CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Retinol, trans-β-carotene, zeaxanthin, 13-cis-β-carotene, α-carotene, lycopene, lutein and cryptoxanthin, were purchased from Hoffmann-La Roche (Switzerland) and Heavy water (D₂O) from HW Division, BARC Mumbai. BRIT (India) supplied ¹⁴[C]-sodium bicarbonate. Eagles Minimum Essential Medium (MEM), lecithin, triolein and deoxy cholic acid were procured from Sigma Chemical Company, USA. Preformulated medium used for the transportation of stool samples and isolation of colonic epithelial cells was supplied by M/s Noninvasive Technologies, Columbia, Maryland, USA. 35mm culture dishes were purchased from Nunc, Denmark. Spray dried Spirulina obtained as Multinol, Parry Nutraceuticals Ltd., India and Sesbania (Agathi) leaves were purchased locally. High purity solvents were used for all extractions. All other chemicals were high purity analytical grade reagents procured from Merck, India.

2.1.2 Organism

Spirulina platensis was obtained in the form of pure culture on agar slants from Central Food Technological Research Institute (CFTRI), Mysore. Stock cultures were kept at 27°C under low illumination and maintained by serial subculturing done at every 20 days.

2.1.3 Experimental animals

Male Sprague-Dawley rats with average body weight of 150-200 g maintained in the laboratory animal house were used for feeding experiments. New Zealand white
rabbits (*Oryctolagus cuniculus*) with average body weight about 2 kg were used for different studies. Monkeys (*Maccaca radiata*) of average body weight 3 kg were selected for the bioavailability studies. The animals were maintained at the animal house of Annamalai University.

### 2.2 Methods

#### 2.2.1 Culture medium

Zarrouk’s medium (Zarrouk, 1966) was used for the liquid culture of *Spirulina platensis*. The composition is as given below.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NaHCO₃</td>
<td>NaNO₃</td>
</tr>
<tr>
<td>2.</td>
<td>K₂HPO₄</td>
<td>2.5 gL⁻¹</td>
</tr>
<tr>
<td></td>
<td>16.8 gL⁻¹</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>NaCl</td>
<td>1.0 gL⁻¹</td>
</tr>
<tr>
<td>3.</td>
<td>MgSO₄</td>
<td>0.2 gL⁻¹</td>
</tr>
<tr>
<td>4.</td>
<td>FeSO₄</td>
<td>0.01 gL⁻¹</td>
</tr>
<tr>
<td>5.</td>
<td>K₂SO₄</td>
<td>1.0 gL⁻¹</td>
</tr>
<tr>
<td>6.</td>
<td>CaCl₂</td>
<td>0.04 gL⁻¹</td>
</tr>
<tr>
<td>7.</td>
<td>EDTA</td>
<td>0.08 gL⁻¹</td>
</tr>
</tbody>
</table>

Double distilled water was used for the preparation of medium by dissolving the salts A and B separately and combined after complete dissolution of contents. The media was sterilised by autoclaving and used for developing the asceptic cultures in flasks. 2% agar slants were also made with same constituents of Zarrouk’s medium for the maintenance of pure cultures.

#### 2.2.2 *Spirulina* Stock Culture

*Spirulina platensis* available as stock cultures were inoculated on 2% agar-agar nutrient medium. One to two drops of stock liquid culture of *Spirulina* was inoculated and spread uniformly using sterile inoculation loop. The agar slants were kept at ambient temperature (25.0°C ± 2.0°C) in subdued light of 30-50 μmol m⁻²S⁻¹ light intensity. Subculturing was done in 20 to 30 days so that clean cultures were obtained. Suspension cultures were developed by inoculating the scrapings from the agar slants into Zarrouk’s medium in flasks. Incubated the cultures at 25 °C with light phase of 10-12 hours and dark phase of 8-10 hours on an orbital shaker at a rocking speed of 30-40 rpm. Initial biomass was adjusted to 50 mgL⁻¹ dry weight. All operations were done under asceptic conditions.
2.2.3 Bioreactor

A laboratory scale micro bioreactor was fabricated using Borosil glass. Transparent vertical column mini bioreactor with illumination and agitating system having a working volume of 1.0 liter capacity was developed. The design is shown schematically in Figure 3.21. Optimization of conditions for the growth and other parameters, such as light intensity, agitation, CO\textsubscript{2} supplementation etc., for maximum label incorporation and biomass production were done. Main part of the reactor consists of an illuminated region with a column of 100 cm length and diameter of 5 cm, so as to hold 1 liter of the culture. Top of the column opened to desiccators filled with silica gel and the bottom of this column is fitted with stoppered outlet for withdrawal of the culture. Magnetic stirrer was used to agitate the bioreactor, for controlled mixing of the culture at desired speed (150 rpm). The reactor column was illuminated with a set of cool white fluorescent lamps (4x20 W) fitted on a movable stand, which in turn provided appropriate photosynthetic flux density.

2.2.4 Induction of carotenogenesis

Using different methods, carotenoid over production was induced. *Spirulina* cultures grown in normal water were used for these studies. Effect of different light intensities, nitrate starvation and CuSO\textsubscript{4} toxicity on biomass and β-carotene production was investigated.

2.2.4.1 Effect of light on biomass and carotenoid production

Placing the light source at appropriate distances from the cultures created different levels of light intensities. Light intensities of 25, 50, 75 and 100 μmol m\textsuperscript{-2} s\textsuperscript{-1} were exposed. Biomass production and β-carotene content in the cells at definite intervals were assessed as described below.

2.2.4.2 Effect of NO\textsubscript{3} deficiency in the medium on biomass and carotenoid production

Zarrouk’s medium was prepared with different levels of sodium nitrate, 0 mM, 10 mM, 20 mM, 25 mM and 30 mM (control). All other contents remained unaltered. *Spirulina* cultures were grown and the growth kinetics was recorded. The biomass was isolated and the carotenoids were estimated.
2.2.4.3 Induction of stress using CuSO$_4$

Effect of copper ions on the Spirulina growth and production of β-carotene was studied. Three different concentrations of CuSO$_4$ were prepared, viz., $10^{-8}$ M, $10^{-6}$ M and $10^{-3}$ M in Zarrouk’s medium and growth studies were done.

2.2.4.4 Reuse of spent medium

In order to compensate lag in the biomass production of the deuterated cultures and to ensure maximum utilization of costly precursor in the medium, conditions were standardized for the use of spent medium in different cycles. After the initial growth phase of 25 days, $1/5^{th}$ of the biomass was retained in the medium and rest is harvested. These cultures were allowed to grow further until the next stationary phase designated as second cycle. Thus, the same medium was used up to 6$^{th}$ cycle. Total biomass production and β-carotene accumulation in each cycle were determined. This was done initially using the normal water and then extended to $^{14}$[C] and $^3$[H] cultures.

2.2.5 Biomass production

Freshly inoculated medium was allowed to grow and the biomass increase was assessed by withdrawing aliquots of culture from lag phase to log phase to stationary phase on every 5$^{th}$ day. The growth kinetics in terms of biomass (dry weight) of algal cells in suspension cultures was determined by measuring the absorbance (Becker, 1994). 2 ml sample of the culture from the flask was withdrawn and measured optical density at 550 nm non-destructively. Turbidity in terms of optical density was converted to corresponding dry weight. Standard graph was constructed by taking cultures of known cell density. Corresponding dry weight was determined by filtering the cultures with oven dried preweighed filter paper. Determination of dry weight was done in duplicate.

2.2.6 Biomass harvest

Spirulina cells were grown in suspension cultures up to 30-40 days. The cells were harvested at stationary phase by withdrawing the cultures in 50 ml polypropylene tubes and centrifuged at 900 x g for 10 minutes. Removed the medium and the pellets were freeze-dried, weighed and stored under nitrogen at -20 °C.
2.2.7 Extraction of carotenoids

Harvested biomass was suspended in acetone: petroleum ether (1:1) v/v and kept overnight under 4°C for better and easy recovery of carotenoids. Then the mixture was centrifuged at 1000xg for 20 minutes. After repeated extractions (4 times) the supernatants were pooled and washed twice with distilled water using a separating funnel. Washed extracts were dried over anhydrous sodium sulphate and reduced to a minimum volume by evaporating the solvents using \( \text{N}_2 \) stream. The crude extracts were reconstituted in HPLC grade hexane, and kept for further separation of carotenoids in amber coloured containers under nitrogen at -20 °C. All operations were done at subdued light under \( \text{N}_2 \) atmosphere.

2.2.8 Separation and analysis of carotenoids

Crude extract in hexane was subjected to fractionation using different chromatographic techniques. Separated pigments were subjected to spectrometric analysis for the identification of different individual carotenoids.

2.2.8.1 Alumina column chromatography

A glass chromatographic column of size 1 cm x 10 cm was made using activated Alumina (Brockmann–Neutral) and a layer of anhydrous sodium sulphate was kept on the top of the alumina column filled with mobile phase (1% acetone in hexane, v/v). The crude extract was applied on the top of the column and then passed the mobile phase. Different fractions were collected and carotenoids were quantified.

2.2.8.2 Thin layer chromatography

Silica gel 100 coated glass plates (20 x 20 cm) were used for TLC. Using capillary tubes, 5 μl of crude extract was spotted on the base of the silica plate. Authentic standards of different carotenoids were spotted for identification of bands. The plates were developed using mobile phase containing 5% benzene in petroleum ether (v/v). Carotenoids were identified and the respective spots were scraped out, eluted with hexane.

The carotenoid contents of the different fractions were determined by measuring the absorbance using spectrophotometer (Shimadzu, Japan). The Extinction coefficient \( E^{11^\text{th}} \) in petroleum ether (60-80°C) for \( \alpha \)-carotene (2710) at 445 nm, \( \beta \)-carotene (2500)
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at 450 nm, lycopene (3470) at 474 nm and \( \beta \)-cryptoxanthine (2386) at 452 nm and for lutein (2550) at 445 nm in ethanol (Bhaskarachary et al., 1995) was taken.

2.2.8.3 HPLC analysis

HPLC analysis was carried out using chromatographic system consisting of a Shimadzu model LC 10A equipped with variable wavelength detector SPD 10 AV and chromatopack. Stainless steel 25 cm x 4.5 mm separation column (Waters ODS 18) was used. Suitable volume, 5-15\( \mu l \), of sample of extracts were injected on to rheodyne injector of maximum volume 20 \( \mu l \). Isocratic separation of carotenoids was performed with the mobile phase consisting of acetonitrile / chloroform / isopropanol / water in 780: 160: 35: 25 (v/v) proportions and flow rate was adjusted to 2 ml / min. The eluent was monitored with the detector wavelength set at approximate wavelengths and sensitivity was set at 0.01 AUFS. Standards of \( \beta \)-carotene, lutein, lycopene, and cryptoxanthin were used externally. The retention time was recorded and peak areas of standards and tests were noted on each run and used for calculation of concentrations of different fractions. All samples were injected in duplicate.

2.2.8.4 LC-MS analysis

Carotenoid extracts were subjected to liquid chromatographic analysis (Waters LC). Injection volume was 10\( \mu l \) and a total run time of 40 minutes was given and individual carotenoids were separated. Then the \( \beta \)-carotene fraction was passed on to the Mass spectrometer (Waters) attached to the LC. Relative abundance and m/z ratios of each fraction were computed and the mass spectrum was constructed. LC-MS analysis was done at Human Nutrition Research Centre on Ageing, Tufts University, Boston, USA.

2.2.9 \(^{14}\)C labeling

Scrapings from the agar stock culture of Spirulina platensis were dispensed in Zarrouk’s medium (Becker, 1994) prepared in double distilled water. \(^{14}\)C-sodium bicarbonate enriched inoculums were prepared by culturing cells in presence of radioactive sodium bicarbonate. This was used as inoculum for developing liquid cultures in conical flasks. \(^{14}\)C sodium bicarbonate (5\( \muCi/ml \)) was used for the preparation of medium. Cultures were maintained in conical flasks at 27.0\( ^\circ\)C and provided light intensity of 50\( \mu \)mol s\(^{-1}\) m\(^{-2}\). Cultures were kept in continuous shaking conditions on an orbital shaker
with light phase of 10 hrs. The growth of the cultures in radioactive medium was monitored. Biomass was determined as described earlier. Growth of the cultures was compared with that of cultures grown in non-radioactive medium.

2.2.9.1 Measurement of Radioactivity in carotenoids

Aliquots of samples were separated by TLC. β-carotene was identified and subjected to radioactive measurements using liquid scintillation counter (Wallac 1409, USA). Freshly prepared scintillation fluid (Toluene-795 ml, Triton X100-125 ml, Methanol-l80 ml, POPOP-0.2 g, PPO-6 g) was used.

2.2.10 Isolation of colonic epithelial cells

Exfoliated colonic epithelial cells were isolated by a modified procedure of Albaugh et al. (1992). Approximately 500 mg of stool samples were introduced into a container having transport medium (SCSR-T) using a spatula attached to the stopper and kept at 4°C. On arrival, the stool samples were thoroughly mixed using vortex mixer for approximately 15 seconds. Then the suspension was filtered into 50 ml stoppered polypropylene tubes and additional transport medium was added to make the volume to 25 ml. 10 ml of the cushion (SCSR-C) was underlaid beneath the suspension and centrifuged at 200 x g for 10 minutes. Contents turned into different layers, viz., the upper supernatant, lower pellet and an interphase between them. Accumulated cells at the interphase and also that present in the bottom layer were removed carefully using sterile pipette (Figure 2.1). Supernatant up to the interphase was discarded. The recovered cells were washed thoroughly with PBS and collected by centrifugation at high speed (1000 x g). The viability of the cells was determined by Trypan blue exclusion and the protein content was estimated using Lowry’s method (Lowry et al., 1951). About 60-70% of the cells were viable as evidenced by exclusion of Trypan-blue. The yield of the cells was in the range of 1-1.5 x 10^6 cells/g stool sample and the analysis of multiple samples showed less than 10% variation within the samples. During the isolation steps, accumulation of viable colonic epithelial cells was observed in the interphase fraction and cushion-pellet fraction. The majority of viable cells were obtained from the interphase.
2.2.11 Culture of colonic epithelial cells

Isolated cells were suspended in Eagle’s MEM containing 5% fetal calf serum. Approximately $1 \times 10^6$ cells/ml MEM were seeded on 35 mm culture dishes (Nunc, Denmark) and incubated at 95% air 5% CO$_2$ in Forma CO$_2$ incubator set at 37°C. Cells were maintained for 4 hours, unattached cells were removed, fresh medium was added and the cultures were used for subsequent experiments. Cells were harvested by scrapping the culture dishes with rubber policeman.

2.2.12 Extraction of colonic epithelial cells for β-carotene and retinol

Known quantities of isolated colonic epithelial cells were taken and washed with PBS. Freshly isolated cells or cultured cells after different treatments were taken for the extraction of retinol, β-carotene and lutein. Cells were disrupted by sonication for 5 minutes. The lysed cells were saponified using methanolic KOH (10% w/v) for 3 hours at room temperature. Saponified samples were washed and repeatedly extracted with hexane. Pooled hexane extracts were concentrated by passing N$_2$ gas. The hexane layer was collected, redissolved in ethanol (100% v/v) and were kept at -20°C in amber coloured vials until further analysis. In order to protect the samples from the oxidative damage all operations were carried out under subdued light and N$_2$ atmosphere.
2.2.13 Uptake of \( \beta \)-carotene by colonic cells \textit{in vitro}, effect of bile salt (deoxy cholic acid), lecithin and triolein

Isolated colonic epithelial cells were seeded in 35 mm culture plates at \( 1 \times 10^6 \) cells/ml and maintained at 37°C in a Forma \( \text{CO}_2 \) incubator. After 4 hours unattached cells were removed and added fresh medium containing \( \beta \)-carotene (0.08 \( \mu \)g/ml). In order to study the effect of deoxy cholic acid the medium was supplemented with different concentration of deoxy cholic acid. To study the effect of lecithin, the medium was supplemented with different concentrations of lecithin and to study the effects of triolein the medium was supplemented with different concentrations of triolein. The cells were maintained in culture at 37°C for a given time. The medium was removed, cell layer was washed and harvested by scrapping with a rubber policeman. The cells were extracted and determined the amount of \( \beta \)-carotene. In order to study the time course of uptake, cells were maintained in culture in the presence and absence of different supplements for different time intervals. The cells were harvested at the end of each experimental period and analysed for the \( \beta \)-carotene in the cells.

2.2.14. Uptake of \( ^{14}\text{C}\)-labeled \( \beta \)-carotene

The colonic epithelial cells in culture were used to study the uptake of radio-labeled \( \beta \)-carotene produced by \textit{Spirulina} in culture. MEM was prepared with \( \beta \)-carotene having radioactivity of 4550 cpm/ml. Culture dishes were incubated at 37°C for different time periods. Medium was removed, cell layer was washed and the cells were harvested. These cells were extracted and the concentrated extracts were spotted on a TLC plate coated with silica gel-100 and subjected to fractionation as described earlier. \( \beta \)-carotene fractions were identified using authentic standards. Radioactivity in \( \beta \)-carotene fraction was measured, by using a Wallac liquid scintillation counter.

2.2.15 Estimation of protein

Total protein in the colonic epithelial cells was estimated by the procedure of Lowry \textit{et al.} (1951)

\textbf{Reagents}

1. Alkaline Copper reagent (freshly prepared)
   a. 1 part \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \) (1\% w/v)
   b. 1 part sodium potassium tartarate (2\% w/v)
To one volume of the mixture added 50 volume of 2% (w/v) sodium carbonate in 0.1 N NaOH

2. Folin phenol reagent
   Diluted the commercial phenol reagent to 1 N with distilled water.

3. Standard bovine serum albumin

Procedure

To 500 μl sample added 2.5ml alkaline copper reagent, allowed to stand for 15 minutes at room temperature. Added 250 μl of phenol reagent, vortexed and allowed to stand at room temperature for 30 minutes. Optical density was read at 660 nm.

2.2.16 Statistical analysis

Statistical analysis of the data was done using Student’s t test (Bennett and Franklin, 1967).