Extraction of β-Carotene from Locally Dunaliella salina Using Bacterial Lipase Enzyme

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Abstract

The unicellular alga Dunaliella salina prosper in high salt concentration environments. It can accumulate significant amounts of commercially important chemicals such as, lipids, glycerol and carotenoids. β-carotene is the most important carotenoids they produce, it reaches up to 14% of dry wight. Additionally, can use in different applications in cosmetics, nutrition and pharmaceutical. There are a lot of studies on the development of β-carotene extraction methods to produce high purity product with minimum cost and time. The aim of the article is β-carotene extraction using bacterial lipase enzyme.

Keywords: *Dunalilla salina, β-carotene, Lipase, Extraction.*

INTRODUCTION

The product's quality influences purchasing choices. Most people seeking to use natural and organic sources of food or supplements such as algae and plants from a long time ago. Before 2000 years, the Chinese had used Nostoc as a source of nutrition for the first time to survive during famine due to its high protein and pigment contents, and low-fat content (Chu, 2012). Microalgae are among the fastest-growing autotrophs on the earth, which usually utilize available material for growth. Also, they are one of the cheapest organisms that can be used in different biotechnological applications. Several studies demonstrate the microalgae are responsible for over 50% of primary photosynthetic productivity on the earth (Milledge, 2010). In addition, they can grow in a various natural aqueous and terrestrial environment. Still, only a few hundred of the thousands of microalgae species have been studied, and just a handful are cultivated on an industrial scale. University, Australia, believes that microalgae biotechnology has grown and diversified significantly over the past 30 years (Milledge, 2010). Moreover, they produce various natural products including proteins, enzymes, lipids, vitamins, carbohydrates, minerals, and carotenoids. Humans and animals cannot synthesize carotenoids and usually take them through food (Solovchenko and Chekanov, 2014). Their most essential applications include food supplements, food colourant, and feed additives. In addition, carotenoids have anti-oxidant activities, which can protect humans from certain cancers and arthritis (Gong and Bassi, 2016). Also, they can reduce the risks of AIDS, diabetes, and neurodegeneration. Among the known

carotenoids produced by microalga, β-carotene, astaxanthin, lutein, and canthaxanthin are the best-marketed carotenoids. According to forecasts made by Zion Market Research Global in 2016, the market value of carotenoids is anticipated to evolve at a compound annual growth rate of 3.5% from 2016 to 2021 while reaching revenue of USD 1.52 billion in 2021 (Rammuni et al., 2019). Also, depending on last data, the total weight of dry biomass of commercial microalgae in 2004 was 700 tons (Michalak and Chojnacka, 2014).

Amongst the various microalgae, the Species of the genus Dunaliella have economic importance. One of the most important genera of Dunaliella is D. salina because it accumulates significant amounts of valuable chemicals, such as carotenoids and glycerol (Shariati and Hadi, 2011). Several studies have documented that D. salina is the best commercial source of natural β-carotene among all organisms globally, which has a wide range of applications in nutrition, cosmetic and pharmaceutical products. In recent years, mass cultivation of D. salina for β-carotene production has been accomplished in several countries, including Australia, the USA and China. At present, the increasing needs for natural β-carotene led to focus on the development of practical extraction techniques from D. salina. The extraction with the conventional method such as organic solvents provide good yields. However, unfortunately, many unwanted issues that always challenge scientists such as high cost, toxic nature of organic solvents, the residue contamination of final products, and consuming time. One of the best extraction methods is using enzymes such as, cellulase, protease, and lipase. In addition, can obtained enzymes from different plants, mammalian cells, and microorganisms (Marathe et al., 2019). The extraction using enzymes are safe, rapid, and simple method. Also, they safe to use in food and nutraceutical purposes, owing this there are a huge concern about the extraction using enzymes. However, in this study, we are aiming for the development of an extraction techniques using bacterial lipase enzyme, which can, on the one hand, reduce the environmental impact and produce high-quality β-carotene.

Literature review.

1. The genus of Dunaliella:

First sighted of Dunaliella in 1838 in saltern evaporation ponds in the south of France by Michel Felix Dunal, it was named after its discoverer by Teodoresco in 1905 (Oren, 2005a). Also, he the first one who described the genus. Then in the same year Clra Hamburger published a paper presenting an in- depth description of Dunaliella (Oren, 2005a). Researchers in the 18th century thought that Dunaliella is the same as Haematococcus. However, at the beginning of the 19th century, it was reported that this genus clearly differed from Haematococcus and erected the new generic name Dunaliella (Shariati and Hadi, 2011). Now here are 28 species of Dunaliella are recognized (Fig.1). They include five species flourishes in freshwater, whereas 23 species flourishes in saline environments (Fig.1) (Shariati and Hadi, 2011).

Based on morphological and physiological characteristics the taxonomic organization of Dunaliella species is still controversial issue owing to considerable intra-species variation in morphology, which depends on growth conditions. Consequently, many of Dunaliella isolates previously were misidentified. Then Borowitzka and Siva (2007), reorganize the genus based on cell morphology and physiology properties (Ramos et al., 2011). According to the National Center of Biotechnology Information of the National

Institute of Health, algae of the genus Dunaliella belong to (Chlorophyta, Chlorophyceae, Chlamydomonadales, Dunaliellaceae) (Polle et al., 2009). Dunaliella is a halotolerant, photosynthetic green alga, unicellular, flagellated, and comes in varying shapes but it is almost ovoid (Sathasivam and Juntawong, 2013). Also, it is morphologically similar to Chlamydomonas, with the main difference being the absence of a cell wall in Dunaliella species (Borowitzka, 1990). The cells lack a rigid cell wall but are enclosed by a glycoprotein coat called a glycocalyx that swells or shrinks when exposed to hypertonic and hypotonic conditions. Cell size often ranges from 5 to 25 µm in length and 3 to 13 µm in width, but under unfavourable conditions, cells may change shape and size (Hosseini Tafreshi and Shariati, 2009).

The cells have two equal-length flagella at the anterior end of cells, a single cup-shaped chloroplast, with central pyrenoid surrounded by the storage product starch especially in the marine and halophilic species (Fig. 2,3) (Polle et al., 2009). In addition, they have an eyespot located at the anterior peripheral location in the chloroplast, but In D. salina may be hardly visible in the light microscope. Similarly, the cells contain nucleus occupies most of the cell's anterior part and is often surrounded by anterior lobes of the chloroplast. Several studies have reported the Dunaliella genus have a single nucleolus, which is often surrounded by clumped heterochromatin and it has a porous envelope. Furthermore, they contain mitochondria, which can be seen the mitochondrial profiles in various parts of the cell in thin sections. The cells also include 2 to 4 Golgi bodies, each of them consisting of 10 to 15 cisternae. Also, they have endoplasmic reticulum (ER) located underlies the plasmalemma, and they have different type of vacuoles. Cell morphology may change under some environmental growth conditions, which occur between logarithmic and stationary phases (Shariati and Hadi, 2011). The cells in the motile state divide by lengthwise division. Under specific situation, usually at lowered salinities the cells become round non-motile cells embedded in a thick layer of mucilage this called palmella stage, or they may form aplanospores with a thick, rough wall (Borowitzka, 1990). In benthic algal mats of Great Salt Lake, Utah Brock, 1975 scientists observed such palmelloid forms of Dunaliella sp. (Oren, 2005a). Sexual reproduction is by isogamy with conjugation like that in Chlamydomonas.

The zygote is red or green and is surrounded by a very thick and smooth wall of sporopollenin. Then after a resting stage the zygote nucleus divides meiotically to form up to 32 cells liberated through a rupture in the mother cell wall. Several researchers have reported the sexual reproduction more observed in the field, but rarely in culture (Borowitzka, 1990). The zygote has the same size and structural characteristic as growing cells. Also, meiosis occurs during the germination of the zygote (Shariati and Hadi, 2011). In recent years, D. salina the most commonly used model species within the genus of Dunaliella, which is used as model organisms for different researches such as, osmoregulation, carotenoid production, and photosynthesis under extreme conditions, also numerous strains of D. salina are grown commercially for the production of natural β-carotene (Polle et al., 2009).

Figure 1: Species of Dunaliella – from 1 to 5 freshwater species, and from 6 to 28 salty water species, adapted from (Shariati and Hadi, 2011).

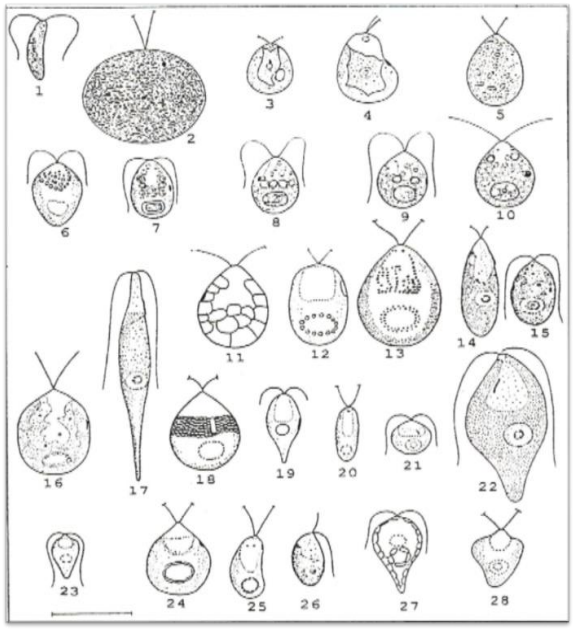


Figure 2: Photograph of Dunaliella, show flagella, the chloroplast fills the posterior region, and the glycocalyx surrounding the cell as a ring, adapted from (Polle et al., 2009)

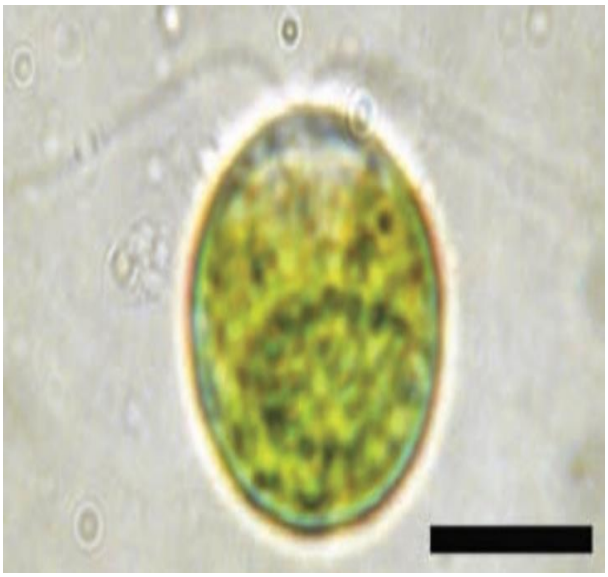
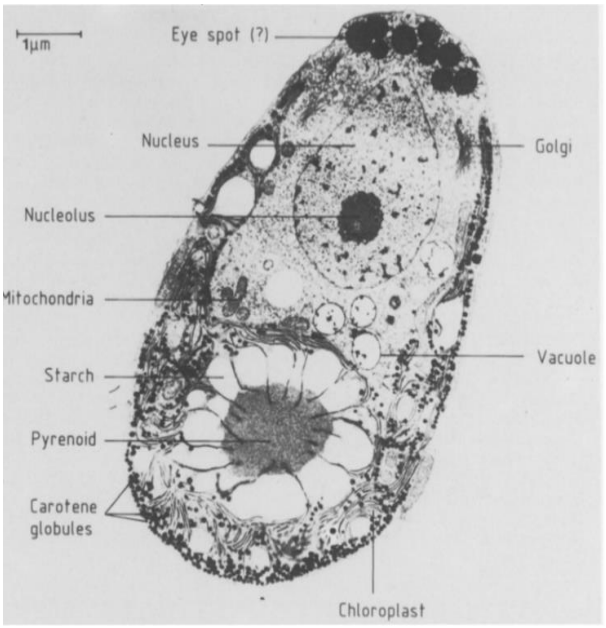


Figure 3: Micrograph of Dunaliella show single, large cup-shaped chloroplast with its photosynthetic inter-thylakoid membranes include numerous of β-carotene globules, pyrenoid, starch, vacuole, mitochondria, nucleolus, nucleus, and Golgi body, adapted from (Ben-Amotz and Avron, 1990).



1.1. Dunaliella environments:

Understanding Dunaliella environment is critical to achieve successful isolation and optimization of the Dunaliella growth. The species often grow in aquatic marine habitats around the world like oceans, brine lakes, salt marshes, and salt water ditches near the sea with a varied chemical compositions and salt concentrations ranging from 0.05 to 5.5M NaCl (Shariati and Hadi, 2011). The most popular examples are the Dead Sea in Israel, the Pink Lake in Australia, and the Great Salt Lake in the United States (Ben-Amotz, 1993). Also, they occur in a wide range of pH tolerance from 1 to 11 (Borowitzka, 1990), and temperature ranging from below 0 °C to 40 °C (Hosseini Tafreshi and Shariati, 2009). The ability of tolerance to a wide range of salinities, light intensities, temperatures, and their ability to

survive for many years among salt crystals contributed to the distribution of Dunaliella species around the world (Polle et al., 2009). Several strains of D. salina were isolated from different locations, for example Romania, Spain, Iran, Egypt and Kuwait.

2. D. salina applications:

There are many applications of D. salina in nutrition of humans and animals, cosmetics, environmental biotechnology and high-value molecules extracted from them. Also, they can act as a nutritional supplement or as a source of natural food colorants duo to their varied chemical properties. In addition, extensive research has been carried out Dunaliella produce several bioactive compounds like enzymes such as dihydroxy acetone reductase (Ben-Amotz and Avron 1990), vitamins, antioxidants (Chidambara Murthy et al. (2005); Milko (1963), Single-cell protein (SCP), minerals (Supamattaya et al. (2005), proteins (Hosseini Tafreshi and Shariati, 2009), and carbohydrates up to 8% (Arun and Singh, 2016). In addition, due to lacking a cell wall and containing high levels of β-carotene (vitamin A), can used Dunaliella as meal in poultry and aquaculture feed or food. Recently, Pentapharm (Basel, Switzerland) suggested an ingredient from D. salina with strong ability to stimulate cell proliferation and turnover and to improve the energy metabolism of skin (Hosseini Tafreshi and Shariati, 2009). It contains sporopollenin organic material, which is similar to amino acids and they can used in sun-protection products for skin and hair care (Pourkarimi et al., 2020). Moreover, when Dunaliella grown under appropriate condition (especially high salinity); they can produce glycerol up to 50%, which is sufficient to account for most of the required osmotic pressures. Under this condition, glycerol acts as a "compatible solute" that protects enzymes against both inactivation and inhibition. Also, is considered as an important commercial organic chemical and osmoregulator, which used in the cosmetic, pharmaceutical and food industries, also it is utilized as an antidrying medium. The dried algal meal, which remains after removal of glycerol and β-carotene from Dunaliella, contains about 40% proteins (Ben-Amotz and Avron 1990). Furthermore, D. salina cells contain high concentrations of polyunsaturated fatty acids (PUFA), which can be used as a supplement or complete food to enhance the performance and state of the human body or improve a specific bodily function (Hosseini Tafreshi and Shariati, 2009). Moreover, it's a promising source of biofuel due to its high content of lipids ranges from 45 to 55% of dry weight (Tornabene et al. 1980). In (1998) Park et al., suggested that hydrocabones productivity of D. salina similar to that from Botryococcus braunii, which was known to economically produce liquid fuels (Hosseini Tafreshi and Shariati, 2009). Also, Dunaliella one of the best options for testing the environmental toxicity among the microalgae because their ability to grow under severe conditions and lack of a rigid cell wall. Previous studies by Thakur and Kumar (1999) suggested a high N and P removal efficiency of the D. salina immobilized in Ca-alginate beads. Also, can exposed a dead or nongrowing living algal biomass to the waste as an absorbent agent for adsorption or absorption metal as Alternative method. This method easy, selective, efficient, and low cost for treating a large amount of wastewater. Also, can used this method in absorption and removal of heavy metals like chromium (VI) ions from saline waters. In addition, several studies have documented the petroleum ether, hexane, ethanolic extracts of D. salina have antimicrobial activity against several micro-organisms of importance for the food industry,

including Escherichia coli, Staphylococcus aureus, Candida albicans and Aspergillus niger. Also, can used D. salina when they grow under stress conditions as a model to study the mechanisms of photo-adaption and light-harvesting, because can modified the alga's liquid culture medium, and can teste the micro-environmental parameters. In addition, Dunaliella is an ideal organism to study environmental stress resistance mechanisms at the molecular level due to the high salinity tolerance, its simple cellular structure, and easy cultivation (Hosseini Tafreshi and Shariati, 2009). Moreover, D. salina considered as one of the most an important commercial source of natural carotenoids such as β-carotene, phytoene, phytofluene, lutein, and zeaxanthin, owing to it is simple life cycle and growth requirements. The phytoene, phytofluene (colorless carotenoids) can protect the skin by acting as UV absorbers, as antioxidants, as modulation of gene expression, and can used in cosmetics and wellness nutrition (von Oppen-Bezalel and Shaish, 2009). Previous published studies about lutein demonstrate used lutein as a pigmentation factor in fish and poultry, as colorant in the drug and cosmetic industries, they also decreasing the risk of chronic diseases like cataracts, arteriosclerosis, age-related macular degradation, and preventing cancer. On the other hand, zeaxanthin is a stereoisomer lutein act as neutraceuticals against macular degradation in the eye, and as an antioxidant (Hosseini Tafreshi and Shariati, 2009). A lot of researches have proven β-carotene, and other carotenoids have many health benefits such as protection against UV and oxidative damage leading to premature ageing, and also have therapeutic benefits such as anti-inflammatory and anti-cancer activities. According to the last updates, the most successful microalgal carotenoids production is astaxanthin from H. pluvialis, and β-carotene from D. salina.

2.1. β-carotene production from D. salina:

Massyuk who first proposed D. salina as a source of natural β-carotene, and then as a source of glycerol (Borowitzka, 1990), which accumulates up to 14% of the algal dry weight (Sui et al., 2019), which is the highest content of β-carotene of any known microorganism. In comparison, a Dunaliella cell can accumulate thousands of times more β-carotene than a carrot cell (Hosseini Tafreshi and Shariati, 2009). Dunaliella β-carotene is composed of a mixture of the cis-isomers and trans-isomers, with composition of 15-cis (10%), 9-cis (41%), all-trans (42%), and other isomers (6%) (Hosseini Tafreshi and Shariati, 2009) (Fig. 4). In contrast, properties of all-trans differ from 9-cis, which all-trans is insoluble in oil, easily crystallized, and 9-cis is soluble in hydrophobic solvents, very difficult to crystallize, and its oily in its concentrated form (Ben-Amotz and Avron, 1990). To avoid cellular crystallization of all-trans β-carotene and survive at low temperatures, Dunaliella delivers a higher proportion of 9-cis to all-trans β- carotene, also 9-cis functions in vivo as an oily matrix for the all-trans form (Ben-Amotz, 2019). Also, depending on the culture conditions Dunaliella can have the most significant amounts of 9-cis isomer among all sources (up to 50% of all isomers), (Hosseini Tafreshi and Shariati, 2009). The amount of the accumulated β-carotene and the 9-cis to all-trans ratio depend on the amount of light absorbed by the cell during the division cycle (Ben-Amotz and Avron, 1990). Also, besides its physiological function each of them has many applications (Table 1) (Raja et al., 2007).

Figure 4: structures of β-carotene isomers a. 9-cis-β-carotene, b. all-trans β-carotene, adapted from (Raja et al., 2007).

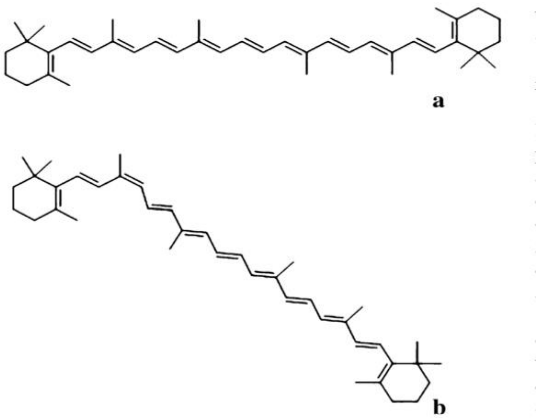


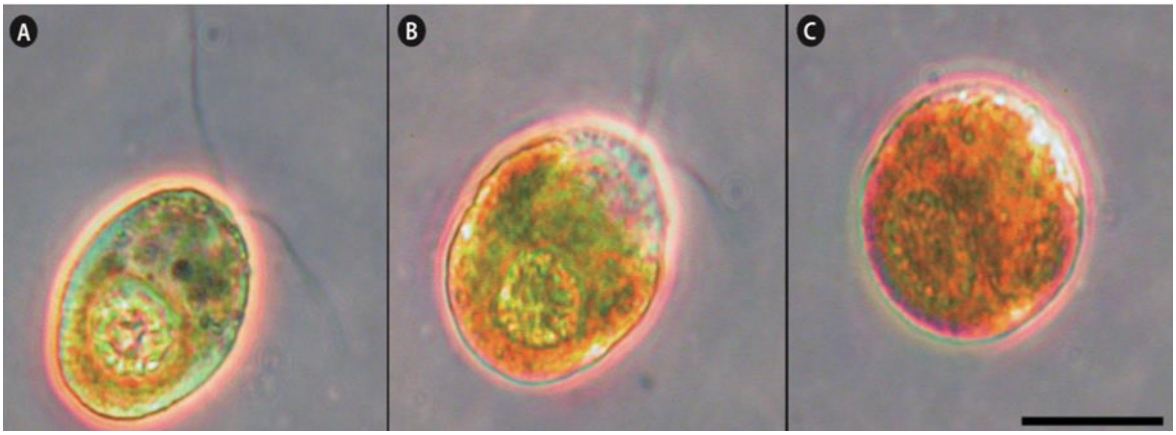
Table 1: Applications of β-carotene isomers extracted from Dunaliella salina, adapted from (Raja et al., 2007).

Principal producer	Isomer	Applications	References
Henkel-Cognis nutrition and Health, Hutt Lagoon and Whyalla, Australia; Cyanotech (Kona, Hawaii) USA; Inner Mongolia, Biological Eng. (Inner Mongolia, China); Nature Beta Technologies (Eilat, Israel); Tianjin Lantai Biotechnology (Tianjin, China); Dutch State Mines (DSM; Food Specialties, Netherlands)	9-cis	Food colouring agent	Berset 1990
	all-trans	Source of vitamin A in animal feed	Gomez and Gonzalez 2004
	9-cis and all-trans	Enhances yolk colour in vitamin A deficient chicken	Gomez and Gonzalez 2004
	9-cis and all-trans	Possess antioxidant activity by quenching singlet oxygen, scavenge peroxyradicals and inhibit lipid peroxidation	Terao 1989
	9-cis	Prevent cancer of various organs like stomach, cervix, pancreas, colon, rectum, breast, lungs, prostate and ovary	Poppel and Goldbohm 1995; Raja et al. 2004b
	9-cis and all-trans	Regulates immune response by increasing the percentage of monocytes and enhances natural killer cell activity in elderly men	Williams et al. 2000; Kazi et al. 1997

	9-cis	Involved in neoplastic transformation and control of growth	Bertram and Bortkiewicz 1995
	9-cis and all-trans	Decrease sensitivity to sun and protect against erythropoietic protoporphyria	Peto et al. 1981

Obtained of β-carotene from D. salina is much easier for many reasons, disruption of cells is much easier than that in other algae because its lack a cell wall, continuous culture in the laboratory is easy and the growth rate is high, and resistance to various environmental conditions is higher than in other algae (Pisal and Lele, 2005). Under non-inducing conditions (usually 1-2 M NaCl) D. salina is green and contain about 0.3 % β-carotene, similar to other algae and plant leaves. After induction and growth under unappropriated conditions, which including high light intensities, high temperature, high salinity and nutrient deficiency, D. salina predominate over all other organisms to a seasonal pigment bloom and cells color change to orange-red, alga can accumulate large quantities of the β-carotene within oily globules in the inter-thylakoid space of the chloroplast, which act as a sun-screen to protect chlorophyll and DNA from high irradiance (Fig. 5) (Shariati and Hadi, 2011), (Borowitzka, 1990). In addition, β-carotene also acts 'carbon sink' to store excess carbon produced during photosynthesis under conditions where growth is limited but photosynthesis carbon fixation must continue (Borowitzka, 1990). Strains unable to accumulate β-carotene die when exposed to high irradiation, while the β-carotene-rich Dunaliella strains flourish (Ben-Amotz, 1993). The optimum salinity of D. salina for growth ranging from 18 to 21% NaCl, while the optimum salinity for carotenoid production is > 27% NaCl β-carotene (Borowitzka and Borowitzka, 1990). Also, many studies documented, to maximum yield of β-carotene the salinity must be intermediate, also the salinity has to higher than the optimum to avoid the predatory protozoa and the non-carotenogenic algal competitor (Raja et al., 2007).

Figure 5: D. salina cells under different culture conditions. A. Green cell, B. Stressed cell turning orange, C. Orange cell (accumulated β-carotene), adapted from (Ramos et al., 2011).



One of the best strategies to obtain high β-carotene production in mass cultures of D. salina is adjusting light and salinity of the culture (Hosseini Tafreshi and Shariati, 2009). In addition, carotenogenesis is greatest under sub-optimal growth conditions when the growth rate is low (Borowitzka, 1990). Carotenogenesis of Dunaliella and cell growth are two separate biological processes of different controls, and that carotenogenesis can be induced physiologically at any stage of the cell cycle (Ben-Amotz, 1995). In Table 2 shows comparisons between β-carotene content and biomass of D. salina cells grown under various stress conditions. β-carotene is oxidized by liver enzymes to produce vitamin A, which is necessary for vision and of the epithelial tissues, (Hosseini Tafreshi and Shariati, 2009), as supplements for human and animal feeds (Milledge, 2010), as colorant, and antioxidant (Pisal and Lele, 2005).

Table 2: Different stress condition effects on β-carotene content and biomass of D. salina, (+ = stimulating effect; - = inhibitory effect; 0 = no effect), *= rate of decrease is very slow, adapted from (Borowitzka, 1990).

Factor	biomass	β-carotene
Salinity increase	--	++++
Salinity decrease	+	(-)*
N deficiency	−−	+++
P deficiency	−−	+
Increase in inorganic carbon	+++	0
Increase in light	+	++++
Decrease in light	−	−−−
Temperature increase	+	++
Temperature decrease	_	_
Increase in oxygen	–	–

In addition, inhibit or prevent various types of tumors in humans and animals such as skin cancers, gastrointestinal tract cancers, pancreas cancer, and breast cancer (Hosseini Tafreshi and Shariati, 2009). Several studies show the importance of β-carotene in controlling cholesterol levels and reducing the risk of cardiovascular diseases, and coronary heart disease. Also, it is having protective ability against harmful free radicals and simulative impact on the immune system. (Hosseini Tafreshi and Shariati, 2009). There are three categories of products derive from D. salina: β-carotene extracts, Dunaliella powder for human use and dried Dunaliella for feed use (Abu-Rezq et al., 2010). Depending on the formulation, the prices of these products ranging from US$ 300 to US$ 3000/kg. In addition, nutritional supplements can be produced by encapsulating of oil suspensions or solutions, or tableting using beadlet forms (Borowitzka, 1990). Supplementation of animal feed by addition of this pigment enhanced the color of the flesh of fish and the yolk of eggs and improved the health and fertility of grain-fed cattle (Hosseini Tafreshi and Shariati, 2009). In addition, β-carotene can accompanied with other carotenoids of Dunaliella, predominantly: lutein, neoxanthin, zeaxanthin, violaxanthin, cryptoxanthin, α-carotene, approximately 15% of the carotene concentration and is marketed as "carotenoids mix" (Dufossé et al., 2005). Also, compound of phytoene, phytofluene, and β-carotene marketed as (PhytoflORAL™) for oral intake, which used for health protection and prevention of premature aging and photo-aging that occurs due to external (sun, oxidants, contaminants) and internal (metabolism and oxidation processes) damage. It is also being marketed now as a nutricosmetics product both to prevent skin aging and as a skin whitener (von Oppen-

Bezalel and Shaish, 2009). In the USSR in 1966, established the first pilot plant for Dunaliella cultivation for β-carotene production (Oren, 2005a), which became the third major microalgal industry in the world. After that in 1986, other pilot projects have being in Australia, the USA, and in Israel (Borowitzka and Borowitzka, 1990). The major producer of β-carotene is Cognis Nutrition and Health, whose farms cover 800 ha in Western Australia. The production of β-carotene from Dunaliella throughout the world is now one of the most success stories of halophile biotechnology (Oren, 2005a). There are many technologies are used, from extensive low-tech cultivation to intensive cultivation at high cell densities under controlled conditions (Oren, 2005a). Also, different strategies have been proposed to maximize β-carotene production per unit time and per culture volume (Hosseini Tafreshi and Shariati, 2009). The main reason for the commercial production of Dunaliella in outdoor cultivation is maximize β-carotene production rather than biomass as in Chlorella or Spirulina culture (Ben-Amotz, 1993). In fact, optimization of cultivation parameters and cultivation systems is necessarily needed to achieve the maximization of β-carotene production.

2.1.1. Neutral β-carotene vs. synthetic β-carotene:

β - carotene is a tetraterpene with eleven conjugated double bonds. They can be obtained from natural sources like microalgae, vegetables, fruits, and the fungus Blakeslea but its concentration relatively low, or can be synthesized chemically (Hosseini Tafreshi and Shariati, 2009). The synthetic form contains all- trans isomer only while the natural has mixture composed of 9-cis, all-trans isomer. It is evident that the 9-cis isomer is a better antioxidant and anticancer than the all-trans. Existing research recognizes the natural β-carotene has better physical properties (such as its solubility in fat) than the manufactured product, make Dunaliella β-carotene much more valuable and attractive for the consumers than before (Hosseini Tafreshi and Shariati, 2009). Moreover, some studies documented there is a toxicity risk of synthetic β-carotene supplements due to the possible chemical contamination derived from the production and purification processes. In addition, the price of natural β-carotene from D. salina is much higher than that of synthetic, which is ($1000 to $2000 kilogram-¹) for natural and ($400 to $800 kilogram-¹) for synthetic (Abu-Rezq et al., 2010). In fact, costs must be significantly lower to account for losses at each processing step, capital expense, marketing, packaging and distribution costs. The different price between natural β-carotene and manufactured reflects that the consumers prefer natural.

3. Carotenoids Extraction techniques:

Carotenoids are one of the three prominent groups of natural pigments present in microalgae other than chlorophylls and phycobiliproteins (Rammuni et al., 2019). In recent years, there are many researches about the extraction methods of carotenoids from algae. The principle goal of extraction techniques is to achieve a high yield of desired compounds, to preserve co-products, minimize energy consumption, investigate the recycling methods to reduce waste generation, optimize the process (operational temperature, pressure, carrying capacity, side reactions, and separations), and increase the scale (Michalak and Chojnacka, 2014). The extraction includes several important points such as sample pretreatment, choosing the proper solvent, saponification, and selecting the best extraction method. Microalgal cells are usually enclosed by cell wall or coat. Some other physical and

chemical barriers present in the cells prevent the transfer of carotenoids during extraction. Also, the presence of diverse sets of carotenoids with varied levels of polarity makes their simultaneous extraction difficult. Additionally, the oxidative property of carotenoids limits exposure to excess heat, light, acids, and long extraction times (Saini and Keum, 2018). All of this makes the extraction of the desired compound more difficult. Therefore, the sample pretreatment are beneficial in the disruption of algal cells, breakdown of the physical barriers present in the algae, and making biologically active compounds more available, thus significantly improving the extraction yield (Michalak and Chojnacka, 2014). In addition, there are several methods of cell disruption like, physical (osmotic shock, cooking, freeze-thaw, drying), chemical (base, acid), and enzymatic (Saini and Keum, 2018). The selection of a suitable method for cell disruption is based on algae species-specific. Conventionally, carotenoids are extracted using organic solvents, such as acetone, chloroform, hexane, isopropanol, methanol, methylene chloride and diethyl ether. Also, can used combinations of solvents, which provides a synergistic effect on the extraction of carotenoids. One of the most critical factors for carotenoids extraction is choice an appropriate solvent or solvent combination (Saini and Keum, 2018). However, the benefits of saponification are removing any other compound other than the desired compound, such as chlorophyll from the sample, because this compound interferes with the direct extraction of carotenoid. For example, in the extraction of β - carotene from D. salina can saponified the biomass with calcium hydroxide facilitates the conversion of chlorophyll into calcium salts which are insoluble in the solvents utilized for the extraction. Hence, it becomes feasible to extract carotenoid without chlorophyll contamination (Rammuni et al., 2019). In general, there are different methods used for the extraction of carotenoids from natural sources and can be classified as following: the atmospheric liquid extraction with Soxhlet or maceration, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), pulsed electric field (PEF) assisted extraction, the supercritical fluid extraction (SFE) which is often based on the use of supercritical CO2 (SC-CO2) as a solvent, with minimal use of organic co-solvent such as ethanol, the enzyme-assisted extraction (EAE), and green extraction. These extraction methods differ in the mode of cell disintegration, degree of applied pressure, and temperature. For instance, Soxhlet extraction utilizes solvents at boiling temperature and ambient pressure, while (SFE) are operated at low temperature and high pressure. In contrast, (UAE), (PEF) and (EAE) utilize ultrasound waves, high voltage pulses and cellulolytic enzymes, respectively for the disintegration of cells, which facilitates the release of intracellular carotenoids (Saini and Keum, 2018). The extraction should be characterized by high efficiency, low cost, time savings, and environmentally friendly (Michalak and Chojnacka, 2014). In next (Table 3) shows advantages and disadvantages of each method.

Table 3: Advantages and disadvantages of extraction methods, adapted from, (Saini and Keum, 2018) and (Michalak and Chojnacka, 2014).

Extraction method	Advantages	Disadvantages
Atmospheric liquid extraction with maceration	High extraction yields without utilizing sophisticated instruments	Uses large quantities of toxic solvents, which increases the cost of production
Soxhlet extraction	Simple The recovery of carotenoids is high Didn't required sophisticated instruments	Consuming time Uses large quantities of solvents, which increases the cost of extraction Can cause thermal degradation and cis-trans isomerization of carotenoids
Microwave-assisted extraction	Fast and improved extraction rate Use low amounts of solvents and is cost effective High extraction yields	Energy input-assisted extraction method Required additional separation step to remove solid residues Not suitable for heat-sensitive products
Ultrasound-assisted extraction	Rapid, simple, non-thermal Use low amount of solvents Lower equipment costs than other extraction methods	Energy input-assisted extraction method
Pressurized liquid extraction	Fast (few minutes) Requires minimum amount of organic solvent Highly applicable to a laboratory-scale context	Not suitable for thermolabile compounds sensitive to high temperature and pressure conditions Is not as selective as supercritical fluid extraction
Pulsed electric field extraction	High extraction yield Non-thermal process Low energy usage	High costs of instrumentation Bubbles in the samples may cause technical problems PEF parameters may differ with change in electrical conductivity of the sample
Supercritical fluid extraction	Fast, efficient, economical, high yield Useful for extraction of thermolabile compounds Provide carotenoids with high purity	High cost of instrumentation Not suitable for samples containing high amounts of water Low yield of polar carotenoids
Enzyme-assisted extraction	Facilitated release of the desired bioactive to the medium Ecofriendly, nontoxic, without toxic chemicals High extraction yield	High cost of some enzymes

3.1. Extraction of β-carotene:

As mentioned before; β-carotene is commonly used in the food as coloring agent. The synthetic colorant is releasing harmful chemicals which are highly polluting, allergic, carcinogenic and injurious to humans (Nadar et al., 2018). Therefore, the β-carotene natural colorant can replace harmful synthetic dyes. Also, consumption of this compound by

humans are harmful; thus, the development of an efficient extraction is necessary to obtain this compound from natural sources (Hosseini et al., 2017). Owing to this, need to use more safe extraction methods. In general, there are several methods for β-carotene extraction from algae, where the β-carotene is extracted directly or after breaking cells by osmotic or mechanical shock (Mojaat et al., 2008). Almost, the most effective methods are using organic solvents or supercritical carbon dioxide (SC-CO2). Conventional solvent extractions usually utilize solvent obtained from non-renewable sources, highly flammable, volatile and often toxic, causing environmental pollution and the greenhouse effect (Saini and Keum, 2018). These extraction techniques are considered simple, but expensive, used large quantities of solvent, and consuming time, which requires multiple extraction steps to achieve the desired level of carotenoids recovery (Rammuni et al., 2019). This method usually required high temperatures to remove the solvents, which can lead to degradation of pigment, and the final product may contain trace amounts of solvent and, consequently, reduce their potential for use in food products (Mezzomo and Ferreira, 2016). The sample pretreatment is required to be employed for disruption of the cell, improving the mobility of carotenoids into the extraction solvent, thereby enhancing extraction efficiency (Rammuni et al., 2019). Kagan and Braun (2001) have proposed a general method using a multi-phase solvent mixture composed of water, a hydrophobic solvent and an aqueous co-solvent, selected based on molecular polarity and extraction efficiency (Rammuni et al., 2019). However, direct contact between microalgae and a solvent may result in significant biomass losses. In addition, one of the most critical points is solvents selection, which must satisfy certain requirements such as biocompatibility, maximum solubility for β -carotene and important extraction ability. For the extraction of β -carotene from D. salina usually using the solvents which have a (log P oct) value higher than 5 or molecular weight higher than 150 g/mol, such as tetrahydrofuran (THF), also the solubility of β-carotene in this solvent is higher than in another solvent. (Mojaat et al., 2008).

On the other hand, among all the extraction methods employed for β-carotene, supercritical carbon dioxide (SC-CO2) technology using carbon dioxide (CO2) near the critical point as a co-solvent the extraction has been considered as an alternative to employment in food and pharmaceutical industries. The higher diffusion coefficient and lower viscosity of (SC-CO2) allows for rapid penetration into cells pores, thus enhancing extraction efficiencies (Rammuni et al., 2019). Under optimized conditions, this extraction method leads to optimum yield with the highest purity, more efficient extraction, simpler, and faster. Also, this method does not require separation step to recover the products, so it is saving cost, time and labor requirements. Moreover, does not need to use toxic solvents and thus leading to leaving the final product without solvent contamination. Furthermore, it is suitable for using with thermo-sensitive compounds. In addition, this extraction considered as an environmentally friendly (Green extraction), which can recycle the (CO2) gas stream. In recent study the recovery of carotenoids from D. salina was up to 47% and 115.43 μg of β-carotene per g of dry biomass at 400 bar and 55 °C (Rammuni et al., 2019), (Saini and Keum, 2018). Finally, application of this technique for extraction of carotenoids from D. salina has been widely studied in the last decade.

3.2. Extraction using enzymes:

The main principle of extraction using enzyme is using enzymes as a catalyst under optimum conditions, which disrupted the microalgal cells or cell walls to release the intracellular components (Nadar et al., 2018). This extraction depends on the characteristic property of enzymes to carry forward reactions with accurate specificity, region-selectivity, and ability to conduct reactions under mild conditions with the retention of their biological potencies of bioactive compounds. It is alternative to conventional solvent extraction methods and become more attention.

Also, this method has faster extraction rate, higher yield, lower energy consumption, and simpler recovery with minimum solvent usage as compared to traditional methods (Nadar et al., 2018). In addition, it is complemented by extraction with organic solvents such as hexane and ethyl acetat, which are approved for use in foods in most countries (Arvayo-Enríquez et al., 2013). Operational conditions such as the temperature of the reaction, time of extraction, pH of the system, and enzyme concentration are critical for the extraction process. The temperature condition in this extraction can be under controlled, so it is beneficial for extraction of thermo-sensitive molecules such as flavors, pigments, and oil, and its eco-friendly method (Nadar et al., 2018). In general, there are many examples of hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases. Also, common enzymes are used in the food industry, such as neutral and alkaline protease, α-amylase, cellulase, and pepsin. For example, the extraction of proteins from the algae, Chondrus crispus, was reported, where the algal cell wall was degraded using polysaccharides such as k-carrageenase, β-agarase, xylanase, and cellulase. This demonstrates the importance of the specificity of enzymes toward their substrate in the extraction (Marathe et al., 2019). Several experiments have shown that applying a mixture of an enzyme is necessary for the extraction of active compounds from the biomass of algae, duo to algal cell wall is chemically and structurally more heterogeneous than in other cells like a plant. For example, the extraction of protein from algae Gracilaria verrucosa using mixture of cellulase and agarose increases the protein yield (Marathe et al., 2019). Also, extraction of astaxanthin from the microalgae Haematococcus pluviali using Glucanex® (containing β-1,3-glucanase and protease from the fungus Trichodermaharzianum) give the highest extractability of astaxanthin (83.90%) (Saini and Keum, 2018). Some microorganisms like Aspergillus niger can produce many enzymes, which is considered as one of the most complete multienzyme producers to produce cellulases, hemicellulases, glucoamylases, and pectinases.

Natural enzymes use is much better than the commercial because of their cost-effectiveness rate and a substantial reduction in processing time can be achieved while attaining high carotene content. Moreover, the use of enzymes produced naturally from microorganisms can reduce the processing time by ~95% compared to commercial enzymes. (Arvayo-Enríquez et al., 2013). Also, recovery of carotenoids by this method was up to 97%. In addition, this method increased the yield without affecting the physical and chemical propitiates of carotenoids (Nadar et al., 2018). Several researchers observed the enzymatic extraction, increase in various biological activities of bioactive compound. For instant, Hardouin et al. used enzymes to extract bioactive compounds from Ulva armoricana and observed anti-viral activities against herpes simplex virus type-1 (Marathe et al., 2019).

This method of extraction can be comping with another extraction method such as ultrasound-assisted enzyme extraction (UAEE), enzyme-assisted supercritical extraction (EASCFE), microwave- assisted enzymatic extraction (MAEE), and high pressure-assisted enzymatic extraction (HPAEE), in order to improves the yield of extraction and to exploit the advantages of both the techniques in extraction biomolecules (Nadar et al., 2018), (Marathe et al., 2019). The advent of enzymology represents an important breakthrough in the biotechnology industry, with the worldwide usage of enzymes being nearly U.S. $ 1.5 billion in 2000 (Gupta et al., 2004).

3.2.1. Lipase enzyme:

Lipases are enzymes that catalyze the total or partial hydrolysis of fats and oils. They are ubiquitous in nature and can be isolated from various sources like bacteria (45%), fungal (21%), animal (18%), plant (11%), and algal (3%) (Farzad Mardani kataki, 2016). Lipases of microbial origin, especially bacterial and fungal represent the most widely used class of enzymes in biotechnological applications and organic chemistry due to higher catalytic activity, seasonal changes independent production, ease in genetic manipulation for desired characteristics, production in bulk quantity, and use of cheaper growth culture media (Javed et al., 2018). Many different Gram-positive and Gram-negative bacterial strains produce lipase enzyme and the next (Table 4) show some important species. The lipases from Pseudomonas bacteria are widely used for various of biotechnological applications (Gupta et al., 2004). Also, they are can be intracellular, attached to membrane, or mostly extracellular. The number of available lipases has increased since the 1980s (Thakur, 2012). This is mainly a result of the substantial-achievements made in the cloning and expression of enzymes from microorganisms, in which increasing demand for these biocatalysts with novel and specific properties (Thakur, 2012). In general, lipases do not require cofactors, can act in a wide range of pH, stable at high temperatures, have high specificity, exhibits regio-, chemo- and enantioselectivity (Celligoi et al., 2016), and have high stability in organic solvents. Also, lipases can carry out hydrolytic reactions and synthetic reactions like esterification, acidolysis, and alcoholysis (Farzad Mardani kataki, 2016).

Table 4: Some important bacterial species considered as lipases producers, adapted from (Gupta et al., 2004).

Bacterial species.	Reference.
Pseudomonas aureofaciens	Koritala et al. 1987
P. mendocina	Jaeger et al. 1999; Surinenaite et al. 2002
P. fragi	Jaeger et al. 1994; Schuepp et al. 1997; Ghanem et al. 2000
Bacillus sp.	Sidhu et al. 1998a, 1998b; Pandey et al. 1999; Sharma et al. 2002a; Nawani and Kaur 2000
B. subtilis	Jaeger et al. 1999
B. alcalophilus	Ghanem et al. 2000
Burkholderia glumae	Jaeger and Reetz 1998; Reetz and Jaeger 1998
Lactobacillus curvatus	Brune and Gotz 1992
Proteus vulgaris	Jaeger et al. 1999
Staphylococcus aureus	Simons et al. 1996; Jaeger et al. 1999
S. epidermidis	Simons et al. 1996; Jaeger et al. 1999
S. haemolyticus	Oh et al. 1999
Serratia marcescens	Matsumae et al. 1993,1994; Pandey et al. 1999; Abdou 2003
Streptomyces exfoliates	Arpigny and Jaeger 1999
Chromobacterium	Koritala et al. 1987

violaceum	
Achromobacter sp.	Mitsuda et al. 1988
Acinetobacter sp.	Wakelin and Forster 1997; Barbaro et al. 2001
Arthrobacter sp.	Pandey et al. 1999

These can carry out reactions in both aqueous and organic media. Due to their characteristics, lipases are widely used in diverse industrial fields, such as textile, biofuels, food industry, detergent, cosmetics, pharmaceutics, paper manufacturing, waste-water treatment and others (Table 5). They can also be used in many processes, such as synthesizing structural lipids, synthesizing of flavor esters, low calorie lipids and milk fat and in ripening cheese. The catalytic potential of lipases can be further enhanced and made selective by the novel phenomena of molecular imprinting and solvent engineering and by molecular approaches like protein engineering and directed evolution (Gupta et al., 2004). Moreover, they are non-toxic and eco-friendly; lipase is considered more suitable than other chemical or synthetic catalysts. Lipase currently ranks third among the most currently commercialized enzymes after proteases and carboxylases.

Table 5: Commercial bacterial lipases, sources, applications and their industrial suppliers, adapted from (Gupta et al., 2004).

Commercial lipase	Source	Supplier	Application	References
Lumafast	*Pseudomonas menodocina*	Genencor International, USA	Detergent	Jaeger et al. 1994; Jaeger and Reetz 1998
Lipomax	*P. alcaligenes*	Gist-Brocades, Netherlands; Genencor International, USA	Detergent	Jaeger et al. 1994; Jaeger and Reetz 1998
Lipase AH(Oren, 2005b)	*P. cepacia*	Amano Pharmaceuticals, Japan	Organic synthesis	Jaeger and Reetz 1998
Chromobacterium viscosum lipase	*Chromobacterium viscosum*	Asahi Chemical Biocatalysts	Organic synthesis	Godfrey and West 1996
Lipase 50P	*Chromobacterium viscosum*	Biocatalysts, UK	Biotransformations, chemicals	Godfrey and West 1996
Lipase 56P	*P. fluorescens*	Biocatalysts, UK	Biotransformations, chemicals	Godfrey and West 1996
Lipoprotein lipase	*Alcaligenes sp.*	Meito Sankyo Co., Japan	Research	Godfrey and West 1996
Alkaline lipase	*Achromobacter sp.*	Meito Sankyo Co., Japan	Research	Godfrey and West 1996
Lipase AL, ALC/ALG	*Achromobacter sp.*	Meito Sankyo Co., Japan	Technical grade	Godfrey and West 1996

Conclusion

Due to the increase in population and continence research about natural sources of food or supplement, researchers focused on the microalgae especially Dunaliella salina as a natural source, because it produces various the bio-active compound such as β-carotene. So, they concern about the extraction of β-carotene from D. salina via effective methods with high yield, high purity, low cost, and simple method. One of the most effective methods of extraction is using natural enzymes.

Conflict of interest

The authors have not declared any conflict of interest.

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