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Bioproduction of carotenoid compounds using two-phase olive mill waste as the substrate

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ABSTRACT

The olive oil production process by the two-phase centrifugal system generates a waste named "alperujo", which has a high percentage of water (65%) and contains phenolic compounds. These compounds are phytotoxic and pollute the soil and waterways, hindering the disposal of the alperujo. However, a diverse microbiota with biotechnological applications, such as the carotene-producing bacteria *Microbacterium* sp., was isolated from these wastes. The aim of this work was to evaluate the ability of an aqueous extract from alperujo (AE) to sustain the growth and carotene production of *Microbacterium* sp. in an attempt to valorize this waste. An inverse relationship between *Microbacterium* sp. growth and carotene production and AE concentration was observed. The bacterial growth was accompanied by a decrease in nitrogen, total sugar and glucose levels. In addition, total polyphenol content decreased, whereas pH of the AE increased. These results demonstrate that AE can be used as a substrate for carotene production, being an alternative strategy for alperujo valorization.

1. Introduction

Regardless of the method, olive oil extraction generates by-products that are potential pollutants to the environment, a problem that has not yet been satisfactorily solved [1]. Olive oil extraction can be performed by the traditional non-continuous process, which uses a hydraulic press, or by the continuous two- or three-phase processes, in which the oil is obtained using centrifugal decanters. Nowadays, two- and three-phase centrifugation systems are the most commonly used methods. In the three-phase process, water is added to improve the separation, and two residues are generated: olive-mill wastewater (OMW) or "alpechin", composed of vegetation water and added water, and the olive cake or "orujo" [1]. In the two-phase process, in contrast, little or no water is added, and a semi-solid waste composed of olive pomace and vegetation water is generated, called "alperujo" or two-phase olive mill waste (TPOMW). Alperujo is generated in a proportion of 80 tons per 100 tons of processed olives, it has high water content (~65%), a slightly acidic pH and almost 90% of its dry weight is organic matter [1]. It is also highly polluting to soil and water because of its high content of phenolic compounds [1–3].

Among the treatments proposed to remediate these wastes, their use as substrates for the growth of microorganisms seems to be the most convenient method because they provide the possibility of producing high value added products such as biopolymers and enzymes, with a concomitant reduction of the environmental impact of these wastes [1,4,5].

Carotenoids are lipid pigments with 40 carbon atoms and a variable number of conjugated double bonds in their structure. This characteristic gives them antioxidant properties as well as a color ranging from yellow to orange [6]. They are synthesized by plants, yeast, fungi and bacteria, but not by vertebrates, which must obtain them from the diet [7]. Carotenes are biologically important to prevent oxidative damage to lipid membranes, and some of them are also precursors of vitamin A [7]. Studies show that a diet rich in carotenoids decreases the risk of cardiovascular, neurodegenerative and vision diseases [8]. Nowadays, carotenoids are obtained by chemical synthesis from the precursor β-ionone, a process that accounts for more than 85% of the global β-carotene market [9]. However, the growing concerns about the pollutants generated by chemical industries and the shift in consumer preferences to products with natural additives make the search for natural sources of carotenes commercially interesting [9]. In addition, the use of a waste as the growth medium makes the microbial production economically viable. However, research is needed to establish the feasibility of using TPOMW as a microbial substrate because its physicochemical characteristics do not favor

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microorganism growth. Thus, in this work we propose the use of TPOMW as a substrate for the production of carotenoid compounds by *Microbacterium* sp. isolated from TPOMW. This strategy will promote TPOMW valorization by generating a high value added product, hence contributing to a better management of this oil waste.

2. Materials and methods

2.1. Microorganism

Microbacterium sp. was isolated from alperujo (two-phase olive mill waste) by an enrichment method and maintained on Trypteine Soy Agar slants (Britania, Buenos Aires, Argentina) at 4 °C. Identification was performed by CIBIC, lab (Centro de Diagnóstico Medico de Alta Complejidad, Rosario, Argentina).

2.2. Two phase olive mill waste (TPOMW) samples

Fresh TPOMW samples of Arbequina olives that were collected and immediately processed in April 2015 were provided by an oil processing plant located in Coronel Dorrego, province of Buenos Aires (Argentina). The samples were stored at −20 °C until further use. Moisture content was analyzed by drying the samples in a vacuum stove at 50 °C until constant weight was achieved.

2.3. Culture medium

A previously weighed amount of TPOMW (alperujo) was extracted with distilled water using a TPOMW:water ratio of 1:4 (w:V) for 2 h at 4 °C with continuous agitation. The obtained aqueous extract (AE) was centrifuged at 3500 rpm for 30 min, filtered and sterilized by autoclaving at 121 °C for 20 min. The sterile AE was used either undiluted (25% w/V) or diluted to 12.5% and 6.25% with sterile distilled water.

2.4. Production, extraction and quantification of carotenoids

Cultures were performed in 250 mL Erlenmeyer flasks containing 50 mL of the desired concentration of AE and incubated for 10 days at 30 °C under orbital shaking (100 rpm). Samples were taken on day 0, 1, 2, 3, 6 and 10. In order to measure glucose consumption, total sugar, phenolic compounds, pH, biomass and carotenes, 2.5 mL of culture were removed. After separation of biomass by centrifugation at 3000 rpm for 15 min, the supernatant was filtrated and stored at −20 °C until required.

For carotenes extraction, the pellets were incubated with dimethyl sulfoxide (DMSO) at 50 °C for 30 min, and thereafter extracted with 3 mL acetone for 2 or three times until the residual cell pellets were colorless. The supernatant was separated by centrifugation at 3000 rpm during 15 min and acetone extracts were combined. Pooled extracts were transferred into a centrifuge tube and 5 mL of hexane and 1 mL of distillated water were added. To separate the phases, tubes were vortexed for 10 s and centrifuged at 3000 rpm during 15 min. The clear colored hexane phase was removed with a glass Pasteur pipette and collected in amber flasks. The procedure was repeated, until the lower phase was colourless. The hexane layer was evaporated under nitrogen stream and carotenes were suspended in 3 mL acetone [10]. Quantification was performed by measuring absorbance at 450 nm in a Shimadzu UV-160A spectrophotometer, considering $A_{1\%}$ 2500 [6]. Each culture condition was run in triplicate.

Table 1
Some physicochemical properties of different concentrations of AE.

Determination	AE (% w/V)		
	6.25	12.5	25.0
pH	5.71 ± 0.18^a	5.61 ± 0.07^a	5.30 ± 0.03^a
Total sugar (g/L)	4.1 ± 0.8^a	8.3 ± 0.9^b	14.3 ± 1.4^c
Glucose (g/L)	0.62 ± 0.05^a	1.43 ± 0.06^b	2.28 ± 0.11^c
Polyphenols (mg/L)	245 ± 39^a	411 ± 16^b	742 ± 53^c
Nitrogen (mg/L)	38 ± 3^a	71 ± 4^b	126 ± 5^c

Data represent mean ± S.D., n = 4. Different letters within rows indicate statistically significant differences ($p < 0.05$).

2.5. Analytical procedures

Total phenolic content was determined spectrophotometrically by the Folin-Ciocalteu method using caffeic acid as the standard [11]. Total sugar content was determined by the meta-hydroxydiphenyl method [12] using glucose as the standard. Glucose was measured with an enzymatic-Glicemia kit, kindly provided by Wiener Lab (Rosario, Argentina). The pH was measured with an Altronix TPX-I electrode. Nitrogen was determined by Kjeldahl method. Biomass was determined by measuring the optical density (O.D.) in 10 mm cuvettes at 660 nm, followed by conversion to mg of dry biomass with a calibration curve. A non-inoculated flask (W/In) was included in all experiments and treated as the inoculated samples.

Separation of carotenoids was achieved by HPLC on a C-18 reverse phase analytical column (Waters Spherisorb ODS2 5 μm, 4.6 mm × 250 mm), using acetonitrile:water 9:1 (solvent A) and ethyl acetate (solvent B) at a flow rate of 1 mL/min. Peaks were monitored with a Waters Alliance e2695 HPLC equipped with Waters 2998 Photodiode Array Detector. The gradient for separation was 0–100% ethyl acetate in acetonitrile/water (9:1) over 25 min with flow rate of 1.2 mL/min [14].

2.6. Statistical analysis

Analysis of variance (ANOVA) followed by post-hoc Tukeyís multiple comparisons test were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com). A threshold of $p = 0.05$ was set as significance level to compare means.

3. Results and discussion

3.1. Substrate characterization

In Table 1, some physicochemical properties of the AE significant for microorganism development are shown. Total sugar concentrations of the three dilutions of AE are similar to the sugar concentrations reported for OMW [4,13,15] and for the liquid obtained from the aqueous extraction of stored and dry alperujo [16,17]. Among sugars, glucose, which is the most common substrate used for the growth of heterotrophic microorganisms, is present in the AE at significant levels, representing about 16% of total sugar. Kjeldahl nitrogen content is also in the range reported for OMW and is considered to be low for microorganism development [1,18].

Part of the environmental hazards arising from olive mill wastes is attributed to the presence of polyphenols, which are antimicrobial and phytotoxic agents [1–3]. As shown in Table 1, polyphenol content of the AE even at the lowest concentration (6.25%) reached levels previously reported to have an inhibitory effect on microorganism growth [19]. It has been proposed that, besides polyphenols, other factors such as low pH also contribute to OMW toxicity, and

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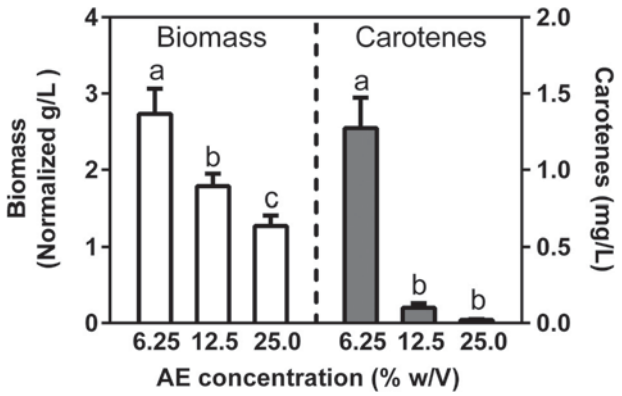

Fig. 1. Effect of AE concentration on *Microbacterium* sp. growth and carotene production. *Microbacterium* sp. was grown in different concentrations of AE for 10 days. At the end of the culture (day 10), biomass (left panel) and carotenes (right panel) were determined. Data represent mean ± S.D. of 4 independent experiments. Different letters within each panel plot represent statistically different values, ($p < 0.05$).

the same effect is expected to occur with AE because it presents an acid pH [13].

The characteristics of the AE taken together (low pH, low nitrogen content and high polyphenol content) are not favorable for microorganism development. However, a diverse microbiota composed of yeast, molds and bacteria was isolated by many authors from olive mill wastes [20]. In a previous work we reported the presence of 1 mold, 6 yeast and 5 bacterial strains with CFU counts in the order of 10^8 UFC/ml AE for bacteria and 10^6 UFC/ml AE for yeast [21]. Similar results were obtained by other authors for OMW [20,22]. Among the bacteria, the strain identified as *Microbacterium* sp. was selected because of its ability to produce carotenoid pigments. Since this microorganism was isolated from alperujo, it was expected to be well adapted to grow in the stressing conditions of the AE.

Besides bacteria, fungi, yeasts and algae can be used as potential sources of carotenoid. Moreover, the production of β-carotene by the alga *Dunaliella* sp. and the fungus *B. trispora* are well developed; however, in both cases technical requirements persist that make the process complex [23]. In contrast, bacteria are less demanding with regard to culture conditions and exhibit faster growth rates than algae and fungi, and thus they are good candidates for the biotechnological production of carotenoid compounds [24].

3.2. *Growth of* Microbacterium *sp. and carotene production with different AE concentrations*

The biomass and carotene contents after 10 days of culture of *Microbacterium* sp. in media with different AE concentrations is shown in Fig. 1. Although the growth of *Microbacterium* sp. was poor at 25% w/V AE, at 12.5% AE the microorganism almost duplicated, and at 6.25% the growth was significantly higher. Thus, *Microbacterium* sp. isolated from TPOMW was able to grow on non-supplemented AE, and biomass production was optimal on 6.25% (w/v) AE. The same trend was observed with total carotene production, which was maximal at 6.25%.

It is important to note that pH was not buffered and neither carbon nor nitrogen sources were added to the medium, in contrast to previous reports [4,15,25,26]. Morillo et al. [27] observed an optimal growth of *P. jamilae* in an AE of alperujo at 20% w/V, whereas *X. campestris* growth was reported with 10% w/V AE and 40% OMW [5]. OMW was used as substrate for microorganism growth at concentrations between 30 and 100%. This was suggested to be related to the need for a balance between soluble nutrients and toxic compounds present in the AE [27]. At high dilution rates, the medium lacks the required amount of nutrients, while increasing the AE concentration precludes microorganism growth because of the presence of high levels of inhibitors. [4,5,13,15,26]. As *Microbacterium* sp. growth and carotene production were better at 6.25%, we selected this AE concentration for further experiments.

3.3. *Time-course of* Microbacterium *sp. growth in AE*

The time course of *Microbacterium* sp. growth on 6.25 (w/v) AE was investigated as a function of different initial inoculum densities (In 1 = 0.6 g/L; In 2 = 1.2 g/L; In 3 = 2.4 g/L). A lag phase was not detected for In 3, probably due to the sampling period used. In contrast, for In2 and In1 the lag phase lasted approximately 1 day (Fig. 2A). Cultures reached stationary phase at different times from the inoculation in dependence on initial biomass: 3 days for In1 and In2, and 2 days for In3. Maximum biomass concentration was similar for the three inocula (Fig. 2A), suggesting that substrate composition limits the amount of cells that can be obtained.

The maximal specific growth rate was higher for In1, which exhibited the best biomass gain (~four times initial biomass), followed by In2 (~three times) and In3 (~two times) (Fig. 2B). These results show that AE is suitable for *Microbacterium* sp. growth in a time scale of a few days.

3.4. *Carotene production by* Microbacterium *sp. growth in AE*

Carotenoid compounds are secondary metabolites, and thus they are produced at later stages of the culture. Fig. 3 shows that carotene production increased in the last stage of exponential growth and in the stationary phase, as previously reported [24,28]. Although the relative carotene increase at the end of the culture was higher for In1, the amount of total carotenoids obtained for In3 was the largest (Fig. 3). In addition, the time needed to reach the maximal carotene production was shorter for In3 (2 days) than for In2 (6 days) and In1 (10 days). Thus, the increase in inoculum density led to an increase in carotene volumetric productivity.

One function of carotenoids is to serve as membrane integrated antioxidants, protecting cells from oxidative stress [29]. Therefore, environmental factors that contribute to oxidative damage stimulate microbial carotenoid biosynthesis [30]. Trace amounts of metal ions increase carotenoid production in alga, yeast and bacteria, since they generate an oxidative environment and activate specific carotenogenic enzymes [30,31]. TPOMW contains low levels of iron, cooper and zinc, which in combination with agitation could act as stimulants for carotenoid production [1,18]. In addition, polyphenols disrupt membrane integrity and in aerobic conditions can generate hydrogen peroxide contributing to the oxidative challenge [32]. Thus, the observed carotenoid increase might be part of the antioxidant response of *Microbacterium* sp. to the adverse conditions presented by the AE [32,33].

Carotenoids are obtained at diverse levels depending on the microorganism strain, substrate characteristics and culture conditions [9]. In the case of yeast, a broad range of carotenoid yields has been reported, ranging from 0.5 to 185 mg/L of culture [34]. The maximum level of total carotenoids produced in the present work (2 mg/L AE) is comparable to that obtained with *Sporidiobolus salmonicolor* grown in shaken flask on a medium containing peptone, glycerol, glucose and malt extract [10] and higher than that obtained in the same study using corn steep liquor and parboiled rice waste as substrate. As for bacteria, *Micrococcus* sp. isolated from marine sediment samples grown in marine broth produced 5.72 mg of total carotenes/L [28], and 7.7 mg/L of β-carotene were obtained upon optimization of *Flavobacterium multivorum* grown in a medium with glucose, yeast extract, peptone, urea and sodium carbonate [24]. In the present work, similar levels of total carotenoids from *Microbaterium* sp. growth were obtained in AE

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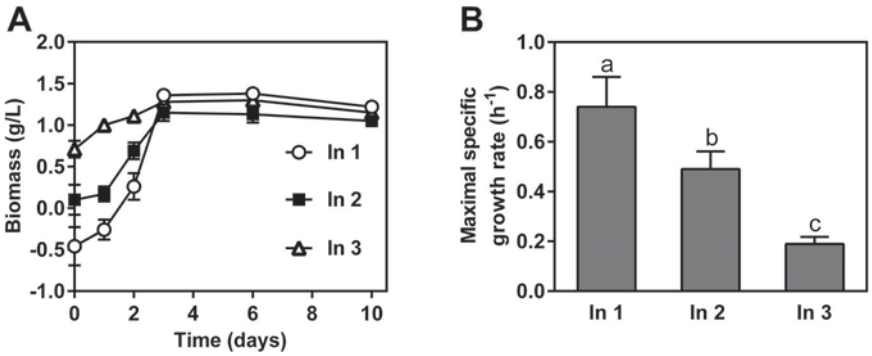

Fig. 2. Time-course of *Microbacterium* sp. growth in AE. (A) Different inoculum concentrations of *Microbacterium* sp. were grown in AE at 6.25% for 10 days, and biomass was determined for each culture at different times. (B) The specific growth rate was determined for each culture from the exponential growth phase. Data represent mean ± S.D. of 4 independent experiments. Different letters represent statistically different values, ($p < 0.05$).

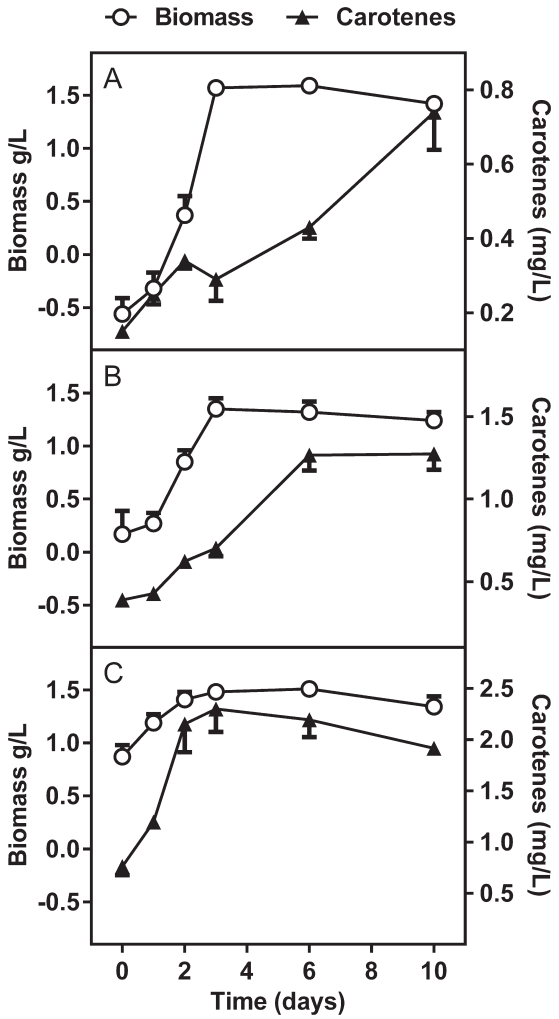

Fig. 3. Time courses of g/L of biomass (open circles) and carotene production (filled triangles) in *Microbacterium* sp. cultures grown on 6.25% AE and inoculated with 0.6 (A), 1.2 (B) and 2.4 (C) g/L.

without any supplements or optimization, with this situation being an excellent starting point for further studies to improve this yield.

Total sugar and glucose consumption of different cultures was then evaluated. *Microbacterium* sp. growth was associated with sugar consumption (Fig. 4A). Although total sugar levels decreased, a significant residual concentration remained in the medium even after 10 days of culture. In3 consumed more total sugar than In1 and In2, with only ~20% being left in the medium, compared with ~50% and ~40% left by the other two. Oxidation of mannitol, sorbitol, arabinose, rhamnose, ribose, xylose, and starch is variable for members of the genus *Microbacterium* [35]. Most of these simple, water-soluble sugars and sugar alcohols are present in alperujo [21], and thus they could be responsible for the residual value of total sugar observed at the end of the culture. In addition, mannitol is a recalcitrant compound in olive fermentation brines and is not consumed by indigenous microbiota in stored alperujo even after 6 month of storage [17].

As expected, glucose was preferentially used by *Microbacterium* sp. since its concentration decreased faster than the drop in total sugar concentration (Fig. 4B). Moreover, glucose was almost completely consumed by the three inocula, but after different times (Fig. 4B). Once the glucose was metabolized, no major increase in biomass was observed, even when total sugar still decreases (Figs. 3 and 4). This is more evident in the case of In3, in which the faster consumption of glucose (less than one day), slows microorganism growth giving a smaller specific growth rate (Fig. 2). Therefore, it seems that although the inoculum is able to consume other sugars, these are used to maintain biomass and carotenoid synthesis and to a lesser extent to biomass production. Besides, nitrogen is consumed during the fermentation, a fact that could also preclude microorganism growth (Table 2).

Nitrogen acts as a negative regulator of the carotenoid pathway [31,36,37]. In *Fusarium fujikuroi* higher levels of carotenoids are produced in low-nitrogen medium. Moreover, nitrogen removal induced a transient increase of the mRNA amounts for specific structural genes of the carotenoid pathway [36]. In *Phaffia Rhodozima* culture in low nitrogen medium, the activity of the enzyme ATP-citrate lyase, which is responsible for synthesizing the carotenoid precursor acetil-CoA, increases [38]. Furthermore, the algae *Haematococcus pluvialis* accumulates astaxanthin only when cell division is stopped and all nitrogen in the culture medium has been consumed [39].

Carotene production by marine *Micrococcus* sp. was high in a poor nutrient medium, without any improvement in the amount of biomass [28]. Moreover, it has been observed that glucose concentrations above 0.5 g/L repress carotenoid synthesis in metabolically engineered *E. coli* [40]. Interestingly, in our system carotene levels boosted after glucose and nitrogen were exhausted in the medium (Figs. 3 –4B and Table 2).

Total polyphenol content decreased at the end of the culture, reaching similar values for In1, In2 and In3 (Fig. 4C). A reduction of ~40% was achieved after 10 days of culture. The delay in polyphenol consumption by *Microbacterium* sp. may be due to the preference of the strain to metabolize other carbon sources such as glucose and simple sugars present in the AE.

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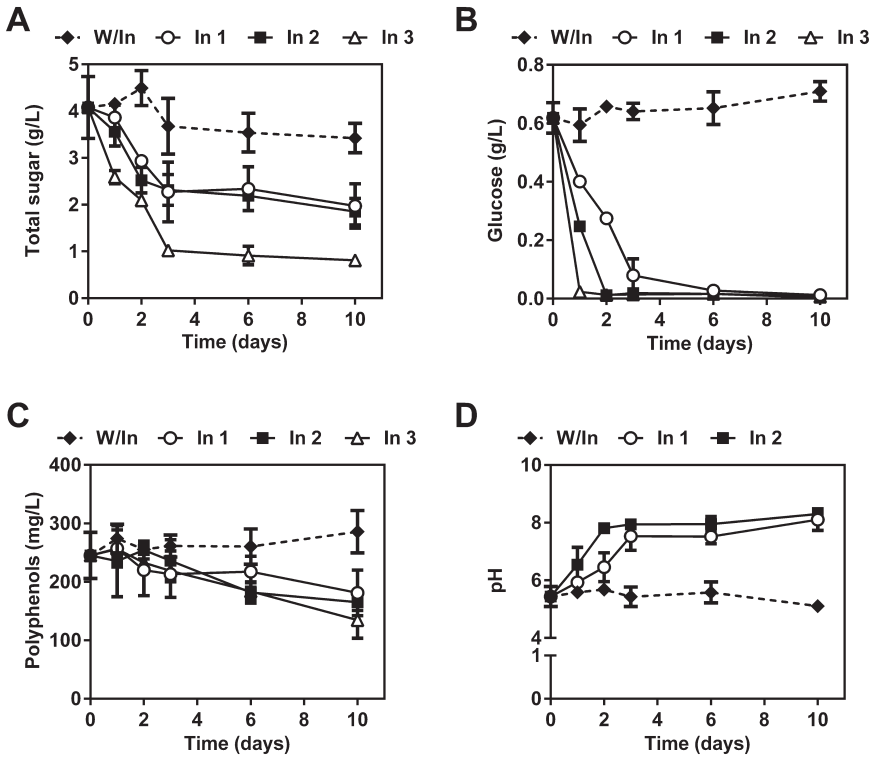

Fig. 4. Substrate characterization during *Microbacterium* sp. growth in AE. Different inoculum concentrations of *Microbacterium* sp. were grown in AE at 6.25% for 10 days, and total sugar (A), glucose (B), polyphenols (C) and pH (D) were determined for each culture at different times. Data represent mean ± S.D. of 4 independent experiments.

Table 2
Final physicochemical properties of AE after 10 days of culture.

	Total sugars (g/L)	Glucose (g/L)	Polyphenols (mg/L)	pH	Nitrogen (mg/L)
W/In	$3{,}4 \pm 0{,}3^{a}$	0.71 ± 0.03^{a}	286 ± 36^{a}	5.10 ± 0.03^{a}	44 ± 3^{a}
In1	2.0 ± 0.5^{b}	0.122 ± 0.005^{b}	181 ± 39^{b}	8.11 ± 0.37^{b}	3.3 ± 0.1^{b}
In2	1.9 ± 0.3^{b}	0.005 ± 0.002^{b}	165 ± 14^{b}	8.29 ± 0.18^{b}	2.5 ± 0.4^{b}
In3	0.8 ± 0.2^{c}	0.007 ± 0.001^{b}	134 ± 31^{b}	8.28 ± 0.60^{b}	3.6 ± 0.2^{b}

W/In: non inoculated sample. Data represents mean ± S.D., n = 4. Different letters within a column indicate statistically significant differences ($p < 0.05$).

As *Microbacterium* sp. developed, a rise in pH was observed (Fig. 4D). All the cultures reached the same pH after 10 days. A similar response in pH was also obtained during the fermentation of agro-industrial wastes by the carotenoid-producing yeast *S. salmonicolor*, which was attributed to the lysis of the microorganism when the substrate is consumed [41]. Remarkably, the pH increase was coincident with the drop in glucose and total sugar (Fig. 4). Moreover, nitrogen was almost completely consumed during the fermentation by the three levels of inoculum (Table 2). Therefore, it is also plausible to speculate that nitrogen utilization could be the responsible of the rise in pH [42].

Table 2 summarizes the final physicochemical properties of the AE after 10 days in culture.

3.5. Characterization of carotenoids produced by Microbacterium sp.

In an attempt to characterize the carotenoids produced, we performed reverse phase HPLC in conjugation with a photo diode array detector [14]. The chromatograms present seven peaks, all with retention times shorter than β-carotene (retention time 17.61 min), which suggest that carotenoids present in the sample are more polar, probably having a hydroxyl or carbonyl group (Fig. 5).

Table 3
Retention times and absorption maxima of different carotenoids resolved by HPLC.

Peak #	Retention time (min)	λ1 (nm)	λ2 (nm)	λ3 (nm)
1	9,832	450	479	504
2	10,92	354	376	386
3	14,773	452	480	504
4	15,339	452	480	504
5	15,873	452	480	504
6	16,392	450	479	504
7	16,896	450	479	504

Typically the carotenoid pigments present a three peak spectrum, in which the shape and location of the main absorption bands are determined by the structure of the chromophore. Thus, the analysis of the UV–vis spectrum of carotenoids provides information about their structure [6]. Carotenoids eluting in peak 1 and 3–7 all present a similar spectrum with maximum absorption at 480 nm and 504 nm, and with a shoulder at 450 nm (Fig. 5 and Table 3). The similarity in their spectra indicates they have the same chromophore in the molecule. Moreover, the maximum absorption of

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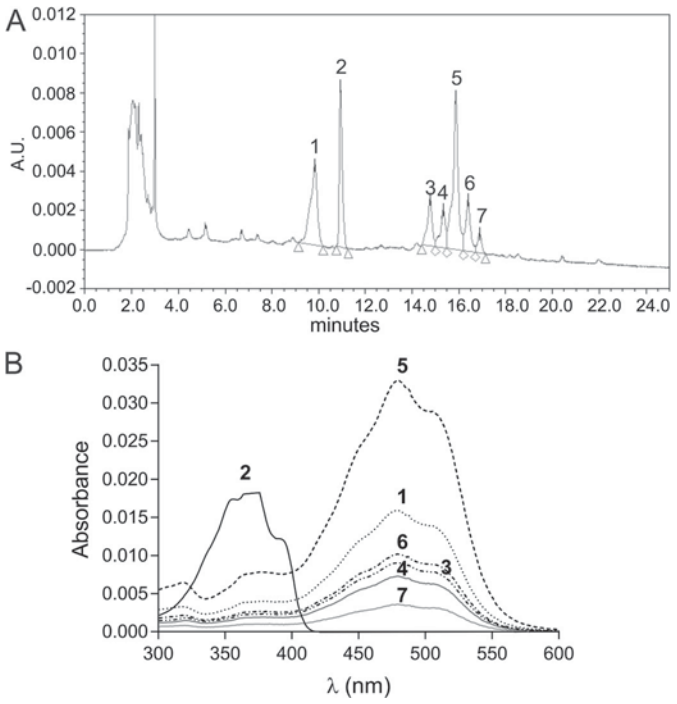

Fig. 5. Carotenoids produced by *Microbacterium* sp. after 10 days growing in AE. (A) HPLC chromatogram. (B) UV–vis spectra of each chromatogram peak.

480 nm suggests a chromophore length of 12 conjugated double bonds, and the presence of a weak band at ~368 nm may indicate the existence of a *cis* bond. The poor inner structure of the spectra and the shorter retention time in the chromatogram suggest the presence of groups adding polarity to the molecule like carbonyl groups, which are known to produce a bathochromic shift in the maxima absorption wavelength and a decrease in the fine structure of the spectra [6]. On the other hand, carotenoid eluting in peak 2, presents a spectrum which is different from the others indicating the presence of a different chromophore. The maximum absorption at 376 nm suggests a chromophore length of 6 conjugated double bonds. Moreover, due to the absorptions at wavelengths in the UV region, this compound is probably colorless. Among bacteria, the genus *Micrococcus*, *Mycobacterium* and *Rhodococcus* contain carotenoids with conjugated keto functions [14,43]. This type of carotenoid are more polar, a property that influence, their absorption and distribution once ingested as well as their antioxidant activity in membranes [44–46].

4. Conclusion

Microbacterium sp. was able to grow and produce carotenes in an AE of alperujo as substrate, with a maximal carotene production of 2 mg/L AE. Microorganism growth was accompanied by a complete glucose consumption and a reduction in total sugar concentration. The development of *Microbacterium* sp. decreased phenolic compound content and increased pH. The results presented in this study for the production of value added compounds will serve as a feasible strategy for alperujo valorization.

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