate achieved an average specific carotenoid production of 450 μg/g, representing a value 88% higher than FT-5.12 strain (51 μg/g). Thus, the cellular biomass production by isolate FT-5.12 was ten times higher (on average 30.33 g/L) than the isolate FT-7.22 (3.3 g/L). The pigment production analysis in bacterial isolates revealed that there is an inverse relationship between cell growth and specific carotenoid production. Overall, our analysis indicated that the isolate FT-7.22 has the highest effect on specific carotenoid production, with an estimated value of −343.49 (Fig. 1c). In the context of achieving better carotenoid yields, analysis of the specific carotenoid production variable proved to be more critically important than the total carotenoid production variable.

3.3. Effect of gas transfer rate on carotenoid production

Based on the results of the first factorial design and to further increase the pigment production, we conducted a second 2^3 factorial design (Table 3), for analyzing the oxygen transfer rate and the bacterial isolate type.

For agitation and culture aeration, the results were significantly different in the total carotenoid production ($p < 0.05$), with an estimated effect of 36.33 and 16.78, respectively (Fig. 2a). In our study, maximum pigment production was for the two isolates at 2340 μg/L, being 1.36-fold higher ($p < 0.01$) than what we observed for the previous design, under the best conditions (1718 μg/L). This value was 131% higher than that obtained in a carotenoid optimization study using *Sporidiobolus salmonicolor* (1019 μg/L) [5] revealing the high potential of the bacteria *K. palustris* as a carotenoid producer.

The data from the second factorial design were employed to generate a mathematical model describing the profiles of total carotenoids production at 48-h by *K. palustris* isolates at 95% confidence. The variables were fitted to a first-order model equation and examined in terms of the goodness of fit [Equation 7].

Total carotenoid production ($\mu g/L$)

$$= 2071.500 + 75.500S + 163.500\ A + 55.500\ Kpa + 29.500\ S\ A + 1.500\ S\ Kpa\text{-}40.500\ A\ Kpa \quad \text{[Equation7]}$$

Table 3
Factorial design 2^3 with values and experimental results obtained from carotenoid production of *Kocuria palustris* with varying oxygen transfer rate (agitation and absence/presence of plastic spiral in the flask).

Essay	Independents Variables			Response		
	Aeration (S)	Agitation (A) (rpm)	Bacterial isolated type (Kpa)	Total carotenoids (μg/L)	Specific carotenoids (μg/g)	Dry weight biomass (g/L)
1	−1 (Absence spiral)	−1 (180)	−1 (FT-7.22)	1772.00	556.41	3.18
2	+1 (presence spiral)	−1 (180)	−1 (FT-7.22)	1852.00	586.85	3.15
3	−1 (Absence spiral)	+1 (250)	−1 (FT-7.22)	2112.00	628.35	3.36
4	+1 (presence spiral)	+1 (250)	−1 (FT-7.22)	2328.00	687.09	3.39
5	−1 (Absence spiral)	−1 (180)	+1 (FT-5.12)	2056.00	72.23	28.46
6	+1 (presence spiral)	−1 (180)	+1 (FT-5.12)	1952.00	69.08	28.25
7	−1 (Absence spiral)	+1 (250)	+1 (FT-5.12)	2148.00	70.14	30.62
8	+1 (presence spiral)	+1 (250)	+1 (FT-5.12)	2352.00	75.95	30.96

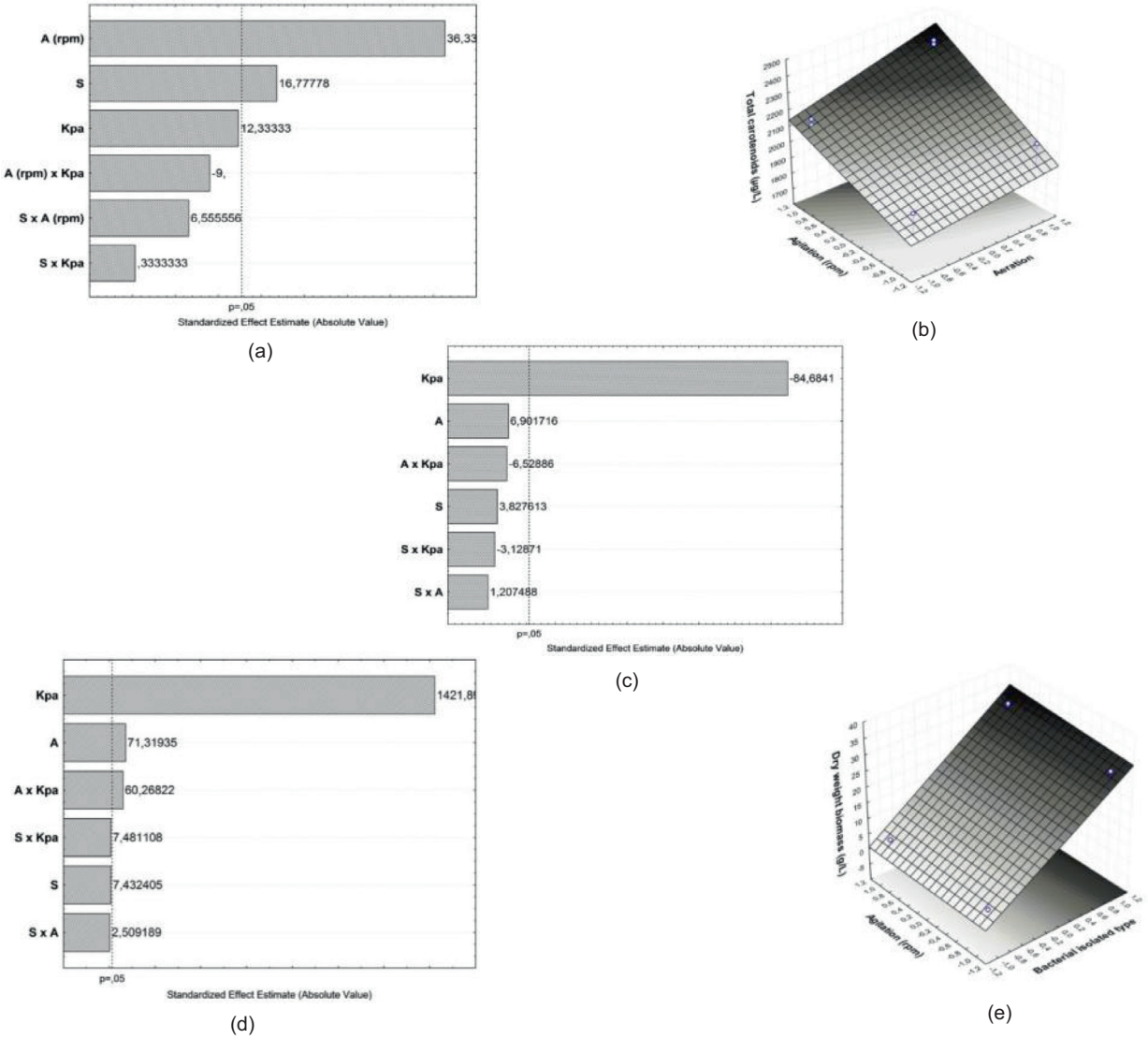

Fig. 2. Factorial design 2^3 with effects of agitation (A), aeration (S), and bacterial isolated type (Kpa) on carotenoid production. (a) Pareto diagram with effects of these variables on total carotenoid production (μg/L). (b) Response surface with the effect of agitation and aeration on total carotenoid production. (c) Pareto diagram with the effects on specific production of carotenoids (μg/g). (d) Pareto diagram with the effects on biomass (g/L). (e) Response surface of agitation effect and of bacterial isolated type in the production of dry weight biomass.

where S is the homogenization strategy of the cell culture, A is the agitation, and Kpa is the bacterial isolate type.

The correlation coefficient (R^2) obtained was 0.99, close to 1, indicating that the models fit with 99% accuracy to the results obtained. As expected, Fig. 2b denotes that forcing an increase in the gas transfer rate through an augmentation in the agitation and turbulence by spirals, resulted in an evident increase in total carotenoid production. This observation corroborates the conclusion presented by Shatila et al. [53] that aeration enhances carotenoid production in *Exiguobacterium aurantiacum*.

Comparative analysis of specific carotenoid production by the isolate FT-7.22 displayed an 8.5-fold higher specific production

than FT-5.12, whereas the total production remained similar for both strains. As identified in the first factorial design, there was an inverse relationship between growth and specific carotenoid production. The specific carotenoid production was highly dependent on the type of isolates used in the study (Fig. 2c).

In this context, statistical analysis using biomass as a response indicated that the type of isolate used (estimated effect of 1421.89) and agitation (estimated effect of 71.32) (Fig. 2d) were significant contributing factors. Kovács et al. [42] reported that *K. palustris* depends on oxygen supply for growth. The results presented here strongly indicated that biomass production depends directly on oxygen availability, by differentially affecting growth in *K. palustris* (Fig. 2e).

3.4. Pigment production in bioreactor

Considering potential industrial production, we used the optimal parameters in a scale-up bioreactor. Fig. 3 presents the kinetics of growth, carotenoid production, and substrate consumption (glucose and nitrogen) after 48-h of cultivation. It also depicts that the FT-5.12 isolate exhibited a higher maximum growth rate (μmax) than the FT-7.22, being 0.4194 h^{-1} and 0.3479 h^{-1}, respectively.

In general, the TSB culture medium provided efficient growth of bacterial isolates and their production of carotenoids. It is worth highlighting the importance of the culture medium in the production of carotenoids, because its composition as sources of carbon, nitrogen, and presence of salts, among others, are factors that influence the type of carotenoid synthesized and its concentration [5,49].

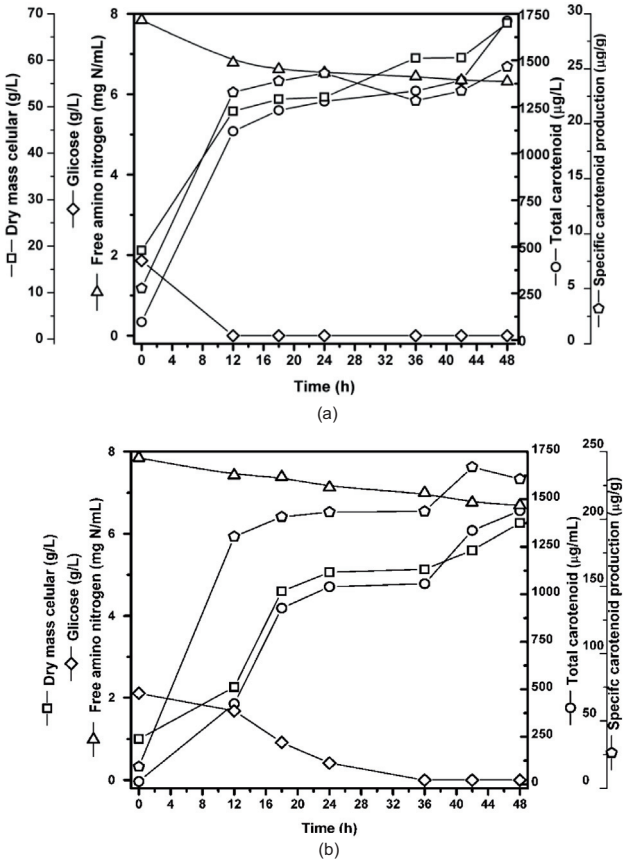

Fig. 3. Biomass, substrate consumption, and carotenoids production in a bioreactor by *Kocuria palustris* at optimized conditions (250 rpm, 30°C, light environment. 1.0 vvm, initial pH 7.0 in medium TSB) (a) Isolated FT-5.12 (b) Isolated FT-7.22.

Carotenoid bioproduction from *K. palustris* showed an increased rate during the initial 0–18 h of culture, and a second augmentation in the 36–42 h to the 48-h period (Fig. 3). It appears that the cell growth and pigment production profile followed an increase in two phases, suggesting a diauxic change, starting at 24-h. Increased accumulation of total carotenoid production was reached at 48-h, during the stationary phase of isolated growth. This was also observed by Masetto et al. [54] for carotenoid production in a bacterial culture of *Flavobacterium* sp. (ATCC 21588) in the stationary phase. Concerning the consumption of substrates, isolate FT-5.12 consumed all the glucose within 12-h of culture, whereas isolate FT-7.22 required 36-h (Fig. 3). The nitrogen was not totally consumed by either isolate. In concordance with the observations of Orosa et al. [55] in their experiments with *Haematococcus pluvialis*, the nitrogen source does not have much influence on the production of carotenoids. However, isolate FT-7.22 consumed 34.53% more nitrogen than isolate FT-5.12 at 48-h. Hence, the consumption of both nitrogen and glucose was dependent on the type of the isolate.

Thus, we observed that the maximum yield of carotenoids (Pp) for the isolate FT-5.12 was 33.66 $\mu g.L^{-1}.h^{-1}$, and 29.66 $\mu g.L^{-1}.h^{-1}$ for FT-7.22 (Table 4); whereas the specific carotenoid production (YP/X) for the isolate FT-7.22 was 9 times higher than the isolate FT 5.12 (Table 4). From the extraction, 465.2 mg (FT-5.12) and 391.3 mg (FT-7.22) of enriched carotenoids were obtained. When the maximum carotenoid production by the two isolates in the optimized condition in Erlenmeyer flasks (2.34 mg) and the production in a bioreactor for both isolates (428.25 mg) were compared, it is observed that the scaling in bioreactor denoted a 183 times increase in production.

3.5. Identification of carotenoids

The pigment extracts indicated the presence of a major peak C1 [retention time (RT): 3.75 min] and three minor peaks with RT of 4.21 (C2), 4.68 (C3), and 8.09 (C4) min (Fig. 4a). The spectral (scanning 200–600 nm) and LC-MS analyses of the three principal peaks displayed three maximal adsorption values: 415.5 nm, 439.7 nm, and 468.9 nm (Fig. 4b), which are characteristic of the sarcinaxanthin carotenoid (705 Da), and the minor derivative compounds (C2 e C3), sarcinaxanthin diglucoside (1029 Da) and monoglucoside (887 Da) [11].

In the isolate FT-7.22, compound sarcinaxanthin was produced at 112480 μg/L (2340 μg/g dry weight biomass), but FT-5.12 production was 50390 μg/L (750 μg/g dry weight biomass). The com-

Table 4
Parameters calculated during the carotenoid production in bioreactor with TSB medium at optimized conditions (250 rpm, 30°C, light environment, aeration 1.0 vvm, initial pH 7.0).

Parameter	*Kocuria palustris*	
	FT-5.12	FT-7.22
YP/N[a] (μg/g)	1062.41	531.01
YX/N[b] (g/g)	42.20	2.31
YP/S[c] (μg/g)	918.45	681.49
YX/S[d] (g/g)	36.48	2.96
YP/X[e] (μg/g)	25.17	229.78
Px[f] ($g.L^{-1}.h^{-1}$)	1.02	0.11
Pp[g] ($\mu g.L^{-1}.h^{-1}$)	33.66	29.66

[a] Nitrogen conversion factor in carotenoid.
[b] Nitrogen conversion factor in cells.
[c] Glucose conversion factor in carotenoid.
[d] Glucose conversion factor in cells.
[e] Relationship between the carotenoid production and cells.
[f] Yield in cells.
[g] Yield in carotenoid.

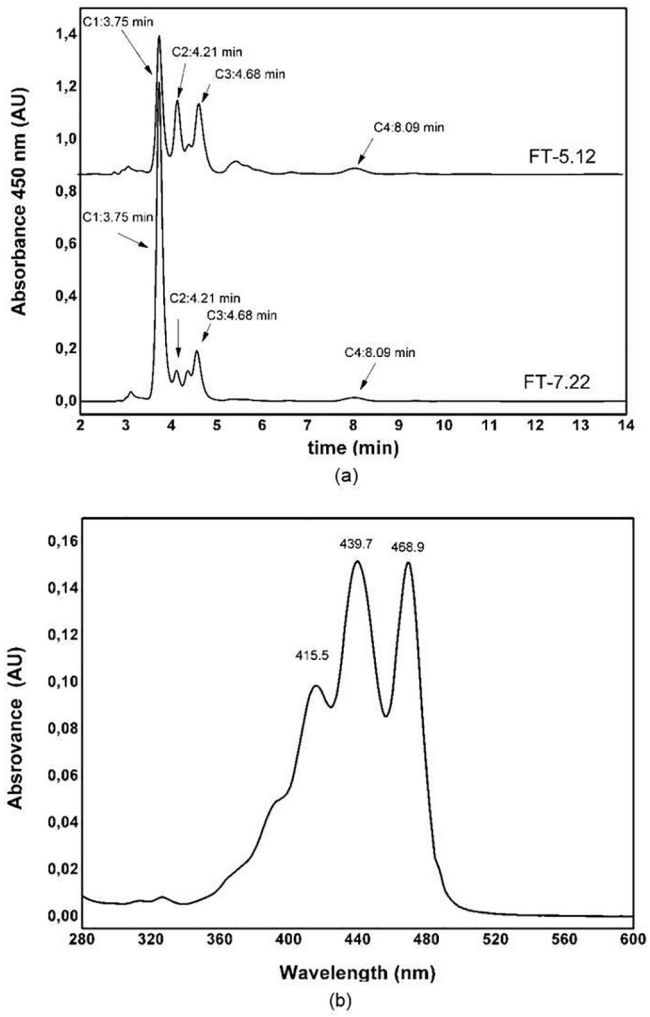

Fig. 4. (a) HPLC elution profiles of the carotenoids extracted from *Kocuria palustris*. (b) Absorption spectra of the major carotenoids from *Kocuria palustris* identified using spectral data.

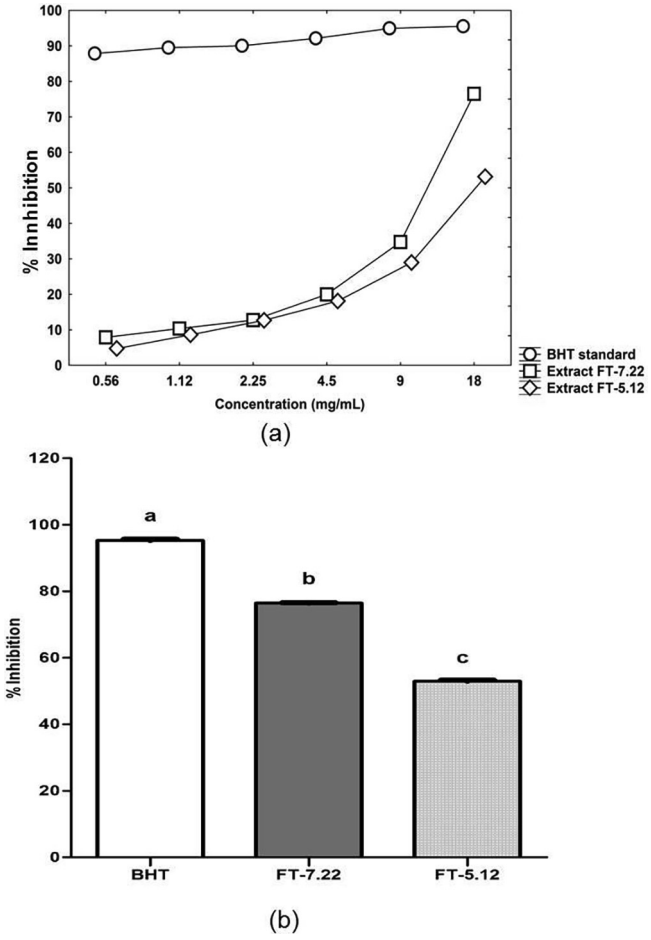

Fig. 5. (a) Antioxidant activity curve of standard BHT, extracts of *K. palustris* FT-7.22 and FT-5.12, determined by β-carotene/linoleic acid system. (b) Significant difference between the BHT standard and the extracts analyzed at their highest concentrations. Bars with different letters differ from each other, as determined by the Tukey test ($p < 0.01$).

pound C4 was putatively identified as lycopene [11,56], at concentrations of 2280 μg/L (FT-7.22) and 4770 μg/L (FT-5.12). Lycopene was also identified in a study by Netzer et al. [11], in the biosynthetic pathway during the production of sarcinaxanthin. The highest concentration of sarcinaxanthin produced by FT-7.22, in our study, was similar to that produced by a genetically modified *Escherichia coli* (2500 μg/g) [11].

3.6. Biological activity as antioxidant

Antioxidant activity of crude carotenoid extracts displayed elevated percentages of inhibition; 76.53 ± 0.09% (FT-7.22) and 53.2 ± 0.32% (FT-5.12), at the highest concentration (18 mg/mL), while the BHT standard exhibited the highest percentages in all the tested concentrations and was significantly different from the others ($p < 0.01$) (Fig. 5a, b).

The FT-7.22 extract demonstrated a 1.45-fold higher activity than that of FT-5.12 in 18 mg/mL. Probably, the antioxidant activity is directly related to the abundance of sarcinaxanthin in the extract. Interestingly, this extract presented 76.53 ± 0.09% of inhibition; 4.5% more than astaxanthin carotenoid extract produced by *Exiguobacterium* sp. [33].

3.7. Photoprotective activity in vitro

The crude carotenoid extract of FT-7.22 displayed an SPF of 9.36 ± 0.52, while FT-5.12 it was 8.66 ± 0.23. Again, significant differences were observed between them, suggesting that a high photoprotective activity is related to the sarcinaxanthin-rich extract (FT-7.22). In Brazil, these values are above the levels required for sunscreens; according to Brazilian Resolution RDC No. 30 of June 1, 2012, the minimum value of SPF sunscreen should be 6.0 [57]. Many lines of research have been encouraged to find natural products with photostability, retention capacity on the skin, and lack of/lower allergenic potential. These extracts of microorganisms with rare C50 carotenoid molecules deserve special attention because, in addition to the photoprotection capacity, the solubility and stability of oil emulsions indicate the use in sunscreens and other cosmetic applications [5].

4. Conclusions

This study identified two novel bacterial isolates from the Caatinga Domain – FT-5.12 and FT-7.22, as *K. palustris* – that can produce a significant amount of a rare carotenoid identified as sarcinaxanthin. The FT-7.22 extract is a $^1O_2$ potent scavenger and exhibited a higher value of FPS. This is a promising discovery to

obtain carotenoids from a new natural source, which can be used in combination with sunscreen filters for applications in cosmetic formulations.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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