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Production of Natural β-Carotene from *Dunaliella* Living in the Dead Sea

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Abstract

The main aim of this work was to investigate the possibility of Beta Carotene production using micro algae *Dunaliella salina* existing in the Dead Sea. The research being carried out covers a number of interdependent steps and, is focusing on laboratory scale cultures. Under stress conditions such as high light intensity or nutrient starvation, cells of the unicellular alga *Dunaliella salina* overproduce β-carotene, which is accumulated in the plastids in newly formed triacylglycerol droplets. *Dunaliella* was isolated from the Dead Sea and cultivated using a certain media until the cell count was 500,000 cell/mL. The cultivation step was monitored and video captured using a digital microscope with USB camera, then it was centrifuged and extracted using different kinds of organic solvents. The amount of dry weight was 3 g/L of which 3-6% was β-carotene. Freeze drying step was performed to obtain β-carotene as powder. The analysis of the powder was carried out by the research and development department at one of the pharmaceutical companies. It was evident that a pilot plant investigation should be the next step on the way of commercialization of such a profitable process.

Keywords:*Dunaliella*; Cultivation; Harvesting; Centrifugation; β-Carotene;β-Carotene Extraction; Freeze drying; Dead Sea.

1. Introduction

The green unicellular flagellate *Dunaliella* salina is the richest natural source of the carotenoid β-carotene. The halophilic species of *Dunaliella* also accumulate very high concentrations of glycerol. *Dunaliella salina* was first proposed as a commercial source of β-carotene and latter as a source of glycerol. β-carotene from *Dunaliella* is now being produced on a commercial scale in Australia, the USA and Israel. *Dunaliella* is a unicellular, bi-flagellate, naked green alga which is morphologically similar to chlamydomonas with the main difference being the absence of a cell wall in *Dunaliella* [1]. In *Dunaliella* salina and *Dunaliella* parva the chloroplast accumulate large quantities of β-carotene (as droplets) so that the cells appear orange-red rather than green. Teodoresco described tow species: *D.salina* and *D. viridis* .*D.salina* has somewhat larger cells and under suitable conditions it synthesizes massive amounts of carotenoid pigments, coloring the cells brightly red while *D.viridis* never produces such red cells [1, 6].

To investigate the apparently strict interdependence of carotenoid overproduction and the formation of sequestering structures, we exploited the unicellular alga *Dunaliella Salina* as a laboratory system inducible for enhanced carotenoid formation [7]. When exposed to stress conditions such as high light intensity or nutrient starvation, two stereoisomers of β-carotene, all-*trans* and 9-*cis* β-carotene, accumulated, reaching up to 14% of the cell's dry weight, with the pigment being deposited into plastid. We now know that not all *Dunaliella* species produce massive amounts of carotene and those that can do so only under suitable conditions [1].

1.1. Historical overview:

Hundred years have passed since the description of the genus *Dunaliella*, the unicellular green alga which is responsible for most of the primary production in hypersaline environments worldwide. First sighted in 1838 in saltern evaporation ponds in south France by Michel Flix, *Dunaliella*, was named after its discoverer by Teodoresco in 1905. In the century that has elapsed since its formal description of the genus *Dunaliella* has become a convenient model organism for the study of salt adaptation in alga. The establishment of the concept of organic compatible solutes to provide osmotic balance was largely based on the study of *Dunaliella* species. Moreover, the massive accumulation of β-carotene by some strains under suitable growth conditions has led to interesting biotechnological applications [8, 9].

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1.2. Dunaliella in the Dead Sea:

Systematic quantitative studies on the biology of the Dead Sea have been performed only since 1980. It is now well established that the Dead Sea biota are domained by the unicellular green alga *Dunaliella.* The pigment responsible for the brightly red coloration displayed by *D. salina* was recognized already very early as a carotenoid. The carotenoids are of fundamental importance to life. Not all their functions are fully understood, but it is evident that carotenoids are intimately involved in the protection of organic molecules from oxidative destruction and in light-induced energy production via photosynthesis [10].

In the alga, this β-carotene seems to act a sun screen to protect the chlorophyll and the cell DNA from high irradiance which characterizes the normal habitat of *D. salina*. β-carotene is a valuable chemical, in high demand as a natural food coloring agent, as a pro vitamin A as additive to cosmetics and as a health food [10]. The purified β-carotene compositions may be administrated for a variety of therapeutic or prophylactic uses to humans or in veterinary applications, the compositions may used as a vitamin preparation, alone in conjunction with other vitamins to supplement dietary intake of vitamin A and in thus to treat or prevent conditions associated with vitamin A insufficiency. The compositions of invention can also be used in prevention or treatment of a variety of diseases pathological conditions associated with free radicals and lipid auto-oxidation. In other uses the high purity natural β-carotene is administrated therapeutically or prophylactically for a variety of cancers particularly epithelial cell cancers [10]. Preparations have shown significant protection against various tumors in animals. Accordingly, the beta carotene preparation is indicated for use in therapeutic methods for a variety of cancers including in humans. β-carotene preparations have shown to be useful in treating, inhibiting or otherwise preventing tumors in humans. β-carotene is generally regarded as the most commercially important and widely used carotenoid. It is used as a food coloring agent, an antioxidant and an important and safe pro-vitamin A source.

2. Methodology

2.1. Cultivation

The development of culture conditions is very important step in terms of favoring growth of the chosen strain and, at the same time, discouraging growth of contaminants. It has already been concluded that carotenoid production has to be carried out under a different set of conditions from those favoring the increase in biomass. Therefore the optimization of both sets of conditions is necessary, see Figure (1), the cultivation was monitored and video captured by a digital microscope with USB camera, Figure (2). *Dunaliella* also has a very wide pH tolerance ranging from pH 1 t o pH 11. In fact *D.*salina is one of the most environmentally tolerant eukaryotic organisms known and can cope with a salinty range from seawater 3% NaCl to NaCl saturation 31% NaCl, and a temperature range from < 0ºC to >38ºC [2, 3, 4, 5, 7].

When the sunlight, fertilizer, and water temperature are excellent photosynthesis is often limited by the concentration of carbon-dioxide. Extra carbon-dioxide could be added to aquaculture waters and this could increase production, but it is necessary to keep the carbon-dioxide from escaping to the atmosphere.

Fig 1. Dunaliella Culture.

Fig 2. Digital microscope with USB camera.

The following table illustrates the optimized cultivation media for the Dunaliella species:

Table 1. Modified Johnson media

To 5 L of distilled water add :	
NaCl	24% per unit volume
$MgCl_2.6H_2O$	7.65 g
$MgSO_4.7H_2O$	2.6 g
KCl	1.02 g
$CaCl_2.2H2O$	1.02 g
KNO_3	5.1 g
$NaHCO_3$	0.2 g
KH_2PO_4	0.2 g
Fe-solution	51 mL
Na_2EDTA	945 mg
$FeCl_3.6H2O$	1220 mg
Trace element solution	51 mL
H_3BO_3	305 mg
$(NH_4)6Mo_7O_{24}.4H_2O$	190 mg
$CuSO_4.5H_2O$	30 mg
$CoCl_2.6H_2O$	25.5 mg
$ZnCl_2$	20.5 mg
$MnCl_2.4H_2O$	20.5 mg
Adjust pH to 7.5 with HCl	

2.2. Cell Counting

When the algal culture reaches the desired density (about 0.25 - 0.5 g dry weight/L), the algae were harvested from the tank by pumping out the water slurry containing the dispersed algae. Transfer 1 mL of each cell type to different test tubes, and fix the Tetrahymena by adding 1 mL of formaldehyde. First count the algae. Position the hemocytometer on the microscope stage and adjust the objective so you are foucsed on the grid lines. Count the number of cells in the 4 A squares plus the central C square and average them by dividing by 5. Each value, multiplied by 10 gives you the cells per mL.

2.3. Using a Counter Chamber

A device used for determining the number of cells per unit volume of suspension is called a counting chamber. The most widely used of chamber is called a hemocytometer, since it was originally designed for performing blood cell counts.

2.4. Harvesting

Harvesting the crop is one of the most critical steps. Swimming species are usually harvested by partially draining a tank then sieving it. Sessile crops are usually removed manually. Large plankton can be filtered from the water. Harvesting techniques vary depending on the nature of cultivation media and the degree of management employed [8]. Different methods were tested including flocculation, centrifugation and flotation, and it was found that centrifugation is the best one, but it will be very costly for large scale application. The dry weight content was 3g/L of which 3-6% was β-Carotene.

Fig 3 Extraction of Beta Carotene.

2.5. Concentration

The slurry is then concentrated in algal content, typically by evaporation, centrifugation, flocculation, ultra filtration, flotation, etc. Results have shown that the flocculation using alum followed by centrifugation will be efficient and less energy consuming rather than direct centrifugation [8]. Following the concentration step of the slurry the salt was largely removed by the addition of fresh hot water and ultra filtration. This also breaks the algal cells, liberating the beta-carotene.

2.6. Centrifugation

The concentrate was then centrifuged in a suitable continuous flow centrifuge, such as cream separator, to yield an upper cream phase of carotene and lipid, which is continuously removed [9]. The block flow diagram of the process is shown in Figure (4).

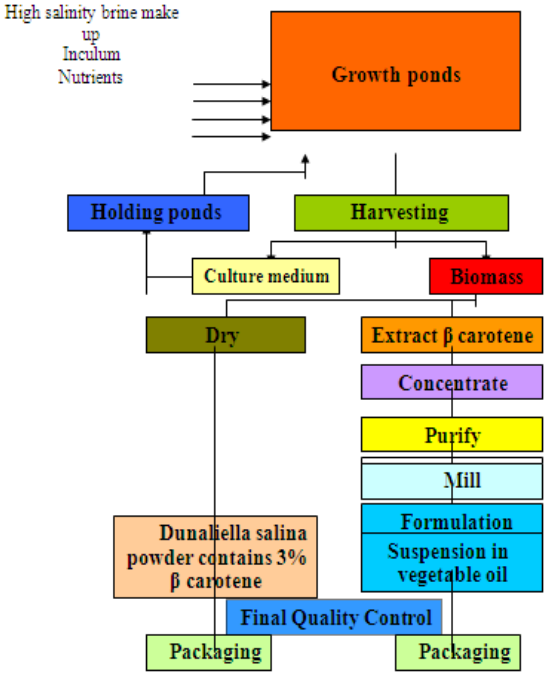

Fig 4. Block flow diagram.

2.7. Extraction

Another critically important aspect of *Dunaliella* β carotene production is the extraction of β carotene, see Figure (3). The process used for extraction depends, in part, on the harvesting procedure used and on market requirements. Extraction using conventional organic solvents is efficient, but may not be acceptable to customers seeking a natural product [6, 9]. More acceptable alternative extraction methods use hot vegetable oil or jojoba oil.

2.8. Freez Drying

In chemical synthesis, products are often freez dried to make them more stable, or easier to dissolve in water for subsequent use. In bio separation, freeze drying can also be used as a late-stage purification procedure, because it can effectively remove solvents. Furthermore, it is capable of removing molecules with low molecular weights that are too small to be filterd out by a filtration concentration membrane. Freeze drying has a long process time, because the addition of too much heat to the material can cause melting or structural deformations [6, 8]. Therefore, freeze drying is often reserved for materials that are heat sensitive, such as proteins, enzymes, microorganisms, and blood plasma. The low operating temperature of the process leads to minimal damage of these heat sensitive products. The freeze drying process was carried out under -50^{o}C and 1.63 hecta pascal using the freeze dryer of type Heto Power Dry PL 3000. The obtained powder was analyzed by the research & development department at one of the Pharmaceutical companies.

3. Results

3.1. Cultivation

Dunaliella cells were found in samples collected from the Dead Sea. The cells were observed under digital microscope with USB camera, so that *Dunaliella* cells were cultivated in an inorganic media (Johnson media). Ordinary air was supplied at room temperature 25±2^{o}C under high illumination.

3.2. Cell Counting

The number of *Dunaliella* cells was found to be about 500,000 cell/mL using Hemocytometer slide under microscope. Cell filtration was performed for five samples and a sequential curve was plotted with a slope near to 0.0407 (approximately horizontal line resulted) and an intercept of 2.1129 indicating a cell density of 2.1129 g dry weight per litter, Figure (5). The number 2.1129 indicates that harvesting can be started. A 1 m^3 cultivation media is found to contain approximately 114.1 g β-carotene. The fluctuation in the dry weight can be attributed to the manual filtration of the samples.

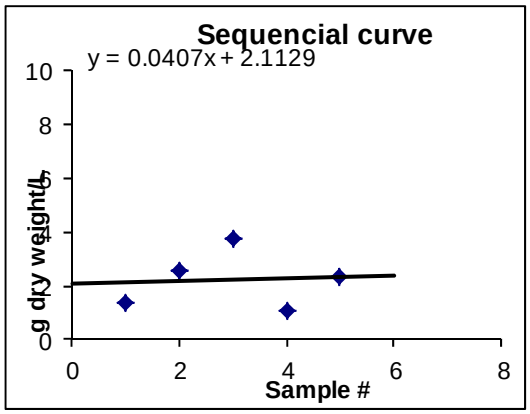

Fig 5. Sequential curve of filtered samples.

3.3. Extraction

After cells were harvested by centrifugation with 4000 rpm, β-carotene was extracted from *Dunaliella* cells using 2 solvents, 96% n-hexane and hot vegetable oil /jojoba oil separately, see Figure (6). Jojoba oil was chosen as extraction solvent due to:

- Jojoba Oil is used medicinally. It contains vitamins E and B complexes. It also has the minerals silicon, chromium, copper and zinc. It has a very high percentage of iodine. The iodine concentration gives the Jojoba oil a great power to heal.
- Jojoba is liquid wax highly penetrating and closely resembles human serum.
- Inflammatory, anti-oxidant, prolong shelf life.
- Jojoba Oil is highly rich in Vitamin C, as well as processing sulfur, copper, and cobalt and has traces of tin, and a volatile oil.

Fig 6. β-carotene extracted with jojoba oil

3.4. Freeze Drying

A centrifuged suspended in distilled water samples were dried using a freeze dryer device at a temperature of -85 °C and 0.32 hpa pressure, the run time was four hours to obtain powder. 1g *Dunaliella* powder was obtained, Figure (7).

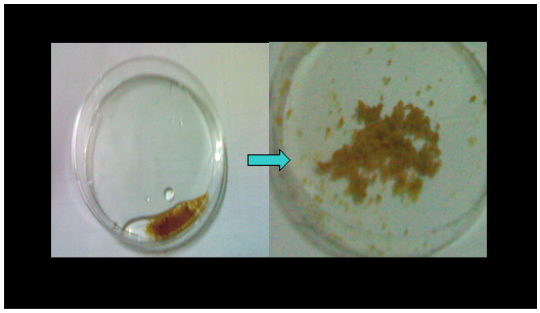

Fig 7. Freeze dried Dunaliella powder.

Scanning spectrum of the extracted pigments is shown in Figure (8).The highest peak was appeared at 440 nm wave length and indicated an optical density value of 2.7. The shorter wave length peak is related to other carotenoids .

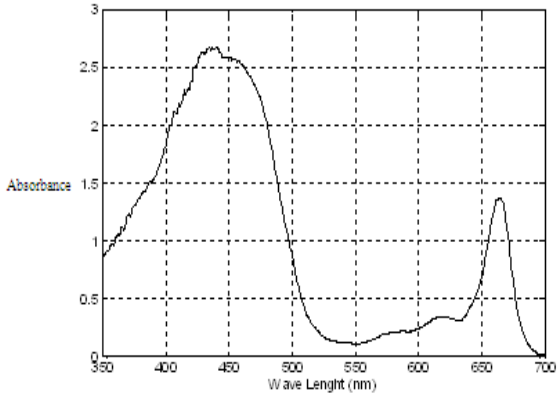

Fig 8. Absorption Spectra of β-carotene extracted with hexane .

4. Discussion of Results

Although β-carotene can be synthesized or extracted from other natural sources, *Dunaliella* is still the richest and best natural source of this cartenoid. The most suitable media for the isolated *Dunaliella* species at lab scale cultivation was found to be modified Johnson media, which gave the highest growth and β-carotene production. Pigment analysis using spectrophotometer indicates the presence of β-carotene, in addition to other related pigments. As can be seen from Figure (5), the dry weight per litter cultivation media was 2.1 gram of which 5.8% was β-carotene. Then in $1m^3$ cultivation media 114.1 g highly pure β-carotene exists. To obtain 1 kg of highly purified β-carotene 8.8 m^3 cultivation media is needed, its selling price is about 3000 US$/kg.

The best method for concentrating the algal material without smashing its clusters was found to be gathering it in aseparatory funnel and permit it to sediment by gravity force. The best extraction solvents were hexane, ether, jojoba oil or light vegetable oil for dietary uses. It was noted that sun-dried or heat dried samples of *Dunaliella* were rapidly degraded in terms of keeping β-carotene not oxidized, so freeze drying process employed and showed high efficiency in drying samples without oxidizing any part of β-carotene [8].

Furthermore, the use of β-carotene as a food or food additive and a nutritional supplement means that a high quality product is required. This means that great care must be taken in the extraction and formulation steps.

5. Conclusions

The following points can be concluded as the main results of this study:

1. Natural Beta Carotene can be produced from *Dunaliella* microalgae existing in the living Dead Sea.
2. Tanks are adequate for the cultivation of microalgae *Dunaliella*.
3. Harvesting by centrifugation and solvent extraction are necessary steps for the production of natural β-carotene.
4. Adjusting the ratio of 9-cis to all-trans is of vital importance as natural β-carotene is a precursor of vitamin A.
5. Freeze drying step is necessary to obtain β-carotene as powder without affecting its sensitive structure.

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