

Production of Natural Pigments From Novel local Psychrotolerant *Kocuria* spp.

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Abstract: Two local psychrotolerant coloured bacteria, *Kocuria polaris* MO and *Kocuria carniphila* MY, which were isolated from Nile river during winter in Kafer El-Shakhe-Egypt. They were investigated for natural pigments production as *K. carniphila* MY was a yellow isolate and *K. polaris* MO was an orange one. These natural pigments were extracted using acetone after cultivating both isolates on King's medium and being incubated at 10°C for 7 days. Low incubation temperatures for both bacterial isolates were found to enhance production of large amount of pigments which was seen to be reduced by increasing temperature till it was completely vanished above 30°C. On the other hand, a pH value of 7-8 was optimum for pigmentation in both strains. Light was found to be suppressive for pigmentation in both isolates where in dark places pigment production was improved rather than in lightened ones. No significant difference was shown on pigmentation after incubating cells once static, and then shaken. Bacterial pigments were then identified by TLC, where it was found that *K. carniphila* MY contains β -carotene and xanthophylls, while *K. polaris* MO contains β -carotene and echinenone. Pigments were then separated by column chromatography and it was found that *K. carniphila* MY contains 0.127 mg/g of β -carotene and 4.073 mg/g xanthophylls while *K. polaris* MO contains 0.308 mg/g of β -carotene and 0.2 mg/g of echinenone. β -carotene, echinenone and xanthophylls were determined spectrophotometrically after separation.

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Key Words: Psychrotolerant bacteria, *Kocuria carniphila*, *Kocuria polaris*, natural pigments.

1. Introduction

Natural pigments and synthetic dyes are extensively used in various fields of everyday life such as food production, textile industries, paper production, and agricultural practices (Cserhati, 2006). Nowadays customers demand natural products as a consequence of proven toxicological effects of some synthetic compounds (Sowbhagya and Chitra, 2010). Pigments isolated from microorganisms have increased interest in recent years, such as molecules belonging to the polyisoprenoid group (i.e. β -carotene, astaxanthin, and canthaxanthin) (Dufosse, 2005). β -carotene and astaxanthin (a xanthophyll) are produced by many kinds of bacteria, and are essential in maintaining the yellow color of the retinal macula, giving it the ability to act as sunblock on certain parts of the retina (Venil et al, 2009). The carotenoids are widespread amongst bacteria and undoubtedly play an important role in protecting them from the damaging effects of light and, in an aerobic environment, the oxidative damage from activated forms of oxygen. Carotenoids may also play a role as light-gathering pigments in photosynthetic bacteria (Moss, 2002).

Pigments produced by cold-loving microorganisms can be used instead of synthetic ones as alternative safe and natural colors in food industry (ice cream, candies, and other colored food products and pharmaceuticals (Kapoor, 2006). Some species of the psychrotolerant genus *Kocuria*, such as *Kocuria carniphila* (yellow) and *Kocuria rosea* (red), produce

yellow or pink colonies when grown on solid media (Reddy et al, 2003; Yong et al, 2009).

Kocuria have been isolated from human skin, animal and dairy products, and beer. They are found in many other places in the environment, including water, dust, and soil. *Kocuria* can grow well in environments with little water or high salt concentrations (Kloos et al, 1974; Kocur, 1986; Kovacs et al, 1999).

Carotenoids are classified as follows: Carotenoid hydrocarbons are known as carotenes and contain specific end groups, oxygenated carotenoids are known as xanthophylls. Examples of these compounds are a) zeaxanthin and lutein (hydroxy), b) spirilloxanthin (methoxy), c) echinenone (oxo), and d) antheraxanthin (epoxy) (Sergio et al, 1999). Carotenoids are required in animals as a source of pro-vitamin A (or retinol), even though they cannot produce them, but must obtain them through their diet. Plants primarily use carotenoids as photosynthetic accessory, pigments associated with chlorophyll in the membrane. They act to regulate the flow of energy in the photosynthetic system by either contributing energy for use in photosynthesis through light absorption, or by removing excess energy from the system to modulate energy flow by quenching singlet oxygen (photo protection). Carotenoids can also provide photo protection in the animals that consume them, such as found with lutein in the human eye, where it serves to protect it against UV damage (De Carvalho et al, 2008).

The studies were investigated the supplemental effect of β -carotene on antioxidant capacities in plasma, liver and longissimusdorsi muscle (He et al, 2010; Vardi et al, 2010). The most prominent carotenoids used by humans are alpha-carotene, lycopene, lutein, β -cryptoxanthine, and especially β -carotene. In both plants and animals, carotenoids can be broken down into functionally important molecules, including apocarotenoids in plants that function as hormones, pigments, flavors, floral scents and defense compounds, and retinoid in animals that function as vitamins, visual pigments and signaling molecules (Sherma et al, 1992). Enzymatic oxidative cleavage of carotenoids is also found in bacteria and plants (Johannes and Klaus, 1999). *K. polaris* produces red-orange pigments when cultured on laboratory media, while *K. carniphila* produces yellow pigments (Reddy et al, 2003). Pigments produced by cold-loving microorganisms can be used instead of synthetic ones as alternative safe and natural colors in food industry (ice-cream, candies, and other colored food products) and pharmaceuticals (Kapoor, 2006). Enzymatic oxidative cleavage of carotenoids also found in bacteria and plants (Johannes and Klaus, 1999).

2. Materials and Methods.

Materials

Microorganisms

The two local psychrotolerant strains used in this study are *Kocuria polaris* MO and *Kocuria carniphila* MY with accession numbers JX485386 and JX485387, respectively. They were isolated and identified by El-Sharouny et al (2013) using 16S rDNA (Sambrook et al, 1989; Ausubel et al, 1999; Hall, 1999).

Media

Nutrient agar (Lenore et al, 1997) for maintenance of bacterial strains.

King's medium (King et al, 1954) for pigment enhancement.

Methods

Environmental conditions for maximum pigments production

Effect of temperature

To determine the optimum temperature for pigment production, portions of 50ml King's medium (pH 7) were inoculated with 1 ml of 65 CFU/ml (OD = 0.5) of each bacterial strain and incubated at different temperatures (4°C to 36°C). Finally, total carotenoids were measured. Growth was also monitored at different time intervals.

Effect of pH

The effect of different starting pH values on the pigment production of isolated *Kocuria* spp. was investigated after 7 days incubation. Aliquots of the King's medium were initially adjusted to different pH values ranging from (4-11). Both *Kocuria* spp. were incubated at the optimum temperatures achieved from the previous investigation.

Effect of light

The effect of light intensity on the pigment production was investigated after 7 days incubation once in light and other in darkness. Aliquots of the King's medium were initially adjusted to the optimum pH value deducted from previous step and incubated at the optimum temperatures too.

Effect of aeration

The effect of aeration was investigated after 7 days incubation once shaken and other static at optimum growth conditions deducted from the previous investigations.

Extraction of pigments

Extraction of pigments from bacteria was done according to methods used by Medicharla et al (1991) and Lorenz (2001). The two bacterial isolates *K. carniphila* MY and *K. polaris* MO were cultured by inoculation of 1 ml of 65 CFU/ml (OD = 0.5) in 50 ml in King's broth for 7 days and incubated at 10°C and at pH 7 at static conditions in dark. Cells were then centrifuged at 1300 rpm for 15 min, and then washed with distilled water. Pigment in fresh bacterial samples was extracted by adding 15 ml acetone and 15 ml of 10% NaCl solution and shaken for 2 min, left in the dark over night to sediment the solids then centrifuged at 2500 rpm for 10 min. The extract was washed 3 times with 200 ml of distilled water, then add anhydrous sodium sulfate (~15 g), and then transferred to separation funnel with petroleum ether. Petroleum ether combined with pigment extract was filtered through glass wool, then concentrated by rotary evaporator and used in present work.

Quantitative estimation of total carotenoids

Approximate total carotenoids detection was performed as follows: total carotenoids produced intracellularly in the cells of two isolates, and previously extracted, measured in spectrophotometer at wave length with maximum absorbency (approx. 471-477nm) (Lorenz, 2001). The maximum absorbency against an acetone blank on the spectrophotometer was recorded. The total carotenoids were expressed as the sum of β -carotene and other carotenoids. Concentrations were determined by following equation:

$$\text{Total carotenoids (\%)} = \frac{\text{Abs max X (Volume X Dilution)}_{\text{acetone}}}{250(\text{Specific absorption of total carotenoids}) \text{ X Sample wt (mg)}} \text{ X 100}$$

Identification by TLC

A line in the bottom of (TLC) plate coated with neutral alumina was drawn. The pigment extracted previously was applied as a drop on this line. The plate was allowed to stand in TLC chamber containing petroleum ether. TLC plate was allowed to develop for approx. 30 min then removed from TLC chamber and quickly marked the solvent front and center of the carotenoid spots with a pencil. Retention factor, Rf value, is used to characterize and compare components of various samples (Yuangsoi et al, 2008).

$$R_f = \frac{\text{Distance travelled by the pigment}}{\text{Distance travelled by the solvent}}$$

Column Chromatography

A micro-chromatography column prepared using silica gel. About 5 ml of petroleum ether runs through the column, a few drops of the pigment solution were added and left to run through the column, elution done with solvent or solvent mixture (3-5% acetone + petroleum ether at the end add acetone + methanol). Compounds should move as small horizontal bands. On reaching the bottom of the column, each band collected in a small test tube. Fractions dried individually in rotary evaporator and resuspended in acetone (Grung et al, 1992). Separated pigments were examined in spectrophotometer at wave length 486 nm for β -carotene, 474 nm for xanthophylls and 466 nm for echinenone. Standards used for identification were prepared in acetone (4 μg /ml) and results were recorded according to following equation :

$$\text{Pigment mg/g} = \frac{\text{Abs max} \times V \times 1000}{a \times W}$$

Where A is the absorbance, V is the volume of the solvent, W is the dry weight of bacterial cells in grams, and a is the specific absorbance coefficient of carotenoid.

3. Results and Discussion

Environmental conditions for maximum pigment production

King et al (1954) found that King's medium enhances pigment production. On the other hand, any change in the concentration of the medium ingredients was found to have no effect on pigmentation but only on bacterial growth. This proves that these carotenoids are secreted as secondary metabolites, regardless of the level of medium contents. They advised to use glycerol, 10, peptone, 20, K_2HPO_4 , 1.5 and of MgSO_4 , 1.5 for better growth. Medicharla et al (1991) used medium containing peptone (0.5%), yeast extract (0.2%), and soil extract (5%) for the isolation of the

psychrophilic pigmented *Micrococcus roseus* strain (*Kocuria polaris*) (Fig.1a and 1b). The pigment was hardly shown to appear on the fifth day of incubation and reaches its maximum intensity at the seventh one.



Fig.1a. *Kocuria carniphila* MY isolate cultivated on King's agar at temperature of 10°C, pH of 7 and for 7 days.

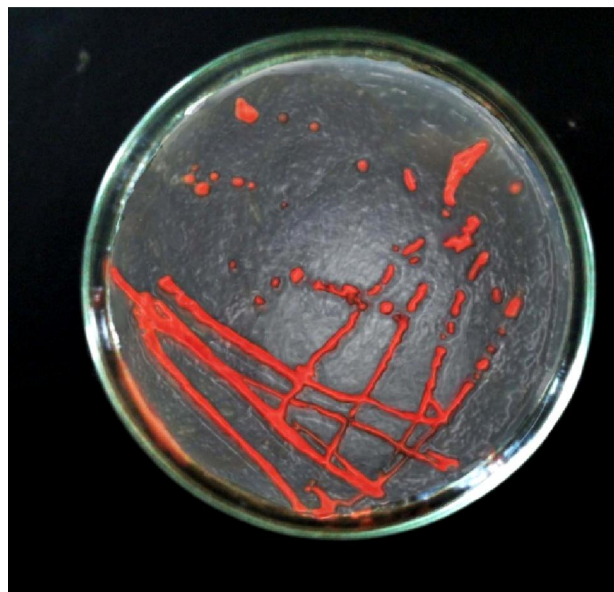


Fig.1.b. *Kocuria polaris* MO isolate cultivated on King's agar at temperature of 10°C, pH of 7 and for 7 days.

Effect of temperature, pH, light and aeration on pigmentation

The incubation conditions which influenced the pigment production by bacteria were studied. The evaluation of effect of incubation temperature on pigmentation production revealed that at low temperatures (4°C and 10°C) both bacterial isolates were found to produce large amount of pigments which was seen to be reduced by increasing temperature till it

was completely vanished above 30°C (Fig.2). No significant difference noticed between pigment production at 4 and 10°C but only bacterial growth was more at the later. This was reasonable to choose 10°C for more investigations. When the bacterial cells are shifted to temperatures below their ambient temperature, they respond by increasing the proportion of unsaturated fatty acids in the membrane, thereby

increasing the fluidity of membranes by increasing the disorder in the lipid bilayer. Such fluidity changes occur only at suboptimum temperatures, not at temperatures near the optimum, at which a more rigid membrane is probably required. Hence, carotenoids, by their ability to rigidify membranes, may have an important role to play at temperatures near the optimum (Medicharla et al, 1991).

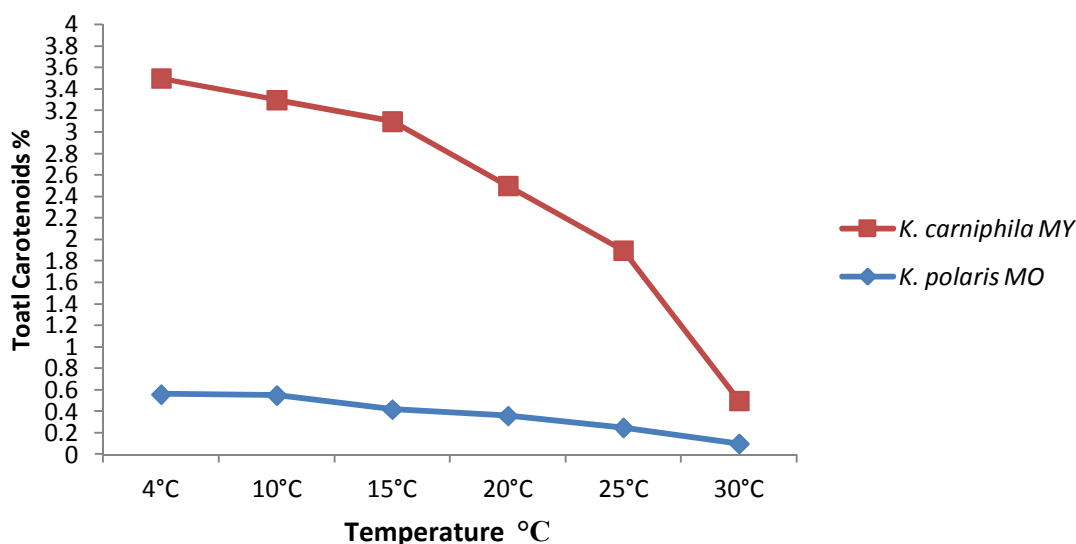


Fig.2. Effect of different temperatures on total carotenoids produced by *K. carniphila* MY and *K. polaris* MO.

On the other hand, neutral pH values were optimum for pigment production in both strains with gradual decrease as one moves towards either acidic or alkaline values (Fig. 3) (AbuSaraa et al, 2011).

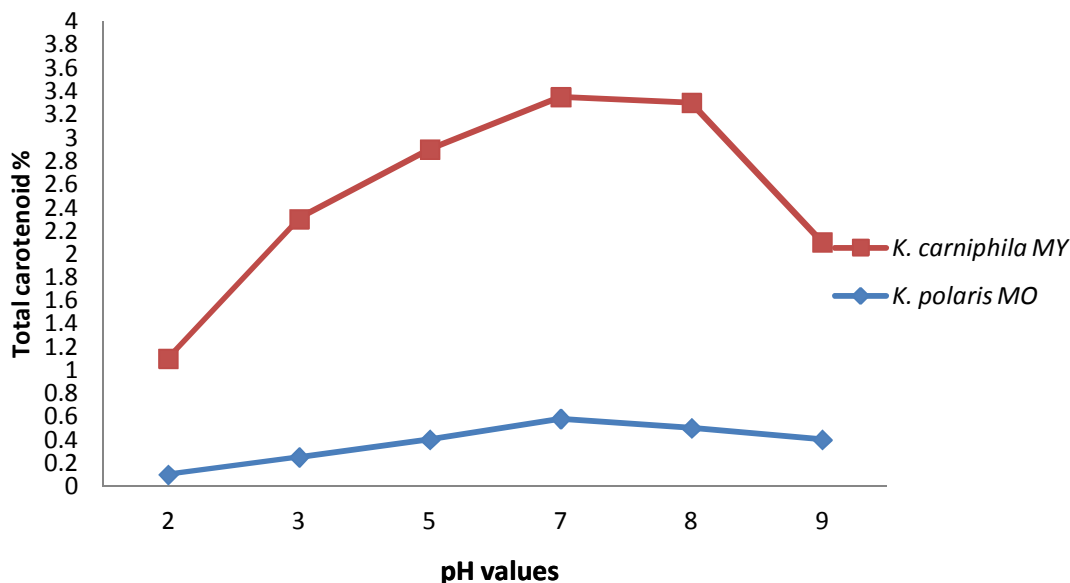


Fig 3. Effect of different pH values on total carotenoids produced by *K. carniphila* MY and *K. polaris* MO.

Light was found to suppress pigment production in both *Kocuria* spp. The maximum productivity was achieved in complete darkness (Fig. 4). However, these results appear to be contradicted with those of AbuSaraa et al (2011), who mentioned that the light was useful for both growth and β -carotene production in the strains of *Dunaliella* (micro-algae). They

reported that light intensity is the major inducing factor for β -carotene production, which is highly effective in protecting *Dunaliella* cells against photoinhibition due to the ability of β -carotene to quench damaging singlet oxygen and hydroxyl radicals (Ben-Amotz et al, 1989; Prescott et al, 2005).

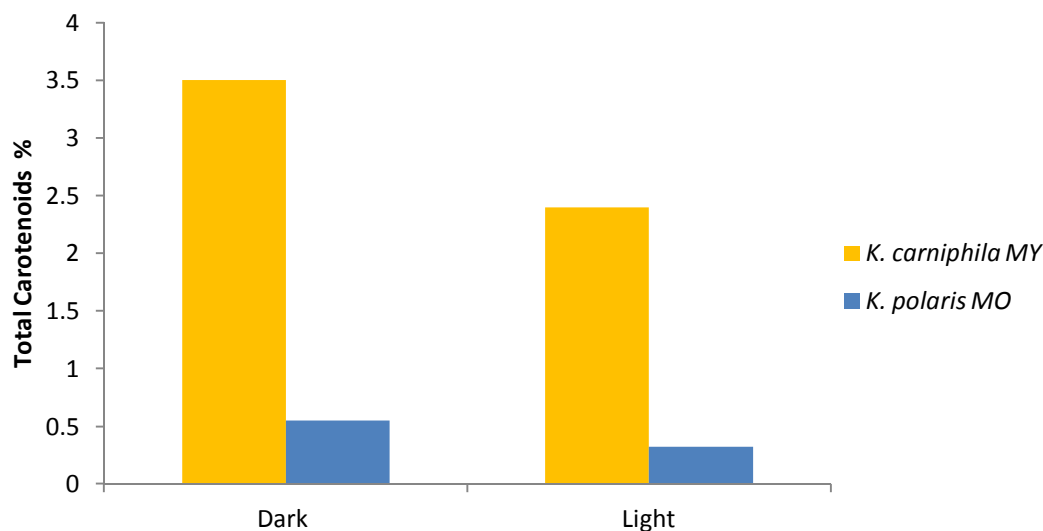


Fig 4. Effect of light intensity on total carotenoids produced by *K. carniphila* MY and *K. polaris* MO.

Results reported by Medicharla et al (1991) stated that studies have indicated that carotenoids in *Micrococcus roseus* (*K.polaris*) do not protect the bacterium against photodynamic killing. They also added that cells of *M.roseus* in which carotenoids biosynthesis was stopped with diphenylamine showed very small amounts of carotenoids but exhibited greater resistance to photodynamic killing than did the pigmented wild-type *M.roseus*.

No significant difference was shown on pigmentation after incubating cells once static, as applied in the previous experiments, or shaken (Data are not shown). This could be considered convenient data concentered with the fact that carotenoids produced as a secondary metabolite from both locally isolated *Kocuria* spp. which are in the first place aerobic bacteria. Shatila et al (2013) extracted carotenoids from an orange pigmented strain of *Exiguobacterium aurantiacum* FH which was isolated from air in Lebanon. They found that pigments were highly produced after 2 days of incubation at 30°C in shaken conditions. They also stated that aeration enhanced carotenoids production by the isolate.

Estimation of total carotenoids

Total carotenoids were estimated after each experiment and finally after applying the optima levels of temperature, pH, light intensity and aeration.

Approximate total carotenoids for pigments extracted from *K.carniphila* MY represented 3.32% of the bacterial weight, while for pigment extracted from *K.polaris* MO were 0.526% of the bacterial weight.

K. carniphila MY and *K. Polaris* MO were proved to produce carotenoids (Reddy et al, 2003; Savini et al, 2010) (Fig. 5a and 5b)(Fig. 6a and 6b).

Bacterial carotenoids identification by TLC

For *K.carniphila* MY two bands were eluted together with petroleum ether. The golden yellow band was eluted with 3 to 5% acetone in petroleum ether. The orange band was developed with 1 to 2% acetone in petroleum ether. The colored zones were resolved and result recorded: a golden yellow pigment, Rf 0.93 and a pale yellow pigment, Rf 0.763 (Contain β -carotene and xanthophylls) (Fig.5a)

For *K.polaris* MO two bands were eluted together with petroleum ether. The golden yellow band was eluted with 3 to 5% acetone in petroleum ether. The red band was eluted by acetone with methanol (1:1). The

colored zones were resolved and result was recorded: a golden yellow pigment, Rf 0.92 and a pink pigment, Rf 0.17 (Contain β -carotene and echinenone) (Fig. 5b).

Column Chromatography

Total pigments extracts when applied to column chromatography was separated into β -carotene, echinenone and xanthophylls. *K.carniphila* MY was found to contain lower amount of β -carotene (0.127 mg/g) (Yellow band) than *K.polaris* MO (0.308 mg/g). Whereas, a considerable amount of xanthophylls (Golden yellow band) was found in *K.carniphila* MY (4.073 mg/g), which was completely lacked in *K.polaris* MO. On the other hand, a small amount of echinenone (Red band) was found in *K.polaris* MO (0.2 mg/g), while *K.carniphila* MY lacked the presence of echinenone (Table 1).

Reddy et al (2003) found that *Kocuria* produces natural pigments, which can be used instead of synthetic ones as alternative safe and natural colors in food industry and pharmaceuticals. Natural pigments could be highly produced on growing the bacteria on different low cost raw materials. They also stated that *Kocuria* spp. was found to produce β -carotene as natural pigment in addition to other types of pigments.

Table 1. Total amounts of carotenoids extracted from both *Kocuria* spp.

Bacterial isolate	β -Carotene	mg/g dry wt	
		Xanthophylls	Echinenone
<i>K.carniphila</i> MY	0.127	4.073	0
<i>K.polaris</i> MO	0.308	0	0.200

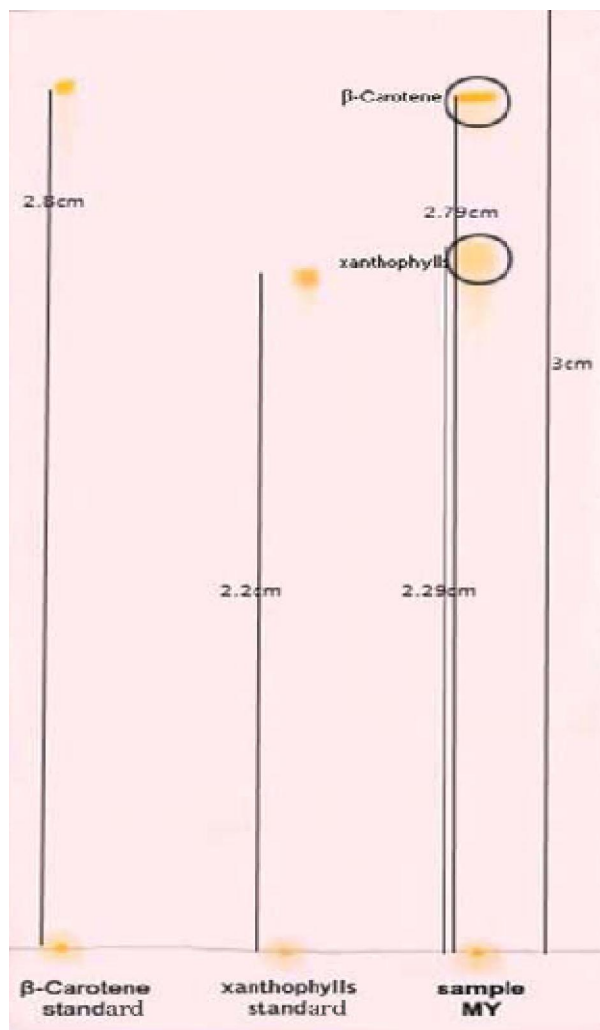


Fig.5a. TLC for pigments extracted from *K. carniphila* MY

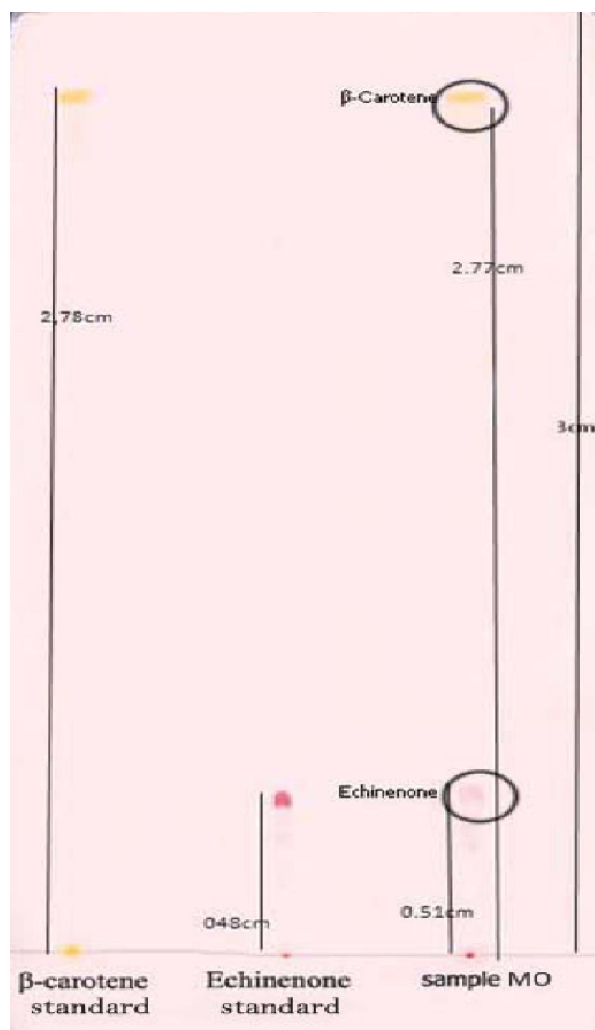


Fig.5b. TLC for pigments extracted from *K.polaris* MO.



Fig.6a. Yellow pigment extracted from *K. carniphila* MY by acetone.



Fig.6b. Orange pigment extracted from *K. polaris* MO by acetone.

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