

Production of a Carotenoid-Rich Product by Alginate Entrapment and Fluid-Bed Drying of *Dunaliella salina*

Gareth Leach, Gisela Oliveira and Rui Morais*

Laboratório de Biotecnologia Vegetal, Escola Superior de Biotecnologia-UCP, Rua Dr António Bernardino de Almeida, 4200 Porto, Portugal

Abstract: A new carotenoid-rich product was formed by entrapment of *Dunaliella salina* in calcium alginate beads of different alginate concentrations, followed by drying in a fluid-bed dryer. The drying process yielded β -carotene recoveries of between 79 and 89% and produced a change in the 9-*cis*/*all-trans* ratio of β -carotene isomers. The carotenoid stability of the product was dependent on both the storage conditions and alginate content (range 3.3–7.3 g litre⁻¹) of the beads. In the presence of light and oxygen total carotenoid degraded following a first order kinetic model with degradation constants between 0.016 and 0.039 days⁻¹, with the lowest degradation occurring with the lowest alginate concentration. Product stored in the dark and flushed with nitrogen produced first-order degradation constants of 0.012 and 0.020 days⁻¹ for the two higher alginate concentrations; that with the lowest alginate content showed no noticeable degradation after 58 days storage. During storage, the 9-*cis* isomer was significantly more unstable showing a relative loss under all conditions, degrading almost completely when stored in the presence of light and oxygen and reaching an equilibrium ratio with the *all-trans* isomer when stored in the dark and flushed with nitrogen. © 1998 SCI.

Key words: alginate; β -carotene; cell entrapment; *cis/trans*-isomers; *Dunaliella salina*; fluid-bed drying; microalgae

INTRODUCTION

β -Carotene is an orange pigment occurring naturally in many plants particularly vegetables and fruit (Bureau and Bushway 1986). However, by far the highest concentrations of β -carotene are found in the halotolerant microalgae *Dunaliella salina*, reaching levels of up to 100 g kg⁻¹ on a dry weight basis (Ben-Amotz 1993). β -Carotene has important nutritional characteristics as the most effective precursor of vitamin A (National Research Council 1980). It is also a potent antioxidant, scavenging potentially harmful oxy radicals which are commonly associated with the induction of certain cancers (Mordi 1993; Germann 1994). β -Carotene can

occur as one of several stereoisomers, the most stable is the *all-trans* form; however, the *cis*-forms are significantly more lipid soluble and may be better absorbed by the human body (Ben-Amotz *et al* 1989; Ben-Amotz and Avron 1990; Ben Amotz 1993). Zechmeister (1962) referred to the fact that *cis* forms present a provitamin A activity of less than 50% of the activity of *all-trans*- β -carotene. However, recent studies carried out with stable isotope labelled 9-*cis*- β -carotene revealed that 9-*cis*- β -carotene can be isomerised almost completely into *all-trans*- β -carotene in the human small intestine. This implies that 9-*cis*- β -carotene is as good as *all-trans*- β -carotene in being a source for vitamin A (Parker 1996). β -Carotene can be chemically synthesised.

The extreme halotolerance of *D salina* has enabled it to be cultivated on industrial scale under appropriate

* To whom correspondence should be addressed.

conditions for the production of natural β -carotene with approximately equal proportions of *all-trans* and *9-cis*-stereoisomers (Ben-Amotz and Avron 1990; Phillips 1993). β -Carotene is either extracted into vegetable oil and subsequently encapsulated in a gelatine capsule (Nonomura 1987), or the biomass spray-dried and processed to form a β -carotene-rich biomass tablet or capsule. There is little information available in the public domain concerning either of these processes or products. It is particularly important to obtain a good recovery of a stabilised product since carotenoids are highly unstable and may undergo oxidative degradation during processing (Cysewski 1994) or subsequent storage (Goldman *et al* 1983).

The formation of thermally stable calcium alginate gel has been applied to foods for texture improvement (Sime 1990), flavour encapsulation (King 1988) and to produce phage free starter cultures (Champagne *et al* 1992). There are many examples of the use of alginate gels in biotechnology for the entrapment of living cells for bioconversions using immobilised cell bioreactors (Bailey and Ollis, 1986). This approach has also been applied to *Dunaliella* to be used for the biological synthesis of glycerol (Grizeau and Navarro 1986).

In this work, the objective was to encapsulate *D salina* in calcium alginate beads and to form a novel product by rapid drying of the beads using a fluidised bed dryer. This form of dryer has been successfully applied in the food industry for the drying of particulate foods (Shilton and Niranjani 1993) and within the pharmaceutical industry for heat sensitive products. The effect of this drying process and subsequent storage on the degradation and isomer composition of β -carotene was of particular interest.

EXPERIMENTAL

Culture

Dunaliella salina culture (CCAP 19/30) grown in an outdoor raceway pond of 4 m³ at a pilot plant in Algarve was supplied by Necton Lda (Belamandil, Portugal). Culture agitation was provided by a paddle wheel with a rotation velocity of 30 rpm. With this conditions the mean circulation velocity of the culture was 0.24 m s⁻¹. The culture medium was composed of sea water, sea salt at a concentration of 3 M, KNO₃ at 5 M and K₂HPO₄ at 0.2 M. CO₂ was bubbled into the raceway pond at a rate to maintain culture pH at 8.0. At the pilot plant, the mean light intensity in April 1996 was 650 $\mu\text{E m}^{-2} \text{s}^{-1}$. Temperature ranged between 22°C at noon and 12°C during night. Prior to inoculation in the pond, a culture of *D salina* was produced in plastic sleeves at the same outdoor conditions. Initial cell concentration in the raceway pond was 2×10^5 cell ml⁻¹. After a residence time of 10 days, 25 litres of *D*

salina culture in carotenised stage and with a cell concentration of 4.5×10^5 cell ml⁻¹ was harvest and processed.

Harvesting and preparation of the culture

The microalgae culture was concentrated by centrifugation to a concentration of 5×10^7 cell ml⁻¹. It was then washed in two stages to a final salinity of 50 g litre⁻¹. The β -carotene content in the *D salina* biomass was 50 g kg⁻¹. The isomer composition was *all-trans*- β -carotene 58%, *9-cis*- β -carotene 36.5% and α -carotene 5.5%.

Preparation of *D salina* entrapped in alginate beads

Sodium alginate (PROTAN-Biopolymer A/S Norway Protanal LF 10/60 SLP 785) was solubilised with deionised water to concentrations of 11.0, 7.0, 5.0 g litre⁻¹ and combined with cell concentrate in the ratio of 2:1 (v/v), whilst mixing thoroughly, resulting, respectively, the following concentrations 7.3, 4.7, 3.3 g litre⁻¹. The resultant mixtures were then extruded through a syringe with a needle diameter of 0.8 mm into a 75 g litre⁻¹ CaCl₂ solution forming calcium alginate beads containing entrapped cells. The beads were harvested from the CaCl₂ solution and excess surface moisture removed by filter paper. The size of the capsules was measured using a Olympus B061 magnifying glass with an objective of $\times 20$. The moisture content of the beads was obtained by drying the beads overnight at 105°C.

Drying of beads

The beads were dried in a laboratory fluid bed dryer from Armfield (FT 31) by co-fluidisation with aluminium rings of internal diameter of 11 mm and external diameter of 21 mm. Co-fluidisation was necessary to prevent alginate beads sticking to the walls of the dryer. It is possible to use any inert particles larger than the dried beads for this purpose provided they can be fluidised. The drying conditions were: temperature 70°C; airflow 3.5 m s⁻¹ and drying time of 10 min. After drying, the alginate beads were separated from the aluminium rings using a metallic sieve DIN 4188 (from Fritsch GmbH). The dimensions of the dried beads were measured using the same precision magnifying glass. Moisture content after drying was also determined at 105°C overnight.

Storing of samples

Dried beads of each alginate concentration were separated into two sets and placed in identical 10 ml sample bottles. One set was flushed with nitrogen for 60 s and stored in the dark. The other was left

TABLE 1
Comparison of solids and β -carotene content of beads of different alginate concentration

Alginate concentration (g litre ⁻¹)	Wet bead solids (g kg ⁻¹)	β -Carotene concentration of wet beads (g kg ⁻¹ DM)	Dry bead solids (g kg ⁻¹)	β -Carotene concentration of dried beads (g kg ⁻¹ DM)	β -Carotene mean loss during drying ^a (%)
7.3	59.9 ± 0.2	19.1 ± 1.7	699.4 ± 0.9	15.8 ± 1.5	17.3
4.7	62.2 ± 1.4	27.6 ± 4.8	698.2 ± 7.9	23.9 ± 1.4	13.0
3.3	75.4 ± 0.5	45.4 ± 2.5	785.9 ± 0.2	36.0 ± 0.1	20.7

^a Losses calculated as % of carotene on a solids basis in dry compared to wet beads.

unflushed and exposed to fluorescent light from a Philips lamp (TLD 15W/33) giving a light intensity of 35 $\mu\text{E m}^{-2} \text{s}^{-1}$.

β -Carotene analysis

β -Carotene was extracted from beads with a primary extraction with pure ethanol, followed by subsequent extractions with acetone until all the colour was removed. If acetone (a stronger solvent for β -carotene than ethanol) was used alone, rapid dehydration of the gel matrix in the first extraction step where pigment concentration is at its highest, would make complete removal of the pigment difficult in subsequent steps. Dried beads required partial rehydration with water for 10 min prior to extraction with the same sequence of solvents. Extracts were evaporated to dryness with nitrogen and stored at -15°C until determination. Extractions were performed in triplicate. The carotenoid content of samples in acetone was determined by UV-spectrophotometer (Shimadzu) at 450 nm using an extinction coefficient of 2500. Changes in the isomer content of the carotenoid as a result of drying and storage were followed by reverse phase HPLC. Stored extracts were redissolved in methanol/diethyl ether (4:1, v/v) and injected into a Beckman Gold HPLC system equipped with a diode array detector. Isomers were separated by a Vydac 201TP54 C18 reverse phase column using a binary gradient of methanol (solvent A) and acetonitrile (solvent B). The course of the gradient was 100% A for 5 min, 90% A and 10% B at 5.1 min for 30 min, followed by 100% A at 35.1 min for 20 min. The flow rate was 0.7 ml min⁻¹. The column temperature was maintained at 30°C. Isomers were identified by their absorption spectra and comparison of retention time with isomer standards (obtained from Roche).

RESULTS AND DISCUSSION

Effect of fluid-bed drying on the bead characteristics

The drying process caused a loss of water from the gel matrix and a consequent shrinkage of the beads from

2 mm diameter to 1.4 mm diameter; the dried beads were hard but still contained between 214 and 300 g kg⁻¹ moisture (Table 1). Lower alginate concentrations may lead to beads of a less compact three-dimensional lattice. This might account for an increased number of entrapped cells with decreasing alginate concentration and explain why a weak increase of the beads mass leads to a high increase of β -carotene on the dried beads. The total carotenoid losses of between 13 and 20% during fluid bed drying were weaker than those obtained by Cysewski (1994) for conventional spray-drying of *Spirulina* spp.

Stability of total carotenoid during storage of the beads

The carotenoid content in all but one of the experiments declined during storage, following a first order degradation pattern (Table 2). The low degradation constants demonstrate that alginate entrapment can stabilise carotenoid comparing with degradation reported for carotenes in a variety of different matrices (Goldman *et al* 1983; Pesek and Warthesen 1988; Wagner and Warthesen 1995). Interestingly, in both storage environments the degradation was lowest in the beads made with the lowest alginate concentration. With spray-dry microencapsulation, it has been shown that an increase in polymer levels can reduce degradation of carotenes (Wagner and Warthesen 1995). In the

TABLE 2
First-order degradation constants for total carotenoid of *D. salina* entrapped in fluid bed-dried calcium alginate beads stored with and without exposure to light and oxygen

Alginate concentration (% (w/v))	Storage condition	Degradation constant (days ⁻¹)	R ²
7.3	Light/oxygen	0.028	0.988
4.7	Light/oxygen	0.039	0.982
3.3	Light/oxygen	0.016	0.925
7.3	Dark/nitrogen	0.020	0.965
4.7	Dark/nitrogen	0.012	0.952
3.3	Dark/nitrogen	NS	NS

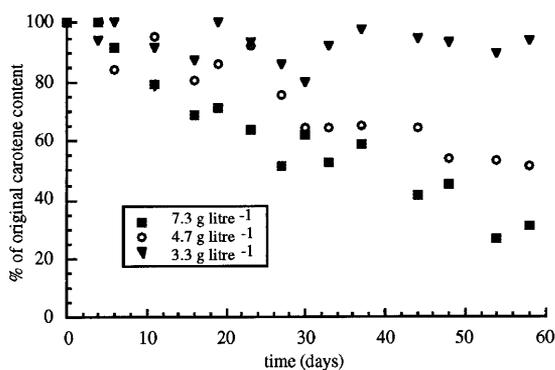


Fig 1. Effect of alginate concentration on the stability of total carotenoid from *D salina* entrapped in dried calcium alginate beads stored in darkness and flushed with nitrogen.

case of encapsulating with a gel, the effect of drying on the porosity of the gel is likely to be of key importance, since access of oxygen to carotenoid will be the significant factor affecting degradation. The results may indi-

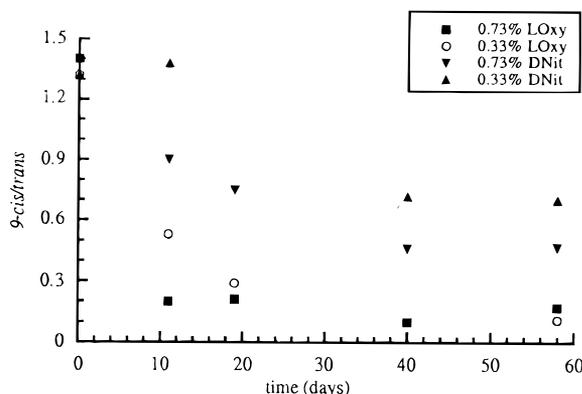


Fig 2. Effect of storage in light and oxygen on the 9-cis/trans β -carotene ratio of *D salina* entrapped in dried alginate beads.

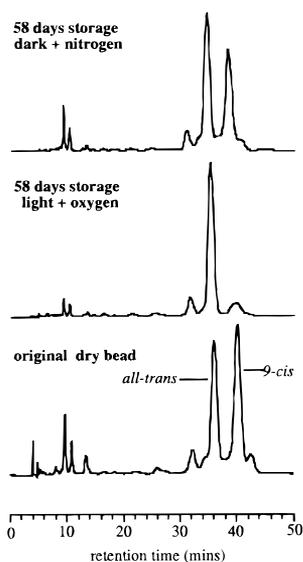


Fig 3. Changes in 9-cis/all-trans isomer ratios for β -carotene from dried alginate beads containing entrapped *D salina*, in 2 storage conditions.

cate that upon dehydration, a less porous structure may occur with a lower alginate concentration. This aspect of our work should be the subject of further investigation.

Effect of fluid-bed drying and subsequent storage on isomer composition of carotenes

The drying process itself resulted in significant changes in isomer composition of the biomass. The isomer composition of the dried beads was *all-trans* 42%, 9-*cis* 50% and α -carotene 8.2%. In result of drying process increases on the 9-*cis* and α -carotene isomers were observed, apparently as result of isomerisation of *all-trans*- β -carotene. The carotenoid stability of all bead types was improved by storage in a reduced oxygen environment and in the dark. The beads formed with the lowest alginate content were particularly stable in terms of total carotenoid, showing little decline even after 58 days storage under these conditions (Fig 1). During storage, both bead types showed a relative loss of the 9-*cis* isomer compared to the *all-trans* form (Fig 2). The loss was greatest in the early stages of storage and significantly greater for the beads exposed to light and oxygen. For beads stored in the dark and flushed with nitrogen, the 9-*cis*/*all-trans* ratios seemed to reach an equilibrium after 40 days, with significant proportions of the *cis* isomer still present at day 58. This difference is emphasised by comparison of the carotenoid chromatograms in Fig 3.

It can be concluded that entrapment of a concentrate of *D salina* cells in alginate followed by fluid-bed drying has potential for producing a carotene-rich nutraceutical product with good total carotenoid stability characteristics. However, if significant loss of the 9-*cis* isomer is to be avoided, a low oxygen, low light environment is to be recommended.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Commission of the European Communities AAIR programme (AIR2-CT94-1283). We also would like to thank the technical assistance given by Sandra Sá and Sergio Viegas and the preliminary work with alginate performed by Isabel Silva. Gareth Leach gratefully acknowledges the support from an EC post-doctoral AAIR bursary.

REFERENCES

- Bailey J E, Ollis D F 1986 Immobilised cells. In: *Biological Engineering Fundamentals* (2nd edn). McGraw-Hill International Editions, Singapore, pp 595–600.
- Ben-Amotz A 1993 Production of β -carotene and vitamins by the halotolerant alga *Dunaliella*. In: *Marine Biotechnology*

- (Vol 1: Pharmaceutical and Bioactive Natural Products), eds Attaway D H & Zaborsky O R. Plenum Press, New York, USA, pp 411–417.
- Ben-Amotz A, Avron M 1990 The biotechnology of cultivating the halotolerant alga *Dunaliella*. *Trends Biotechnol* **8** 121–126.
- Ben-Amotz A, Mokady S, Edelstein S, Avron M 1989 Bioavailability of natural isomer mixture as compared with synthetic *all-trans* β -carotene in rats and chicks. *Am Inst Nutr* **22** 1013.
- Bureau J L, Bushway R J 1986 HPLC determination of carotenoids in fruits and vegetables in the United States. *J Food Sci* **51** 128–130.
- Champagne C P, Morin N, Couture R, Gagnon C, Jelen P, Lacroix C 1992 The potential of immobilised cell technology to produce freeze dried phage protected cultures of *Lactococcus lactis*. *Food Res Int* **25** 419–427.
- Cysewski G R 1994 Ocean-chill drying of microalgal and microalgal products. USA Patent 5276977.
- Germann I 1994 Beta-carotene, inducer of cell-cell communication. *Int Food Ingr* **4**.
- Goldman M, Houv B, Saguy I 1983 Decolorization of β -carotene in model systems simulating dehydrated foods. Mechanism and kinetic principles. *Food Sci* **48** 751–754.
- Grizeau A D, Navarro J M 1986 Glycerol production by *Dunaliella tertiolecta* immobilized within calcium alginate beads. *Biotechnol Lett* **8** 261–264.
- King A H 1988 Flavour encapsulation with alginates. In: *Flavour Encapsulation*, eds Risch S J & Reineccius G A. American Chemical Society, Washington, USA, pp 122–125.
- Mordi R C 1993 Carotenoids: Functions and degradation. *Chem Ind* **2** 79–83.
- National Research Council 1980 *Recommended Dietary Allowances* (9th edn). National Academy of Sciences, Washington, DC, USA.
- Nonomura A M 1987 Process for producing a naturally derived carotene-oil composition by direct extraction from algae. USA Patent 4680314.
- Parker R S 1996 Absorption, metabolism and transport of carotenoids. *FASEB J* **10** 542–551.
- Pesek C A, Wartheson J J 1988 Characterisation of the photo-degradation of β -carotene in aqueous model systems. *J Food Sci* **53** 1517–1520.
- Phillips T D 1993 Stress manipulation in *Dunaliella salina* and dual-stage β -carotene production. PhD thesis, Rhodes University, Republic of South Africa.
- Shilton N C, Niranjana K 1993 Fluidization and its applications to food processing. *Food Struct* **12** 199–215.
- Sime W J 1990 Alginates. In: *Food Gels*, ed Harris P. Elsevier Applied Science, London, pp 53–77.
- Wagner L A, Wartheson J J 1995 Stability of spray-dried encapsulated carrot carotenes. *J Food Sci* **60** 1048–1053.
- Zechmeister L 1962 *Cis-trans Isomeric Carotenoids, Vitamin A and Aryl Polyenes*. Academic Press, New York, USA.