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Research Paper

Carotenoid Biosynthesis in *Micrcoccus luteus* Grown in the Presence of Different Concentrations of Nicotine

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Abstract: *The results of the present study have shown that in Micrococcus luteus, light was not an absolute requirement for growth and carotenoid biosynthesis, while molecular oxygen was found to be an absolute requirement for both growth and carotenoid biosynthesis. On the other hand, at stationary phase of growth, the yield of the cells grown in the presence of (0 -24 mM) nicotine was not significantly affected when the cells approached the stationary phase, but concentrations of nicotine in excess of 12 mM caused an initial lag phase which was increased steadily in duration with the increase of nicotine concentration, while no significant differences in the growth –rate amongst treatments within the above range of nicotine concentrations could be seen once the lag-phase was over. It was also found that a complete suppression of the long-chain polar carotenoids was not achieved when the cells grown in the presence of above mentioned concentrations of nicotine. Nicotine did however, resulted in up to 50% inhibition of the long chain polar carotenoids on behalf of the C_{40} – hydrocarbon carotenoids (carotenes), namely, phytoene, phytofluene, £-carotene and neurosporene. Lycopene however, was an exception. Diphenylamine (DPA) at concentrations of 20 and 25µg / ml culture medium almost completely suppressed the formation of the polar long–chain carotenoids where they found to account to only 1.53 and 0.86 % for total polar epiphasic carotenoids (TPEC) and 0% for the total polar hypophasic carotenoids (TPHC) accompanied by very high increase in C-40 carotenoids level (phytoene in particular). Based on these results it may be suggested that there is a biosynthetic link between the $C\text{-}_{40}$ conventional hydrocarbon carotenoids (carotenes) and the long-chain polar carotenoids (xanthophylls). Lycopene was found not to be on the biosynthetic pathway which leads to the formation the polar long –chain carotenoids.*

Keywords: *Micrococcus luteus*, carotenoids, biosynthesis, effect of nicotine and culture conditions.

Introduction

The alkaloid, nicotine, has shown to be a potent and reversible inhibitor of cyclohexylidine ring formation in some biosynthetic sequences leading to ring initiation as incase of ß-carotene formation in mycobacterium marinum. (Howes, C.D. and P.P. Batra, 1970, Batra, P.P., Gleason, R.M. and J.W. Louda, 1973). Since then, the inhibitory effect of this compound has been investigated in several other carotenogenic systems (Kushwaha, S.C. and M. Kates, 1976, Ishikawa, E. and H. Abe, 2004). On the other hand, our previous study, (Al-Wandawi, H., 2000) has shown that the cells of *Micrococcus* luteus grown in nicotine-free growth media, form the acyclic hydrocarbon carotenoid, lycopene as the only C40 – carotenoids (1.35 %), while the major carotenoids pole consisted of polar long – chain carotenoids (98 %) containing one or two cyclohexylidine У- rings (Al- Wandawi, H., 1977, 2000). In general, with the exception of few studies (Krubasic, P., M. Kobayashi, and G. Sandman, 2001, Umeno, D.A. and F.H., Arnold, 2004, Umeno, D., A.V. Tobias and F.H., Arnold, 2005, Netzer, R. and H. Maret, 2010) which are almost exclusively based on cloning and genetically engineered microbial carotenogenic systems, no direct evidence so far available to show the presence of a relationship between the C40 - s carotenoids and the long-chain (C 45 and C50) carotenoids. The present study was conducted, as a part of a series of studies as an attempt to explore the biosynthetic pathway leading to the formation of these long-chain carotenoids.

Materials and Methods

Organism

Micrococcus luteus used in this study, was referred to in some earlier studies as *Sarcina flava* (Thirkell, D., and M.I.S. Hunter (1969) and / or *Sarcina lutea* (Mathews, M.M., and N.K. Krinsky (1970), but in a latter study (Schleifer, K.H., W.E. Kloos and M. Kocur, 1981) both *Sarcina flava* and *Sarcina lutea* were considered the same organism, and renamed as *Micrococcus luteus*, and this new name has been used since then (Al-Wandawi, H, 2000, Ding, L.L., Alexader, A., Lei, S. and Anders, J. 2010).

The organism is aerobic, non–photosynthetic, chrome - yellow pigmented, Gram-positive cocci and water and soil inhabitant.

The Basal Liquid Culture Medium

The basal liquid culture medium used for the routine cultivation of the organism (unless otherwise stated) contained the individual components of Trypticase soy broth (Difco) supplemented with yeast extract, biological grade dextrose (Oxoid) and phosphate. The final composition of the components in (g / liters) was as follows: Bacto - tryptone (Difco), 17.0, Bacto - soytone, 3.0, sodium chloride, 5.0 di-potassium hydrogen phosphate, 2.50 sodium di-hydrogen phosphate, 1.60, di-sodium hydrogen phosphate, 0.90, yeast extract, 1.0 and bacteriological grade dextrose (Oxoid), 2.5. The pH was very close to 7.0. Sterilization was achieved by autoclaving at 121^{o} C and 15 ppi (103 Kpa) for 15 min before use.

Static, Static –Shake and Shake Culture

In an attempt to establish a justifiable growth condition, a series of experiments were conducted including growing the cells under static, static-shake and shake - culture conditions. For this part of the study, cultures of the organism were grown at 30^{o}C in the presence of selected nicotine concentrations, namely, 0, 7, 12 and 24 mM using the basal liquid culture medium (1 liter growth medium in 2 liter capacity flask) , with continuous illumination at 6135 lumen per square meter. The shake - cultures were agitated on an orbital shaker at 160 rpm. The static - cultures were grown to stationary phase (176 hrs). The cells of each pair of flasks were harvested and the wet weight (g / l)

was determined, while the other flask was re-incubated under standard growth conditions (shake-culture conditions until stationary phase of growth was reached (50 hours). This treatment was designated as static - shake culture. The cells were then recovered by centrifugation and the wet weight of the bacterial biomass was determined .The results which were obtained under static and static- shake culture conditions were compared with those of cultures grown under normal shake-culture conditions.

The Effect of Light on Growth of *Micrococcus luteus*

The cells were grown aerobically for 36 hrs in the basal culture medium on an illuminated cooled orbital incubator, equipped with 6 quick - start fluorescent tubes with a total output (as measured at the bottom of the incubator) of 6135 lumen. Dark conditions were obtained by placing each flask in a light – proof bag and the dark–grown cultures were obtained by inoculating the culture in darkness from cell suspensions prepared in dark. Other culture conditions for both light and dark grown cultures were similar. The extent of growth was measured absorptiometrically (E_{660} nm) at different time intervals during the whole period of incubation.

Standard Culture Conditions and Stock Culture

Based on the results obtained from the above preliminary experiments, a standard growth conditions employed whereby a high biomass of the bacterium can be obtained from a relatively small volume of growth sufficient for the various parameters required in this study to be invested. Based on the results of this part of study, a standard culture conditions was developed, namely growing the cells at 30 ^{o}C with continuous illumination at 6135 lumen in a Galenkamp illuminated cooled orbital incubator fitted with 6 quick start florescent tubes and with continuous agitation at 160 rev/min. The ratio of culture volume to the flask containing the standard growth medium was 1: :2 (v /v), while the inocu - lum consisted of 2.5 bacterial colonies (previously grown on nutrient agar in a Petri-dish suspended in 2.5 for 3 days at 30^{o}C) in a bench incubator. The inoculates were added as homogenous cell suspension to a pair of flasks, each containing one liter of sterile basal culture medium. After 24 hrs, a few nutrient agar plates were inoculated using a loop full amount from such culture and incubated for 3 days at 30^{o}C and then stored at 4^{o}C and was used as stock culture, while all the flasks were re-incubated for additional 12 hrs, followed by harvesting the cells at 20,000 x g for 15 minutes in a Sorvall RC-2 B at 4 ^{o}C. The cell–pellet was washed once with 0.9% (w / v) sodium chloride solution and once with distilled water using 200 ml each per cell-pellet per liter culture volume . The wet weight was determined. Preliminary experiments showed that the organism can be maintained as such for several months, nevertheless, fresh cultures were made fortnightly.

Absorptiometry Method for Routine Monitoring of Bacterial Growth

For routine measuring of bacterial cells, an optical extinction (absorbance) method at a particular wavelength (660 nm) was used. The extent of growth at different time intervals during the whole incubation period which covered growth phases was determined in terms of extinction (E_{660}nm) using an EEL colorimeter (Evan Electro-Selenium Ltd., England) using filter No.608 (red, 660 nm).

Nicotine

Bacterial cells were grown under standard growth conditions. Nicotine was added as ethanolic solution (5 ml per liter growth medium) at the time of incubation to give a final range concentrations of 1-24 mM. The cell mass at different time intervals was measured spectrophotometrically as mentioned above.

Diphenylamine (DPA)

Is a crystalline pleasant – smelling compound used chiefly in the manufacture of dyes and as an indicator. One of its vital uses is in the detection of apoptosis. Chemically, it is an aromatic secondary amine containing two phenyl substituent and has a role as antioxidant, carotenogenesis inhibitor (phytoene dehydrogenase inhibitor).

Lysis of Bacterial Cells and Carotenoid Extraction

Preliminary experiments using different procedures revealed considerable difficulty to break the cells and extract carotenoids from this Gram-positive bacterium. Such difficulty may be attributed to the presence of teichuronic acid (TUA) in the cell wall of this organism (Ding, L.L., Alexander A., Lei S., and Anderson J. (2010). It worthy to mention that TUA is composed of a long – chain polysaccharide composed of disaccharide repeating unit. Therefore, lysis of the cells was tried and found to be very successful. This was achieved by suspending the bacterial cell-pellet in 2 volumes of saline solution. Lysozyme (muramidase) was added to a final concentration of one mg lysozyme per gram bacterial wet weight. Lysis was allowed to proceed for 90 min at 30^{o}C in an orbital shaker with continuous agitation (140 rev / min). A lysis curve was obtained by following the drop (E_{660} nm) as a result of lysis of the cells during the various time intervals throughout the whole lysis course. The carotenoids were then extracted from the lysed cells with redistilled acetone (10 volumes per packed cell pellet) using Ultra–Turax homogenizer. (UNKE & Konkel, Germany) for a period of 2 min (4 x 30 seconds with a pause of 30 seconds for cooling) The homogenate was cooled throughout the handling period on ice and was then centrifuged at 20,000 x G g for 20 min at 2 – 4^{o}C . The supernatant was recovered and the residue was re-extracted with fresh acetone (as above) and centrifuged the combined supernatant acetone –extracts were combined and concentrated to about one quarter of litter of their original volume on a rotary evaporator (Buchi Technik, AG, Switzerland) at 35^{o}C under reduced pressure. The pigments were then transferred to diethyl ether in a separating funnel by adding and 2% (w/v) aqueous sodium chloride solution to a final ratio of : diethyl ether : acetone : sodium chloride solution (10:10:8 (by vol.)). The contents of the funnel were shaken gently (vigorous shaking might have resulted in emulsion formation). The funnel was left to stand for equilibrium and phase separation to be achieved. The epiphase (diethyl ether layer) was recovered. While the hypophase (acetone – NaCl solution mixture) was re-extracted twice with fresh volumes of diethyl ether. The combined ether extracts were then freed from acetone by washing twice with equal volumes of 2% sodium chloride solution. Because of the very polar nature of most of the carotenoids extracted the ether extract was concentrated under reduced pressure at 35^{o}C and dried directly (vacuum pump) or by azeotropic distillation with benzene (Liaan-Jensen, 1971). Preliminary experiments showed that the traditional anhydrous sodium sulphate method when tried for drying causes significant loss of the carotenoids.

Saponification and Phase Separation of Carotenoids

In order to obtain total un-saponifiable pigments (carotenoids) the bacterial cell–lysates from four liters culture were dissolved in 20 ml diethyl ether (peroxide – free) and 20 ml of 10% (w /v) methanolic potassium hydroxide was added. Saponification was allowed to proceed in an inert atmosphere of pure nitrogen for 2-3 hrs. at room temperature in dark, followed by overnight storage at 4^{o}C in a cold room. Sodium chloride solution (40 ml, 2%) was added followed by 20 ml peroxide–free diethyl ether and 20 ml of freshly redistilled absolute ethanol. The mixture was gently shaken in a 250 ml capacity separating funnel and left to stand at room temperature until separation of two phases was achieved. The epiphase was kept in the dark while the hypophase was re-extracted twice with fresh volumes of diethyl ether. The ether extracts was then bulked and freed from alkali by washing 5 times with equal volumes of 2% sodium chloride solution and evaporated (azeotropically) at 30^{o}C to obtain the total dry unsaponifiable fraction (TUF, which is almost carotenoids).

Resolution of the Carotenoids

The total unsaponifiable fraction (TUF) was dissolved in 50 ml of 95% methanol and transferred into 250ml capacity separating funnel. The container which had been used for storing the unsaponifiable fraction was rinsed with 50 ml of light petroleum and the washing was added to the separating funnel and the combined contents were shaken gently rather than vigorously and left to stand at room temperature until two phases hah separated. The epiphase (light petrolium layer) was recovered and the methanol hypophase was re-extracted (2-3 times) with fresh volumes of light petroleum. The bulked light petroleum extract was washed free of methanol using (2-3 equal volumes) of 2% (w/v) of sodium chloride solution, evaporated and dried as above, dissolved in 10 ml of light petroleum, transferred to brown-air tight bottle, flushed with high purity nitrogen and designated as "total epiphasic carotenoids TEC" and kept in a refrigerator until required for analysis.

Separation of the Total Hypophasic Carotenoids Fraction (THC)

The total hypophasic fraction (THC) was recovered from methanolic layer in a separating funnel by the addition of 2 volumes each of 2% (w / v) sodium chloride solution and diethyl ether (epiphase) and the contents of the separating funnel were mixed well and left to stand until the ether phase was well separated from the methanolic water–solution (hypophase). The ether phase was retained and the hypophase was re-extracted once with equal volume of fresh diethyl ether. The hypophase was discarded and the ether extracts were combined and washed twice with equal volume each of 2% chloride solution and evaporated to dryness, stored in a brown container and high purity nitrogen and designated as total polar hypophasic carotenoids (TPHC).

Resolution of Carotenoids of Total Epiphasic Carotenoids

As a routine procedure, the total epiphasic fraction was dissolved in a minimum volume of light petroleum and chromatogrphed on alummina grade II column. The various fractions, 3-5 ml each are eluted using (0-5% acetone in light petroleum and examined spectroscopically. Based on the elution pattern and the absorptiometric characteristics of the individual carotenoids a preliminary identification of the carotenoids components of this fraction was achieved. Due to the polar nature of the remaining carotenoids of TEF), they were eluted collectively (as a single fraction) with 80% acetone in light petroleum and designated as total polar epiphasic carotenoids (TPEC) and stored under an inert atmosphere of high purity nitrogen, in dark in a cold room until required for analysis.

Quantitation Determination of Individual Carotenoids

As a routine procedure for quantitation of individual carotenoid, ultra violet and visible spectra of each compound were recorded on SP 800, SP 1800 and SP 2000 spectrophotometers (Pye Unicum, Cambridge, UK) from solutions in light petroleum, diethyl ether, acetone, ethanol, methanol, dichloromethane and benzene using 1 cm light path quartz cuuvate cells. The wavelength scale of the spectrometers was checked for each spectrum recorded characteristic lines (453.5, 418.4 360.9 nm etc.) of aholmium oxide filter. Fractions judged to impure by their $^{\lambda}$max values or degree of persistence were re-chromatographed. The quantitation of a mixture of carotenoid pigments or individual carotenoids with an unknown E value was obtained by measuring extinction of a known amount of their or its solution using the following equation:

$$x = \frac{E \,.\, Y}{E^{1\%}_{1cm} \,.\, 100} \,.\, 10^{6}$$

Where:

x = weight of carotenoid (s) in ug
E = the measured extinction of carotenoid (s) against the solvent
Y = volume of carotenoid (s) solution (ml)
E = the extinction coefficient of (1%) carotenoid in 1 cm light path quartz cuvette
Since the E values for most carotenoids in various solvents are available in literature (eg. Davies, 1976) and by using the above equation the amount of each carotenoid was quantitatively determined.

Mass Spectrometry

The mass spectra of the various chromatographically pure individual carotenoid fractions were determined on an "A.E.I MS-30 single beam, double focusing spectrometer interfaced with a DC 50 (A.E.I) DATA acquisition direct sample insertion technique was used. A probe temperature of 100-250 ^{o}C, an ionization potential of 24 or 70 ev, a cage temperature of 150 C, an ion current of 300 uA and a pressure of 10 $^{-7}$ tour comprised the standard condition throughout the protocol of the analysis.

Results and Discussion

The results of the effect of different concentrations of nicotine on *M. luteus* grown under normal shake-culture condition and under static, static–shake culture conditions are shown in (Table. 1). The results show that in spite of nicotine concentrations, the yield (biomass) estimated absorptiometrically (E_{660} nm, 6.7–6.8) after an incubation period of 36 hr was almost similar for all concentrations of nicotine including 12 mM, but nicotine concentration in excess of 12 mM caused an initial lag phase which was proportional with nicotine concentration in the culture media. On the other hand, results presented in (Fig. 2) suggest no significant differences in the rate of growth can be seen once the lag phase was over (e.g., during the logarithmic phase of growth) From the results presented in (Tables 1-3) it can be seen that molecular oxygen is an absolute requirement for growth (Table 1), while, light seems to be not essential for growth and carotenoid biosynthesis in this organism (Tables 2 & 3), nevertheless, since it has been reported (Harding and Shropshire, 1980, Bhosale, P., 2004) that light enhances and or, stimulates carotenogenesis ,therefore, illumination was included among within the standard growth conditions employed in this study. The results presented in (Table. 4) suggest that sufficient biomass can be obtained through an incubation period not exceeding 36 hours. The results presented in (Table. 5) show that the amount of the biomass was not affected when the cells were grown in the presence of 20 µg/ml culture medium, although cultures grown in the presence of 5 ml ethanol and 10µgDPA per liter growth have showed some increase in the biomass. Table 6 shows the effect of different concentrations of DPA on TC, TPEC, TPHC and on the profile of the newly emerged individual carotenes (other than lycopene). It can be seen that DPA at concentrations of 20 and 25ug/ml culture medium almost completely suppressed the formation of the TPEC and TPHC where they found to account to only 1.53 and 0, 86% and 0% of the total carotenoids, accompanied by the emergence of phytoene and phytofluene and to least extent other C40 - carotenoids. From the results shown in (Table 7) it may be concluded the highest levels of the total carotenoids (44, 40, 47.5 and 50, 30%) was obtained from total epiphasc fraction (TEF) in the cultures grown in the presence of 7, 1 and 12 mM nicotine.

The synthesis of total polar epiphasic carotenoids (TPEC) was only slightly inhibited with the exception of the treatment 12 mM nicotine, where the TPEC only accounted for (40.4%) of those of control. Formation of the total polar hypophasic carotenoids (TPHC) was significantly decreased in treatments with 7, 1 and 12 mM nicotine and accounted for 50.1, 52.7 and 59.7% respectively. An additional finding was that the presence of ethanol at a concentration equivalent to 0.5% in the culture media had stimulating effect on carotenes formation in general and lycopene in particular. Regarding the effect of different concentration of individual carotenoids, it can be seen that phytoene was the predominant carotenoids and constituted 36.5, 33.5 and 26.0% of the total carotenoids within treatments 1, 12 and 7 mM respectively. Phytofluene and £- carotene followed the same sequence as phytoene but their sequence of their was slightly altered. The highest value for neurosporene was

observed when the treatments with 7, 12, 0 and 2 mM nicotine. Lycopene however, showed the highest within the treatments 0 and 3 mM nicotine and within the control.

Conclusions

The results obtained as an outcome of this study, suggest that molecular oxygen is prerequisite for the growth of Micrococcus luteus, while light was not. The concentrations of nicotine in excess of 12 mM caused an increase in initial lag - phase which was found to be proportional with the increases of its concentration, while no significant differences with nicotine concentration on the rate of growth could be seen once the lag phase was over (e.g. during logarithmic phase of growth). The results of investigating the effect of different concentrations of nicotine on carotenoid biosynthesis suggest that a complete inhibition of the polar long – chain carotenoids (TPEC & TPHC) was not possible when different concentrations of nicotine in the range of (0 -24 mM were tested. Nicotine however, resulted in 50% accumulation of saturated of saturated hydrocarbon carotenoids on behalf of the long – chain carotenoids. Diphenylamine at a concentration of 20 µg/ml culture medium induced a complete suppression of the long–chain carotenoids and accumulation of the C-40 carotenoids, which simply means that the formation of the long–carotenoids depends totally on the structural alteration (desauration) of the C -40 hydrocarbon carotenoids (carotenes). Finally, the structural elucidation of the carotenoids encountered in this study was based on various analytical methods such as, UV-visible absorption spectra, thin layer and column chromatography and by their fragmentation pattern during the mass spectral analysis.

Figures and Tables:

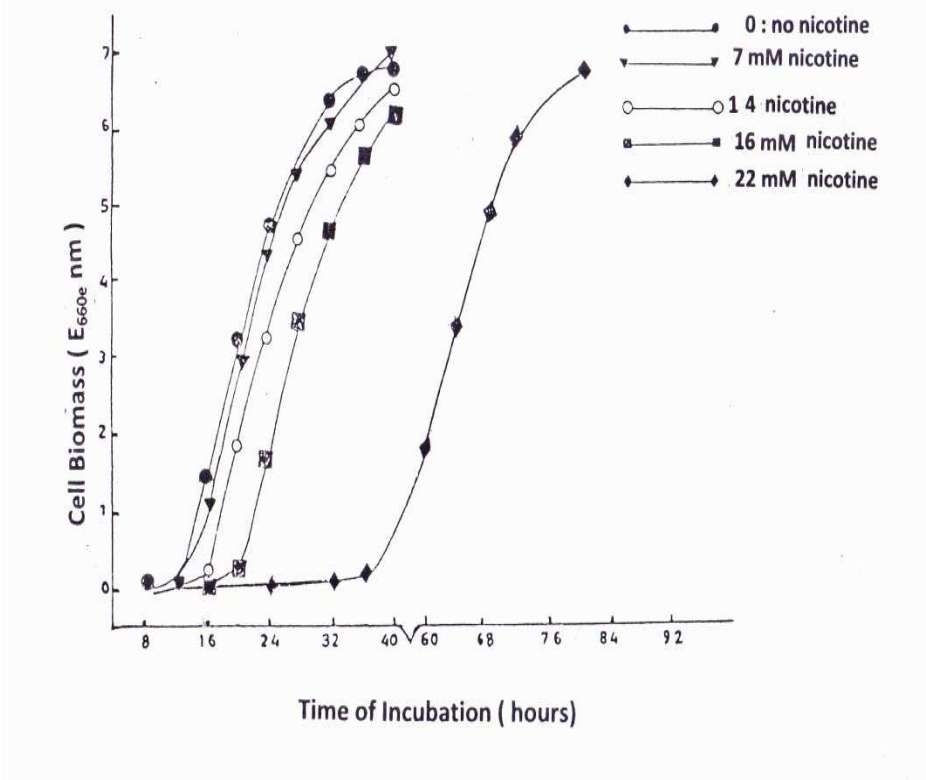

Fig.1: Effect of different concentrations of nicotine on growth of *Micrococcus luteus*

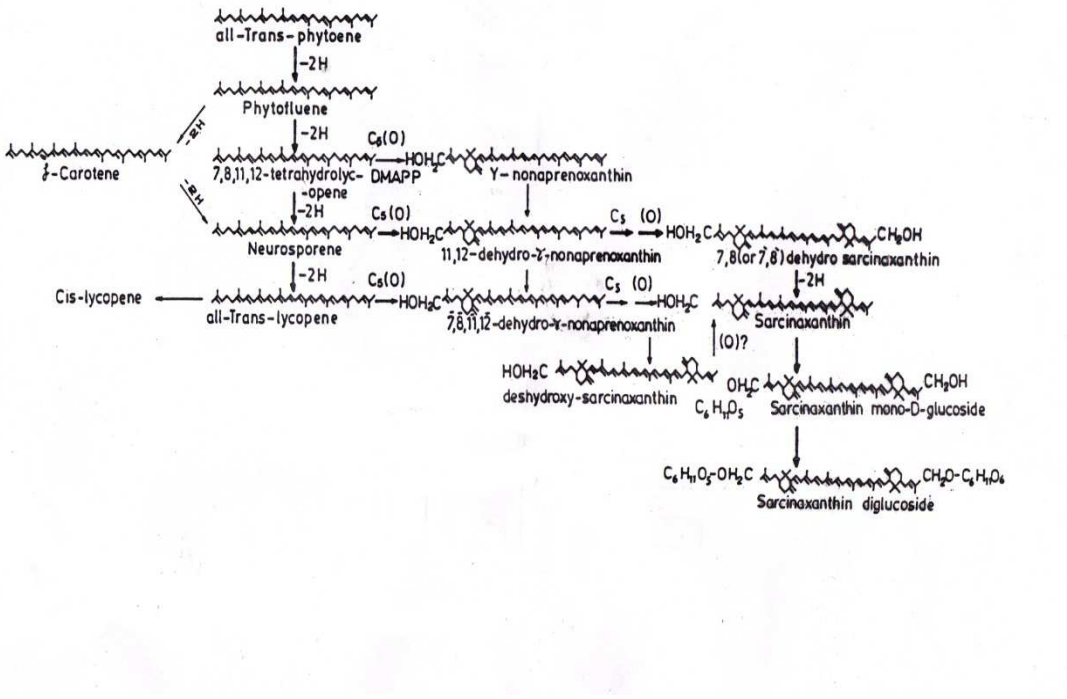

Fig. 2: The possible biosynthetic pathway of carotinoids in *Micrococcus luteus*

Table 1: The of effect of different nicotine concentrations on biomass yield of *Micrococcus luteus* cultures grown under static, static and shale culture conditions

Nicotine concent. Mm	g. bact (wet.wt.) . Per liter Growth time of Incubation (hours)			
	Static culture, 176	Static –shake, 176 + 50	Shake- culture,36	
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 = ethanol and 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 treatment.

Table 2: Effect of light and dark on C- 40 hydrocarbon carotenoids biosynthesis and biomass of *Micrococcus luteus* grown for 36 hours in the presence of 25 µg per ml culture medium

	Percentage of total carotene					**g.bact .(wet wt.) /liter growth**
Treatment	Phytoene	Phytofluene	£-carotene	neurosporene	Lycopene	
Light	95.2	4.2	0.7	0	O	**27.16**
Dark	93.6	5.5	0.9	0	0	**25 .48**

Table 3: The effect of light and dark on biomass (average of 10 liter growth) at stationary phase of growth of *Micrococcusluteus* grown in the presence of 7 mM nicotine

	gm . bacteria (dry wt.) / liter growth										Average	
	F1	**F2F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**			
Light	5.27	4.93	4.92	4.77	5.08	4.84	5.07	4.98	5.16	5.22	5.02	
Dark	4.82	4.71	5.07	5.70	5.79	5.51	5.57	4.95	4.59	4.78	5.15	

: F= flaskF 1 – F 10

Table 4: The influence of incubation period on carotenoids biosynthesis in Micrococcus luteus grown in nicotine –free culture medium

Incubation period (hr)	µg carotenoids	Per 10gm	(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 5: 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 6: The effect of different concentrations of diphenylamine (DPA) on carotenoid biosynthesis in *Micrococcus luteus*

DPA/ ml cultur e	Percent *of Total Caroteno ids*								
	µg carote noids/ gm.bact. .(wetwt.)	TC	*TPEC*	*TPH C*	*Phytoe ne*	*Phytof- luene*	*Caro- etne*	*Nurosp- orene*	***Lycop- ene***
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***

Table 7: The influence of varying concentrations of nicotine on carotenoid biosynthesis in *Micrococcus luteus* (% of Total Carotenoids)

Nicotine mM	gm.bac /2Lgro-wth	TUF	TC	TPEC	TPHC	Phyto-ene	Phytofl-uene	£-carotene	Neurosp-orene	Lycopene
C	72.19	360.76	7.14	29.72	63.14	O	0	0	0	7.14
D	70.68	326.38	22.89	28.09	49.09	5 .76	2.87	1.15	1.51	11.59
1	60.80	434.90	44.37	24.57	31.07	34.68	6.71	3.29	0.51	0
2	67.40	807.48	23.91	23.58	52.51	12.66	4.31	1.41	1.36	4.16
3	62.30	304.00	11.78	21.90	66.32	0	1.67	0.58	1.05	9.47
7	57.80	986.30	44.44	23.93	31.63	26.59	8.88	2.89	2.05	4.03
12	58.20	996.00	50.30	12.05	37.65	33.47	8.82	2.73	1.79	3.49
24	60.50	1122.32	14.82	26.73	58.45	8.38	2.09	0.76	0.56	3.04

C; control; D; 5 ml ethanol per liter growth medium, total carotenoids = (TC + TPEP + THP).

All cultures were incubated for 36 hour, (stationary phase) except the treatment (24Mm nicotine) which required 108 hours to reach stationary phase of growth.

TUF: Total unsaponifiable fraction; Total carotenoids = total carotene (TC) + Total polar epiphasic carotenoids (TPEC) + Total polar hypophasiccarotenoids (TPHC)

Table 8: Absorption spectra of acetone – extracted carotenoids of *Micrococcus luteus* grown in the presence of 7mM nicotine. The individual carotenoids are arranged in order of increasing polarity

Carotenoid	Color	λ max in Hexane	Chemical – Formula
Phytoene	colorless	276 286 298	$C_{40}H_{64}$
Phytofluene	flurescene	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	
Neurosporen	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.

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