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# A NOVEL SYSTEM FOR BIO – INCORPORATION OF [ 1-$^{14}$C ]ISOPENTENYL PYROPHOSPHATE INTO C $_{40}$ CHAIN CAROTENOIDS USING A CRUDE CELL-LYSATE OF *MICROCOCCUS LUTEUS*

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## ABSTRACT

The early steps up to the formation of acyclic C-$_{40}$ carotenoids are presumed to be common to the biosynthesis of all carotenoids with 40 or more carbon atoms (long chain carotenoids). No direct evidence so far available to confirm the early steps for the formation of the long – chain $C_{45}$ and $C_{50}$ carotenoids and their glycosides, which simply means that at least, the earlier steps of their biosynthesis are still poorly understood. In the present study, a novel biological system was developed capable of incorporation of [1-$^{14}$C]-isopentenyl pyrophosphate into the carotenoids of *Micrococcus luteus*. The results revealed that the novel long – chain carotenoids are sharing the same precursor with the conventional $C_{40}$-carotenoids. Moreover, the unique bio- radiolabeling developed in this study may be invaluable for labeling the individual carotenoids and related compounds as alternative to the complicated, time consuming chemical synthesis.

**Keywords**: *Micrococcus luteus*, carotenoids , bio-radiolabeling using {1-$^{14}$C} IPP.

## INTRODUCTION

Carotenoids represent a class of diverse compounds naturally present in both photosynthetic and non-photosynthetic bacteria, fungi, algae and all green plants. They serve a variety of functions such as antioxidants which impart anticancer properties to some carotenoids such as lycopene (Kavanaugh *et al*., 2007), colorants (Berry *et al*., 1971; Al-Wandawi *et al*., 1985), disease prevention in humans (Mayne, 1996) and precursors for many bioactive molecules such as vitamins, enzymes, hormones etc. Chemically, carotenoids are subfamily of the isoprenoids and are among the widest spread natural pigments (Umeno *et al*., 2005). The vast majority of about more than 750 known natural carotenoids arise as a result of different types and levels of modification of the $C_{-40}$ backbone catalyzed by promiscuous downstream carotenoid biosynthetic enzymes (desaturases, oxygenases, hydroxylases, cyclases, etc (Britton, 1998). On the other hand, bacteria such as *Corynebacterium*, *Halobacterium*, *Arthrobacter glacialis* and *Micrococcus* species are known to accumulate $C_{50}$–carotenoids, sometime as a major carotenoid pole with a very small amount of C-$_{40}$ carotenoids , sometimes only lycopene as in *Micrococcus luteus where lycopene amounts*( 1.35 %) of the total carotenoids (Al-Wandawi, 2000). These longer- chain structures are presumed to be synthesized starting from the $C_{40}$– structure by addition of two $C_5$ units. With the exception of very few number of studies e.g., Krubasik *et al*. (2001), Umeno and Arnold (2004), Das *et al*. (2007) and Netzer *et al*. (2010) which almost exclusively based on genetically engineered and cloning techniques in microbial systems, no direct evidence so far available to support this assumption. However, tetraterpenoid carotenoids (8 isoprene units representing their backbone) are formed like other terpenoids from the universal biological isoprenoid precursor, namely, isopentenyl pyrophosphate (IPP; C $_5$). The isomerization of (IPP) to dimethylallylpyrophosphate (DMAPP) , takes place through condensation of these two $C_5$ molecules , namely, (IPP and DMAPP) to yields geranyl pyrophosphate ( GPP;C-10 ). The subsequent addition of a $C_5$ (e.g., IPP) unit results in the formation of geranylgeranyl. pyrophosphate (GGPP;$C_{20}$) The common $C_{40}$ carotenes are formed by the dimerization of "GGPP" ($C_{20}$) to yield $C_{40}$ carotenoids. On the other hand, carotenoids with isoprenoid skeleton (backbone) larger than 40-carbon atoms (homocarotenoids) were found fairly recently in comparison with the very well recognized $C_{40}$- carotenoids such as, lycopene, β-carotene, and lutein for example , the first naturally occurring ($C_{50}$-carotenoids) to be encountered was "decaprenoxanthin" isolated from light - grown culture of a Gram positive, aerobic, non-photosynthetic bacterium; *Flavobacterium dehydrogenans* Arnaudi (Liaaen–Jensen *et al*., 1967). It has also been isolated from psychrophilic bacterium, *Arthrobacter glacialis* (Arpin *et aI*., 1975). In the present study, a novel bio-incorporating system using crude cell –

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lysate of *Micrococcus luteus* and [1-$^{14}$C] IPP was developed to see if these long - chain carotenoids are sharing the same precursor with the conventional $C_{40}$-carotenoids.

## MATERIALS AND METHODS

### Organism

The bacterium used in this study, was previously known as *Sarcina flava* (Thirkell and Hunter, 1969) and or *Sarcina lutea* (Arpin *et al*., 1975), but in more recent literature both of these organisms were considered one organism and re-named as *Micrococcus luteus* (Schleifer, 1981). The organism is a Gram–positive, strict aerobe, non-photosynthetic, chrome–yellow pigmented cocci, a common inhabitant of soil and fresh, and the carotenoids are located within the cell membrane.

### Preliminary experiments

A series of preliminary experiments were conducted to explore the influence of some culture conditions including the effects of static, static–shake, and ordinary shake culture, light, and nicotine (7 mM ) and diphenylamine (DPA) in the range of (0–25µg per ml culture medium on the biomass yield and carotenogenesis in *Micrococcus luteus* prior to the incorporation study to be undertaken.

### Culture and growth conditions

The cells of *Micrococcus luteus* were grown to exponential phase of growth at 30$^{O}$C with continuous agitation (160 rev/min and illumination (3423 lux) in tripticase soy – broth (Difco) supplemented per liter with dextrose (Oxoid),1 g; sodium dihydrogen orthophosphate, 1.6g disodium hydrogen orthophosphate (anhydrous), 0.9 g. Nicotine was added as ethanolic solution (5ml per liter culture medium) and diphenylamine (DPA) was added in an amount equals to 20 ug per ml culture medium .The growth was allowed to proceed for about 36 hr, with continuous agitation (160 rev /min) and illumination (3423 lux). The cells were then harvested at logarithmic phase of growth (as has been indicated absorptiometrically ($E_{600}$) where was found to exert minimum effect on the biomass measurement. The cell pellet thus obtained was washed once with 0.1 mole phosphate buffer (pH 7) and twice in distilled water, both at 1:10 (w/v) and the cells were recovered by centrifugation, and wet weight was recorded prior to lysis. A series of preliminary experiments were conducted to investigate the influence of the chosen concentration of diphenylamine (DPA) on growth of *Micrococcus luteus* .The other experiment was conducted to find out an efficient method to obtain a biologically active cell – lysate for incorporation. It was found that the various conventional methods such as French-pressure cell, ultrasound, grinding with abrasive materials, glass beads etc, failed to breakdown the bacterial cells. Therefore, enzymatic lysis of the cells using lysozyme (muramidase) was tried and found to be very efficient method.

### Preparation of crude cell- lysate

Lysis was achieved by suspending the bacterial cell–pellet in 0.9 % sodium chloride solution, lysozyme was added to a final concentration of 1 mg lysozyme per gram bacterial wet weight. Lysis was allowed to proceed with continuous agitation (140 rev /min), for 90 min.

### Extraction of carotenoids for use as a carrier

As a standard procedure, carotenoids were extracted from the lysed cells with acetone (10 volumes per packed cell – pellet using ultra Turax homogenizer and was then centrifuged at 20,000 x g for 20 min at 2-4$^{O}$C in an automatic refrigerated Sorval centrifuge). The supernatant was recovered and the residue was re-extracted once as above with fresh volume of acetone and centrifuged. The combined acetone extracts were concentrated to about one fourth of the original volume on a rotary evaporator at 35$^{o}$C under reduced pressure. The pigments (almost carotenoids ) were transferred to peroxide – free diethyl ether in a separating funnel by adding diethyl ether ; acetone and 2% (w/v )aqueous solution to a final ratio of 10:10:8 (by volume), respectively. The contents of the funnel were shaken gently (vigorous shaking might have resulted in emulsion, which if happens can be treated by adding few crystals of sodium chloride or few drops of ethanol). The funnel was then allowed to stand (in dark) for equilibrium and phase separation to be achieved. The epiphase (diethyl ether layer) was recovered, while the hypophase (mixture of acetone and water which may contain may very small amount of the pigments), was re-extracted twice with fresh volumes of diethyl ether. The combined ether extracts were then freed from acetone by washing two times with equal volumes of 2% NaCl solution. Because of the very polar nature of the some carotenoids of this bacterium, the ether extract thus obtained, was concentrated under reduced pressure at 35$^{o}$C rather than using conventional drying salts, such as anhydrous sodium sulphate which cause significant loss of the pigments. Sometimes, azeotropic distillation with benzene (Liaaen–Jensen, 1971) was also found to be necessary.

### Saponification

The total carotenoids obtained from 4 liters of growth was dissolved in 20 ml diethyl ether (peroxide–free) and a 20 ml portion of 10% methanolic–KOH was added. saponification was allowed to proceed in an atmosphere of pure nitrogen gas for 2–3 hours ( gentle stirring), followed by overnight storage at 4$^{o}$C in a cold room (Al-Wandawi, 2000).

### Preparation of unsaponifiable fraction

The total unsapoifiable fraction (TUF) was separated into two sub–fractions by dissolving it in 50 ml of 95% methanol and transferring it to a 250 ml capacity separating funnel. The flask which was used for the storage of the TUF was rinsed with 50 ml of light

petroleum (b.p.40–60$^{O}$C) and was added to the same funnel. The combined contents were shaken gently (to avoid emulsion formation) and left to stand until phase separation.The epiphase was recovered and the hypophase was re-extracted twice with fresh volumes of light petroleum. The bulked light petroleum was washed free of methanol with several equal volumes of 2% NaCI solution, and dried (as above) to yield the total epiphasic fraction (TEF).The total hypophasic fraction was transferred to a separating funnel, followed by addition of 2 volumes of 2% NaCI solution and diethyl ether. The ether phase was retained. The remaining hypophase re-extracted once with fresh volume of diethyl ether. The hypophase was then discarded. The ether extracts were combined, washed twice with equal volumes of 2% NaCI solution and evaporated to dryness (as above) to yield the total hypophasic fraction (THF) to mention that this procedure was developed to ensure the recovery of all carotenoids with various degree of polarity.

**Identification and Quantitation of Carotenoids:**

**Resolution of the TEF**

The TEF was dissolved in a minimum volume of light petroleum and chromatographed on an alumina grade-II column. The various fractions (3 -5 ml each) were eluted with 0–5% acetone in light petroleum (in an increasing order of polarity). Each fraction was examined spectroscopically (Davies, 1976; Britton,1995) . Impure fractions were combined and re-chromatographed. The polar carotenoids which remained on the column were eluted collectively wit1h 20% light petroleum in acetone and designated as total polar epiphasic pigments (TPEP).

The individual carotenoids were identified by their UV-; Visible spectra in appropriate solvents; adsorption characteristics; co-chromatography with authentic compounds using thin layer chromatography (TLC), mass spectrometry techniques. Quantitation of the carotenoids was mainly based of their absorption characteristics (Davies, 1976; Britton, 1995). Mass spectra of the various chromatographically pure carotenoids were determined on an (AEI-MS-30, single beam, double focusing mass spectrometer, interfaced with (DS-50 AEI; AEI) data acquisition system. Direct probe insertion technique, a probe temperature of 100–250$^{o}$C, an ionization potential of 24 or 70 eV a cage temperature of 150$^{O}$C, an ion current of 300 μA, and a pressure of $10^{-7}$ were considered the standard conditions for mass spectral analysis

**Radioactivity measurements**

The radioactivity of each portion (aliquot) eluted from the alumina column was assayed by liquid scintillation counter. The association of the radioactivity with the actual pigment zone was confirmed by, a. scanning a thin layer chromatogram using a Panax thin layer scanner, b. preparation of a radiogram and comparison of the position of the radioactive zone (in terms of $R_f$ value) with those of the pigments resolved on the thin layer; c. scanning the actual pigment zone and extraction with a suitable solvent or with mixture of solvents (based on the polarity of the

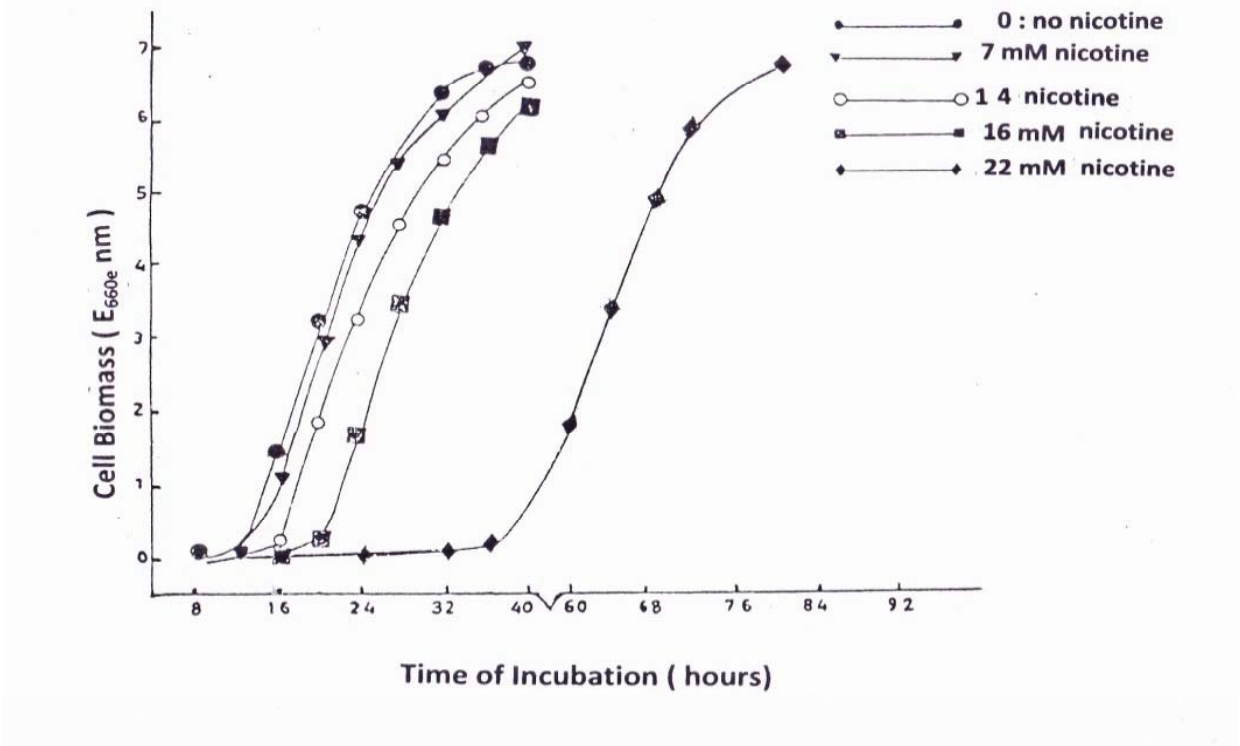


Fig. 1. Effect of different concentrations of nicotine on growth of *Micrococcus luteus.*

fraction extracted) followed by assaying on a scintillation counter.

**Incorporation of [1– $^{14}$C]IPP into carotenoids of *Micrococcus luteus***

The crude cell - lysate of *Micrococcus luteus* was prepared as mentioned above (used with no further purification) mixed with [1–$^{14}$C]IPP. The cofactors added were as follows; ATP (adenosine–5–triphosphate; disodium salt); NADPH (reduced nicotinamide adenine dinucleotide); and $MgCl_2$ in one ml of 0.1 mole phosphate buffer (pH7.0) to concentrations of 10, 1.2 and 1mM respectively. The incubation system thus prepared was flushed with high purity nitrogen gas, Stoppard tightly to create an anaerobic condition and allowed to proceed for one hour in an orbital shaker (140 rev/min) at 30$^{o}$C under continuous illumination (1238 lux). The stopper was then replaced by a cotton–wool plug and incubation continued for 8 hours. Carotenoid carrier (about 2 mg) consisted of mixture of carotenoids representing individual carotenoids of TC, TPEC, and TPHC obtained from culture of *Micrococcus* luteus grown in the presence of 7 mM nicotine was used as a carrier.

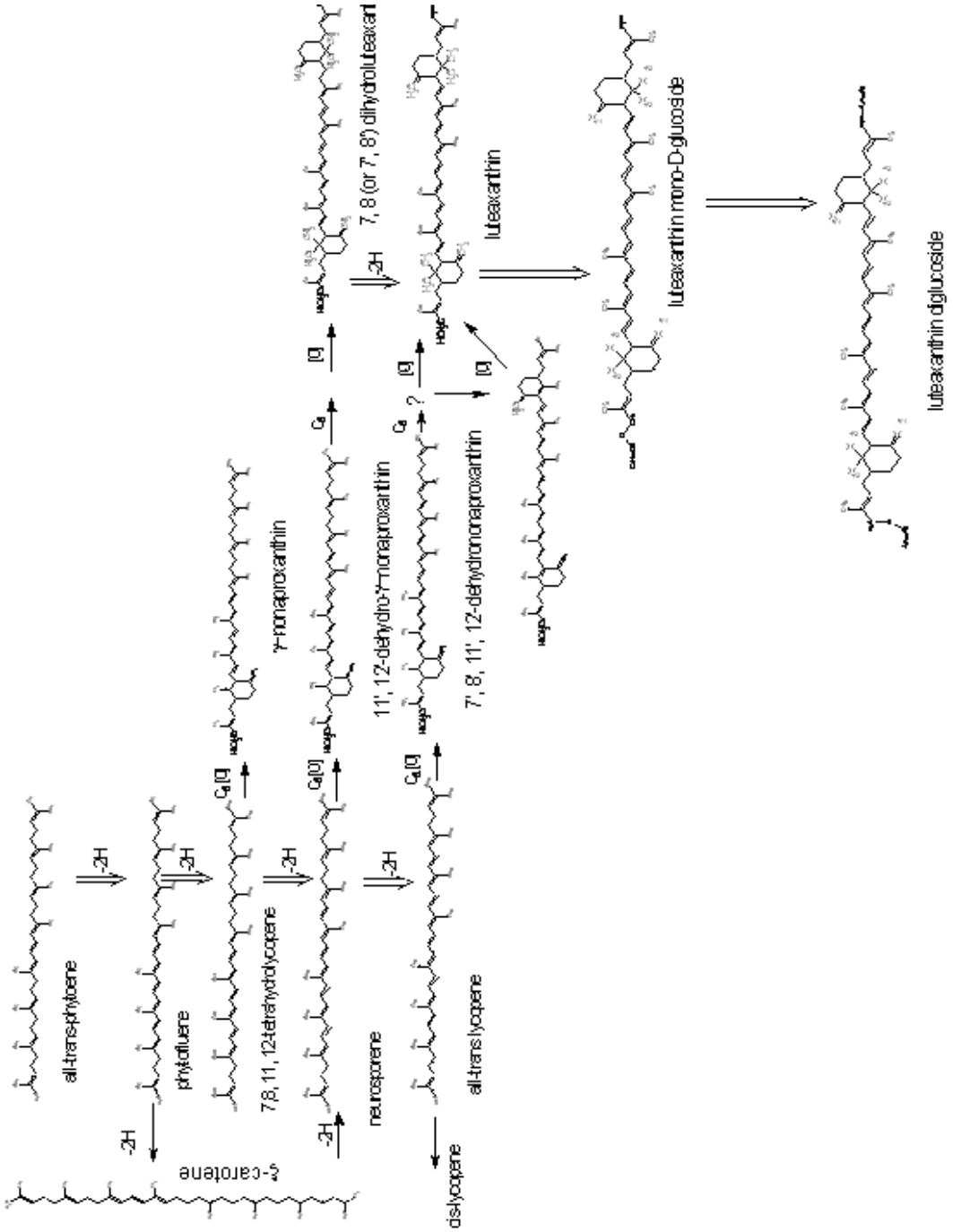


Fig. 2. Proposed biosynthetic pathway(s) for carotenoids in *Micrococcus luteus*.

## RESULTS AND DISCUSSION

All the novel long-chain carotenoids which have so far been discovered such as decaprinoxanthin; Sarcinaxanthin; have "branched isoprenoid skeleton consisting of 10 isoprene units". These carotenoids presumed to share a common biosynthetic origin as "conventional $C_{-40}$ carotenoids such as lycopene β–carotene or lutein as example. The extension of their carbon–backbone beyond the conventional 8 isoprene

Table 1. The of effect of static, static-shake, and normal shake culture on bio-mass at stationary phase of *Micrococcus luteus* cells grown in the presence of different concentrations of nicotine.

| Nicotine cocent. mM | Biomass (gm) wet, weight Per liter growth | | |
|---|---|---|---|
| | Time | Of | Incubation |
| | Static culture, 176 hr. | Static-shake, 176 hr. + 50hr* | Normal shake- culture, hr. |
| C | 2.51 | 40.00 | 30.10 |
| D | 3.75 | 42.60 | 36.40 |
| 7 | 1.96 | 32.04 | 30.00 |
| 12 | 2.71 | 45.20 | 36.30 |
| 24 | 0.90 | 40.30 | 31.40 |

C-nicotine (free growth medium), D=5 ml ethanol per liter growth medium. Nicotine was added as an ethanolic solution (5ml per liter growth medium) amongst the different treatments. *cells were grown for 176 hours as static culture, followed 50 hours as shake- culture.

Table 2. The influence of incubation period on carotenoids biosynthesis in *Micrococcus luteus*.

| Incubation period (hrs) | µg carotenoids | Per 10 gm | (wet.wt.) | Cells | |
|---|---|---|---|---|---|
| | TUP | TEC | TPEC | TPHC | Lycopene |
| 26 | 92.29 | 33.21 | 21.69 | 61.02 | 3.90 |
| 36 | 152.89 | 48.89 | 34.03 | 72.30 | 8.18 |
| 40 | 169.11 | 48.44 | 33.06 | 101.50 | 9.35 |

Table 3. The effect of different concentrations of diphenylamine (DPA) on growth pattern of *Micrococcus luteus*.

| µg DPA per ml culture medium | gm bacterial cells per liter growth |
|---|---|
| 0 | 15.30 |
| 5 | 17.00 |
| 1 0 | 16.30 |
| 15 | 15.35 |
| 20 | 15.40 |
| 25 | 14.40 |

Table 4. The effect of different concentrations of diphenylamine (DPA) on carotenoid biosynthesis in *Micrococcus luteus*.

| DPA/ml culture | Carotenoids Total of Percent | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | µg carotenoids/g bact. (wet wt.) | TC | TPEC | TPHC | Phytoene | Phytofluene | Zeta-Caro-tene- | Nuro-sporene | Lycopene |
| 0 | 15.50 | 1.35 | 57.34 | 41.32 | 0 | 0 | 0 | 0 | 1.35 |
| 5 | 24.92 | 1.32 | 63.27 | 35.41 | 0 | 0 | 0 | 0 | 1.32 |
| 10 | 28.84 | 44.36 | 39.31 | 16.34 | 23.99 | 9.32 | 5.73 | 3.03 | 2.28 |
| 15 | 41,69 | 84.48 | 10.64 | 4.88 | 70.97 | 8.06 | 2.45 | 0.86 | 0. 57 |
| 20 | 45.21 | 98.47 | 1.53 | 0 | 92.97 | 4.77 | 0.73 | 0 | 0 |
| 25 | 62.71 | 99.14 | 0.86 | 0 | 91.87 | 6.50 | 0.78 | 0 | 0 |

TC: total hydrocarbon (C-40) carotenoids, TPEC: total polar epiphasic carotenoids, TPHC: total polar hypophasic carotenoids

units is likely to be initiated by attack by electrophilic $C_5$ units which could arise from dimethylallyl pyrophosphate (DMAPP). If one excludes the alleged $C_{50}$-carotene; sarcinene, which has not been detected in this study and in most of the relevant studies, the substitution of the $C_{40}$ carotene at $C_2$ and or $C_2^{/}$with $C_5$ group is usually (but not always); e.g. "dehydroxydecaprenoxanthin, dehydroxy sarcinaxanthin" accompanied by the oxidation of the terminal methyl group to a primary alcohol. On the other hand, although long-chain carotenoids are presumed to share the early biosynthetic steps with $C_{40}$carbon atoms, but with exception of some few recent studies which mainly based on cloning and genetic engineering carotenogenic and noncarotenogenic microbial systems, no direct evidence so far available to confirm this. Prior to incorporation study to be under taken, a series of preliminary experiments were carried out to establish appropriate conditions for possible incorporation of [1-$^{14}$C] IPP into various carotenoid fractions including the selection of ideal culture medium composition (Table 1), suitable culture conditions and incubation period (Table 2). The preliminary experiments also involved the study of effect of different concentrations of diphenylamine (DPA) on the growth and carotenogenesis (Table 3 and Fig. 1). The results presented (Table 4) shows the effect of different concentrations of DPA on carotenoids biosynthesis, and a highest amount of phytoene was accumulated when DPA was present at a concentration of 20µg/ml culture medium. The data presented in (Table 5) show the absorption spectra of different carotenoid compounds obtained as a result of growing the cells of *Micrococcus luteus* in the presence of 7 mM nicotine to in order to be used as a carrier in the subsequent incorporation step. The data presented in (Table 6) reveal the levels of radioactivity imparted by [1–$^{14}$C]-isopentenyl pyrophosphate into in total hydrocarbon carotenoids (TC), total polar epiphasic carotenoids (TPEC), and total polar hypophasic carotenoids (TPHC).

The data presented in (Table 7) show the levels of radioactivity in individual hydrocarbon carotenoids (TC). The levels of radioactivity in the most polar carotenoids, namely, $C_{45}$, $C_{50}$ carotenoids and their glucosides are shown in (Table 8). From these results a number of conclusions may be derived. Thus, it seems that the growth culture medium and culture conditions (Tables 1, 2) was quite suitable such study as indicated by high biomass and carotenoid biosynthesis. The results presented in (Table 3, Fig. 1) show that the presence of DPA at a concentration of 20 µg per ml culture which was necessary for accumulation of high quantity of phytoene in particular which is in turn a precursor for all other

Table 5. Absorption spectra of acetone – extracted carotenoids of *Micrococcus luteus* grown in the presence of 7 mM nicotine. The individual carotenoids are arranged in order of increasing polarity.

| Carotenoid | Color | max in Hexane | Chemical –Formula |
|---|---|---|---|
| Phytoene | Colourless | 276 286 298 | $C_{40}$ $H_{64}$ |
| Phytofluene | Fluorescence | 331 347 366 | $C_{40}$ $H_{62}$ |
| *£-carotene :fr.A* | faint yellow | 354(infl) 376 397 422 | $C_{40}$ $H_{60}$ |
| *fr.B* | = = | 356(infl) 377 397 422 | |
| *fr.C* | = = | 358(infl) 378 399 422 | |
| *Neurosporene* | Yellow | 392(infl) 412 435 465 | $C_{40}$ $H_{58}$ |
| Lycopene: cis | orange-pink | 358(infl.) 438 464 495 | $C_{40}$ $H_{56}$ |
| all – trans | pink | 418(infl) 443 468 500 | |
| Nonaprenoxanthin | Yellow | 392(infl.) 412 435 465 | $C_{45}$ $H_{68}$ O |
| 11,12-hydro-Nonaprenoxanthin- | Yellow | 374(infl.) 351 371 392 | $C_{45}$ $H_{66}$ O |
| 7,8 (7/,8/)-dihydrosarcinaxanthin- | Yellow | 376(infl.) 396 419 447 | $C_{50}$ $H_{74}$ $O_2$ |
| Sarcinaxanthin | Yellow | 390(infl.) 413 437 467 | $C_{50}$ $H_{72}$ $O_2$ |
| Sarcinaxanthin –monoglucoside- | Yellow | 391(infl.) 413 436 466 | $C_{50}$ $H_{82}$ $O_7$ |
| Sarcinaxanthin-di-glucocide | Yellow | 394(infl.) 415 437 466 | $C_{50}$ $H_{92}$ $O_{12}$ |

Note: since there is currently no bacterium by the name *sarcina*, therefore, we recommend the name "luteusxanthin" may be more relevant.

Table 6. Incorporation by crude cell - lysate *of Micrococcus luteus* of [1– $^{14}$C ] IPP into various carotenoid fractions.

| Fraction | TC | TPEC | THP |
|---|---|---|---|
| Radioactivity (d.p.m)* | 1729256 | 278674 | 20898 |
| % radioactivity** | 85.23 | 13.74 | 1.03 |

Disintegration per minute * , ** % of total radioactivity (2028828)

carotenoids and necessary for downstream structural alteration by desaturases, oxygenases and cyclases enzymes, and offers reasonable biosynthetic conditions for incorporation of [1-$^{14}$C] IPP into the carotenoids including the $C_{40}$ and long-chain $C_{45}$ and $C_{50}$ carotenoids. In addition, DPA at 20µg/ml culture medium had no significant reduction effect on the biomass at the time of harvesting for carotenoids characterization as well. The data presented in (Table 4, Fig. 2) reveal that growing the cells in the presence of 7 mM in culture medium resulted in formation of individual carotenoids covering the whole spectrum including the individual carotenoids of TC, TPEC, and TPHC fractions without inducing significant reduction effect in biomass formation at the time of harvesting So these carotenoids were used as carrier for the subsequent incorporation studies .The data presented in (Table 5) show that the incorporation of radioactivity into the various fractions of carotenoids with different polarity including long- chain carotenoids gave an early indication that all these carotenoids are sharing the same precursor origin represented in this case by [1$^{14}$C ] IPP. This conclusion was supported further by the data presented in (Tables 6, 7, 8)

## CONCLUSION

In the present study a bio-radiolabeling system consisted of [1–$^{14}$C]-IPP and crude cell-lysate was developed and found to be capable to incorporate radioactivity into the structurally different carotenoids of *Micrococcus luteus* including the $C_{40}$-hydrocarbon carotenoids and the $C_{45}$-, $C_{50}$-carotenoids and their glucosides. This may simply indicate the long-chain polar carotenoids are same precursor with the conventional $C_{40}$-carotenoids, and they totally dependent for their formation on the structural alteration in phytoene through the activity of the enzymes, desaturases, oxygenases, and cyclases.

The early steps up to the formation of acyclic C-$_{40}$ carotenoids are presumed to be common to the biosynthesis of all carotenoids with 40 or more carbon atoms (long chain carotenoids). Nevertheless, if we exclude a few number of recently published papers which almost exclusively based on cloning and genetic engineering, no direct evidence so far available to confirm the early steps for the formation of the long–chain $C_{45}$ and $C_{50}$ carotenoids and their glucosides, which simply means that at least, the earlier steps of their biosynthesis are still poorly understood. In the present study, a novel system was developed whereby the cells of a non-photosynthetic, Gram–positive bacterium, namely, *Micrococcus luteus* were grown in the presence of 7 mM nicotine to affect the accumulation of the saturated carotenoids (if any?) from the early biosynthetic pathway leading and subsequent formation of the long-chain carotenoids in addition to the presence of significant amounts of long-chain carotenoids in order to be used as a carrier in the subsequent incorporation study. Diphenylamine (DPA) at a concentration of 20 µg per ml culture medium was used in an attempt to encourage the accumulation of phytoene in particular to ensure thes presence of some specific active enzymes such as, desaturases, hydroxylases, cyclases, etc., which are requisites for the downstream desaturation and structural alterations A crude cell-hydrolysate was then used to incorporate isopentenyl pyrophosphate [1-$^{14}$C]-IPP into the carotenoids of this

Table 7. Incorporation of [1–$^{14}$C] IPP into hydrocarbon carotenoids by crude cell lysate of *Micrococcus luteus* cells.

| Radioactivity | Phytoene | Phytofluene | £-Carotene | Neurosporene | Lycopene |
|---|---|---|---|---|---|
| d.p.m | 164925 | 1042482 | 174622 | 10017 | 247210 |
| % total radioactivity-A | 9.54 | 60.29 | 10.10 | 5.78 | 14.30 |
| % total radioactivity-B | 8.13 | 51.38 | 8.61 | 4.93 | 12.18 |

Radioactivity = disintegration per minute (d.p.m.), total radioactivity –A=total radioactivity incorporated into the C-$_{40}$–carotenes fraction, total radioactivity incorporated into total carotenoids.

Table 8. Incorporation of [1-$^{14}$C] IPP into the various carotenoids of total polar hypophasic carotenoids (TPHEC) of *Micrococcus luteus.*

| Fraction | Color | % acetone in petrol | $^{\lambda}$Max in hexane | Radioactivity (d.p.m.) | % of total radioactivity |
|---|---|---|---|---|---|
| C | Yellow | 1- 2 | 378 397 419 447 | 71769 | 41.57 |
| D | Yellow | 1- 2 | 392413 435 446 | 12436 | 7.20 |
| E | Yellow | 2 | 374 396 418 443 | 17398 | 10.08 |
| F | Yellow | 2 | 376 397 420 447 | 12975 | 7’52 |
| G | Yellow | 2 | 392 413 436466 | 9854 | 5.71 |
| H | Yellow | 10 | 334 351 371 392 | 33896 | 20.79 |
| I | Yellow | 10-20 | 394 416 441 466 | 12198 | 7.32 |

organism. The results clearly revealed that the novel long-chain carotenoids are sharing the same precursor with the conventional C40-carotenoids. Furthermore, the unique labeling developed in this study may be of value for radiolabelling of individual carotenoids and related compounds as alternative to the complicated, and time consuming chemical synthesis of carotenoids which is challenging and costly

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