Biotechnology Letters, Vol 19, No 5, May 1997, pp. 401–405

Production of ^{13}C-labelled β-carotene from Dunaliella salina

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A 4.5 litre photobioreactor was developed and used to produce ^{13}C-labelled β-carotene by feeding $^{13}CO_2$ to growing cells of the marine alga Dunaliella salina. Three runs were carried out using different feeding regimes producing β-carotene containing 40, 56 and 76 Atom% ^{13}C. Approximately 145 mg β-carotene was recovered from each run. Incorporation of ^{13}C into β-carotene ranged from 1.4 to 2.8%. The labelled β-carotene produced would allow multiple tracer experiments for the study of human carotenoid metabolism.

Introduction

Dietary carotenoids are attracting a great deal of interest regarding the protective effect they may afford (see, for example, Mayne, 1996). In the study of the mechanisms and kinetics of uptake from the diet, a labelled tracer is of great use as the measurement of tracer enrichment allows the determination of absorption and clearance kinetics against a background concentration which may change little following the administration of a nutrient dose. Whilst radioactive tracers provide more sensitivity and easier detection, stable isotopes offer a safe means of labelling tracers for use in human studies. This paper reports the production of ^{13}C-labelled β-carotene by cultivation of the marine alga *Dunaliella salina*. This organism accumulates large amounts of β-carotene in response to the stress of nutrient depletion and high light intensity (Shaish *et al.*, 1993). As it is photosynthetic, it offers the opportunity of incorporating labelled carbon into its metabolic products by feeding with labelled CO_2. This may be readily produced from labelled $NaHCO_3$ which is easy to handle and is available at a much lower cost than many labelled organic compounds such as glucose. In order to make most efficient use of the labelled precursor in the bioreactor system, a gas recycling regime was employed during periods of illumination when photosynthesis was active. In order to prevent the build-up of excessive concentrations of dissolved O_2 produced by photosynthesis, an external O_2 sink (acidified water) was used.

Materials and methods

Organism

D. salina (CCAP 19/18) was obtained in liquid culture from the Culture Collection of Algae and Protozoa, CCAP (Oban, Scotland) and maintained on ASW medium (Artificial Sea Water, CCAP) by serial subculture every 10 days. The stock cultures were maintained at 20°C under illumination levels of 150 μmol photosynthetically active photons $m^{-2}s^{-1}$ (LI-190SA Quantum Sensor, with LI-1000 data logger, LI-COR Inc., Nebraska, USA).

Extraction of carotenoids

Cell suspension was harvested from the bioreactor and centrifuged to concentrate the algae to about 250 ml. Aliquots of the concentrate were mixed with an equal volume of methanol:tetrahydrofuran (1:1 w/w) to liberate the carotenoids from the cells. This mixture was then extracted with hexane, the carotenoids partitioning into the non-polar phase. The high biomass concentration hindered the breakage of the emulsion formed during extraction, and it was necessary to add more methanol to the aqueous phase to reduce the viscosity. The hexane extracts were pooled and butylated hydroxytoluene added to a final concentration of 0.1% (w/v) as an antioxidant. This extract was stored in the dark at 4°C before analysis.

Analysis of carotenoids

Carotenoids were analysed by HPLC (Hart and Scott, 1995) for purity and *9-cis : all trans* ratio, and by spectrophotometry (450 nm, 1 cm pathlength in hexane, $E^{1\%}=2560$) for total yield. Samples were also analysed by low resolution electron ionisation mass spectrometry (MS890 with DS-90 data system, Kratos, Manchester, UK) for determination of ^{13}C labelling. The β-carotene was vaporised into the mass spectrometer ion source using a heatable probe, programmed from 60 to 500°C at a rate of 40°C/min. The ion source temperature was held at a constant 250°C.

Bioreactor design

A photobioreactor was constructed using a cellular polycarbonate sheet joined by a flange at the top to a bubble

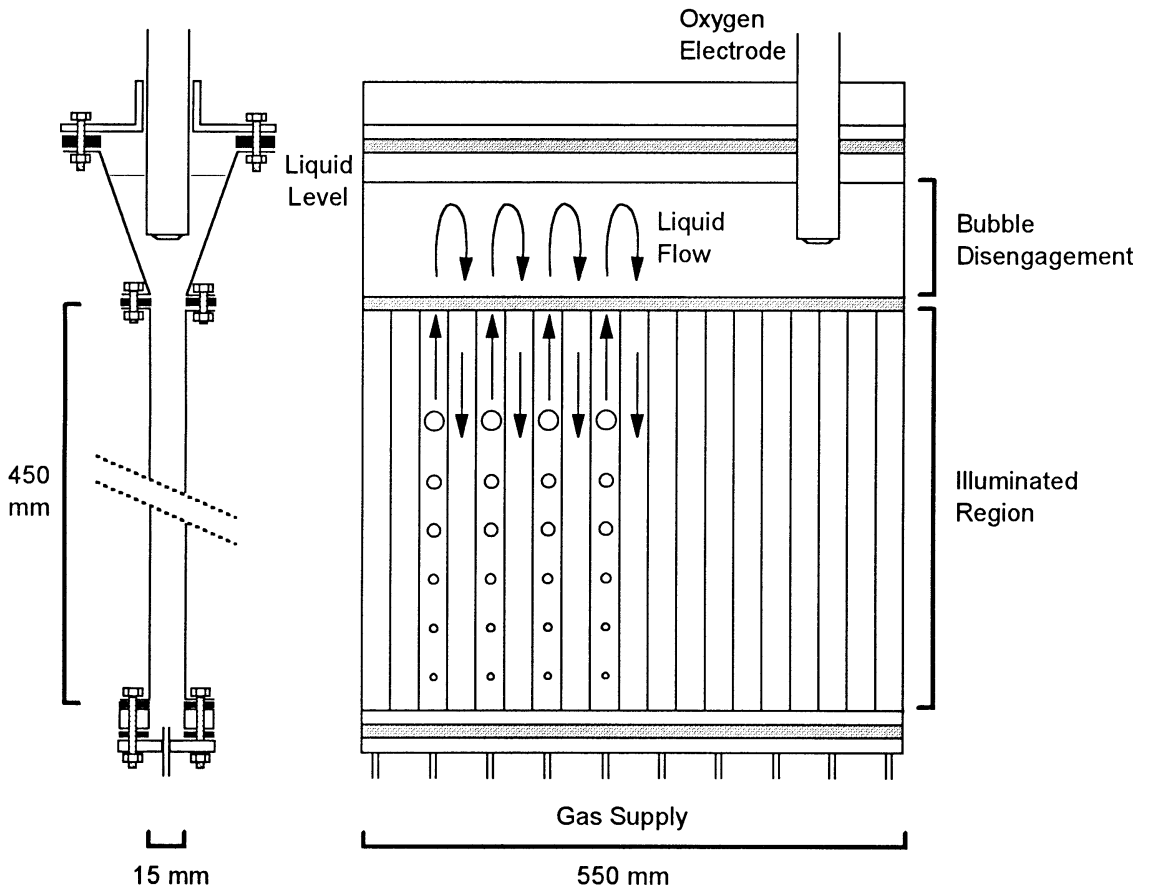

Figure 1 Schematic diagram of algal photobioreactor.

disengagement unit and instrumentation plate and at the bottom to a sparge unit. The reactor has a working volume of 4.5 litres. The reactor design is illustrated schematically in Figure 1.

Gas was introduced in alternate sections of the polycarbonate sheet to induce the flow pattern illustrated – effectively that of a multiple channel bubble column. The reactor was illuminated from one side with a Metal Halide lamp (Osram Powerstar 400 HQI T W/D) producing a photosynthetic photon flux density varying between 300 and 900 μmol $m^{-2}s^{-1}$ at the face of the reactor. The average photon flux density was estimated to be 500 μmol $m^{-2}s^{-1}$. A tank of water (path length 20 cm) was placed between the lamp and the reactor to absorb some of the infra-red radiation and thus mitigate temperature rises in the vessel. During the dark period, an air/$^{12}CO_2$ mixture was sparged through the growth vessel and vented to atmosphere. During the light period, gas was recycled through the growth vessel via an oxygen sink (10 litres of water acidified to pH 2.2 with 4.2% w/v citric acid, to absorb O_2 but not CO_2). Immediately prior to the light period, this oxygen sink was stripped of oxygen by gassing with nitrogen to increase the sink capacity. The recycled gas was supplemented by $^{13}CO_2$ produced by dosing $NaH^{13}CO_3$ solution into 1M HCl. A schematic diagram showing the layout of the cultivation systems is given in Figure 2, with gas flows indicated for the light period.

Three bioreactor runs were carried out (Runs 1–3). In the first run, using a 12 h light:12 h dark photoperiod, 0.7 g/day of $NaH^{13}CO_3$ was fed between days 5 and 23, followed by 1.4 g/day between day 23 and the harvest of the cells on day 30. The growth medium was double strength Artificial Sea Water medium (ASW) with soil extract (CCAP, Oban, Scotland). The second and third runs were carried out using a 16 h light:8 h dark photoperiod and triple strength ASW with soil extract. In the second run labelled carbonate was added

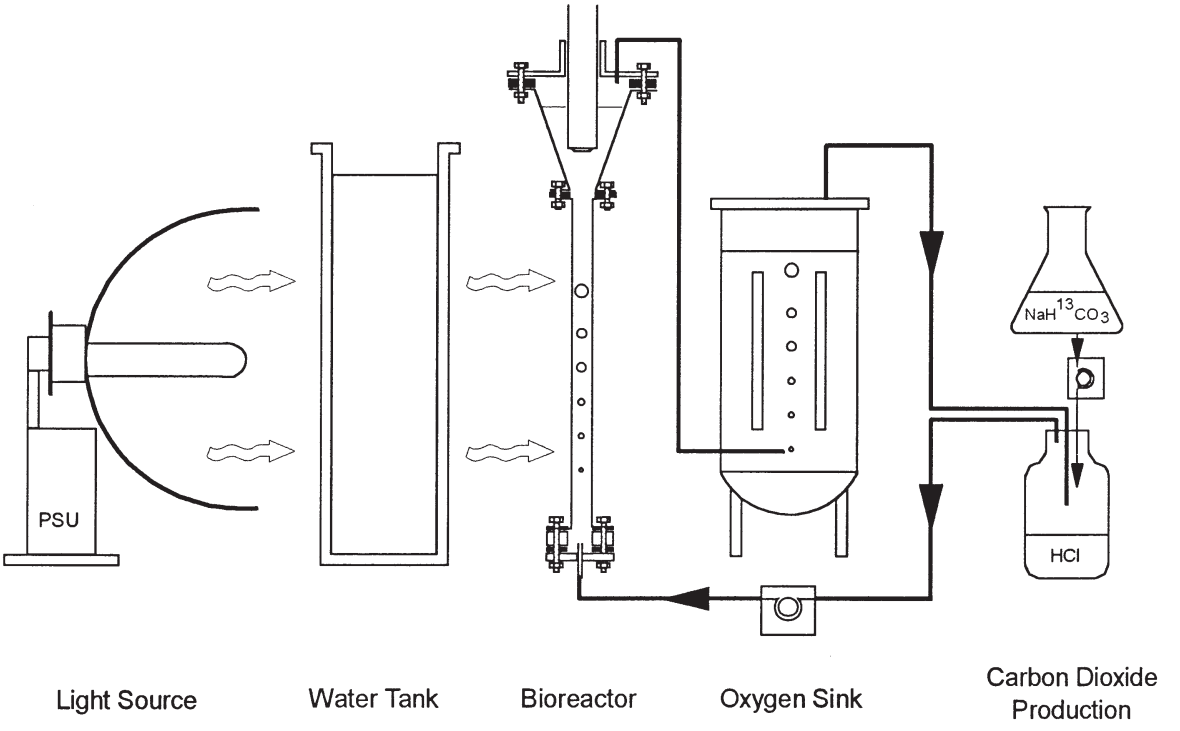

Figure 2 Schematic diagram of algal cultivation system.

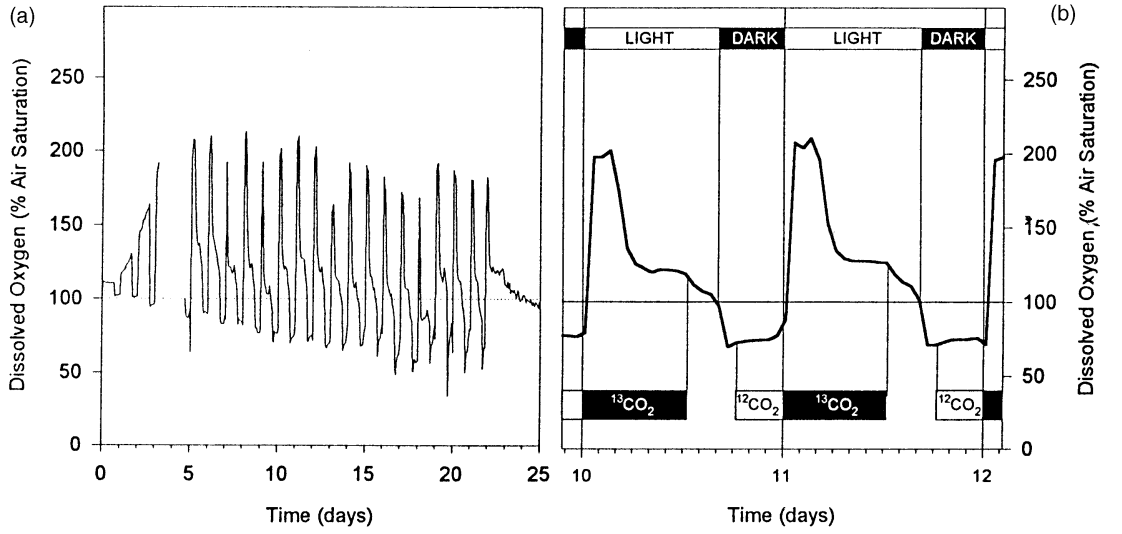

Figure 3 (a) O_2 time course for Run 2. (b) Detail of diurnal cycle.

throughout the cultivation at a rate of 0.7 g/day for 6 days, and then increased to 1.4 g/day until harvest after 25 days. In the third run, labelled bicarbonate was added at a rate of 1.4 g/day from day 3 until harvest at day 21. The dissolved oxygen concentration in the bioreactor was measured using a polarographic oxygen electrode. The oxygen time course was similar in all cases, and that for Run 2 is shown in Figure 3.

Table 1 Results from cultivations of Dunaliella salina

	Total β-carotene Produced (mg)	Cis:Trans Ratio	^{13}C content (Atom %)	^{13}C Incorporation (%)	Final Cell Density (cells/ml)
Run 1	130	1.01	40	1.4	1.8×10^6
Run 2	145	1.11	56	1.6	2.1×10^6
Run 3	155	0.94	76	2.8	2.1×10^6

Results

After growth, the cells were harvested and the carotenoids extracted and analysed as described above. The efficiency of incorporation of ^{13}C from the bicarbonate into the β-carotene was calculated from the β-carotene yield and mass spectrum data. The cell density was determined by direct microscopic counting of the algal cells using a haemocytometer. These results are presented in Table 1 and Figure 4.

Discussion

One issue which arises when attempting to produce labelled compounds by feeding organisms with a labelled gaseous substrate is that of effective use of the often expensive label. It is for this reason that a gas recycling protocol was employed during the feeding of label in this system. A consequence of this, however, is the buildup of oxygen in the growth medium with consequent bubble formation and possible toxicity to the organisms. For stoichiometric and complete conversion of CO_2 to O_2 by photosynthesis, the largest daily charge of CO_2 from the bicarbonate added in this series of experiments would be expected to produce approximately 16.5 mmoles of oxygen. Assuming an oxygen solubility in the ASW medium to be similar to that in 2M NaCl (0.131 mmol/l) (Dawson *et al.*, 1969), and that at the start of the light period the growth medium is already in equilibrium with atmospheric O_2, photosynthesis would lead to a final O_2 concentration approximately 28 times that of normal saturated values. In this system, an O_2 sink was used which consisted of 10 litres of degassed water (O_2 solubility 0.224 mmol/l), acidified to reduce the solubility of CO_2. With this in place, the O_2 oxygen concentration is ca. 5.8 times normal saturated values. Both these values assume no gas headspace in the system, which in reality will reduce the concentration achieved. The time course for the dissolved oxygen concentration in the reactor presented in Figure 2 demonstrates the production of O_2 from photosynthesis by the algae. During the light period, the dissolved oxygen concentration rises to approximately twice the normal air saturation value. This represents approximately one third of the maximum expected value. The balance is likely to be accounted for by either accumulation in the headspace, of diffusion to atmosphere through the silicone rubber tubing used in the system.

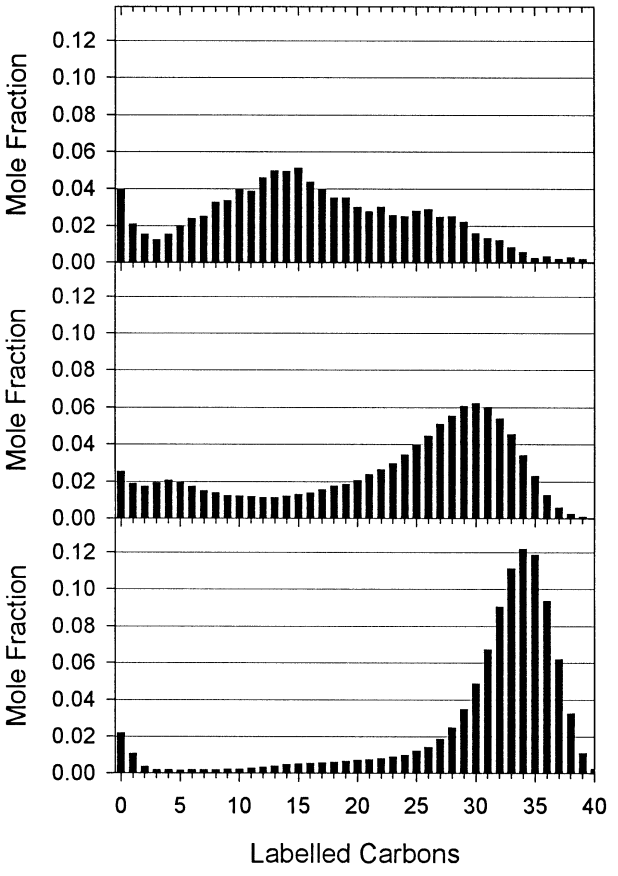

Figure 4 Mass spectra of β-carotene from Runs 1–3.

The data in Table 1 demonstrate a very large incorporation of ^{13}C into the β-carotene produced by the algal cells. For Run 3, the average molecular formula of the β-carotene is $^{13}C_{30}{}^{12}C_{10}H_{56}$. This high level of label is useful for tracer studies in human nutrition when only very small amount of β-carotene may be recovered from blood samples following a feeding experiment. Of further interest is the difference in the spectrum of label incorporation between the three runs, resulting from the

different labelling regimes employed. The statistics of random incorporation of $^{13}CO_2$ into β-carotene would predict a binomial distribution for the spectrum for a constant proportion of $^{13}CO_2$ in the carbon source. The spectrum for Run 1 clearly indicates two overlapping distributions, one with a mode around 14 labelled carbons and a second with a mode around 26 labelled carbons. It seems likely that these result from doubling the feed rate of labelled bicarbonate after 18 days of feeding. The spectrum for Run 2 shows a predominant mode around 30 labelled carbons, and that for Run 3 showing a mode of 34 labelled carbons. The latter two runs both show a more unimodal distribution, resulting from a more constant feed rate of bicarbonate. The mode at around 4 labelled carbons in Run 2 presumably results from the initially low dose rate of labelled bicarbonate, diluted with exogenous carbon dioxide. All three runs have some material at natural abundance, resulting from β-carotene production from unlabelled carbon dioxide.

The ability to control the isotopic spectrum of β-carotene produced by this system has great benefit for the production of tracers for nutritional experiments. The distinct mass spectra of the β-carotene produced by different feeding regimes would allow each isotopomer to be used an an independent label in the same experimental system. The proportion of each isotopic species in an experimental sample could then be determined by mass spectrometry, so long as the β-carotene molecules could be analysed intact. In this way, for example, different isotopomers could be used to label different components of a diet, to determine their relative absorption in a single experiment, avoiding the confounding effects of intersubject variation resulting from performing the experiment on different days. The different isotopomers could also be used as internal standards for quantification of β-carotene by mass spectrometry.

Finally, β-carotene from *Dunaliella salina* has been taken as a nutritional supplement for many years and is likely to be given ethical approval for use in human nutritional trials.

Acknowledgements

This work was part funded by the U.K. Ministry of Agriculture, Fisheries and Food. The authors would like to thank the IFR Engineering Workshop for constructing the bioreactor; John Eagles and Fred Mellon for the mass spectrometry; Dave Hart and John Scott for HPLC analysis of carotenoids, and Richard Faulks for helpful discussions about labelling.

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Received as Revised 6 March 1997;
Accepted 7 March 1997