Seventh Symposium on Salt, Vol. I 679–684 (1993)
Elsevier Science Publishers B.V., Amsterdam

Pilot β-Carotene Production: The Northern Territory Experience

Nathan Sammy

Department of Industries and Development, Darwin, Northern Territory, Australia

ABSTRACT

A pilot study is continuing on the potential of β-carotene production from the brine alga, *Dunaliella salina* in Central Australia at Alice Springs. Using a local strain from natural salt lakes near the South Australian border, β-carotene production has been maintained at 7μg ml^{-1} of brine annually in spite of very low winter temperatures of 0°C or less. Lack of persistent strong winds resulted in salinity stratifications in the artificially constructed ponds; the resulting solar pond effect dramatically reduced growth and yield of β-carotene. However, modifications have been implemented to overcome this problem. In the mild wind conditions experienced in Alice Springs, the algae continually stratify near the brine surface and this phenomenon is used as a preconcentrating factor for harvesting by conventional flocculation methods. A preliminary method of extracting β-carotene into oil has been investigated.

INTRODUCTION

In 1964, Craigie and McLachlan demonstrated the presence of glycerol in the cytoplasm of the marine species *Dunaliella tertiolecta*. Ben Amotz of the Weizmann Institute, Israel, studied the photosynthetic system of *Dunaliella parva*, and Lesley Borowitzka isolated a strain of *Dunaliella viridis* from South Australia and began a study of the mechanism of salt tolerance. During the period 1972–3, both Ben Amotz and Borowitzka demonstrated that contrary to previous reports, *Dunaliella* species did not accommodate their highly saline environment by accumulating inorganic ions. However, the species they studied accumulated the compatible solute, glycerol, to osmoregulate in widely fluctuating environments from marine to hypersaline (Borowitzka, 1974; Ben Amotz et al., 1980).

In 1978, Roche Research Institute of Marine Pharmacology in Sydney and the Weizmann Institute in conjunction with Koor Foods were independently investigating *Dunaliella* species as potential sources of glycerol and β-carotene. Dampier Salt Ltd, quite separately, and in the same year began an investigatory programme under my leadership to consider β-carotene production at Lake MacLeod in Western Australia where the alga occurs naturally in vast quantities. Similarly other Australian salt companies like Cheetham Salt, Shark Bay Salt, Dry Creek, and Leslie Salt conducted their own investigations but only ICI Australia Ltd published some information (Jones et al., 1981).

In 1980 Roche established a pilot plant at Hutt Lagoon in Western Australia and in 1981, Roche Algal Biotechnology was established as a separate division to conduct field, laboratory and piloting studies until the end of 1983. Roche did not continue with the project. The Commonwealth Department of Science and Technology became interested in the potential of an algal biotechnology-based industry in Australia and in 1984, awarded a contract to Wesfarmers, a Western Australian company with broad rural based interests, to undertake a Public Interest Project. The object of this grant was to examine the feasibility of establishing an algal biotechnology-based industry for Australia using the original Roche pilot at Hutt Lagoon in Western Australia. The project was completed in 1985 with the Commonwealth Government of Australia owning the rights to two patents and the technology for an industry in Australia. A summary of this technology was published by Moulton et al. (1987a).

Currently, two Australian companies are producing and further investigating natural β-carotene production from *Dunaliella salina*. Hoffmann La Roche bought into the pilot project at Hutt Lagoon in Western Australia and obtained a licence from the Commonwealth Government to continue β-carotene production. Betatene in Whyalla, South Australia is the only other Australian operation with β-carotene product on the market.

TAXONOMY

The genus *Dunaliella* was first described by Téodoresco (1905) and he later studied in detail the morphology and life cycle of the two species, *D. salina* and a new species he described as *D. viridis* (Téodoresco. 1906). While this taxonomy was adopted widely for hypersaline brines, the review by Masyuk (1973) expanded the species recognised in the genus to 23. According to him there are three species producing β-carotene, *D. parva*, *D. salina* and *D. pseudosalina*. The accumulation of β-carotene is a result of environmental stress, as examples, due to depletion of nutrients, high insolation and salinity increase. However *D. salina* is the best producer of β-carotene which according to Masyuk causes red "flowering" of the brine in hypersaline reservoirs. According to him *D. viridis* remains green at all salinities. The species described as *D. bardawil* is conspecific with *D. salina* (Avron, pers. commun., 1982).

BEHAVIOUR

In natural salt lakes and in some Australian solar salt fields maintaining high numbers of *Dunaliella*, the two species *D. salina* and *D. viridis* are very common. The green species is common in the brine when the density is below 1.10 kg m^{-3}. Above that density, the red species predominate in the brine column and the green species accumulate mainly in bottom salts deposits, in the interstices of gypsum crystals, or in sodium chloride crystals. This accumulation occurs adjacent to the purple sulphur bacteria and the anaerobic zone. Although the dissolved oxygen levels in brines are very low as previously reported by Sammy (1985), measurements of the dissolved oxygen level in the crystal interstices is at saturation during the photosynthetic period of a day (Tyler, pers. commun., 1988). The green species may occur as motile cells but more often occur as the palmella stage. A sample of high density brine will invariably contain a mixture of the two species with *D. salina* predominating.

The use of bulk sodium chloride salt produced by solar evaporation to manufacture artificial brine for the cultivation of *Dunaliella salina* will also introduce *D. viridis*. While much effort may be expended in obtaining pure cultures of *D. salina*, this effort may be wasted in outdoor extensive cultures because of contaminations with the green species. For extensive outdoor cultures, manipulation of environmental growth parameters based on the understanding of the biology of *Dunaliella* species is a more efficient use of resources. A basis of this understanding of the biology of the species has been published by Moulton et al. (1987b). However, for intensive culture methods, pure cultures of *D. salina* may prove an efficient and effective way of mass production of β-carotene.

PRODUCTION TECHNIQUES

Chen and Chi (1981) outlined a scheme for process development of glycerol from *Dunaliella*. They postulated that animal protein feed and β-carotene could be recovered as coproducts of glycerol production. Growth of the algae would be initially maintained in brine enriched with potassium nitrate and ammonium dihydrogen phosphate with the sodium chloride concentration maintained at 1.5 M. The batch of algae grown would then be subjected to increased salinity with the sodium chloride maintained at 4.0 M to stimulate glycerol production. This method of relatively fast growth at low salinity followed by an increased in salinity was dismissed by Borowitzka et al. (1984) based on operational experience of extensive cultures.

The method of extensive batch culture of the algae has been adopted for commercial production in Australia, and although the fertilizers used and the techniques of addition are considered propriety, the principle has been adapted from published information (Ben-Amotz et al., 1983). Like all algae, growth is encouraged by the addition of nitrogenous and phosphorus-containing fertilizers. The fertilizers may be obtained cheaply, but because of the quantities required to dose the batch cultures, the inorganic impurities contained in the fertilizers would play a significant part in the health and growth of the culture.

In the Alice Springs experiments, the growth of *Dunaliella salina* was modelled on observations made in solar salt field crystallizers, and using the extensive culture method because of restrictions placed on resources and personnel. In Australian solar salt fields, the crystallizer brine depth is always maintained at 20 cm and with an initial brine density of 1.215 kg m^{-3}. This depth was adopted in the Alice Springs pilot study to encourage β-carotene production because of exposure of the algae to a higher degree of solar radiation. An inoculum of algae and salt was obtained from Lake "Suzi" on Erldunda Station south of Alice Springs, a lake with a recognisable bloom of the algae (Fig. 1). The salt was used to form an artificial salt bed in two rubber-

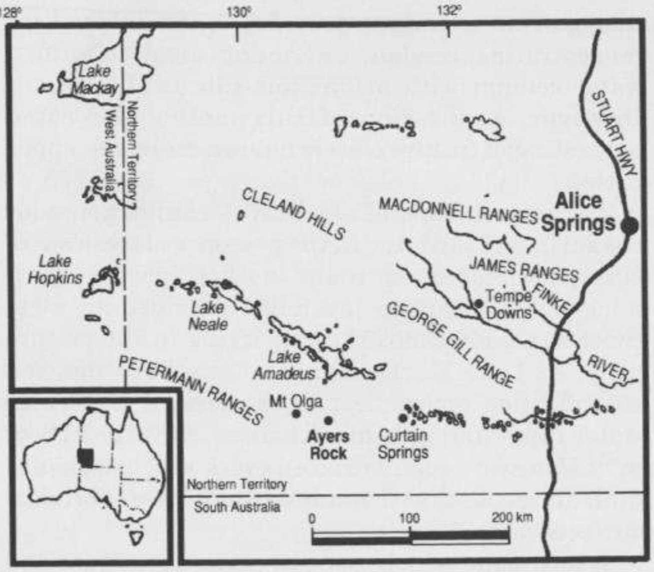

Fig. 1. Distribution of natural salt lakes in Central Australia.

lined ponds sized 30 x 10 m constructed at the Arid Zone Research Institute, Department of Primary Industry and Fisheries in Alice Springs. Brine of density 1.17–1.18 kg m^{-3} was reconstituted with bore water. Fertilizer formulation was adapted from Johnson et al. (1968) and Semeneko et al. (1980). After chemical analyses of the reconstituted brine and of brines from a natural salt lake (Table 1), it was decided that the formulation would consist of potassium nitrate, potassium dihydrogen phosphate and iron chelate. No attempt was made to isolate the red species of *Dunaliella* or culture the inoculum to increase the biomass before seeding the outdoor ponds.

METHODS

During the growth of the outdoor culture, records were kept of pond temperature, brine depth, brine pH and brine density. Biological monitoring was initially based on β-carotene content per volume of brine but then extended to cell counts as a means of microscopically examining for predators and the health of the cells. Brine culture β-carotene content was determined spectrophotometrically (452 nm) on cold acetone extracts of glass fibre filtered samples standardised against pure samples of synthetic β-carotene (Sigma Chemicals). Cell counts of brine culture were determined manually with a Neubauer haemocytometer immediately after killing the motile cells with weak formalin solution. Brine density measurements were made with a hand-held Anton Parr density meter reading to three decimal places, or alternatively determined using hygrometers.

TABLE 1

Analyses of brines from Alice Springs pilot ponds and natural salt 'Lake Suzi' by inductively coupled plasma spectrophotometry

Elements	Pilot ponds	Lake Suzi
Na	115800	67640
K	1870	6640
Mg	4710	27560
Ca	676	169
Fe	<0.01	<0.02
Al	<0.17	<0.34
Si	1.58	0.88
P	<0.40	<0.80
Li	1.36	<0.34
Be	<0.001	<0.002
Ba	0.16	0.11
Sr	3.50	5.20
La	<0.19	<0.67
Ce	<0.3	<1.99
Sm	<0.41	<2.02
Eu	<0.053	<0.21
Gd	<0.10	<1.39
Yb	0.014	<0.02
Zr	<0.03	0.50
B	<0.40	<0.08
Mn	<0.01	0.27
Co	0.10	0.24
Cr	0.17	0.45
Cu	<0.02	0.32
Ni	<0.04	1.18
V	0.26	1.57
Zn	0.48	2.26
Ag	<0.04	0.32
Cd	<0.01	0.21
Mo	1.40	3.10
Pb	<0.30	0.66
Density	1.180	1.260

RESULTS

The outdoor pond culture increased biomass on July 1988, and maintained a β-carotene level of 7 μg ml^{-1} and cell count of 8×10^4 ml^{-1} throughout the cold winter months. Because the bore water used for evaporative loss make-up tended to stratify on the surface, a solar pond effect resulted. During the peak of summer, brine temperatures were in excess of 40°C, and algal cell death in the underlying brine was evident by surface scum formation. This stratification was attributed to the lack of strong winds

TABLE 2

Climatic averages and low temperature extremes for Alice Springs. (Information provided by the Bureau of Meteorology)

Month	Mean evap. (mm)	Mean rain (mm)	Mean day max (°C)	Mean day min (°C)	Low day min (°C)
Jan	391.2	38.0	35.9	21.1	10.0
Feb	364.6	46.0	34.9	20.6	8.5
Mar	309.4	34.0	32.4	17.4	6.1
Apr	240.6	15.0	27.9	12.5	1.7
May	144.6	18.0	22.8	8.2	–2.5
Jun	110.0	13.0	19.8	5.1	–4.8
Jul	112.9	14.0	19.4	4.1	–7.5
Aug	152.7	12.0	22.4	6.1	–3.9
Sept	232.9	10.0	26.5	9.8	–1.0
Oct	288.3	22.0	30.5	14.6	1.3
Nov	339.0	25.0	33.4	17.7	3.5
Dec	375.0	36.0	35.3	20.1	9.7

year round to effect vertical brine mixing in the ponds. There were also great difficulties experienced in maintaining the brine density at 1.17–1.18 kg m^{-3} because of the high evaporation rates experienced (Table 2). However, the brine was dominated by *Dunaliella salina* with small numbers of motile green *D. viridis*. The major accumulation of green cells was in the salt bed.

In an effort to overcome the solar pond effect, the artificial salt bed was removed from one of the ponds, and an aeration system installed to induce even vertical mixing in the pond. The pond brine colouration turned green, although the number of red algae cells remained the same. From this experience it was decided that an artificial or natural salt bed is necessary to keep the green algae cells out of the brine.

HARVESTING TRIALS

One of the difficulties in harvesting the algae is that the cells lack a cell wall and easily break with standard filtration aids. Cell breakage has occurred by shear force experienced during standard filtration methods, pressure membrane filtration, and even during rapid pumping through narrow pipes. The result is a rapid oxidation and loss of β-carotene.

Harvesting has been achieved by diatomaceous earth filtration (Ruane, 1974), stationary or moving salt gradients (Bloch et al., 1982) and hydrophobic adhesion (Curtain et al., 1987). Kessler (1985) published a method of harvesting motile, spherical algal cells using a technique he called hydrodynamic focusing. This technique provides a new method for concentrating random swimming algal cells in a water column with a low flow rate of 0.1 cm s^{-1}. However, application of this method for rapid harvesting of culture cells is not commercially applicable.

The phenomenon of algal cell stratification near the surface of the brine in the presence of freshwater is very noticeable year round in Alice Springs. Skimming off this surface layer has provided an algal concentrate six times that occurring in the culture pond. At Lake MacLeod in Western Australia, cell stratification occurs despite the lack of any freshwater input and at brine densities of 1.215–1.26 kg m^{-3}. However, such surface layers are notoriously difficult to collect with minimal quantities of brine or surface water.

A technique widely used in water treatment and well documented in literature is that of flocculation (Eckenfelder, 1966; Degremont, 1979; Hilson et al., 1980; Moraine et al., 1980). Trials have been conducted with a number of commercially available preparations with a high success rate. It is likely that sulphated polysaccharide produced by the naked algal cells impart a negative charge (Borowitzka, 1974) which is neutralised by cationic flocculants. Commercially available preparations such as polyaluminium chloride and synthetic polymers such as Magnafloc E10 (Allied Colloids Pty Ltd) are effective for use in harvesting algae in our pilot study. After flocculation algal cells lose flagellae and assume a spherical shape, cushioned in the floc formed. This cushioning is important for further treatment of the sludge, as no cell breakage was observed especially during the dewatering process. Stored algal cells remain biologically active and photosynthesis of algae in floc continues in presence of sunlight. Depending on modifications to the technique, the harvesting efficiency by flocculation is greater than 95%, and the β-carotene concentration is approximately 100 times that of the pond culture.

EXTRACTION

Sections of chemically-fixed algae examined under the transmission electron microscope show numerous lipid bodies, each enclosed within a protein layer probably chloroplastic in origin. This was previously demonstrated by Ben Amotz et al. (1982). It is highly probable that β-carotene is deposited in these lipid bodies. Preliminary trials have been undertaken with extraction of the concentrated algae directly into edible oil with a mechanical blender. Cell breakage results in the solution of the lipid bodies in the added oil. Depending on the quantity of algal concentrate, the emulsion formed is difficult to separate except with centrifugation. Analysis of the oil extract showed a maximum concentration of 1.5% (w/v) of β-carotene. Samples of these oil extracts have been kept under refrigeration with good shelf life. Methods of increasing the β-carotene concentration in oil are under investigation. HPLC analysis of pure algal concentrates (Table 3) indicate that an oil extract from algae also contained traces of other carotenoids. This has been confirmed by Borowitzka et al. (1990).

POTENTIAL PROBLEMS

No protozoan predators have been detected in the artificial ponds, although this situation may be different in natural salt lakes in the area. In some Australian solar salt fields, the periodic occurrence of the protozoan *Fabrea salina* can dramatically reduce the bloom of red *Dunaliella salina*.

In southern Australia, a region dominated by cool and wet winters, red cultures of *D. salina* have not produced predictable quantities of β-carotene during the winter months. Outdoor pond cultures may appear red but contain extremely low β-carotene content. This is primarily due to the formation of resistant aplanospores containing the carotenoid canthaxanthin. This was a concern for the cultures at Alice Springs where winter temperatures are very low. However, aplanospore formation has not been observed.

CONCLUSION

The pilot study in Alice Springs has confirmed that β-carotene production by extensive outdoor pond culture is possible all year round without any significant seasonal fluctuations. The next phase of study is to evaluate the commercial feasibility of extensive outdoor culture of *Dunaliella salina* for β-carotene production. An alternative investigative approach is to consider intensive culture or alternatives to the approaches to the culture methods adopted in this extensive culture pilot study.

ACKNOWLEDGEMENTS

The Departments of Primary Industry and Fisheries, and Industries and Development, Northern Territory Government are thanked for permission to publish research information. This project was instigated when the author was Director of Aquaculture in 1988, and gratitude is expressed to Mr. Murray Barton of the Arid Zone Research Institute who has diligently continued the study to the present time. Dr. J. Loung-Van of the Northern Territory University prepared the TEM micrographs of sectioned algae cells.

ICP analyses were performed at the Geology Department, University of Melbourne. All other analyses were conducted at the Northern Territory University, Darwin.

TABLE 3

Pigment analyses of laboratory cultures of *Dunaliella* species by HPLC. Specimens obtained from Lake MacLeod (n.d. — not detected)

	D. salina ($\mu g\ ml^{-1}$)	*D. viridis* ($\mu g\ ml^{-1}$)
β-carotene	12.6	0.26
α-carotene	0.84	n.d.
γ-carotene	0.29	n.d.
Lutein	0.18	0.7
Zeaxanthin	0.13	0.07
Other carotenoids	<0.1	<0.25
Chlorophyll a	1.01	3.25
Chlorophyll b	0.19	0.76
Cell counts ($x10^4\ ml^{-1}$)	19	330

REFERENCES

Ben-Amotz, A. and Avron, M., 1980. Glycerol, β-carotene and dry algal meal production by commercial cultivation of *Dunaliella*. In: G. Shelef and C.J. Schroeder (Editors), Algae Biomass. Elsevier/North Holland/Biomedical Press, Amsterdam, pp. 603–610.

Ben-Amotz, A., Katz, A. and Avron, M., 1982. Accumulation of β-carotene in halotolerant algae: Purification and characterization of β-carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). J. Phycol., 18: 529–537.

Ben-Amotz, A. and Avron, M., 1983. On the factors which determine massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil*. Plant Physiol., 72: 593–597.

Bloch, M.R., Sasson, J., Ginzburg, M.E., Goldman, Z., Garti, N. and Peath, A., 1982. Oil products from algae. U.S. Patent 4 341 038, 6 pp.

Borowitzka, L.J., 1974. Physiological aspects of salt tolerance in the unicellular green alga, *Dunaliella*. Ph.D. thesis, School of Microbiology, University of New South Wales.

Borowitzka, L.J. and Borowitzka, M.A., 1990. Commercial production of β-carotene by *Dunaliella salina* in open ponds. Bull. Mar. Sci., 47(1): 244–252.

Borowitzka, L.J., Moulton, T.P. and Borowitzka, M.A., 1984. The mass culture of *Dunaliella salina* for fine chemicals: from laboratory to pilot plant. Proc. Int. Seaweed Symp., 11: 115–121.

Chen, B.J. and Chi, C.H., 1981. Process development and evaluation for algal glycerol production. Biotechnol. Bioengin., 23: 1267–1287.

Curtain, C.C., West, S.M. and Schlipalius, L., 1982. Manufacture of β-carotene from the salt lake alga *Dunaliella salina*; the scientific and technical background. Austr. J. Biotechnol., 1: 51–57.

Degrémont, 1979. Water Treatment Handbook, Fifth Edition. Halsted Press, New York, 1186 pp.

Eckenfelder, W.W., 1966. Industrial Water Pollution Control. McGraw-Hill Book Company, New York, 275 pp.

Hilson, M.A. and Richards, W.N., 1980. Polymeric Flocculants. In: W.M. Lewis (Editor), Developments in Water Treatment-1. Applied Science Publishers Ltd, London, pp. 93–118.

Jones, A.G., Ewing, C.M. and Melvin, M.V., 1981. Biotechnology of solar saltfields. Hydrobiologia, 82: 391–406.

Johnson, M.K., Johnson, E.J., MacElroy, R.D., Speer, H.L. and Bruff, B.S., 1968. Effects of salts in the enzymes of the halophilic alga *Dunaliella viridis*. J. Bacteriol., 95(2): 1461–1468.

Masyuk, N.P., 1973. Morphology, Taxonomy, Ecology and Geographic Distribution of the Genus *Dunaliella* Teod. and Prospects for its Potential Utilization. Naukova Dumka, Kiev (in Ukranian).

Moraine, R., Shelef, G., Sanband, E., Bar-Moshe, Z. and Shvartzburd, L., 1980. Recovery of sewage-borne algae: Flocculation, flotation, and centrifugation techniques. In: G. Shelef and C.J. Schroeder (Editors), Algae Biomass. Elsevier/North Holland/Biomedical Press, Amsterdam, pp. 531–545.

Moulton, T.P., Borowitzka, L.J. and Vincent, D., 1987a. The mass culture of *Dunaliella salina* for β-carotene: from pilot plant to production plant. Hydrobiologia, 151/152: 99–105.

Moulton, T.P., Sommer, T.R., Burford, M.A. and Borowitzka L.J., 1987b. Competition between *Dunaliella* species at high salinity. Hydrobiologia, 151/152: 107–116.

Ruane, M., 1974. Recovery of algae from brine suspensions. Australian Patent 486 999, 12 pp.

Sammy, N., 1985. Biological systems of Northwestern Australian salt fields. In: B.C. Schreiber and H.L. Harner (Editors), Sixth Symposium on Salt, Vol. I. Salt Institute Inc., Alexandria, Virginia, USA, pp. 207–215.

Semeneko, V.E. and Abdillaev, A.A., 1980. Parametric control of β-carotene biosynthesis in *Dunaliella salina* cells under conditions of intensive culture. Fiziologiya Rastenii, 27(1): 31–41 (in Russian).

Téodoresco, E.C., 1905. Organisation et développment du *Dunaliella*, nouveau genre de Volvocacée-Polyblepharidée. Beihefte zum Botanischen Zentralblatt, 18: 215–232.

Téodoresco, E.C., 1906. Observations morpholgiques et biologiques sur le genre *Dunaliella*. Revue Gén. Bot., 18: 353–371.