CHAPTER 3

PRODUCTION OF BIOSYNTHETICALLY LABELED CAROTENOIDs USING SPIRULINA PLATENSIS *

3.1 Introduction

Increasing the availability of dietary provitamin A carotenoids will improve the vitamin A status among deficient population (de Pee et al., 1996). In this context, studies on the bioavailability of vitamin A have much significance. Such investigations in animal models used radiolabeled vitamin A or precursors of vitamin A in high dose (Olson et al., 1979). These methods are however unacceptable in a study involving human subjects. An alternative approach would be the use of vitamin A rich precursors labeled with stable isotope such as $^2\text{H}$ or $^{13}\text{C}$ (Wilson et al., 1997). The use of such labeled derivatives of vitamin A or β-carotene and subsequent LC-MS analysis can lead to the measurement of the specific activity of vitamin A in the plasma that could be useful in the evaluation of the bioavailability and vitamin A status in humans (Furrr et al., 1989). For this appropriately labeled vitamin A or provitamin A is required.

*Spirulina* is a fresh water micro alga that has very high nutritive value (Key, 1991; Vonshak, 1997) and has attracted much attention because of its use as human and animal protein source and it has very high content of β-carotene than in any other assorted vegetables. It contains valuable micronutrients such as biotin, vitamin $\text{B}_{12}$, folic acid (Venkataraman, 1993) and certain strains accumulate large amounts of γ-linolenic acid (Mahajan and Kamat, 1995). This micro alga has the ability to synthesise β-carotene photosynthetically, making it an ideal organism for metabolic labeling of carotenoids. Efficiency in the production of metabolically labeled provitamin A depends largely on the type of culture system, growth conditions and nature of precursor molecules and therefore,

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Production of Biosynthetically labeled Carotenoids using *Spirulina platensis*

3.2 Materials and methods

*Spirulina* maintained as stock, was used to develop different type of the cultures for the production of intrinsically labeled carotenoids. Algal cells were acclimatized in medium containing heavy water and $^{14}$[C]-labeled sodium bicarbonate. Cultures of *Spirulina* were developed by subculturing the cells in Zarrouk's medium containing increasing concentration of heavy water or radioactive sodium bicarbonate. Cultures were developed in flasks for both normal water and heavy water and incubated for growth up to 35 days. When the growth of the cultures reached the stationary phase biomass was harvested. The total carotenoids were extracted, separated and analysed by using liquid chromatography. β-carotene extracted from the biomass of algae grown in heavy water was analysed by using mass spectrometer and the mass spectrum was compared with that of cells grown in normal water. In order to increase the production of β-carotene and labeling intensity, different methods were tested by growing *Spirulina* in the normal water. Effects of different light intensity, nitrate deficiency, supplementation of CuSO$_4$ and reuse of spent medium on biomass and β-carotene production were tested. Optimum conditions for production of β-carotene were selected and utilized in cultures with heavy water and $^{14}$[C] bicarbonate. In order to scale up the production of deuterium labeled compounds, a laboratory scale bioreactor was fabricated and tested for its efficiency of biomass and carotenoids production. Labeling intensity of the carotenoids was determined using LC-MS system. The details of culture conditions and methodologies have been described in the Chapter 2.

3.3 Results

3.3.1 Growth of *Spirulina* in culture

Pure cultures of *Spirulina platensis* were grown in liquid medium. Growth of the cells was assessed at regular intervals. The culture was grown under a light period of 14 hours and dark period of 10 hours a day. Growth of the cultures was observed. The algal cells were harvested when the number of cells were increased and reached the conditions have to be optimized for producing intrinsically labeled provitamin A. This chapter envisages the investigations on (a) production of biosynthetically labeled carotenoids especially deuterated and $^{14}$[C]-labeled β-carotene using *Spirulina platensis* and (b) development of a laboratory scale bioreactor and optimization of culture conditions for growth of *Spirulina*.
stationary phase. Average dry mass of the cells was about 300 mg/L on 30th day of the culture. The morphology of the cells was also examined. Figure 3.1 (a) shows *Spirulina* filaments under normal conditions of growth in liquid cultures.

### 3.3.2 Biomass production and kinetics

*Spirulina* culture in the liquid medium was allowed to grow for different periods. Figure 3.2 (a, b, c) shows the different stages of cultures in flasks, grown under normal ambient conditions. On every 5th day, the biomass was measured by assessing the optical density at 550 nm and the results are shown in Figure 3.3. Up to 10th day after the inoculation, the cultures showed lag in growth and subsequently cultures attained logarithmic phase on 25th day. Between 30-35 days, growth of the cultures attained a maximum and produced an average biomass of 310 mg/L. No further increase in biomass was obtained, after 35th day and the cultures remained in the stationary phase.

**Fig. 3.3**

Kinetics of growth of *Spirulina* in culture

Stock cultures of *Spirulina* were inoculated in Zarrouk's medium prepared in H₂O and allowed to grow in flasks under controlled light and temperature. Growth of the cells was assessed every 5 days. Values are the average of three different cultures analysed in duplicate.
Legends to Figure 3.1

*Spirulina* cells under phase contrast microscope

Pure cultures of *Spirulina platensis* grown in Zarrouk’s medium under 14 hrs of light period. Morphology of cells under normal growth conditions (x 100) (a). Sporulated *Spirulina* cells after storage for six months without subculturing (x 500) shows accumulation of yellow pigments (b). Regeneration of spores into normal filaments (x 500) after subcuturing in fresh medium (c & d).

Legends to Figure 3.2

Growth of *Spirulina in flask cultures*

*Spirulina* cultures in Zarrouk’s medium, different stages of *Spirulina* cultured in flask, lag phase (a), log phase (b) and stationary phase (c).
3.3.3 Kinetics of β-carotene production

Culture samples were harvested at every 10 days during the different stages of growth and the β-carotene content was determined after extraction and fractionation by HPLC. Figure 3.4 shows the kinetics of β-carotene production at different time periods. Total β-carotene content increased from 30 μg to 170 μg during the period up to 25 days. After 25th day, the β-carotene production did not show any significant variation.

![Fig. 3.4](image)

Spirulina cells were cultured in liquid medium prepared in normal water under controlled conditions. Algal cells were harvested at regular intervals and the β-carotene content were analysed using HPLC. Values given are the average of three different cultures analysed in duplicate.

3.3.4 Biomass extraction and analysis of carotenoids

Harvested biomass was subjected to solvent extraction. Total carotenoids were isolated and crude extracts were fractionated using liquid chromatography as described in chapter 2 and the elution profile is given in Figure 3.5. Seven different carotenoids were identified from the liquid chromatographic analysis. Table.3.1 shows the results of analysis of individual carotenoids. These were *trans*-β-carotene, *9-cis*-β-carotene, *13-cis*-β-carotene, α-carotene, cryptoxanthin and zeaxanthin. Of these carotenoids, *trans*-β-carotene formed about 21.8% and *9-cis*-β-carotene about 6.6% and *13-cis*-β-carotene about 12.7%. Significant fraction of lutein was also obtained.
Production of Biosynthetically labeled Carotenoids using *Spirulina platensis*

Fig. 3.5  
Liquid chromatographic profile of different carotenoids present in *Spirulina*

Liquid chromatographic analysis of carotenoids of *Spirulina* grown in Zarrouk's medium prepared in H₂O. Carotenoids were isolated from *Spirulina* and subjected to liquid chromatography. Elution patterns of different carotenoids are shown. Lutein (1), Zeaxanthin (2), Cryptoxanthin (3), 13-cis-β-carotene (4), α-carotene (5), trans-β-carotene (6) and 9-cis-β-carotene (7) were eluted in 8, 9, 15, 17, 18, 20 and 22nd minutes respectively.

Table 3.1  
Relative amount of different carotenoids in cultures of *Spirulina* grown in H₂O medium in flasks

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein</td>
<td>27.42</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>13.80</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>05.73</td>
</tr>
<tr>
<td>13-cis-β-carotene</td>
<td>12.72</td>
</tr>
<tr>
<td>α-carotene</td>
<td>00.75</td>
</tr>
<tr>
<td>trans-β-carotene</td>
<td>21.80</td>
</tr>
<tr>
<td>9-cis-β-carotene</td>
<td>06.69</td>
</tr>
<tr>
<td>Unidentified</td>
<td>11.04</td>
</tr>
</tbody>
</table>

Algal cells grown under optimum conditions of light and nutrients were harvested and the carotenoids were extracted and analysed using LC. Mean values are percentages of total carotenoids (Mean total carotenoid content was 650 µg g⁻¹ dry weight).

3.3.5 Optimisation of growth conditions for maximum β-carotene production

In order to optimize the production of β-carotene by *Spirulina*, cultures were maintained at different conditions. Effects of change in light intensity, different levels of...
NO3 and CuSO4 on biomass and \( \beta \)-carotene production were studied and the results of these experiments are given below.

3.3.5.1 Effect of light intensity on biomass and \( \beta \)-carotene production

Growth of the algae and \( \beta \)-carotene production were tested, by exposing the flask cultures to different light intensities. Cultures were incubated at photon flux densities, 25, 50, 75 and 100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). Kinetics of biomass production was studied and the results are shown in the Figure 3.6. With increase in light intensity, there was increase in biomass production. Biomass production increased up to about 330 mg/L at stationary phase when cultures were exposed to light intensities of 75 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) and further increase in light intensity to 100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) resulted in slight reduction in the biomass. But the cultures exposed to low intensities, viz., 25 and 50 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) produced significantly low biomass.

![Figure 3.6](image-url)  
**Effect of change in light intensity on biomass production**

Suspension cultures of *Spirulina platensis* were allowed to grow in flasks in Zarrouk’s medium, exposed to different light intensities of 25, 50, 75 and 100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). Dry biomass production was assessed at every 5 days up to 35 days. Values are the average of three different cultures analysed in duplicate.
Kinetics of production of \( \beta \)-carotene by \textit{Spirulina} cultures exposed to different light intensities was also studied and the results are shown in Figure 3.7. The total \( \beta \)-carotene content was about 160 mg when 75 \( \mu \)mol m\(^2\) s\(^{-1}\) light was given, compared to cultures exposed to 100 \( \mu \)mol m\(^2\) s\(^{-1}\) where 200 \( \mu \)g \( \beta \)-carotene accumulated. The amount of \( \beta \)-carotene produced was very low at lower light intensities.

![Fig.3.7](effect-of-light-intensities.png)

**Fig.3.7**  
*Effect of different light intensities on \( \beta \)-carotene production by \textit{Spirulina} in flask cultures*

Suspension cultures of \textit{Spirulina platensis} were allowed to grow in flasks in Zarrouk's medium exposed to different light intensities of 25, 50, 75 and 100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). Cells were harvested and \( \beta \)-carotene content was estimated at every 10 days up to 35 days. Values given are the average of three different cultures analysed in duplicate.

### 3.3.5.2 Effect of nitrate on biomass production

Effect of change in the concentration of sodium nitrate in the culture medium on biomass and \( \beta \)-carotene production was investigated. Concentrations of sodium nitrate in the medium ranged from 0 mM to 30 mM. Biomass accumulation and \( \beta \)-carotene content in cells were estimated and the results are presented in the Figure 3.8. There was a lag phase of 15 days particularly at lower concentrations of nitrate with an average
Production of Biosynthetically labeled Carotenoids using *Spirulina platensis*  

Highest biomass production was obtained when 30 mM sodium nitrate was used in the growth medium compared to other treatments. At lower concentrations, 20 mM and 10 mM, reduction in biomass accumulation was observed. Biomass of algal cells in the completely deficient medium showed a lag in growth; however, it attained biomass of 150 mg/L in 30 days.

**Fig. 3.8**  
**Effect of change in concentration of sodium nitrate on biomass production**

Suspension cultures of *Spirulina platensis* were allowed to grow in Zarrouk’s medium containing different concentrations of sodium nitrate under controlled conditions of light (75 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and temperature (27°C). Dry biomass production was assessed at every 5 days up to 35 days. Values given are the average of three different cultures analysed in duplicate.

\( \beta \)-carotene content in algal cells was also analysed and the results are shown in the Figure 3.9. Higher rate of \( \beta \)-carotene accumulation was observed when low concentration of \( \text{NO}_3 \) was used. On the 30\textsuperscript{th} day, amount of total \( \beta \)-carotene accumulation ranged from 275 \( \mu \text{g} \) to 325 \( \mu \text{g} \) when 20 and 10 mM NaNO\(_3\) were used in the medium. In presence of 25 mM Na NO\(_3\), about 160 \( \mu \text{g} \) \( \beta \)-carotene was obtained. About 180 \( \mu \text{g} \) of \( \beta \)-carotene was produced in cultures where no nitrate was supplied.
Suspension cultures of *Spirulina platensis* were allowed to grow in Zarrouk's medium containing different concentrations of sodium nitrate under controlled condition of light (75 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and temperature (27°C). \( \beta \)-carotene production was assessed at every 10 days up to 35 days. Values given are the average of three different cultures analysed in duplicate.

### 3.3.5.3 Effect of CuSO\(_4\) on biomass and \( \beta \)-carotene production

Effect of copper ions on the biomass and \( \beta \)-carotene production was investigated by changing the concentrations of CuSO\(_4\) in the growth medium. The kinetics of biomass production was studied and the results are presented in the Figure 3.10. Treatment with CuSO\(_4\) significantly reduced the biomass when compared to the cultures without CuSO\(_4\) (control), which produced about 300 mg/L dry biomass. In CuSO\(_4\) treated cultures the biomass was in the range of 75-125 mg/L.

Accumulation of \( \beta \)-carotene in the algal cells was also studied and the results are shown in Figure 3.11. Treatments with \( 10^{-8} \text{M}\) and \( 10^{-6} \text{M}\) CuSO\(_4\) caused an increase in total \( \beta \)-carotene production, which ranged from 125-150 \( \mu \text{g/L}\). However in algal cells cultured in medium containing high concentrations of CuSO\(_4\) (\( 10^{-3} \text{M}\)), no significant increase in the \( \beta \)-carotene content was observed.
Suspension cultures of *Spirulina platensis* were allowed to grow in Zarrouk’s medium supplemented with different concentrations of CuSO$_4$ under controlled conditions. Dry biomass production was assessed at every 5 days up to 35 days. Values given are the average of three different cultures analysed in duplicate.

Suspension cultures of *Spirulina platensis* were allowed to grow in Zarrouk’s medium supplemented with different concentrations of CuSO$_4$ under controlled conditions of light (75 μmol m$^{-2}$s$^{-1}$) and temperature (27°C). Cells were harvested at regular intervals extracted and the β-carotene content was estimated by using HPLC. Values given are the average of three different cultures analysed in duplicate.
3.3.5.4 Growth of Spirulina in spent medium

*Spirulina* was grown using the spent medium in repeated cycles. Biomass production was monitored in different cycles and the results are shown in the Figure 3.12. During the first cycle total biomass production was about 370 mg/L whereas, in the second cycle biomass yield was about 350 mg/L. Significant reduction in the quantity of biomass (300 mg/L) was observed in 3rd cycle onwards and in the 5th cycle productivity was as low as 175 mg/L.

β-carotene content in the biomass obtained from each cycle was also analysed and the results are shown in the Figure 3.13. No significant change in the β-carotene level was observed up to 4th cycle. Between first and fourth cycle, amount of β-carotene ranged from 300 – 375 μg/L. Significantly low level of β-carotene was produced in the fifth cycle compared to first two cycles.

**Fig. 3.12**

Biomass production of *Spirulina* subcultured in spent medium

*Spirulina* cultures in flasks were harvested by centrifugation and 1/5th of the cells were reinoculated into the spent medium and incubated under the same conditions of light (75 μmol m⁻² s⁻¹) and temperature (27°C) for 30 days. Same process was repeated for up to 5th cycle. Biomass content of each passage was determined. Values are the average of three different cultures analysed in duplicate.
**3.3.5.5 Long-term storage of Spirulina**

*Spirulina* stock cultures were grown without sub culturing and under subdued light (25 μmol m⁻²s⁻¹) in Zarrouk’s medium for six months and observed the carotenoid accumulation in the cells. After six months of growth, β-carotene content in the cells was determined and was about 200 μg/g compared to normal culture in flasks, where the average β-carotene content, was only 160 μg/g dry weight. Sporulation of cells and fragmentation of algal filaments were observed after 6 months. Figure 3.1 shows the nature of the algal cells in the sporulated state, which showed yellow pigmentation. These cells were transferred to the half strength Zarrouk’s medium. The sporulated cells were reverted back to the long filaments gradually, in a time span of two months. Figure 3.1 (b,c,d) show the changes in the morphology of the algal filaments, where the β-carotene accumulation in the normal cells were reduced to a level of 150 μg/g dry mass.
3.3.6 Growth of *Spirulina* in $^{14}$C bicarbonate medium

Due to the inherent capacity of this micro algae to accumulate β-carotene in the cells, *Spirulina* was used for the production of $^{14}$[C] labeled β-carotene by growing the algal cells in the medium containing $^{14}$[C]–labeled sodium bicarbonate under different conditions. $^{14}$[C] acclimatized *Spirulina* inoculum was prepared for the development of the culture system as described in the chapter 2. $^{14}$[C] acclimatised algal cells were grown in liquid cultures prepared in Zarrouk’s medium containing $^{14}$[C] labeled sodium bicarbonate. The kinetics of growth was determined and compared with that of normal non-radioactive cultures and the results are shown in the Figure 3.14. Biomass production increased up to 200 mg/L and attained a log phase. Over 350 mg/L biomass obtained, when the cultures were at the stationary phase, which was not significantly different from that in the non-radioactive control cultures.

![Fig. 3.14](image)

**Fig.3.14**

Kinetics of growth of *Spirulina* in $^{14}$[C] bicarbonate containing medium

Suspension cultures of *Spirulina platensis* enriched in $^{14}$[C] bicarbonate cultures was inoculated in Zarrouk’s medium containing $^{14}$[C] sodium bicarbonate (5.0 μCi/ml) and allowed to grow under controlled light (75 μmol m$^{-2}$ s$^{-1}$) and temperature (27°C). Growth of cells was assessed every 5th day. Cells grown in normal water was used for comparison. Values are the average of three different cultures analysed in duplicate.

3.3.7 Production of $^{14}$[C] β-carotene

In the light of the results obtained from the previous experiments metabolic labeling was tried to produce $^{14}$[C] labeled β-carotene with maximum specific activity. Metabolic
Production of Biosynthetically labeled Carotenoids using *Spirulina platensis*

labeling was done by growing *Spirulina* under different intensities of light and under different nutrient conditions like nitrate deficiency and CuSO\textsubscript{4} supplementation and the results are presented in the following sections.

3.3.7.1 *Effect of change in light intensity on labeling intensity*

*Spirulina* culture maintained in Zarrouk’s medium containing \textsuperscript{14}C-labeled sodium bicarbonate were allowed to grow under two different levels of photon flux densities viz., 75 and 100 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} at room temperature for 35 days on an orbital shaker. The biomass production and labeling intensity of \textsuperscript{14}C \(-\) carotene were determined and the results are shown in Table 3.2.

<table>
<thead>
<tr>
<th>Photon flux density density (\textmu mol m\textsuperscript{-2}s\textsuperscript{-1})</th>
<th>Biomass (mg L\textsuperscript{-1})</th>
<th>(\beta)-carotene (\textmu g g\textsuperscript{-1} dry weight)</th>
<th>Total \textsuperscript{14}C incorporation in (\beta)-carotene (cpm \textmu g\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>321.00 ± 1.41</td>
<td>191.26 ± 2.01</td>
<td>65000</td>
</tr>
<tr>
<td>100</td>
<td>203.00 ± 2.94</td>
<td>249.37 ± 2.56</td>
<td>129000</td>
</tr>
</tbody>
</table>

*Spinllina* cultures grown in medium containing \textsuperscript{14}C sodium bicarbonate (5 \textmu Ci/ml) were exposed to light intensities of 75 and 100 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1}. After 35 days, biomass was harvested and \(\beta\)-carotene extracted. Using TLC, extracts were separated and the incorporation of radioactivity into the \(\beta\)-carotene fraction was determined using liquid scintillation counter. Values given are the average of three different cultures analysed in duplicate.

Maximum dry biomass was obtained from \textsuperscript{14}C cultures where a photon flux density of 75 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} was used when compared to the cultures with 100 \textmu mol m\textsuperscript{-2}s\textsuperscript{-1} where 203 mg/L biomass was produced. After fractionation by TLC, \(\beta\)-carotene fraction showed a specific activity of about 1,29,000 cpm \textmu g\textsuperscript{-1} when high light intensity was used, whereas the cultures exposed to 75 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} light, radioactivity in \(\beta\)-carotene fractions was only 65000 cpm \textmu g\textsuperscript{-1}.
3.3.7.2 Effect of NO$_3$ starvation on labeling intensity

Effect of change in nitrate content in the growth medium on the incorporation of $^{14}$C bicarbonate label into the β-carotene was studied and the results are shown in the Table 3.3. Highest biomass and lowest β-carotene production was observed in cultures when sodium nitrate was at a level of 30 mM. Whereas at lower levels, β-carotene content was very high. Total $^{14}$C incorporation into β-carotene was also increased to 135000 cpm μg$^{-1}$. Although the biomass content decreased on reducing Na NO$_3$, there was an increase in β-carotene content.

<table>
<thead>
<tr>
<th>NO$_3$ concentration (mM)</th>
<th>Biomass (mg/L) (Mean ± SD)</th>
<th>β-carotene (μg) (Mean ± SD)</th>
<th>Total $^{14}$C incorporation in β-carotene (cpm μg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>380.3 ± 9.8</td>
<td>150.0 ± 13</td>
<td>48960</td>
</tr>
<tr>
<td>20</td>
<td>345.1 ± 8.9</td>
<td>310.7 ± 7.0</td>
<td>55000</td>
</tr>
<tr>
<td>10</td>
<td>210.7 ± 10.2</td>
<td>295.3 ± 5.6</td>
<td>135000</td>
</tr>
</tbody>
</table>

*Table 3.3 Effect of nitrate starvation on $^{14}$C β-carotene production*

*Spirulina* cultures were grown in Zarrouk's medium containing $^{14}$C sodium bicarbonate (5 μCi/ml) and different concentrations of sodium nitrate ranging from 0.0 to 30 mM. Cultures were kept at 27°C and exposed to light intensity of 75 μmol m$^{-2}$. After 35 days, biomass was harvested and β-carotene extracted. Using TLC, extracts were separated and the incorporation of radioactivity into the β-carotene fraction was determined using liquid scintillation counter. Values given are the average of three different cultures analysed in duplicate.

3.3.7.3 Effect of CuSO$_4$ on labeling intensity

*Spirulina* cultures were grown in the $^{14}$C bicarbonate containing medium supplemented with different concentrations of CuSO$_4$. Table 3.4 shows the results of this experiment. Maximum incorporation of $^{14}$C label into the β-carotene, 125300 cpm μg$^{-1}$ was recorded when $10^{-8}$ M CuSO$_4$ was used. Whereas at $10^{-6}$M level, labeling intensity in the β-carotene fraction was about 86250 cpm μg$^{-1}$ when compared to the control, which incorporated only about 47500 cpm μg$^{-1}$ where no CuSO$_4$ was used.
Production of Biosynthetically labeled Carotenoids using *Spirulina platensis*

Table 3.4
Effect of copper sulphate on $^{14}$[C] $\beta$-carotene production

<table>
<thead>
<tr>
<th>CuSO$_4$ concentration (M)</th>
<th>Biomass (mg/L) (Mean ± SD)</th>
<th>$\beta$-carotene (µg) (Mean ± SD)</th>
<th>Total $^{14}$[C] incorporation in $\beta$-carotene (cpm µg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>450.3 ± 7.8</td>
<td>182.3 ± 2.3</td>
<td>47500</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>405.8 ± 11.0</td>
<td>200.4 ± 14.0</td>
<td>86254</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>425.2 ± 10.1</td>
<td>194.5 ± 10.0</td>
<td>125300</td>
</tr>
</tbody>
</table>

*Spirulina* cultures were grown in medium containing $^{14}$[C] sodium bicarbonate (5µCi/ml) supplemented with different concentrations of CuSO$_4$. Cultures were kept at 27°C and exposed to light intensity of 75 mM m$^{-2}$s$^{-1}$. After 35 days, biomass was harvested and $\beta$-carotene extracted. Using TLC, extracts were separated and the incorporation of radioactivity into the $\beta$-carotene fraction was determined using liquid scintillation counter. Values given are the average of three different cultures analysed in duplicate.

3.3.8 Production of deuterium labeled $\beta$-carotene from *Spirulina*

Apart from the radioactive $^{14}$[C] labeled carotenoids produced from the *Spirulina*, non-radioactive, stable isotopic labeling was also done. Various factors of biomass and $\beta$-carotene production and labeling intensity were studied and the results are presented in the following sections.

3.3.8.1 Development of D$_2$O acclimatized inoculum and growth kinetics

Algal cells from the stock cultures in agar slants were used to develop deuterium acclimatized inoculum for the mass production in deuterated water. Serial sub culturing in the deuterium-containing medium was done. Fast growing cultures were evolved in fully deuterated medium which, provided inoculum for all subsequent experiments.

The kinetics of biomass production was studied and the results are presented in the Figure 3.15. *Spirulina* culture grown in normal water medium was kept as control. There was a lag phase up to 10 days, on 15$^{th}$ day, algae grown in normal water attained steady log phase with average biomass production of 200 mg/L, while only 100 mg/L of biomass was produced by the D$_2$O culture. Biomass obtained was about 150 mg/L from the D$_2$O culture compared to 250 mg/L by the normal culture at the stationary phase.
Suspension cultures of *Spirulina* enriched in D$_2$O were inoculated in D$_2$O enriched medium in flasks and allowed to grow under controlled light (75 μmol m$^{-2}$s$^{-1}$) and temperature (27°C). Growth of cells was assessed every 3 days. Cells grown in normal water were used for comparison. Values given are the average of three different cultures analysed in duplicate.

3.3.8.2 Biomass and β-carotene production in different cycles and use of spent medium

In order to compensate lag in the biomass production of the deuterated cultures and for complete utilization of costly label in the medium, use of spent medium was tried in different cycles. Figure 3.16 (A) shows the biomass productivity at different cycles. Dry biomass production was found to be stable until 5$^{th}$ cycle in the range of 140-145 mg/L and then decreased to 100 mg/L at 6$^{th}$ cycle.

Biomass isolated from different cycles was extracted and carotenoids were analysed. Figure 16 (B) shows the total β-carotene obtained from the biomass of each cycle. β-carotene content was not significantly different up to 6$^{th}$ cycle and was in the range of 141-160 μg. But it was significantly reduced in the 6$^{th}$ cycle (p<0.05) when compared to 1$^{st}$ cycle. *Spirulina* cultures were grown in D$_2$O containing medium and labeling efficiency was studied by controlling the atmospheric air.
**Fig. 3.16**

Biomass and \( \beta \)-carotene content of *Spirulina* subcultured in spent \( D_2 O \) medium in different cycles

![Graph showing biomass and \( \beta \)-carotene content](image)

*Spirulina* cultures in flasks were harvested by centrifugation and \( 1/5 \)th of the cells were reinoculated into the spent medium and incubated under controlled conditions of light (75 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and temperature (27°C). Same process was repeated for up to 6th cycle. Biomass content of each passage was determined after 28 days. Biomass (A) was collected and \( \beta \)-carotene (B) was analysed by using HPLC. Values are the average of three different cultures analysed in duplicate. (p<0.05).

### 3.3.9 Analysis of labeled carotenoids

Biomass obtained from the different cycles of growth was subjected to solvent extraction and analysis. Individual carotenoids were separated using liquid chromatography and the results are presented in the Table 3.5. Lutein and trans-\( \beta \)-carotene formed the predominant carotenoids. Concentration of individual carotenoids varied with respect to different cycles of the growth. Percentage of lutein ranged from...
27-36% and that of trans-β-carotene was about 15-25%. The mean trans-β-carotene content over six cycle was about 19.5% and that of lutein was 30.1%. The stable isotope incorporation was assessed using mass spectrometer.

Table 3.5
Relative amounts of different carotenoids in *Spirulina* grown in D$_2$O enriched medium in different cycles

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>No. of cycles</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Lutein</td>
<td>27</td>
<td>31</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>trans-β-carotene</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>9-cis-β-carotene</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>a-carotene</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>3-cis-β-carotene</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Unidentified</td>
<td>37.5</td>
<td>37.0</td>
</tr>
</tbody>
</table>

*Spirulina platensis* were grown in D$_2$O enriched medium in different cycles for 28 days and the biomass was analysed for various carotenoids using liquid chromatography. Analysis was done in triplicate and the mean values were expressed as percentage of total amount of carotenoids.

### 3.3.9.1 Liquid chromatographic analysis

Liquid chromatographic analysis, of extracts of biomass obtained from different D$_2$O cultures were done. Extracts of carotenoids were separated into different components. Figure 3.17 shows the chromatographic pattern of carotenoids extracted from pooled biomass of *Spirulina* grown under open conditions in D$_2$O medium. Peaks identified as lutein, zeaxanthin, cryptoxanthin, 13-cis-β-carotene, α-carotene, trans-β-carotene, and 9-cis-β-carotene, eluted at different retention times. From the peak areas, percentage of each carotenoid fraction was computed.

*Spirulina* biomass obtained from the cultures grown under closed conditions where exchange of moisture was prevented, was pooled and extracts were subjected to liquid chromatography. The elution pattern is shown in Figure 3.18 where trans-β-carotene was about 18%. For further analysis, trans-β-carotene fraction obtained from the LC was passed on to mass spectrometer for measuring the stable isotope abundance of deuterium incorporation.
Production of Biosynthetically labeled Carotenoids using *Spirulina platensis*

Fig. 3.17

Liquid chromatographic analysis of carotenoids of *Spirulina* grown in D$_2$O medium under open conditions

Carotenoids were isolated from *Spirulina* grown, in the cultures where atmospheric exchange was allowed and subjected to liquid chromatography. Elution patterns of different carotenoids are shown. Lutein(1), Zeaxanthin(2), Cryptoxanthin(3), 13-cis-β-carotene(4), α-carotene(5), trans-β-carotene(6) and 9-cis-β-carotene(7) was eluted in 8, 9, 15, 17, 18, 20 and 22$^{nd}$ minutes respectively.

Fig. 3.18

Liquid chromatographic analysis of carotenoids of *Spirulina* grown in D$_2$O medium under closed conditions

Carotenoids were isolated from *Spirulina*, grown in the cultures where atmospheric moisture exchange was not allowed, subjected to liquid chromatography. Elution patterns of different carotenoids are shown. Lutein(1), Zeaxanthin (2), 13-cis-β-carotene (3), α-carotene (4), trans-β-carotene (5) and 9-cis-β-carotene (6) was eluted in 8, 9, 17, 18, 20 and 21$^{st}$ minutes respectively.
3.3.9.2 Mass Spectrometric analysis

Replacement of H by deuterium in the β-carotene synthesized by *Spirulina* grown in heavy water medium was analysed by mass spectrometric analysis. Mass to charge ratio (m/z) analysis of deuterated β-carotene obtained from the *Spirulina* cultures grown in open conditions are shown in the figure 3.19 (A) where the m/z ratio were shifted from its original position (534) to a range of 545 to 575 indicating 40-60% replacement of H atoms with $^2$H in the C$_{40}$H$_{56}$ molecule of β-carotene when compared to the normal unlabeled β-carotene as shown in the (Figure 3.19B).

![Mass Spectrometric analysis of deuterated β-carotene of *Spirulina* grown in D$_2$O medium in flask cultures under open conditions](image)

Carotenoids isolated from the cells grown in D$_2$O enriched medium, where atmospheric exchange was allowed were subjected to LC-MS analysis. The relative abundance of labeled β-carotene shows a shift in m/z ratio from 534 to a range of 545 to 575 indicating the replacement of about 40 to 60 % of H atoms with $^2$H (A), compared to unlabeled molecule C$_{40}$H$_{56}$ (B).
Similarly, mass spectrometric analysis of $\beta$-carotene extracted from the algal cells grown in the closed conditions was done. Figure 3.20 A shows deuterium enrichment up to 99% with a shift in m/z ratio from 534 to 595 compared to the unlabeled normal $\beta$-carotene with m/z ratio of 534 as shown in the Figure 3.20 B.

Carotenoids isolated from the cells grown in D$_2$O enriched medium, where atmospheric exchange was not allowed were subjected to LC-MS analysis. The relative abundance of labeled $\beta$-carotene shows a shift in m/z ratio from 534 to 594 indicating the replacement of about 100 % of H atoms with $^2$H (A), compared to unlabeled molecule C$_{40}$H$_{80}$ (B).
3.3.10 Design of a laboratory scale bioreactor

In order to increase the volume of biomass and labeled carotenoid production, a culture system was developed. Methods were standardized to scale up *Spirulina* culture using a bioreactor. Figure 3.21 shows design of mini laboratory scale bioreactor.

![Schematic outline of bioreactor](image)

Schematic outline of the bioreactor showing cylindrical body of volume 1.0 liters and movable light source with cool white fluorescent lamps on either side and 5% CO$_2$ in air was bubbled. Magnetic stirrer was used to agitate the culture inside the bioreactor and moisture trap for preventing atmospheric exchange of deuterium is attached.

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
Production of Biosynthetically labeled Carotenoids using *Spirulina platensis*

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 (4x 20 W) fitted on a movable stand, which in turn provided appropriate photosynthetic flux density. It was tested for its potential under different culture conditions. The reactor was used for the culture of *Spirulina* in Zarrouk’s medium prepared in normal water and in D$_2$O. 5% CO$_2$ in air was supplemented to increase the biomass production.

### 3.3.10.1 Growth studies-biomass production in bioreactor

Biomass production was studied using normal water by growing the *Spirulina* cultures in bioreactor and flask cultures were kept as control. The kinetics of the biomass production in the bioreactor and in flask cultures is shown in the Figure 3.22. Result shows that in the growth cycle, a lag phase of 15 days was observed in bioreactor and in flask cultures. Significantly more biomass (350 mg/L) was produced, when *Spirulina* was grown in the bioreactor compared to that in the flasks in the stationary phase (320 mg/L).

**Fig 3.22**

**Kinetics of growth of *Spirulina* in, bioreactor in normal water**

Suspension cultures of *S. platensis* were allowed to grow under controlled light and temperature in culture flasks and in bioreactor. Dry biomass production was assessed every five days. Cells grown in flasks were used for the comparison. Values given are mean ± SD (n=3)
The bioreactor was used for the culture of *Spirulina* in Zarrouk’s medium prepared in D$_2$O for the production of intrinsically labeled β-carotene. D$_2$O cultures were developed in the bioreactor and in flasks, for comparison. Figure 3.23 shows kinetics of biomass production in bioreactor and in flasks. Biomass production and growth of algae in the reactor was found to be high when compared to that in flask culture. There was a lag period in growth of the cells in both treatments. On the 35th day, the reactor produced an average dry biomass of 300 mg/L whereas in the flask cultures, the biomass content was about 180 mg/L in the deuterated medium. Different stages of growth of the algae in bioreactor is shown in the Figure 3.24 (a,b,c).

**Fig 3.23**  
Kinetics of growth of *Spirulina* in bioreactor using deuterated water medium

Suspension cultures of *Spirulina platensis* were allowed to grow under controlled light and temperature in culture flasks and in bioreactor. Dry biomass production was assessed every five days. Cells grown in flasks were used for the comparison. Values given are mean ± SD (n=3).

3.3.10.2 Repeated use of spent D$_2$O medium in bioreactor

In order to maximize the utilization of the costly deuterium label, the reuse of spent heavy water medium was tried. The spent D$_2$O medium was used in repeated cycles and effectiveness in biomass and β-carotene production was studied. Figure 3.25 shows the kinetics of biomass production. The quantity of biomass produced was not affected up to 4th cycle. Mean dry biomass production was about 360 mg/L in the first cycle. However, the amount of biomass produced was less in the 6th passage when compared to 1st and 5th cycle. Labeling intensity in the β-carotene obtained from the different cycles was also determined separately using LC-MS.
Legends to Figure 3.24

*Spirulina* in bioreactor

*Spirulina* cultures in Zarrouk’s medium, different stages of *Spirulina* cultured in bioreactor, at lag phase (a), log phase (b) and stationary phase (c).
3.3.11 Analysis of carotenoids

Biomass obtained from different cycles of growth was extracted and carotenoids were separated using liquid chromatography. Mass spectrometric analysis was done for assessing labeling intensity.

3.3.11.1 Liquid chromatographic analysis

Algal cells grown under optimum conditions of light (75 μmol m$^{-2}$s$^{-1}$) and nutrient in bioreactor were harvested and the components of total carotenoids were analyzed using LC. Chromatographic pattern of analysis of carotenoids from cultures in bioreactor in D$_2$O medium is shown in the Figure 3.26. Lutein and trans-β-carotene were the prominent carotenoids. Table 3.6 shows the relative amounts of different carotenoids present in the *Spirulina* grown in D$_2$O medium in bioreactor. The mean trans-β-carotene content was about 18.8%; α-carotene 0.86%; 13-cis- β-carotene 1.0%; 9-cis-β-carotene 4.1%; cryptoxanthin 1.3% zeaxanthin 9.2% and lutein 29%.
Carotenoids were isolated from *Spirulina* biomass and subjected to liquid chromatography. Elution patterns of different carotenoids are shown. Lutein (1), Zeaxanthin (2), Cryptoxanthin (3), 13-cis-β-carotene (4), α-carotene (5), trans-β-carotene (6) and 9-cis-β-carotene (7) were eluted in 8.4, 10.1, 15.3, 18.9, 19.4, 20.9 and 21.8 minutes respectively.

### Table 3.6

**Relative quantities of different carotenoids in *Spirulina platensis* grown in D₂O enriched medium in different cycles in bioreactor**

<table>
<thead>
<tr>
<th>Cycles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total carotenoids (μg g⁻¹ dry mass)</td>
<td>740</td>
<td>670</td>
<td>560</td>
<td>590</td>
<td>525</td>
<td>512</td>
</tr>
<tr>
<td>Individual carotenoids (as % total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lutein</td>
<td>27</td>
<td>31</td>
<td>29</td>
<td>36</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>10</td>
<td>8</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>13-cis-β-carotene</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>α-carotene</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>trans-β-carotene</td>
<td>18</td>
<td>17</td>
<td>21</td>
<td>21</td>
<td>17</td>
<td>13.0</td>
</tr>
<tr>
<td>9-cis-β-carotene</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>3.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*Spirulina platensis* cultures were grown in deuterium enriched medium in 6 cycles in bioreactor for 40 days each. The biomass was harvested extracted and analysed for various carotenoids using LC. Analysis was done in triplicate and the mean values were expressed as percentages of the total amount of carotenoids.
3.3.11.2 Mass spectrometric analysis

Separated carotenoid fractions were passed on to MS system for determining the labeling intensity of the β-carotene molecule by replacement of H by deuterium. MS pattern of β-carotene isolated from the *Spirulina* under optimum conditions of growth is presented in the Figure 3.27. Replacement of [H] atom in the β-carotene in terms of charge to mass ratio is about 40-60%, as evidenced from the shift in the m/z ratio from 534 to a range of 555 to 570. Mass spectrum of β-carotene extracted from *Spirulina* grown in D₂O in bioreactor when compared to unlabeled β-carotene with m/z ratio of 534.

![Fig3.27](image)

**Fig 3.27**

**Mass Spectrometric analysis of deuterated β-carotene of *Spirulina* grown in D₂O medium in bioreactor**

Carotenoids isolated from the cells grown in bioreactor in D₂O enriched medium were subjected to LC-MS analysis. The relative abundance of labeled β-carotene shows a shift in m/z ratio from 534 to 565 indicating the replacement of about 60% H atoms with ²[H] (A) compared to unlabeled (B) C₄₀H₆₆. 
3.4 Discussion

*Spirulina* is a blue-green algae which usually grows in warm temperature, especially in tropical regions and is reported to grow abundantly in alkaline waters in salty lakes (Farrar, 1966; Ciferri, 1983). It has very high nutritive value owing to the presence of high content of proteins with the essential amino acids and several other micronutrients. Apart from important pigments like chlorophyll, xanthophylls and phycocyanin, *Spirulina* has a carotene content 10 times that of carrots and iron content 12 times that of any other food and is the richest vegetarian source of vitamin B<sub>12</sub>. *Spirulina* has been easily incorporated in medical formulations owing to its excellent nutrient composition (Becker, 1994). There are different varieties of *Spirulina* with varying content of carotenoids. *Spirulina platensis* and *S. fusiformis* contain significant amount of carotenoids. In the present study *S. platensis* has been used to prepare biosynthetically labeled deuterated provitamin A carotenoids. *Spirulina* containing intrinsically labeled provitamin A carotenoids can be used to study the influence of various factors on the absorption, bioavailability and bioconversion of provitamin A carotenoids.

Conditions for the optimal growth of *Spirulina* have been standardized by growing the algae in normal water in flask cultures for maximum production of β-carotene. Analysis of carotenoids showed that *Spirulina* contain *trans*-β-carotene, 9-*cis*-β-carotene, 13-*cis*-β-carotene, α-carotene, lutein, cryptoxanthin and zeaxanthin. Of these carotenoids, *trans*-β-carotene formed about 21.8% and 9-*cis*-β-carotene about 6.6% and 13-*cis*-carotene about 12.7%. Significant fraction of lutein was also obtained. Ability of this alga to produce carotenoids, especially β-carotene depends on various conditions. Several physical and chemical agents affect carotenogenesis in a number of systems (Goodwin, 1980). A number of factors are known to affect growth, biomass production and carotenoid production. Our objective was to optimize conditions for maximum production of carotenoids particularly β-carotene under conditions where biomass production is maximum. Different methods like effects of light, nitrate starvation and CuSO<sub>4</sub> were used to induce β-carotene production using unlabeled medium in flask cultures. Of the different methods adopted, few treatments were selected based on the optimum biomass and β-carotene production and utilized further in ¹⁴C and ²H β-carotene labeling using bioreactor.

Experiments on light induced production β-carotene showed that at higher light intensities, about three to fourfold increase in β-carotene occurred. Results of this
Production of Biosynthetically labeled Carotenoids using Spirulina platensis

Experiment showed that light intensity of 100 μmol m⁻² s⁻¹ was ideal for optimum biomass and β-carotene production. Cultures exposed to high light intensity produced about 330 mg/g dry biomass and 200 μg β-carotene, during 30 days of growth and then gradually reduced. Vonshak (1987) reported that Spirulina with high light saturation values showed remarkable production rates when grown outdoors and indicated that light is one of the critical rate limiting factor.

Present study also investigated the possibility of using varying nitrogen concentrations in the culture medium aimed at the overproduction of carotenoids. At the normal concentration 30 mM, in the Zarrouk’s medium, the level of biomass production was high with less β-carotene production. But highest β-carotene production of 250-325 μg/L was recorded when very low sodium nitrate was used in the medium. Reason for carotenoid over production may be due to the increased formation of amino acids at the expense of sucrose synthesis. Similar results have been obtained with isolated mesophyll cells of Papaver somniferum. In addition to this, effect of the CuSO₄ on the production of the carotenoids was also undertaken. Results indicate that Cu salt even at very low concentration, 10⁻³ to 10⁻⁸ M, affected normal growth as well as the biomass accumulation. β-carotene present in unit dry biomass was very high at the concentration of 10⁻⁸ and 10⁻⁶ M. Similar reports are available wherein a variety of substances, like antibiotics, ionones, amines, alkaloids and terpenes have been studied for their effect on carotene synthesis. Govind et al. (1982) demonstrated that in Blakeslea trispora, metal ions especially Cu ions in stimulated the production of carotenoids. These results indicate that culturing Spirulina in the presence of sodium nitrate at 10-20 mM concentration and supplementing very low level of CuSO₄ (10⁻⁸M) and a light intensity of 75-100 μmol m⁻² s⁻¹ could produce optimum biomass and maximum carotenoids.

Possibility of using the spent medium was also standardised using normal water for the maximum utilization of labeled precursors. The results showed that same medium can be used even up to 6th cycles with out affecting the biomass and β-carotene production indicating that the medium once prepared was nutritionally sufficient to maintain the growth. These results are useful in the optimum utilization of labeled precursors. These standardised conditions for overproducing β-carotene is useful in the production of labeled β-carotene. Various methods have been proposed for the chemical synthesis of ¹⁴C labeled β-carotene. The disadvantages of these procedures include the fact that the triphenyl-derived triphenyl phosphonium salt is not water soluble, thus making it...
difficult to isolate and also triphenylphosphine reactant required for the synthesis is relatively expensive (Azim et al., 1996). Hence, present study attempted to produce intrinsically labeled β-carotene using *Spirulina* culture system. The results show that besides fairly good biomass production (203 mg/L) total $^{14}$C incorporation into β-carotene was about 129000 cpm μg⁻¹ when *Spirulina* was exposed to high light intensities in culture. Whereas, under nitrate starvation, $^{14}$C labeling rate was about 55000 to 135000 cpm μg⁻¹ when sodium nitrate was in a range of 20 to 10 mM. In the case of copper sulphate induced β-carotene production, highest biomass and labeling intensity was recorded when $10^{-8}$ M CuSO₄ was used. This indicates that these methods can effectively be used in the production of intrinsically labeled $^{14}$C β-carotene.

Similarly, $^2$H β-carotene was produced using *Spirulina* culture system. For this purpose, growth conditions of the algae in deuterated water were standardized. The results showed that there is a marked effect on growth in D₂O when *Spirulina* is transferred from H₂O medium to D₂O medium. A lag phase up to 10 days was observed and biomass also reduced significantly when compared to that of H₂O culture. Tropis et al. (1996) also reported very long lag phase of *Spirulina maxima* and demonstrated that the adaptation of the cells to D₂O did not imply any profound modification of the lipid composition. Therefore small scale algal cultures were done in deuterated water in flasks and such cultures were used as stock for developing further cultures.

In order to save the costly label, a study on the reuse of spent medium was tried. The result indicates that up to 6th cycle, spent medium can be used without much reduction in the biomass. These cultures were used in the production of deuterated carotenoids. Liquid chromatographic analysis of the carotenoids isolated from the cultures grown at different cycle shows that β-carotene production remained almost same in the range of 15 to 25%. Increased production of β-carotene at later cycles may be attributed to nutritional stress especially nitrates as described earlier.

Carotenoids isolated from *Spirulina* grown in D₂O enriched water was subjected to LC-MS analysis to determine the extent of deuterium incorporation into β-carotene. MS data showed 60-70% replacement of hydrogen by $^2$H atoms as indicated by the molecular mass cluster at around m/z 554. LC-MS analysis of carotenoids isolated from cells grown in bioreactor under conditions where exchange with atmospheric moisture was completely prevented, almost all H atoms of the β-carotene were found to be
Production of Biosynthetically labeled Carotenoids using *Spirulina platensis* replaced by deuterium, indicating 100% incorporation. The ability to control the isotopic spectrum of β-carotene produced by this culture system has great benefit for the production of tracers for nutritional studies.

After the standardization of small-scale production of deuterated carotenoids in flasks, a mini laboratory scale bioreactor was designed and tested, for scaling up the production of biomass and labeled compounds. Productivity of the bioreactor was tested by growing the algae up to 40 days and compared it with growth in flasks. Comparatively high biomass was obtained from the bioreactor culture. The difference in biomass production may be due to the high rate of photosynthesis, uniform distribution of algal cell and temperature stability in the bioreactor. This indicates that the proposed bioreactor is more productive than the conventional cultures in flasks. As the bioreactor was designed for the production of intrinsically labeled β-carotene, *Spirulina* growth was monitored and the biomass production was found to be considerably increased when compared with heavy water cultures in conical flasks. It is one of the advantages of this bioreactor where high biomass production may lead to increased label incorporation.

Further optimization of biomass production was achieved by bubbling CO₂-air mixture, as supplementing CO₂ was known to increase the production of biomass (Watanebe and Hall, 1996). Results of the liquid chromatographic analysis showed that, among the individual carotenoids, β-carotene formed about of 20-30%. Changing the growth conditions in the bioreactor can further modify this. This indicates that the proposed bioreactor may be useful for algal culture where high productivity is required for the metabolic labeling. Results of mass spectrometric analysis, demonstrated over 60% incorporation of deuterium into β-carotene produced by algal cells as evidenced from the shift of m/z ratio to 566 compared to the unlabeled β-carotene. The statistics of random incorporation of deuterium label into the β-carotene molecule would predict a binomial distribution for the spectrum for a constant proportion of label in the precursor (Wilson *et al.*, 1997). Unique mass spectra of labeled isotopomer of β-carotene produced from the different conditions of algal growth would provide another advantage as it can be used as an independent label in same experimental system. The proportion of each isotopic species in an experimental sample can be differentiated by using mass spectrometry. Each isotopomer can be used in two ways (a) to label different components of same diet to evaluate their relative absorption in same experiment avoiding the confounding effects. (b) Each isotopomers can be used as internal standard for measurements of β-carotene by mass spectrometry.
Results of mass spectrometric analysis demonstrate over 60% incorporation of deuterium into the β-carotene produced by algal cells as evidenced from the shift of m/z ratio to 566 compared to unlabeled β-carotene. High level of the label incorporation is essential for tracer analysis studies in human intervention trials where only very little amount of the β-carotene may be recovered from the blood samples following a feeding experiment (Wilson et al., 1997). While using heavy water in deuterium incorporation studies, one of the problems is the dilution of heavy water with moisture. This may lead to lower deuterium incorporation. One approach to limit this is to use closed system where exchange with moisture from atmosphere can be used. LC-MS analysis of β-carotene from flask cultures maintained under conditions were moisture exchange was limited, showed that the exchange by deuterium was maximum. Similarly the bioreactor was fitted with moisture trap to limit moisture exchange and the LC-MS analysis of β-carotene prepared from such cultures showed 100% exchange of H of β-carotene by deuterium.

These results indicate that the reactor design is highly useful in the production of provitamin A carotenoids. Earlier, Wilson et al. (1997) reported a bioreactor for the production of $^{13}$[C] β-carotene using Spirulina where photosynthetic O$_2$ generation was a major factor which is related with the total biomass production. This reactor system is either complex or expensive due to nature of precursors used. In our bioreactor system, we report the production of deuterated compounds with over 99.0% replacement in β-carotene molecule when compared to the earlier one which used gaseous precursor. Growth of the Spirulina in heavy water in bioreactor permits the metabolic labeling of provitamin A carotenoids and the intrinsically labeled β-carotene can be isolated. Further the extend of labeling can be modified by adopting different culture conditions especially exclusion of moisture which is useful for the production of highly enriched labeled molecule, The relatively good yield of deuterated provitamin A carotenoid particularly β-carotene from the Spirulina cultures produced in mini bioreactor makes it a useful system for producing deuterated β-carotene for bioavailability studies. The deuterated β-carotene also can be used for the evaluation of vitamin A/ carotenoid status in intervention studies. Repeated use of spent medium in reactor reduces the cost of production. Spirulina cultured in deuterated medium is a rich source of carotenoids other than β-carotene. In addition to this, the algal cultures grown in heavy water may be used as a source of deuterated macromolecules like proteins and lipids which may be unique in their physical properties due to deuterium substitution. It is also important to note that this reactor
Production of Biosynthetically labeled Carotenoids using Spirulina platensis

System has the option to control the degree of isotopic labeling by moisture exclusion. Different levels of isotopic substitution can be possible and each isotopomer with a specific m/z ratio would be useful in tracer analysis studies.

Vitamin A deficiency is one of the public health problems all over the world. Potential importance of vitamin A makes it essential to know more about its absorption, transport and distribution in tissues. Previous studies of the intestinal absorption, transport and uptake into tissues of vitamin A in vivo have relied upon the use of either radiolabeled retinol or large doses of the unlabeled vitamin A. The limitation imposed by these two methods, namely, the hazards associated with the use of radiolabeled substances or the disadvantages of using pharmacological doses have severely affected the progress in obtaining useful information concerning the behaviour of vitamin A in vivo. These problems have now been circumvented with the development of a method using deuterium-labeled vitamin A in conjunction with gas chromatography-mass spectrometric (GC-MS) measurements. The deuterated-retinol-dilution (DRD) technique provides a quantitative estimate of total body stores of vitamin A. However, it is not known whether the technique can detect changes in vitamin A pool size in response to different intake of vitamin A (Haskell et al., 1999). The results show that the Spirulina grown in D₂O enriched water, apart from being a source of deuterated compound also will be useful to study the bioavailability of provitamin A.