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Production of carotenoids by *Brevibacterium linens*: variation among strains, kinetic aspects and HPLC profiles

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This work describes carotenoid pigment production by the red bacterium *Brevibacterium linens* covering strain diversity, kinetic and analytical aspects. Pigment production of 23 *B. linens* strains ranged from 0.05 to 0.60 mg pigments L^{-1} culture, with specific productivity from 0.2 to 0.6 mg pigments per g dry biomass. The pigment production time curve showed a sigmoid shape, that matched cell growth. HPLC analysis revealed three groups of peaks, possibly non-hydroxylated, mono- and di-hydroxylated carotenoids. Polar molecules were mainly represented. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 64–70.

Keywords: aromatic carotenoid; *Brevibacterium linens*; productivity; cheese ripening; smear microflora

Introduction

Carotenoids are yellow to red pigments that occur widely in nature. More than 600 different structures have been described among a variety of animal, plant and microbial species [16]. The exploitation of natural functions of these molecules has led to valuable applications in animal feeds, food technology and pharmacy. As a required nutrient for many animal species, carotenoids have to be added to the diet of some reared poultry, fish or crustacean species, where they also help provide rich pigmentation to the flesh and ova. To a larger extent, carotenoids are used as food colorants to enhance appearance and to give organoleptic value to processed food. The cosmetic and food industries take advantage of the antioxidant properties of some carotenoids, which can lead to their inclusion into nutraceutics or drugs, since some carotenoids may prevent diseases like cancer or atherosclerosis, or stimulate antibody response [1,4,16].

Facing the subsequent economic significance of these ingredients, much interest has been devoted to new supplies of carotenoids. In particular, the development of carotenoid-producing microorganisms is now regarded as a most competitive pathway, since it provides pigments of natural origin at an industrial scale eg β-carotene produced from the micro-alga *Dunaliella salina* [21,22] or the fungus *Blakeslea trispora* [3,20], astaxanthin produced from the micro-alga *Haematococcus* [12,15] or the yeast *Phaffia rhodozyma* [15,26]. Furthermore, a large variety of carotenoid pigments, including original structures, can be expected from exploration of the microbial kingdom. However, some bacteria have been given little consideration, and we therefore paid attention to the carotenogenic corynebacterium *Brevibacterium linens*.

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B. linens frequently contaminates milk, and belongs to the cheese ripening flora. As a strict aerobe, it develops on cheese surfaces and is responsible for orange pigmentation and characteristic sulphuric flavours. According to Kohl *et al* [18], the orange pigmentation of *B. linens* is due to three aromatic carotenoids (Figure 1). Besides its traditional use in cheese processing, which could later facilitate acceptability for ingredients extracted from the bacterium, these structures would enlarge the diversity of carotenoids available as food ingredients. Additionally, characterization of pigment synthesis by *B. linens* would help to elucidate the coloration process of cheeses.

Despite demands for a better knowledge of carotenoid synthesis by *B. linens*, information is scarce and results mostly from empirical observations, without quantification of the pigments [6,7,11]. Recently, Oumer *et al* [24] linked

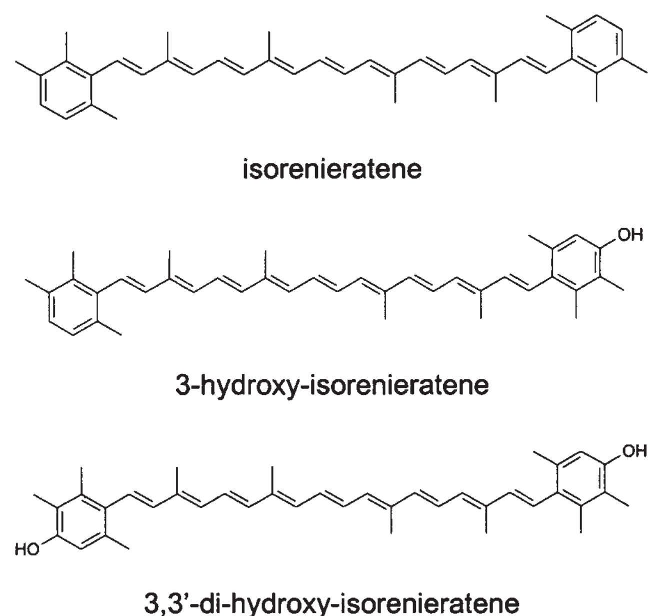

Figure 1 Aromatic carotenoids described in *Brevibacterium linens* [18].

orange pigmentation to the growth of *B. linens*, as a way to estimate cell density. In order to describe the production of pigments by *B. linens* as completely as possible, three complementary aspects of the phenomenon were investigated. First, the diversity of pigmentation among a population of 23 strains was considered, then some kinetic aspects of pigment synthesis were described, and finally pigments extracted from the bacterium were analyzed by spectrophotometry and HPLC.

Materials and methods

Bacterial strains and culture medium

A collection of 23 strains of *Brevibacterium linens* was used, consisting of four reference strains, six wild-type strains and 13 industrial strains. Strains ATCC 9172, ATCC 9175 and ATCC 19391 were obtained from the American Type Culture Collection (Manassas, VA, USA) and NRRL B-4210 was obtained from the Northern Regional Research Laboratory (Peoria, IL, USA). Wild-type strains sav1 and sav2, and sav3 to sav6 were isolated from craft-processed cheeses, and were supplied by, respectively, the Institut National de la Recherche Agronomique (INRA) (Thiverval-Grignon, France) and the Laboratory of Microbiology of the University of Caen (France). Industrial strains ind1 to ind13, were kindly provided by companies producing *B. linens* on a large scale: SKW-Biosystems (La Ferté sous Jouarre, France), Chr Hansen (St Etienne de Chomeil, France), Texel (Dangé St Romain, France), and by the cheese-making Fromagerie des Chaumes (Jurançon, France). The collection was maintained on nutrient agar, stored at 4°C and subcultured monthly.

Cultures were grown in 250-ml Erlenmeyer flasks filled with 50 ml of a medium composed of: 20 g L^{-1} D(+) glucose (Carlo Erba, Val de Reuil, France), 10 g L^{-1} sodium chloride (Carlo Erba), 5 g L^{-1} casamino acids (Difco), 1 g L^{-1} yeast extract (Biokar, Beauvais, France) and 1 g L^{-1} potassium dihydrogenphosphate (Carlo Erba). The pH was adjusted to 6.9 and the medium was heat-sterilized. Flasks were inoculated with 1% 72-h-old preculture (v/v), and incubated at 25°C with 145 rpm agitation.

Pigment extraction and quantitation

Each extract was obtained from cells from 20 ml of homogenised culture. Cells were first centrifuged at $6000 \times g$ for 15 min. The supernatant was checked for residual cells or excreted pigments (see below), then discarded. The cell pellet was rinsed with deionized water, then centrifuged again ($6000 \times g$ for 15 min) and the supernatant was discarded. Cells were mixed with 8 ml of 99.9% methanol (Carlo Erba) and blended to prevent clotting. Samples were then wrapped with aluminium foil to protect them from light, and the extraction was performed under 50 rpm agitation, until cells were bleached (within 2 h). The methanol extract was then separated from biomass after centrifuging the samples at $6000 \times g$ for 15 min. The cell pellet was checked for residual pigmentation (see below), then discarded. The extract was purified from cell debris by further centrifugation at $10\,000 \times g$ for 15 min, and its absorbance at 450 nm was measured. The concentration of pigments (mg pigments L^{-1}) was eventually estimated according to the Beer–Lambert law. However, since the expected isorenieratene and hydroxylated derivatives were unfortunately not available, we were unable to determine ourselves the specific absorption coefficients in methanol, and eventually considered published data of 2080 L g^{-1} cm^{-1} (isorenieratene in petroleum ether [5]).

Checking of biomass after methanol extraction: Jones *et al* [17] showed that pigments of *B. linens*, located in the cell membrane, specifically react with strong alkali, leading to a deep pink-red color. This is explained by the ionization of phenolic rings at high pH, giving a subsequent bathochromic shift [2] and seems to be specific to the diol molecule [18]. Extracted biomass was laid out on an inert white surface (ie Whatman paper), and a few drops of 5 M NaOH were added. The test was positive if a characteristic pink-red color appeared.

Growth and pigment production modeling

The sigmoidal function established by Gompertz [28] was adjusted to experimental data obtained from the time course monitoring of dry matter production and carotenoids estimation in a culture of *B. linens* ind13. Adjustment was made by using Least Squares method on Excel 7.0 software (Microsoft, Redmond, WA, USA).

HPLC analysis

Methanol extracts were evaporated under vacuum, at 75°C and over anhydrous sodium sulphate (Na_2SO_4), in a Büchi rotavapor. The dried pigments were transferred in analytical chloroform (Carlo Erba), until solids were bleached. The chloroform fraction was further evaporated on the same device, and dried pigments were dissolved in 1 ml of methanol. Concentrates were then filtered onto a 0.2-μm pore-sized hydrophilic Millex-GV membrane (Millipore, St Quentin en Yvelines, France), and stored at −20°C in amber glass 10 ml-vials with Teflon-lined caps, until further analysis (within 1 week).

Reverse phase HPLC analysis (20 μl injection) was performed on a 250×4 mm LichroCART RP-18 column (Merck, Chelles, France), sphere diameter 5 μm. All other HPLC apparatus (600 constant flow pump, 996 photodiode array detector and 600 controller) was from Waters (St Quentin en Yvelines, France). The mobile phase of 100% methanol (HPLC quality, Carlo Erba) was run isocratically for 45 min then a gradient was established over the next 35 min with chloroform (HPLC quality, Carlo Erba) to a final ratio of 80/20 methanol/chloroform, followed by a further elution for 50 min with 80/20 methanol/chloroform. The flow rate was 0.5 ml min^{-1}. Monitoring, data recording and processing were led with the Millennium 2.15.01 software (Waters). Carotenoid standards (lycopene, β-carotene, cryptoxanthin, astaxanthin, canthaxanthin, lutein and zeaxanthin) were kindly provided by Hoffman-LaRoche (Basel, Switzerland).

Statistical analysis

When considering more than two levels for one single factor (ie when comparing the 23 strains), one-way analysis of variance was performed under the Statgraphics Plus 3.1 software (Statgraphics, Manugistics, Rockville, MD, USA).

Results

Diversity of pigment production among 23 strains of B. linens

All *B. linens* strains were cultivated for 70 h in shake flasks. Dry biomass varied from 0.23 to 1.92 g L^{-1}, which led to yields less than 9.6% conversion of glucose supplied, indicating low growth. Pigment final production ranged from 0.05 to 0.60 mg L^{-1} (Figure 2), and specific pigment production from 0.20 to 0.60 mg pigments per g dry biomass. The F-ratio calculated for the strain factor was 95% significant on both responses. Despite the 10-fold range affecting the pigment yield of the 23 strains, the LSD test hardly revealed groups of strains with distinct pigment production (Figure 2). This was due to high, but very gradual, diversity among responses, so that diversity (ie significantly distinct types of pigment volumetric production, eg high-producing strains) was low. Specific production varied to a lesser extent, with an even lower diversity.

High pigment production was obtained for strains sav6, ind5, ind11, ind3 and ind4. *B. linens* ATCC 9175 showed the highest pigment production among reference strains.

Kinetics of growth and pigment production

B. linens ATCC 9175, ind3, ind5, ind11, ind13 and sav6 were monitored for biomass and pigment production in liquid medium. After 72 h, dry biomass production ranged from 1.39 to 2.25 g L^{-1}. Biomass yield was low, with a maximum of 11.2% conversion of the glucose supplied. Pigment production by the different strains ranged from 0.96 to 1.37 mg pigment per liter. Shapes of the individual growth and pigment production curves were similar among the six strains (data not shown), with a characteristic sigmoid pattern. There were 95%-significant differences between both biomass and pigment concentrations, as a confirmation of the diversity previously observed. As an example (Figure 3), *B. linens* ind13 cultivated on the above medium showed a 12-h lag-phase. The exponential phase was short (12–24 h), with μ_{max} values ranging from 0.18–0.22 h^{-1} among the six strains. The decelerating phase then lasted until about 90 h, before it gave way to the stationary phase (log plot not shown).

Pigment production plots displayed comparable shapes (Figure 3a: example of strain ind13 cultivated in shake flasks for 102 h). Carotenoid synthesis seems therefore closely related to growth. In order to better appreciate this relationship, the Gompertz model was applied, allowing us to describe both responses continuously. The model fitted the data with a correlation coefficient exceeding 99% in both cases, although the Gompertz model did not seem the best for curves with such a long transition phase (Figure 3a). Model curves for dry biomass and pigment productions were then derived, leading to model dry biomass and pigment productivities (Figure 3b). Pigment production was maximum only 3.5 h after growth was at its apex, and stopped as growth ceased. Over a 102-h long cell development, such an interval is very short. Pigment synthesis was therefore almost simultaneous with cell growth, leading to production kinetics that match typical production of primary metabolites.

Visible spectrum of B. linens methanolic extracts

Pigments extracted from *B. linens* ind5 after 72 h cultivation in shake flasks gave bright yellow solutions. Furthermore: (i) pigment extraction was performed on liquid cultures of every *B. linens* strain; (ii) pigment production of strains ATCC 9175, ind3, ind5, ind11, ind13 and sav6 were

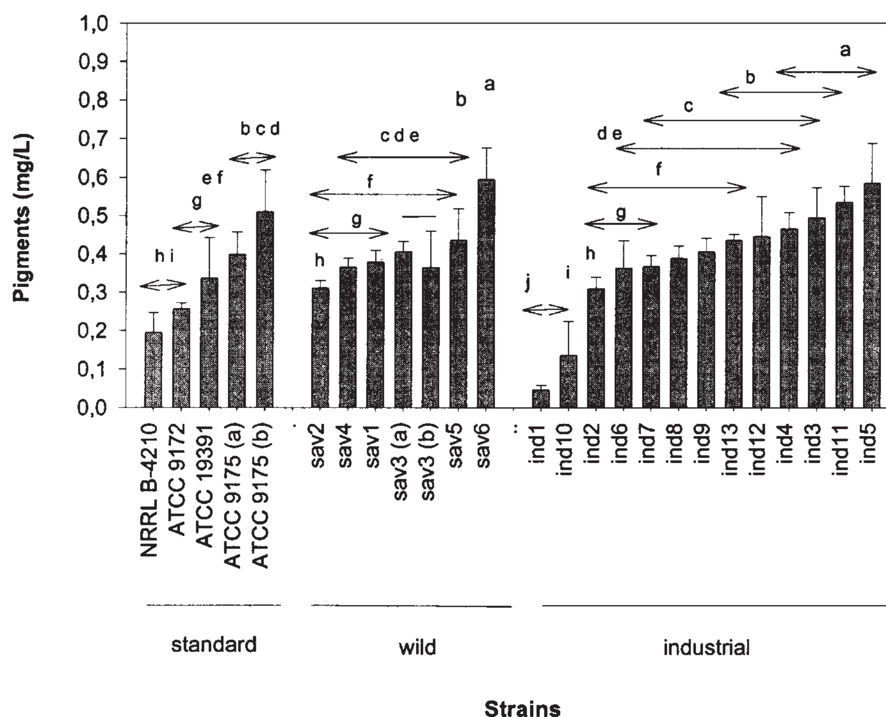

Figure 2 Pigment production observed for 23 strains of *Brevibacterium linens* (70-h cultivation). Results with no letter in common were significantly different with a 95% confidence level.

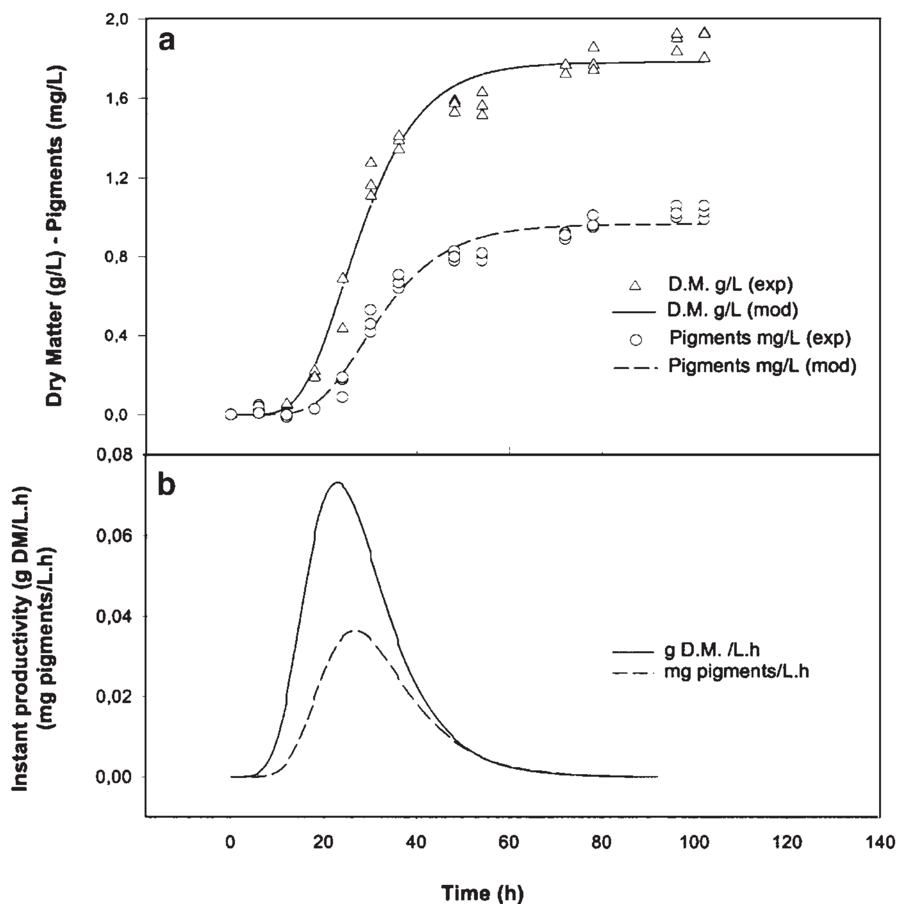

Figure 3 Growth and pigment production by *Brevibacterium linens* ind13 cultivated in 250-ml shake flasks containing 50 ml of liquid medium. Experimental data and Gompertz modeling (a), derivation of the model curves (b). (exp: experimental data) (mod: Gompertz model).

also monitored throughout growth. All the extracts obtained from these experiments gave the same carotenoid-like absorption spectrum (data not shown).

Visible light was absorbed in the 350–550 nm range, with a 454 nm λ_{max}. There was one slight shoulder at 425 nm and a more marked one at 475 nm. Vibrational fine structure was low, evidenced through comparison with the spectrum of standard lutein in methanol. As a test indicated in [13] and prompted by results obtained by Jones *et al* [17], the addition of a few drops of 5 M NaOH to a methanol extract of *B. linens*' pigments turned its color to pink-reddish, with subsequent changes in the spectrum. A bathochromic shift was observed, leading to a λ_{max} close to 475 nm, and fine structure was completely lost.

HPLC profiles of the methanolic extracts

Chromatographic system: Three carotenoid pigments with a variable number of hydroxyl functions might be expected from the work of Kohl *et al* [18]; the chromatographic system we used was designed to separate unambiguously true carotenes, mono-hydroxylated, and di-hydroxylated carotenoids. Retention times of β-carotene and lycopene were approximately 90 min, cryptoxanthin eluted at 39–40 min and both lutein and zeaxanthin eluted between 11 and 15 min (Figure 4a). Since standards were not available, isorenieratene and its hydroxylated derivatives were not tested.

Resolution of the pigments extracted from B. linens: After quantitative extraction of carotenoid pigments from *B. linens* ind13 cultivated for 95 h in a liquid medium containing acetate as the main carbon source, the sample was loaded on the column and further separated into three distinct groups of components (Figure 4b).

Similar chromatograms were obtained from six strains (sav 2, ind3, ind4, ind11, ind13 and ATCC 9175) cultivated for 72 h in a glucose liquid medium. In another experiment, the pigments of strains ATCC 9175 and ind13 were analysed after 95 h cultivation in liquid medium where glucose was replaced by 20 g L^{-1} galactose, saccharose, lactose, sodium DL-lactate, sodium D-gluconate, glycerol (all from Sigma) or sodium acetate (Carlo Erba), and all samples gave comparable results. Furthermore, similar chromatograms were obtained throughout growth of *B. linens* ind13 cultivated for 102 h in shake flasks, with a slightly higher proportion of less polar compounds before 48 h (data not shown).

A typical chromatogram (Figure 4b) displayed three groups of peaks. The first one showed one major peak (1)

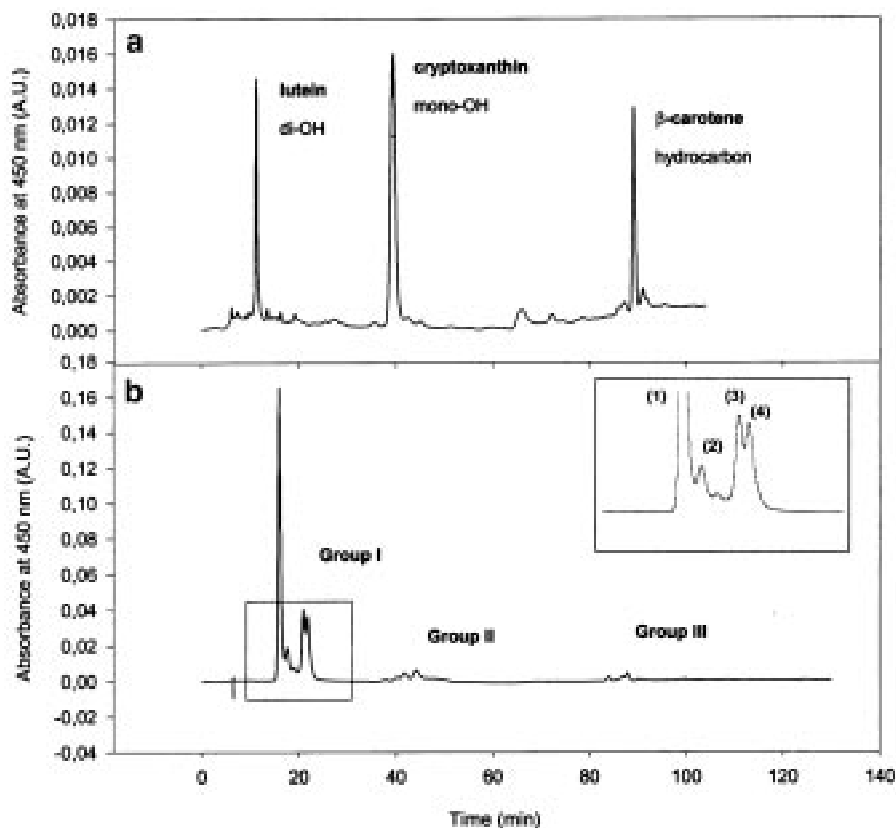

Figure 4 HPLC chromatograms of standard carotenoids (a) and *Brevibacterium linens* ind13 methanolic extract (b).

between 15–17 min (depending on void time), followed 1.6 min later by a small but marked peak (2). After a minor compound had been eluted, two barely separated peaks (3 and 4) ran out the column within 1 min, between 20 and 22 min (for the first). Two other groups of very small peaks were later eluted, between 35 and 45 min first, and then between 80 and 100 min. Spectra of all detectable compounds were carotenoid-shaped (Table 1). A small, but recurrent, 5 nm-translation was observed between the λ_{max} of peak 1 (454 nm) and of both peaks 3 and 4 (λ_{max} = 449 nm), then between the latest and λ_{max} of peak 2 (444 nm). Peak 4 displayed an extra absorption maximum at 345 nm.

Table 1 λ_{max} values of the major compounds separated by HPLC of concentrated methanol extracts of *Brevibacterium linens* carotenoid pigments. Shoulders are in brackets

Peak No.		Retention time	λ_{max} values
(1)	Group I	15–17 min	(425) − 453.6 − (480)
(2)	Group I	16.5–19 min	(420) − 444 − (470)
(3)	Group I	20–22 min	(420) − 448.8 − (475)
(4)	Group I	23–24 min	344.7 − (420) − 448.8 − (475)
Major peak (Group II)		35–45 min	(420) − 453.6 − (480)

Discussion

Kinetic data agreed with observations by Famelart [6], who noticed that growth of *B. linens* ATCC 9175 was low and could run over 80 h, with a short exponential phase compared to the lag and decelerating phases. Similar results have also been reported by Ferchichi *et al* [11]. According to Famelart *et al* [7], such growth curves are due to the large number of nutrients composing the medium, while the high carbon : nitrogen ratio may account for low biomass yields [8].

Although the pigmentation of *B. linens* had for long raised interest in cheese-ripening technology, no experiments, as far as we know, had ever been devoted to examining the occurrence of pigment synthesis during the growth of this red smear bacterium. A growth-associated pigmentation was suspected in *B. linens* ATCC 9175, by Famelart [6] who suggested that pigment intensity might be related to growth rate. In a later publication, Famelart *et al* [7] observed that the orange intensity of cultures in liquid media increased throughout growth, and that it was best under pH and dissolved oxygen conditions favorable to growth. Ferchichi *et al* [11] visually related the intensity of the *B. linens* CNRZ 917 orange pigmentation in liquid media to dissolved oxygen conditions and the occurrence of L-methionine in the medium, and their results showed that pigmentation was intense when growth was best. Oumer *et al* [24] intended to link the pigmentation of *B. linens* ATCC 9175 to cell density in order to quickly esti-

mate growth. However, none of these studies actually published adequate kinetic data relating cell growth and pigmentation, nor compared the pigment production by a variety of *B. linens* strains. For other carotenogenic microorganisms in which such kinetics have already been described, most results agreed with a growth-associated carotenoid synthesis, except that maximum pigment production usually occurred at the end of the exponential phase of growth or even during stationary phase (production of β-carotene by the fungus *Blakeslea trispora* [10]; production of astaxanthin by the yeast *Phaffia rhodozyma* [9,14,25,27]), classifying carotenoid pigments as secondary metabolites.

Oumer *et al* [24] extracted *B. linens* pigments with methanol and found comparable spectra except for a small extra peak at 392 nm. Kohl *et al* [18] separated three carotenoid pigments from *B. linens* cells and reported that they all had virtually identical electron spectra, with a 454 nm λ_{max}. They later identified them as isorenieratene, 3-hydroxy-isorenieratene and 3,3′-dihydroxy-isorenieratene. Nybraaten and Liaaen-Jensen [23], characterizing phenolic carotenoids, indicated that the mono and the di-hydroxy-isorenieratene had rounded spectra in methanol with, respectively, $\lambda_{max} = 450 - (474)$ and 453 nm. Britton [2] gave the following spectra for isorenieratene and its diol derivative in ethanol: (426) − 448 − (475), and (427) − 449 − (476) and indicated that a rounded spectrum may be characteristic of an extended chromophore, which is in agreement with the 15 conjugated double bond chain of the three aromatic structures described by Kohl *et al* [18]. Another interpretation of a rounded spectrum would be that the molecule(s) herein vibrate heterogeneously, eg because of isomeric forms [19]. According to Jones *et al* [17], the color reaction of methanol extracts in the presence of strong alkali is specific to the carotenoid pigments of *B. linens*. It is due to the ionization of phenols (eg in both mono- and di-hydroxylated isorenieratene derivatives) into phenolates [2], with conversion of the corresponding spectrum into the absorption band of the related mono- or di-ketones. Due to the substitutive effects between β or ϕ rings on the absorption spectrum, one can verify that the extract in alkali condition effectively matched the data reported for canthaxanthin [2].

Since only a few studies had been devoted to them, the pigments of *B. linens* had never been separated further than through column chromatography [18]. As far as we know, the present results were therefore the first accurate separation of these carotenoid pigments. Compared to carotenoid standards, the retention times of the three groups of peaks here eluted appeared to match these of, respectively, dihydroxylated carotenoids, mono-hydroxylated carotenoids, and true carotenes. Our results revealed compounds for every polarity level. Since Kohl *et al* [18] identified only one signal per level, these results provided more accurate composition data. The dihydroxyisorenieratene : monohydroxyisorenieratene : isorenieratene ratio calculated from our chromatograms was in the 9 : 1 : 0.5 range, while Kohl *et al* [18] found 9 : 4 : 3. Providing that the separated carotenoids were of a common OH-substitution type within a group, one can suggest that each group, alternatively: (i) included the aromatic form described by Kohl *et al* [18] among a blend of non-aromatic carotenoids; or (ii) was primarily composed of aromatic carotenoids in various isomeric forms. Since they identified the aromatic structures by column chromatography then mass spectroscopy, which methods did not allow detection of isomers, the latest hypothesis remains in accordance with the results of Kohl *et al* [18].

The close relationship between growth and pigment production for *B. linens* may be partly explained by its strict aerobic metabolism. This could explain the production of carotenoid pigments for antioxidant protective effect, in a way suggesting they could be primary metabolites.

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