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Producing Natural Mixed Carotenoids from Dunaliella salina

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Abstract
The aim of this work was to cultivate the micro algae Dunaliella *salina* isolated from the Dead Sea by using a certain media. The cell number was found to be 6 million cells per ml after two weeks of cultivation. The micro algae was harvested and centrifuged, after that it was extracted using ethanol as a solvent. UV-spectrophotometry analysis was carried out for beta carotene and other carotenoids . The analysis showed the presence of different carotenoids, mainly beta carotene and a mixture of different compounds like astaxanthin, which can be considered as an added value.It is evident that this process should find its way for commercialization through a pilot plant at the first step, then by an industrial plant after verifying the results of the pilot plant.
Keywords: Dunaliella *salina*, Beta Carotene, Cultivation, Cell counting, Astaxanthin, Extraction.

1. Introduction

The microalgae which were found in the Dead Sea were 22 different types.

The microalgae Dunalielle salina has been studied since it was found living in the Dead Sea [1]. It has been found that most of the biological- biochemical pathways, all of these things that would occur in a normal environment- are also found in the Dead Sea. The microalgae have several applications:

Enhancing the nutritional value of food and animal feed owing to their chemical composition, playing a crucial role in aquaculture, as sole food source filter feeders, a food additive as many fishes and incorporating into cosmetics.

Dunaliella is unique unicellular green algae. Dunaliella Salina was originally described by Dunal in 1838 as Haematococcus Salinas but in 1905 Teodoresco demonstrated that this species differed from Haematococcus and chlamydomonas; twenty-eight species of Dunaliella are presently recognized[2]

Dunaliella occurs in saline environments and it has vegetative cells without contractile vacuoles. Vegetative cells always have green growth at salinity of 2 to 4 %. The cells are capable of turning orange or red in culture growth at salinity of 6 to 12 %. Vegetative cells always green growth at salinity of 6 to 10 %.The cell shape in species of Dunaliella varies from ellipsoid , ovoid , cylindrical , and fusiform to almost spherical ; cells of given species may change shape with changing conditions , often becoming spherical under unfavorable conditions and light intensity . The general cell organization has been studied in Dunaliella Salina with the light microscope and the electron microscope.

Dunaliella Salina is characterized by lack of cell wall with the presence of only a thin cellular membrane, large cup-shaped chloroplast with its photosynthetic thylakoid membranes, pyrenoid and starch and numerous β-carotene globules within the chloroplast [2] .In addition, two flagella equal in length and usually exhibit a homodynamic pattern of beating, the angle formed between the two flagella bases is usually 90 to 130 degrees and they are connected to each other by one distal and two proximal fibers.

The single chloroplast occupies most of the cell body; it is cup-, dish- , or bell-shaped and has a thickened basal portion containing a pyrenoid. In living cells it has a rugose texture especially in older cells. In Dunaliella Salina the chloroplast accumulates large quantities of β-carotene within oily globules in the inter thylakiod spaces. So, the cells appear orange-red rather than green [2]

Dunaliella Salina looks green, however, in cases of high salinity and light intensity, the microalgae turns red owing to the presence of protective carotenoids in the cells. Most of the harvested micro-algae are now being sourced from one of the most pristine environments in the world, a faraway seaside salt lagoon in Western Australia. The Dunaliella Salina is harvested without any harmful solvents or chemicals and the carotenoids, the highly-valued anti-oxidant pigments responsible for the red color, are then extracted for use in pharmaceuticals, nutritious supplements, aquaculture feeds, food coloring, and cosmetics. The product does not include any pathogens and has very low bacteria levels due to the natural antibacterial qualities of the paste [1]

The β-carotene globules of Dunaliella Salina were found to be composed of practically only neutral lipids more than half of which were β-carotene. Most of the reddish forms may drop their red color when grown at low light intensities.

The flagella apparatus in Dunaliella plays an essential role in its cellular physiology ; its motility allows control of the quantity of light received by the cells that swim toward or away from the light source and detect a pattern in the distribution of light getting to them . It is also of considerable importance in the mating process between gametes [2].

Dunaliella when revealed to stress conditions such as high light intensity or nutrient starvation, two stereo isomers of β carotene, all-trans and 9-cis β-carotene, accumulate, 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 can do it only under suitable conditions.

Today, Dunaliella Salina is cultivated for its high yields of carotene: 2100 mg of β-carotene and 102.4 mg of β-carotene per 100 g, contrasted with 5.8 mg and 2.8 mg respectively for carrots (USDA National Nutrient Database for Standard Reference Release 18 USA). Nowadays it is clear that, although β-carotene-rich Dunaliella salina are indeed present in the unclean ponds, most of the coloration of the crystallizer brine is caused not by the algae but by red halophilic Archaea instead. There are two types of vegetative cells- with contractile vacuoles (subgenus Pascheria) and without contractile vacuoles (subgenus Dunaliella[3].

The identification of dunaliella species according to vegetative cells: always green; optimal growth at a salinity of 2 to 4% (oligohaline/euhaline), or of 6 to 10% (hyperhaline) and capable of turning orange or red in culture; optimal growth at salinity of 6 to 12% (hyperhaline). Also according to shape: radially symmetrical or bilaterally symmetrical, flattened, dorsiventrally curved or slightly asymmetrical [3].The identification of Dunaliella Salina species according to shape: if broadest in the middle or anterior region, posteriorly narrow (ssp. sibirica) ,cylindrical to ovoid, posteriorly broad, anteriorly narrow (ssp.salinan i. Cell cylindrical (fo.oblonga), ii. Cells ovoid; average cell volume more than 1000µm3, average cell length more than 15µm, average cell width more than 11µm (fo.magna); average cell dimensions smaller (fo.salina) [3].

Dunaliella species appears to be able to take up CO2 and HCO3 for photosynthesis. The supplement of inorganic carbon is important to the culture of Dunaliella Salina; at the high salinity at which this algae grows, the solubility of inorganic carbon is low [4].

Condition Effect on Dunaliella Salina:

i) Effect of pH:

The optimum pH for growth for the marine D. tertiolecta is pH 6, while for the halophilic D. Salina and D. viridis is about pH 9 [5].

ii) Effect of Temperature:

The optimum growth temperature for D. Salina is in the range of 20 to 40°C depending on the strain. Dunaliella Salina can bear extensively low temperatures to below freezing but temperatures higher than 40°C are usually lethal [6, 7, 8].

General of Carotenoids

Carotenoids, a nutritional pigmented antioxidant are tetraterpenoid substances (containing 40 carbon atoms, built from 4 terpene units each containing 10 carbon atoms) that are naturally found in plants, microorganisms such as algae, some bacteria, and in some fungus. They are also a common feature in animals, for they impart a distinct color to them, the pink color of flamingos and salmon, and the red color of cooked lobsters are due to such carotenoids. They are usually of two types [9].

1. Carotenes: Which contain no oxygen atoms called lycopene (the red pigment in tomatoes) and beta-carotene (the orange pigment in carrots) are carotenes
2. Xanthophylls: Which contain oxygen atoms: lutein, canthaxanthin (the gold pigment in chanterelle mushrooms), zeaxanthin, and astaxanthin. Carotenoids are efficient free-radical scavengers and hence minimize the oxidative stress and associated cellular damage [9].

β-carotene (beta-carotene) is a strongly colored red-orange pigment abundant in plants and fruits. It is an organic compound and is chemically classified as a hydrocarbon and particularly as a terpenoid (isoprenoid), indicating its derivation from isoprene units. β-carotene is biosynthesized from geranylgeranyl pyrophosphate. It is a member of the carotenes, which are tetraterpenes, synthesized biochemically from eight isoprene units and thus having 40 carbons. Among this general class of carotenes, β-carotene is differentiated by having beta-rings at both ends of the molecule. Absorption of β-carotene is increased if consumed with fats, because carotenes are fat soluble.

Carotene is the matter in carrots, sweet potatoes and pumpkins that colors them orange and is the most common form of carotene in plants.

β-carotene is composed of two retinal groups ,it also has anti-oxidant qualities and it can absorb in the visible region strongly between 400-500 nm, β-carotene is known as provitamin A [10].

β-carotene which is found in plant source can be converted to Vitamin A. And there are two means in which β-carotene can be converted to Vitamin A.

a. By breaking the β-carotene molecule from one end.
b. By cleaving at the center.

In the molecule chain between the two cyclohexyl rings β -carotene cleaves either symmetrically or asymmetrically; at which symmetric cleavage is done by using an enzyme called (beta -carotene-15,15'-dioxygenase) in the human body, this gives two equivalent retinal molecules and each retinal molecule reacts to give retinol (Vitamin A) and retinoic acid [11].

The conjugated chain in carotenoids means that they absorb in the visible region and hence are colored [12]. Natural extracts containing carotenoids, for instance carrot extracts and red palm oil, have been used to give color to foods for ages. Beta-carotene has an advantage over other artificial colors, for example azo dyes, because it occurs naturally in food and is so known to be safe also it has been used to treat various disorders such as erythropoietic protoporphyria , reduce the risk of breast cancer in women, and the risk of age-related macular degeneration (AMD) [13].

The carotenoids also include astaxanthin . Astaxanthin is a carotenoid pigment, which is biologically antioxidant and can be found naturally in algae as well as many plants. It belongs to the same family of fat soluble carotenoids molecules as the yellow/orange colored Beta-carotene**;** but it is different from Beta-carotene as it's molecular structure contains two extra oxygen groups in each ring structure giving it a deep red color and classifying it as a xanthophy ll with up to 10 times stronger free radical scavenging activity. Also unlike Beta-carotene astaxanthin has a no-pro-vitamin A activity**:** It is not converted to vitamin A in the human body**.** And although some humans and animals are able to change carotenoids into other forms, their bodies cannot endogenously produce them. The primary natural source for astaxanthin is the microalgae H. pluvialis, which has the highest levels. Astaxanthin helps stop lipid peroxidation, and advance the preventative abilities of many other antioxidants [14, 15].

Cis and All-trans β-carotene Composition

A carotenoid composition derived from a natural source where at least 50 % by weight of the carotenoid content of the composition is Cis beta-carotene and preferably 9 Cis beta-carotene. Characteristically, the composition beta-carotene content is predominantly 9 Cis beta-carotene.

The invention refers to a carotenoid composition derived from a natural source, with a high Cis beta-carotene concentration and its preparation from natural sources, and more preferably a high 9 Cis beta-carotene composition and its preparation from natural sources.

In this specification it is to be understood that the natural sources of carotene encompass fruits, vegetables plant tissue and animal tissue. A particularly commercial source of carotene is certain types of algae like Dunaliella Salina[16].

Beta-carotene occurs in a number of different chemical isomer forms. Some of these isomer forms are geometrical ones that have a different orientation around one of the double bonds in the conjugated double bond structure of the molecule. This can occur in a number of positions along the conjugated backbone to make a range of different geometrical isomers. In some cases there can even be more than one double bond where change of orientation occurs. The most common geometrical isomer is the all-trans isomer with a structure occurring where the main carbon chain of the molecule occurs in a trans (across) or straight configuration.

However, there are Cis forms of beta-carotene which occur naturally, and can be produced by chemical synthesis, or formed by physical processes like heat on the all trans- isomers, where the main carbon chain of the molecule takes a bend (Cis) or sideways configuration. Naturally occurring Cis forms of beta-carotene are not known to occur over a weight percentage of approximately 30% to less than 50% of the total carotenoid content.

Associated with the different geometric isomers are different properties and possible functions and for this reason there are potential benefits in relatively concentrated forms of the Cis isomers [16].

In natural products such as fruit, vegetables, algae and other plant and animal material the carotenoids are stabilized as part of the cell structure in small micron or sub-micron sized particles in the cell organelles or even by association with other molecules which stabilize the isomeric forms produced by the biochemical pathways of the organism. However, in the preparation of concentrated forms of these materials for commercial products desired from the natural sources, the natural stabilizing capacity of the cellular structure may be removed or reduced in the extraction and concentration of the carotenoids.

Crystallization is a problem in certain applications since the crystalline form may not be available for efficient use in the application because of its relative insolubility.Crystallization occurs particularly with all trans- beta-carotene and as a result it is not, for example, readily available for biological use

Cis isomers on the other hand are much less likely to crystallize and as a consequence are much more soluble than the trans isomers., That is why it is often more preferable to use beta-carotene containing compositions with higher concentrations of Cis isomers for various applications. For example, the 9 Cis isomer is much more readily soluble in oils than the all trans- forms. Actually, it is very hard o get the 9 Cis isomer to crystallize out from naturally derived oils, therefore making it complicated or costly to purify on a large scale.

In naturally occurring products the proportion of Cis isomers is somewhat small, but one of the highest proportions occurs in the halophilic alga Dunaliella Salina where normally 30% to below 50% of the total carotenoid content occurs as the 9 Cis form [17].

The 9 Cis isomer of beta-carotene structure is as follows:

This 9 Cis isomer of beta-carotene is preferably derived from particular natural sources of plant products including green peppers, apricots, flower of certain species of the Acacia genus, cucurbitaceae and in the alga Dunaliella Salina, which has the highest concentration of the 9 Cis isomer of such sources. In this regard, see Ami Ben-Amotz, Amnon Lers and Mordhay Aron: "Steroisomers of Beta-Carotene and Phytoene in the Alga Dunaliella bardawil" Plant Physiol, 1286-1291(Dunaliella bardawil has subsequently been acknowledged by Ami Ben-Amotz as naturally occurring Dunaliella Salina) [17].

The proportion of the total Cis isomers (predominantly the 9 Cis isomer) content in the total carotenoid content of the alga Dunaliella Salina is normally found at around 30% to below 50% of the total carotenoids content on a weight basis as determined by the high pressure liquid chromatography and visible light spectrophotometry techniques. The finished product is preferably dispersed in a natural carrier oil from vegetable, animal and mineral origins and specially olive, soya bean, corn, essential oils, terpene based oils and fish derived oils.

The anti-oxidants may be used to assist to protect the high Cis beta-carotene preparations from oxidation, which is comparatively more significant when in a lower total beta-carotene concentration product, for example, less than 5% of beta-carotene in the preparation. However, even at higher concentrations it is important to protect the beta-carotene preparations from oxidizing.

3. Methodology

3.1 Cultivation:

Two modes of cultivation are being used in large scale bioreactors of Dunaliella. In the more common, the intensive mode is made to control all factors affecting cell growth and chemistry. In the other mode, the extensive growth, Dunaliella grows very slowly in nearly saturated brine where the high salt concentration is used to control consistent production of β-carotene Dunaliella is cultivated in suitable environment like shallow tanks, bioreactors, man-made or natural ponds at a range of temperature of 25-45ºC and a very wide pH tolerance ranging from pH 1 to pH 11. It can cope with a salinity range from seawater (3 - 31% NaCl) [17].

Cultivation process was made in the miracles of Dead Sea factory in a man-made bond by adding Dead Sea salty water and Johnson media that contains 17 chemical substances, the surrounding factors as the temperature, pH and humidity had been controlled.

Factors that determine the growth rate of algae:

Many factors affecting the growth rate of algae like algae type, Light for the photosynthesis process, ideal temperature range, pH in the range of 7-9 to have an optimum growth rate, air for its carbon dioxide requirements, mixing to make sure that all cells are equally exposed to light and photo period- light and dark cycles to show the required condition for the culturing of micro algae.

3.2 Centrifuging and Extraction:

One liter of cultivation water was taken, and centrifugal process with several trials was used to collect the sludge contains in the water. After that, the sludge were extracted by using a separator funnel by adding Ethanol as a solvent (10-15ml), and the extract layer containing carotinoids was separated.

3.3 Freeze drying:

Freeze-drying operates through freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublime directly from the solid phase to gas phase [18] .Lyophilisation consists of a drying substance removing the solvent (typically water):

- freezing the solution into solid phase.
- sublimating the solvent ice crystals to obtain gaseous phase.

β-carotene crystallization was attained at 0.32 mmHg pressure and 85° C temperature; the run time was four hours.

Process of freeze-drying:

The freeze-drying process was achieved through the four following phases:

1-Pretreatment; and its methods include: freeze concentration, solution phase concentration, formulation to stability reactive products, formulation to increase the surface area, and decreasing high vapor pressure solvents.

2- Freezing; its process continues from 1 hour to 24 hours, hinging on the application.

3- Primary drying; on the completion of this first drying cycle, the product will have 3 to 5% moisture content.

4- Secondary drying; on the completion of this secondary drying cycle, the product will have 0.5% moisture content [19, 20].

4. Results & Discussions

The principal target of this project was to produce natural carotenoids from Dunaliella *salina* isolated from the Dead Sea by using Jhonson media.

The cell number was found to be 6 million cells per ml after two weeks of cultivation. The micro algae was harvested and centrifuged, after that it was extracted using ethanol as a solvent. The extracted Beta carotene was subjected to freeze drying at -85 degree Celsius and 1.6 hp for 21 hours in one of the local pharmaceutical companies. The following figure shows the absorption spectrum of the produced beta carotene.

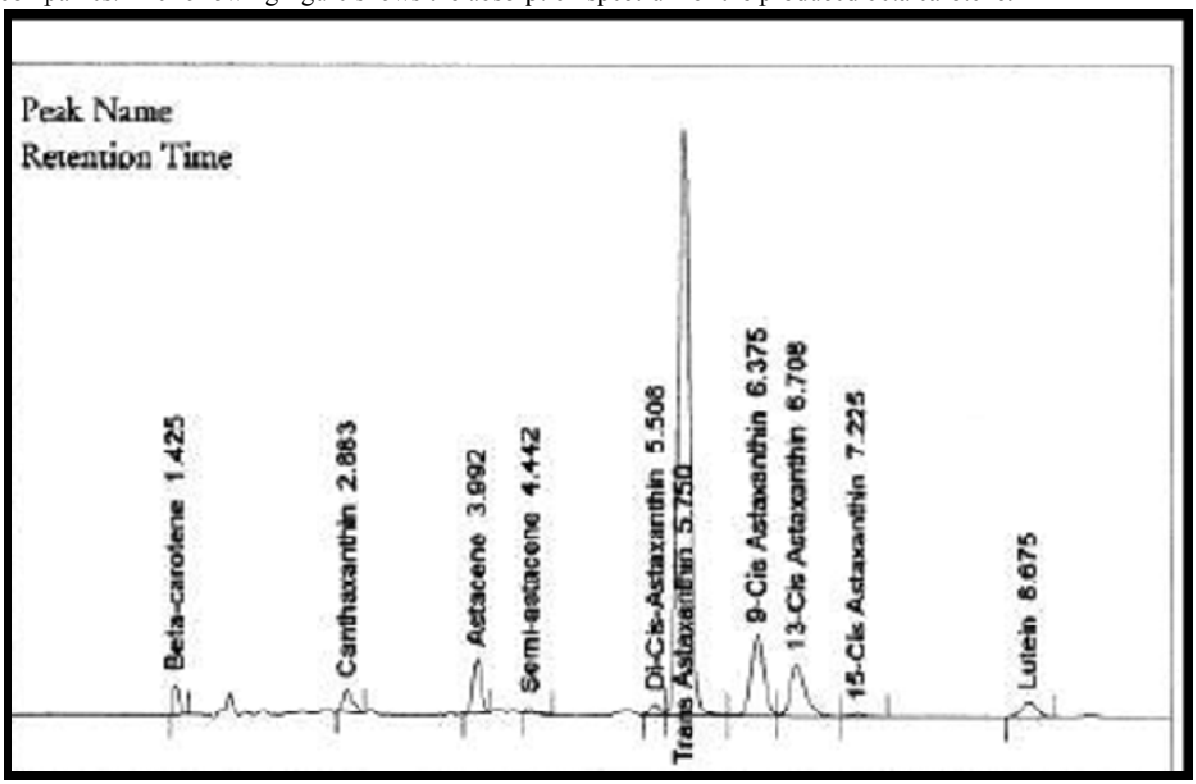

Figure **(1)** Carotenoids absorption spectrum

The absorption spectrum of carotenoids is shown in figure (1), while the absorption spectrum of Beta carotene is shown in figure (2).

The first peak corresponds to Beta Carotene, while the second one corresponds to Astaxanthin. These two absorption spectrums form the rehearsal evidence of the formation of mixed carotenoids during cultivation of Dunaliella *salina* under certain conditions.

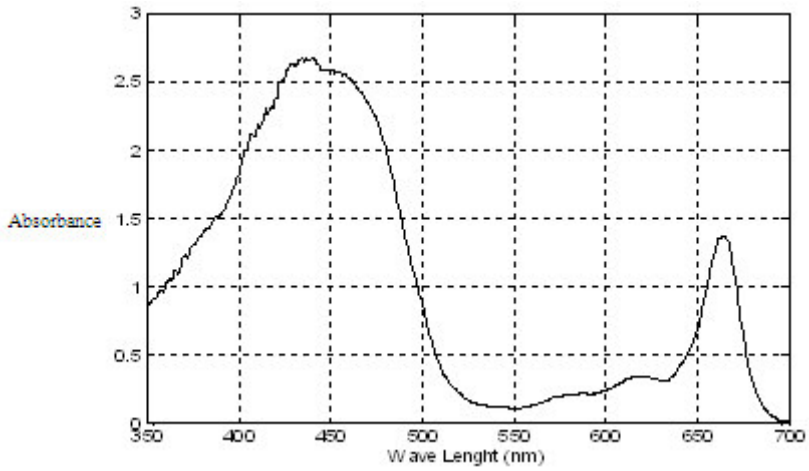

Figure **(2):** Absorption spectrum of Beta Carotene.

Maximum growth was obtained at 20 °C where chlorophyll *a* and β-carotene concentrations were 3.4 and 2.1 mgl-1, respectively, after 10 days. A slight growth was observed at 30 C°, while no growth was observed at 40 C° and 50 C°. The highest growth of *Dunaliella* cells was found at 40 mg Nl-1.

However NaNO3 enhanced the highest growth and β-carotene production compared to other nitrogenous compounds used.
Sodium nitrate (NaNO3) at a concentration of 40 mg Nl-1 gave 5.17 mg l-1 and 4 mg l-1 for chlorophyll *a* and β-carotene, respectively. However, the maximum β-carotene/chlorophyll *a* ratio was found to be 0.82 at 20 mg Nl-1.
Growth and β-carotene production after 15 days of growth using M1 medium supplemented with different nitrogenous compounds: 1, 40 mg NL-1 NaNO3; 2, 40 mg NL-1 Ca(NO3)2; 3, 40 mg NL-1 NH4NO3; and 4, 50 mg NL-1 NH4Cl [21].
The effect of different concentrations of magnesium sulfate on *Dunaliella salina* was studied. *Dunaliella* growth and β-carotene production were found to be the highest at 25 mg l-1 MgSO4. So, chlorophyll *a* and β-carotene concentration were 3.4 mg l-1 and 2.4 mg l-1, respectively.
The maximum chlorophyll *a* and β-carotene production were obtained at 2.5% NaCl with 5 mg/l and 4.2 mg/l chlorophyll *a* and β-carotene respectively, however, the decrease in chlorophyll *a* under laboratory conditions was noticed in 30% and DSw-M1 (3:1)(0.2mg/l) , an increase in β-carotene production was 2.5mg/l noticed in *Dunaliella* grown in DSw-M1 (1:1) as compared to DSw-M1 (3:1). The best ratio of β-carotene /chlorophyll *a* was recorded in culture grown in DSw-M1 (1:1) which was 1.1.
It is evident that carotenoids produced from Dunaliella salina contain a large amount of Beta carotene, and around 15 % 9-cis and 13-cis astxanthin, which can be considered as an added value to the produced beta carotene. It can be considered that the produced Beta- carotene can be used as an alternative source for producing astaxanthin.

5. Conclusions

1. Dunaliella *salina* was cultivated using a certain media and the cell count was measured to be 6 million cells per ml after two weeks of cultivation.
2. Beta- carotene was extracted using ethanol as a solvent.
3. UV- visible spectrophotometric analysis was carried out for beta carotene.
4. The absorption spectrum of the produced Beta carotene showed that it yielded around 15 % 9-cis, and 13-cis Astaxanthin, which can be considered as an important added value to the produced Beta carotene.
5. Microalgae are normally grown in two phases. During the first phase, the cells are provided with plenty of nutrients in order to advance the cells proliferations. In the next phase, the cells are prevented from nutrients and exposed to strong sunlight, when the algae develop high levels of astaxanthin to encounter environmental strain.

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