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**EFFECT OF IRON SALTS ON *Rhodococcus* sp. AND *Gordonia* sp. ON CAROTENOID PRODUCTION****EFFECTO DE LAS SALES DE HIERRO EN *Rhodococcus* sp. Y *Gordonia* sp. EN LA PRODUCCIÓN DE CAROTENOIDES**L. Ortega-Cabello¹, H.I. Pérez-Méndez^{2*}, N. Manjarrez-Alvarez², A. Solís-Oba², A. López-Luna²¹ Doctorado en Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso 1100, Edificio A, Colonia Villa Quietud, PO box 04960, CDMX., México.² Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso 1100, Edificio N, Colonia Villa Quietud, PO box 04960, CDMX., México.

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Abstract

Carotenoids are important pigments, due to their antioxidant activity. The purpose of this research was to observe the effect of GYEA media with FeSO₄ and FeCl₂ on the total carotenoid content (TCC) and the carotenoid profile of *Rhodococcus* sp. and *Gordonia* sp. In the biomass of *Rhodococcus* sp., with 0.025% FeSO₄ a TCC of 14.91±1.66 µg/g was calculated, and two carotenoids were observed; the Carotenoid 2, identified as an astaxanthin dirhamnolide like carotenoid increased from 13.45±0.52% in GYEA media without salts to 24.21±1.48% with 0.025% FeSO₄ (T value of 11.81, α 0.05). With the biomass of *Gordonia* sp. in the presence of 0.025% FeCl₂ a TCC of 28.66±8.21 µg/g was calculated and three carotenoids were observed, no significant increase was determined for these carotenoids. The formation of carotenoid-iron complexes was observed with UV-Vis.

Keywords: marine actinobacteria, carotenoids, iron salts, TCC, carotenoid profile.

Resumen

Los carotenoides son pigmentos importantes por su actividad antioxidante. El objetivo de esta investigación fue observar el efecto del medio GYEA con FeSO₄ y FeCl₂ en el contenido total de carotenoides (TCC) y en el perfil carotenoides de *Rhodococcus* sp. y *Gordonia* sp. En la biomasa de *Rhodococcus* sp. con 0.025% FeSO₄ se calculó un TCC de 14.91±1.66 µg/g y se observaron dos carotenoides; el Carotenoide 2, identificado como un carotenoide similar al dirhamnólido de astaxantina incrementó de 13.45±0.52% en medio GYEA sin sales a 24.21±1.48% con 0.025% FeSO₄ (valor de T de 11.81, α 0.05). Con la biomasa de *Gordonia* sp. con 0.025% FeCl₂ se calculó un TCC de 28.66±8.21 µg/g y se observaron tres carotenoides, no se detectó un incremento significativo para éstos carotenoides. Se observó por UV-Vis la formación de complejos carotenoides-hierro.

Palabras clave: actinobacterias marinas, carotenoides, sales de hierro, TCC, perfil de carotenoides.

1 Introduction

Carotenoids are pigments with biological importance due to their antioxidant activity related to the prevention of diseases such as cancer (lung, colorectal, breast), Alzheimer and Parkinson (Candelas-Cadillo *et al.*, 2005; Yahia and Ornelas-Paz, 2010; Vélchez *et al.*, 2012). They are a natural alternative to synthetic colorants in pharmaceutical, chemical, cosmetic and food industries (Arredondo-Figueroa *et al.*, 2003; Maldonado *et al.*, 2007;

Querellou *et al.*, 2010; Joshi and Rana, 2011; Flores-Miranda *et al.*, 2015). Owing to the toxicity detected on some artificial colorants, the demand for natural alternatives has increased.

Carotenoids can be obtained from plants, fruits, vegetables, microalgae, yeasts and bacteria (Chilkov, 2011; Delgado-Vargas *et al.*, 2000). Marine microorganisms are becoming important because they can synthesize new metabolites (Joshi *et al.*, 2003;

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Lordan *et al.*, 2011). In this regard, actinobacteria are important because of their capacity to produce a wide variety of biologically active compounds, among these are the carotenoids (Bull *et al.*, 2000; Lam, 2006; Subramani and Aalbersberg, 2012).

The biotechnological production of bacterial carotenoid pigments is an important alternative technique for their large-scale production, because the growth of bacteria is season independent, sustainable and highly productive (Kitaoka *et al.*, 1997; Das *et al.*, 2007; Mandelli *et al.*, 2012). Bacterial carotenoid production depends on factors like temperature, the presence of light and nutrient availability, such as carbon and nitrogen sources as well as mineral salts (Ma and Chen, 2001; Bhosale, 2004).

There is evidence that ferrous ion induces oxidative stress to the bacteria (Ma and Chen, 2001; den Hengst and Buttner, 2008), enhancing the carotenogenesis process as an antioxidant defense mechanism (Groves and Lucana, 2010). On the other hand, the presence of metal ions is relevant since they are cofactors in the carotenoid biosynthesis, especially at translational level and interaction with synthases (Weinberg, 1990; Khodaiyan *et al.*, 2007a; Gharibzahedi *et al.*, 2013). The effect of iron salts on carotenoid production has been widely studied in microalgae (Mojaat *et al.*, 2008) and in cyanobacteria (Chakilam, 2012), while marine actinobacteria have remained less studied (Khodaiyan *et al.*, 2007b; Nasri Nasrabadi and Razavi, 2010).

Rhodococcus and *Gordonia* genera, belonging to the class Actinobacteria, have a biotechnological relevance in water and soil bioremediation (Álvarez *et al.*, 2008; Marques *et al.*, 2011) and recently have been studied as potential carotenoid sources (Jeon *et al.*, 2012; Zheng *et al.*, 2013). In this research were studied two marine actinobacteria strains, *Rhodococcus* sp. and *Gordonia* sp. isolated from sediments of the Gulf of México, under control conditions (GYEA medium) and in the presence of FeSO₄ and FeCl₂, to evaluate the influence of iron salts on the total carotenoid content (TCC) and carotenoid profile produced by the two microorganisms. Chávez-Castilla and Aguilar (2015) performed a study of the influence of mineral salts on prodigiosin pigment production by *Serratia marcescens* BS303.

2 Materials and methods

Rhodococcus sp. and *Gordonia* sp. were isolated from

sediments from the Gulf of México obtained during an oceanographic campaign (Maldonado *et al.*, 2009).

2.1 Production of biomass

2.1.1 Growth of strains on Petri dishes

Rhodococcus sp. and *Gordonia* sp. were grown in GYEA medium (control) and GYEA medium supplemented with 0.025, 0.25, 0.5 and 1.00% FeSO₄ or FeCl₂ respectively (Moraleda-Muñoz *et al.*, 2005, Frantz 2009, Nasri Nasrabadi and Razavi 2010) at 28°C. Growth was monitored for 45 days.

2.1.2 Growth of strains on liquid medium

In an Erlenmeyer flask containing 250 mL of liquid GYEA medium with and without iron salts at 0.025%, strains were inoculated with *Rhodococcus* sp. or *Gordonia* sp. and incubated at 28°C, 150 rpm for 6 days.

2.2 Carotenoid extraction

The culture media of *Rhodococcus* sp. or *Gordonia* sp. was centrifuged at 4500 rpm for 15 minutes; the biomass was separated and freeze-dried. The extraction of carotenoid pigment was repeated 5 times according to Romero *et al.* (2012) with a mixture of dichloromethane: methanol: acetone (1:1:2) until the discoloration of the biomass, the solvent was removed under reduced pressure, and extracts were stored in refrigeration until further use.

2.3 Assessment of TCC in extracts by UV-Vis spectrometry

The extract was reconstituted in ethanol, the absorbance was measured at 450 nm and TCC was calculated as Baskar *et al.* (2010) using Eq. (1).

$$TCC(\mu g/g) = \frac{AxVx10^4}{A_{1cm}^{1\%}xM} \quad (1)$$

Where *A* is the absorbance of the sample, *V* is the volume used to dissolve the sample, *A*_{1cm}^{1%} is the molar absorptivity of a carotenoid reference (β-carotene *A*_{1cm}^{1%}=2620) (Rodríguez-Amaya, 2001) and *M* is the weight of the sample. Each measure was in triplicate and ANOVA analysis was performed for each strain to determine significant differences, followed by a Dunnett test compared to control.

2.4 Analysis of the extract by high performance liquid chromatography (HPLC)

The extracts were analyzed by HPLC under the conditions reported by Islas (2010) using an Agilent 1260 chromatograph, equipped with a diode arrangement detector (DAD), a Phenomenex Luna C18(2) column (150x3mm, 5 μ m), using a mixture of methanol: acetonitrile: ethyl acetate: water (80:10:5:5) as mobile phase, the chromatograms were acquired at 450 nm. The peak area % was used to compare the carotenoid profiles with and without iron salts, an independent samples T-test was used to assess differences.

UV-Vis spectra was recorded (190-700 nm) during the chromatographic analysis, the maximum absorption wavelength (λ_{max}) was determined, and if the spectra showed more than one λ_{max} , proportion of λ_{max} III in λ_{max} II (%III/II) was calculated according to Rodríguez-Amaya (2001).

2.5 Analysis of the extract by mass spectrometry (MS)

The extracts were analyzed by direct injection using an ESI-MS under the same conditions as Ortega Cabello *et al.* (2016). Molecular weights were compared against Lipid bank and Carotenoid DB databases.

3 Results and discussion

3.1 Effect of FeSO_4 and FeCl_2 on microbial growth

Rhodococcus sp. and *Gordonia* sp. were grown on GYEA media (control) and GYEA supplemented with 0.025% of FeCl_2 or FeSO_4 . After 10 days it was observed, a large amount of colonies in the case of *Rhodococcus* sp. on both salts comparable with the control. *Gordonia* sp. a minor growth compared to control was observed with both salts, however growth with FeCl_2 was better than with FeSO_4 , this behavior is because according to Ivshina *et al.* (2013) *Rhodococcus* genera is more tolerant to the presence of heavy metals than *Gordonia* genus (Fig. 1).

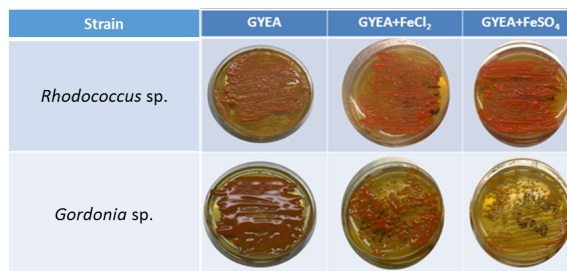


Fig. 1. Comparison of the strains after 10 days of growth under control conditions and in the presence of 0.025% iron salts.

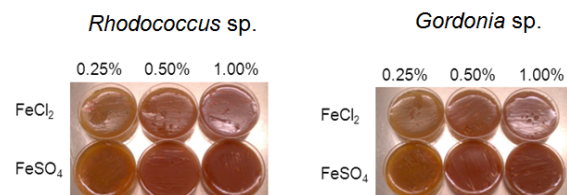


Fig. 2. Growth of strains after 45 days in the presence of iron salts, concentrations from 0.25 to 1%.

However, with the concentrations of 0.25, 0.5 and 1% of the iron salts the growth was extremely low, even after 45 days only a few colonies were observed in both strains, the intensity of coloration of the agar is because of iron salts concentration (Fig. 2). The minimal growth could have been because concentrations of iron salts above 0.025% were toxic for *Rhodococcus* sp. and *Gordonia* sp. (Schmidt *et al.*, 2005).

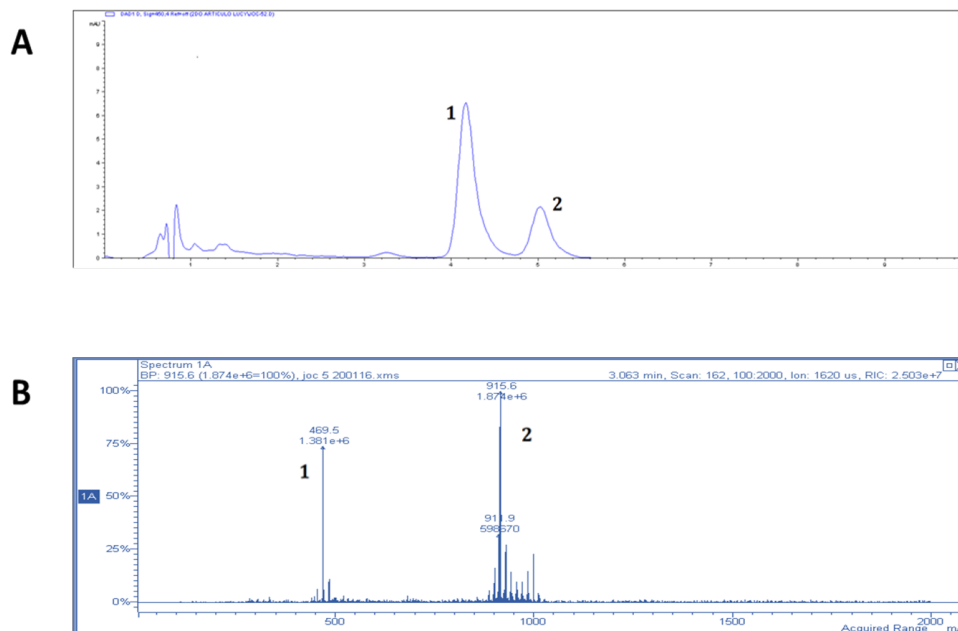
The coloration of the colonies was different in the presence of 0.025% of iron salts to that observed with control media (Fig. 1). In the case of *Rhodococcus* sp. the color of the colonies in the control was orange and changed to coral in the presence of iron salts; for *Gordonia* sp. the change was from orange to red. The change in the color of the colonies could be an evidence of the carotenoids activity in biological binding of metal ions (Hernández-Marin *et al.*, 2012; Ivshina *et al.*, 2013). According to Schmidt *et al.* (2005) and Ivshina *et al.* (2013), to maintain the homeostasis within the cell, microorganisms must keep the reactive heavy metals at an optimal sub-toxic level, by the formation of intracellular metal non-harmful complexes (Wang *et al.*, 2013).

3.2 Effect of the iron salts on TCC

After 10 days of growth on Petri dishes, *Rhodococcus* sp. and *Gordonia* sp. were inoculated on liquid control media and GYEA enriched with 0.025% of iron salts, incubated for 6 days.

Table 1. TCC ($\mu\text{g/g}$) determination after the 6 days fermentation of marine strains in control media and GYEA supplemented with iron salts

STRAIN	GYEA	GYEA + FeSO_4	GYEA + FeCl_2
<i>Rhodococcus</i> sp.	15.52 \pm 0.94	14.91 \pm 1.66	9.04 \pm 1.19*
<i>Gordonia</i> sp.	35.01 \pm 3.60	19.72 \pm 7.18*	28.66 \pm 8.21

* Significant difference ($p < 0.01$)Fig. 3. Carotenoid profile of *Rhodococcus* sp. with FeSO_4 by HPLC (A) and ESI-MS of *Rhodococcus* sp. in control media (B).

The TCC in the extract obtained from *Gordonia* sp. with control media was more than double (35 $\mu\text{g/g}$, Table 1) than that from *Rhodococcus* sp. (15.52 $\mu\text{g/g}$, Table 1). This means that *Gordonia* sp. is a better carotenoid producer than *Rhodococcus* sp.

The production of carotenoids by *Rhodococcus* sp. and *Gordonia* sp. was dependent on the type of iron salt. The TCC in the extract from *Rhodococcus* sp., with FeSO_4 (14.91 \pm 1.66 $\mu\text{g/g}$) was similar to the control (15.52 \pm 0.94 $\mu\text{g/g}$), whereas with FeCl_2 the TCC was significantly lower (9.04 \pm 1.19 $\mu\text{g/g}$) 42% less than with the control (F value of 19.36, $p < 0.01$, Table 1). On the contrary, the TCC in the extract from *Gordonia* sp. in the presence of FeCl_2 (28.66 \pm 8.21 $\mu\text{g/g}$) was similar to the control (35.01 \pm 3.60 $\mu\text{g/g}$), while with FeSO_4 a lower TCC was obtained (19.72 \pm 7.18 $\mu\text{g/g}$) 44% less than with the control (F value of 7.61, $p < 0.01$, Table 1). The

variability observed in TCC could be due to other antioxidant mechanisms, such as the generation of superoxide dismutase (Albarracín et al. 2008, Schulte et al. 2010, de Carvalho 2012). To the best of our knowledge, this is the first report about the influence of Fe salts, FeSO_4 and FeCl_2 , on the *Rhodococcus* sp. and *Gordonia* sp. growth and carotenoid production.

3.3 Analysis of carotenoids obtained with and without iron salts

The carotenoids were analyzed by HPLC, ESI-MS and UV-Vis.

Two carotenoids were detected in the extract from *Rhodococcus* sp. cultivated with control media or in the presence of 0.025% FeSO_4 . The Carotenoid 1 had a retention time of 4.14 min at 450 nm (Fig. 3A) and a single λ_{max} at 480 nm with UV-Vis spectra (Fig. 4).

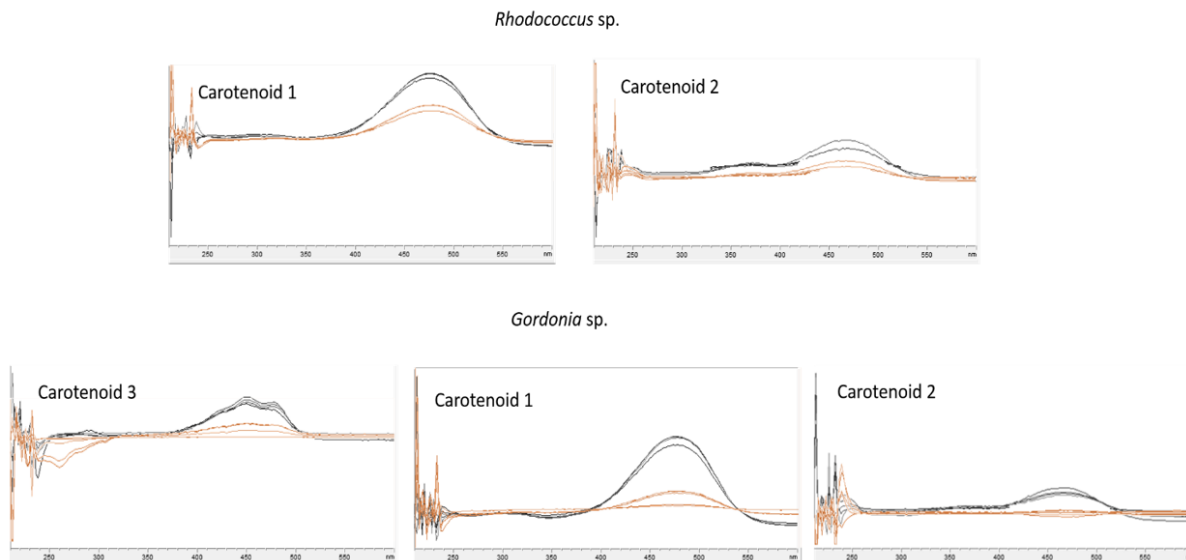


Fig. 4. UV-Vis spectra of carotenoids with control media (black line) and with iron salts (orange line).

Thus suggesting the presence of a carbonyl in conjugation with the olefinic chain, that extends the chromophore and causes a bathochromic effect on the polyene chain, with the concomitant loss in the resolution of the carotenoid UV-Vis spectra (Rodríguez-Amaya 2001). The ion mass of Carotenoid 1 was $m/z = 469$ ($M+Na$) (Fig. 3B) from the results and according to the Lipid bank and Carotenoid DB databases it could be inferred that Carotenoid 1 is an 8'-apoastaxanthin like carotenoid (Etoh *et al.*, 2012; Ortega Cabello *et al.*, 2016). Carotenoid 2 had a retention time of 4.99 minutes at 450 nm (Fig. 3A), showed a single λ_{max} at 470 nm in the UV-Vis spectra, that is in agreement with the presence of carbonyl group in the structure, as mentioned above (Fig. 4). The ion mass of Carotenoid 2 was 915.1 m/z ($M+Na$), according to Lipid bank and Carotenoid DB databases it could be inferred that is a carotenoid glycoside, an astaxanthin dirhamnoside like carotenoid (Asker *et al.*, 2009; Ortega Cabello *et al.*, 2016). Carotenoid glycosides are uncommonly molecules of recent discovery in bacteria, which may have a potential antibiotic activity and can have a role as biosurfactants (Háda *et al.*, 2012; Martínez-Trujillo *et al.*, 2015), which makes this *Rhodococcus* strain interesting on the production of such compounds. With these results, it can be proposed that carotenoids present in *Rhodococcus* sp. are xanthophylls (Ortega Cabello *et al.*, 2016).

Even though the TCC determined in the extracts

from *Rhodococcus* sp. with control media and with $FeSO_4$ is similar (Table 1), the carotenoid profile by HPLC was different. The production of carotenoids was modified; the relative quantity of Carotenoid 1 was $86.54 \pm 0.53\%$ and was reduced to $75.78 \pm 1.48\%$. Whereas the relative quantity of Carotenoid 2 significantly increased from 13.45 ± 0.52 to $24.21 \pm 1.48\%$ (T value of 11.81, α 0.05). The increase of the carotenoid glycoside could have been because carotenoid glycosides provide more stability to the bacteria (Pfander, 1976; Háda *et al.*, 2012).

Gordonia sp. produced three carotenoids with control media and in the presence of 0.025% $FeCl_2$. Retention time of Carotenoid 3 was of 3.26 minutes (Fig. 5A), the UV-Vis spectra shows three λ_{max} of 427, 450 and 480 nm (Fig. 4) and a %III/II of 25%. That suggests the presence of hydroxyl groups on its molecule because this group does not affect the absorption of the polyene chain, and could have a cyclic ending at the end of the molecule, as Rodríguez-Amaya (2001) correlated with the λ_{max} at 427 nm. The ion mass of Carotenoid 3 was 301.0 m/z , which suggests that, is another apocarotenoid with hydroxyl groups (Lipid Bank, Carotenoid DB) (Fig. 5B). Carotenoid 3 is the reduced form of a 4-oxo retinaldehyde like carotenoid reported by Ortega Cabello *et al.* (2016). The other two carotenoids with retention times of 4.14 and 4.99 minutes are the same as carotenoids present in *Rhodococcus* sp. Carotenoids 1 and 2.

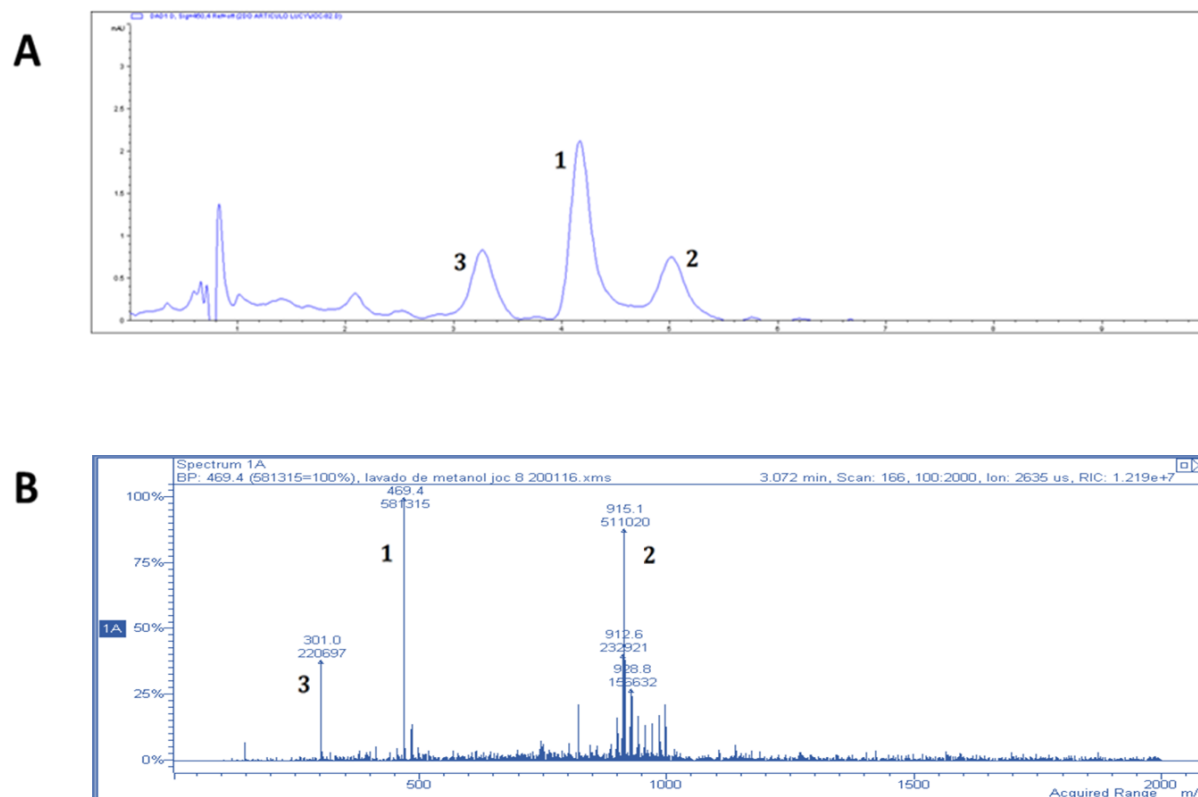


Fig. 5. Carotenoid profile of *Gordonia* sp. with FeCl_2 by HPLC (A) and ESI-MS of *Gordonia* sp. in control media (B).

The carotenoid profile by HPLC of *Gordonia* sp. with FeCl_2 showed a decrease in the UV-Vis absorption compared to the control, however it was not detected a significant change in the relative quantity for any of the three carotenoids. This behavior, could be explained because there is an antioxidant interaction between Carotenoid 3 and Carotenoid 2 as protection in an oxidative environment (Ortega Cabello *et al.* 2016).

3.4 UV-Vis spectra of carotenoids obtained with and without iron salts

A possible mechanism of bacteria to reach homeostasis in the presence of iron salts, is by the formation of non-harmful complexes between xanthophylls and iron (Polyakov *et al.*, 2010; Hernández-Marín *et al.*, 2012; Ivshina *et al.*, 2013). These complexes can also affect the UV-Vis spectra of carotenoids changing the λ_{max} (Polyakov *et al.*, 2010), as was observed in UV-Vis spectra of Carotenoid 2 of *Rhodococcus* sp. and differences in

absorption as were seen in every spectra of Fig. 4.

The chelating properties of carotenoids is an ability conferred to xanthophylls because these kind of carotenoids divert the excitation energy when such complex is formed, with concomitant changes in coloration and absorption as discussed earlier (Schmidt *et al.*, 2005; Polyakov *et al.*, 2010; Hernández-Marín *et al.*, 2012).

3.5 Importance of carotenoid-iron complexes

Even though the TCC was not improved in the presence of iron salts, carotenoid iron complexes have important biological activity. The antioxidant activity of carotenoid-iron complexes cannot be measured by conventional methods because they give higher measures (Prior *et al.*, 2005; Ozgen *et al.*, 2006). Hernández-Marín *et al.* (2012) demonstrated that this complex could be a good electron donor/acceptor of superoxide radical; Polyakov *et al.* (2010) proposed that these complexes might have an important role

in photoprotection. Hess *et al.* (2005), Hurrell and Egli (2010) and Etcheverry *et al.* (2012) have also suggested a possible link between carotenoid and iron intake in the induction of erythropoietic processes, as an immunomodulation mechanism of carotenoids, although it remains unclear.

Conclusions

The growth of *Rhodococcus* sp. with iron salts was similar to control media and with *Gordonia* sp. was lower with iron salts. Concentrations higher than 0.025% of iron salts showed to be harmful on both strains. The increase on TCC of both strains was not significant in presence of 0.025% of iron salts. In the carotenoid profile of *Rhodococcus* sp., an increase on the astaxanthin dirhamnoside like carotenoid (Carotenoid 2) was determined because it provides more stability to *Rhodococcus* sp. with a concomitant decrease of carotenoid similar to 8'-apoastaxanthinal (Carotenoid 1), thus observing the effect of iron salts on this strain. No significant increase was determined for any of the three carotenoids present on *Gordonia* sp, therefore there was no effect of iron salts. The formation of carotenoid-iron complexes was observed, these complexes can be biologically important, as better free radical scavenging agents or to increase iron absorption, thus improving the immune system of users; however further studies need to be done in order to confirm the biological importance of such complexes. A carotenoid glycoside (astaxanthin dirhamnoside like carotenoid) was characterized in both strains with probable biological relevance as antibiotic and surfactant.

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