SUMMARY

CONCLUSION
4.1. Brief background:

*Dunaliella* is a unicellular green alga, generally ovoid in shape 4-10 µm wide and 6-15 µm long (Ben-Amotz and Avron, 1983; 1990). The cells are motile due to the presence of two equal, long flagella with a single chloroplast in the center. Chloroplast contains a large pyrenoid surrounded by polysaccharide granules (a storage product). The chief morphological character of *Dunaliella*, in contrast to other members of chlorophyta is that it lacks a rigid polysaccharide cell wall. Cell is a natural protoplast, enclosed by thin elastic membrane. This makes the *Dunaliella* cells responsible for its rapid change in shape and response to osmotic changes (Ben-Amotz and Avron 1983; Ben-Amotz et al, 1982; Borowitzka and Siva, 2007). There are two commercially important species of *Dunaliella* i.e., *D. salina* and *D. bardawil*. In early years they were valued for the production of glycerol and protein but now the focus is on carotenoid especially the β-carotene and lutein.

The halotolerant alga *D. bardawil* possesses the unique ability to accumulate a very high content of β-carotene. The amount of β-carotene in *D. bardawil* under controlled conditions can be manipulated by different growth conditions, to vary from about 3 to 8% (Ben-Amotz and Avron, 1983). The β-carotene found in *D. bardawil* contains almost equal amount of cis and trans β-carotene owing to 90% of total carotenoids, with the rest composed mostly of lutein and other carotenes (Ben-Amotz et al., 1982). The carotene pigments are usually associated in lipid globules located in the interthylakoid space of the chloroplasts (Ben-Amotz and Avron, 1983). *Dunaliella* produces carotenoid during all stages of growth, and can be manipulated at any stage of physiology (Yong and Lee 1991). Commercial production of *Dunaliella* sp as a source of β-carotene is the major micro algal industry in many parts of the world.

Numerous factors have been shown to induce massive carotenoid accumulation in *Dunaliella*. The important ones are high salinity and high light intensity (Ben-Amotz and Avron, 1983; Loeblich, 1982). It has observed that, an inverse relationship exists related to β-carotene content and the specific growth rate (Ben-Amotz et al. 1982). Hence the major factors of significance in cultivation of algae are nutrients, light, temperature and stress of various natures (Becker and Venkataraman 1982; Raja et al, 2007).

The important function of carotenoid is their potential to prevent chronic disease and vitamin A deficiency (VAD) (Von-Lintig et al, 2005). In humans, VAD leads to night
blindness in milder forms, while more severe progression results in corneal malformations, e.g., xerophthalmia. Besides visual defects, this deficiency affects the immune system, leads to infertility or causes malformations during embryogenesis. Being essential for vision, in vertebrates the vitamin A derivative retinoic acid (RA) is a major signal-controlling molecule in a wide range of biological processes (Underwood, 2004). VAD is a major problem particularly in developing countries. Vitamin A demand can be met either by supplementing vitamin A or carotenoids with provitamin A activity. All naturally occurring vitamin A in the food chain derives from provitamin A conversion and that the world’s population mainly relies on carotenoids from staple food sources to meet vitamin A requirements. The central cleavage mechanism splits β-carotene at the central double bond (Castenmiller and West, 1998) by a specific enzyme, β-carotene 15,15’-oxygenase found to yield two molecule of retinal in intestinal cell and liver cytosol (Goodman and Olsen, 1969). *Dunaliella* is the only organism which produces massive amount of carotenoids and feasible for commercial production. The cultivation of this alga for the carotenoid rich biomass and its biological significance and safety aspects are the main focus of the present work. This study was undertaken in view of β-carotene need and possibility of utilization of algal source for large-scale carotenoid production.

**Objectives of the study:**

- To optimize a cultivation process of *D. bardawil*.
- Analysis of genes involved in light regulated synthesis of carotenoids in *D. bardawil*.
- Studies on the involvement of selected metal ions in the regulation of carotenoid biosynthesis in *D. bardawil*.
- Biological activity of *Dunaliella* carotenoids in *in vitro, in vivo* and cell culture models.
4.2. SUMMARY OF RESULTS

4.2.1. Cultivation, growth, carotenogenesis and nutritional composition of *D. bardawil* biomass:

An indigenous culture of *D. bardawil* was established in both indoor and outdoor culture conditions. The cultures were grown in AS100 medium. During vegetative phase of *D. bardawil* showed a maximum of 20g L\(^{-1}\) wet biomass on 30\(^{th}\) day. On 30\(^{th}\) day the chlorophyll a, b and carotene content was 200mg, 75mg and 136µg /100mg biomass respectively under laboratory culture conditions.

*D. bardawil* cultures grown at 1M NaCl concentration showed good vegetative growth, however for carotene accumulation, 2.0 M NaCl was required. Addition of 2% CO\(_2\) in gaseous to the vegetative phase of *D. bardawil* did not show any significant enhancement in pigment profile. However, feeding of metal ions/micronutrients Fe and Zn to the culture during vegetative growth stage, showed enhanced carotene content in indoor culture condition.

Scale up of cultures up to 2000L in outdoor culture conditions was achieved. During the vegetative phase in outdoor growth condition, *D. bardawil* cultures required a light intensity of < 20 Klux. Hence, the cultures were protected by green house shade nets to obtain optimal light intensity. This helps to maintain the cultures through out the year. The carotenogenesis was achieved in *D. bardawil* by subjecting the culture to high light intensity of 30-35 Klux under outdoor raceway tanks. A maximum of 4% (w/w) β-carotene and 1% (w/w) lutein was observed on wet weight basis in the carotenogenesis induced *D. bardawil* biomass.

After successful mass cultivation of *D. bardawil*, the harvesting, drying and storage conditions were studied. The online centrifuges were used for efficient harvesting of *D. bardawil* cultures. The carotenene induced *D. bardawil* cultures were harvested using online centrifuges. In this 100L cultures were harvested per hour, containing 90-95% cell harvesting. Among the different drying methodologies employed, freeze drying showed a minimum loss of carotenoids during drying. Hence further freeze drying was employed to get a dry biomass. The storage under different temperatures at dark conditions revealed that the dry biomass must be stored at ultra low temperature to retain the carotenoids upon storage.

Analysis of the biomass revealed 22% protein, 27% carbohydrate and 8% fat content on dry weight basis. The unsaturated fatty acids linoleic (35%) and linolenic acid
(81%) was observed among the neutral and glycolipid fractions respectively. In *D. bardawil* biomass, the heavy metals were found to be below the permitted level.

### 4.2.2. Analysis of genes involved in carotenoid biosynthesis pathway during light induced carotenogenesis:

The carotenogenesis in *D. bardawil* cultures was induced by exposing the cultures to high light intensity of 30-35 Klux. During this, the major carotene that accumulated was β-carotene (up to 4%). During this, the transcript analysis of Phytoene synthase (*PSY*), Phytoene desaturase (*PDS*) and Lycopene cyclase (*LCY*) revealed an upregulation of the genes for these enzymes, when the cells were subjected to high light intensity. This upregulation in the genes were positively correlated with the carotenoid production in the cells. In indoor cultures lutein content was high (up to 1%) and an exposure to high light did not exhibit impact on lutein level. In addition to this, there was no significant elevation of transcript level of gene responsible for the conversion of β-carotene to lutein (carotene hydroxylase, *CH*), during carotenogenesis.

### 4.2.3. Safety and toxicity evaluation of *D. bardawil* biomass in albino rats:

The safety of *D. bardawil* biomass after oral administration to short and long period was studied in rats. In short term study (acute oral toxicity), by administration of single dose of *D. bardawil* at the maximum level (5g biomass containing 2% β-carotene Kg⁻¹ b.w) and toxicity symptoms, if any, were monitored for 15 days. In long term toxicological study, the effects of 90 days oral administration of *D. bardawil* biomass (100 and 1000mg biomass Kg⁻¹ b.w) was assessed and compared with the control rats. In both the study, *D. bardawil* biomass at the given doses did not induce any treatment related observable toxic effects, when compared to control group of animals devoid of biomass. Hence *D. bardawil* biomass was found to be safe at the given doses in animal models.

### 4.2.4. Bioaccessibility and bioconversion of carotenoids from *D. bardawil* - *in vitro, in vivo* and cell line models

The bioaccessibility and bioconversion of *D. bardawil* biomass was assessed by *in vitro* and *in vivo* methods. The *in vitro* bioaccessibility of carotenoids was studied by
simulated digestion method. The study revealed that the percent bioavailability of β-carotene and lutein from *D. bardawil* biomass was 22 and 12% respectively after complete (gastric and intestine) digestion.

The uptake and conversion of *D. bardawil* carotenoids to retinol was studied in primary intestinal cells. The study revealed carotene uptake and retinol conversion in primary intestinal cells; hence the primary intestinal cell lines could be efficiently used as an alternative model to study the availability of carotenoids.

The intestinal perfusion study revealed that the retinol conversion, starts within 30 min in the intestine, and there observed a three-fold increase in vitamin A content within 30 min.

Further, the bioavailability of *D. bardawil* carotenoids and vitamin A conversion was studied in experimental rats using single oral dose and multiple doses (7 days), and compared with the synthetic β-carotene treated group. The postprandial response of retinol in serum and liver was studied for 0 to 8hr after single oral dose. The study showed that maximum retinol conversion takes place within 4 hrs after single oral dosage. β-carotene was absent in serum of control group of animals, and found at detectable level in the serum after single oral dose of either *D. bardawil* biomass or synthetic β-carotene. In liver significant (P < 0.05) accumulation of β-carotene was observed. After 8 hrs of intubation, the liver β-carotene levels increased to 1.5µg g⁻¹ and 1.26µg g⁻¹ respectively in the synthetic and *D. bardawil* fed groups compared to initial liver β-carotene (0.35µg g⁻¹). The study also revealed that the accumulation of β-carotene in liver was observed at 8hrs after oral dosage.

Hepatic analysis of the experimental group, after multiple doses, revealed a very high liver store of retinol and β-carotene. The liver retinol content of both the experimental groups was significantly higher compared to control. Enhanced accumulation of retinol upto 3-4 fold was observed in liver among the experimental groups compared to control group. The liver retinol content was higher in the synthetic β-carotene (78%) fed group compared to *D. bardawil* fed group (69%). In contrast to this, β-carotene accumulation in the liver of *D. bardawil* biomass fed group was higher (85%) than synthetic β-carotene (72%) fed group. The study clearly revealed that *D. bardawil* carotenoids are effective in terms of carotene accumulation and retinol formation in the liver.
4.2.5. Biological activity of *D. bardawil* biomass on CCl₄ induced toxicity: Beneficial attributes of *D. bardawil* and its potential to modulate experimentally induced disease conditions

The hepatoprotection and renal protection activity of *D. bardawil* biomass was studied under CCl₄ intoxication. The damage caused by CCl₄ was monitored by parameters of toxicity in serum, liver and kidney. The elevated levels of serum enzymes (serum alanine aminotransferase and serum aspartate aminotransferase) were found to restore in the *D. bardawil* biomass and synthetic β-carotene treated groups. The lipid peroxidation observed in hepatic and renal tissues of CCl₄ treated rats were restored in the rats pretreated with *D. bardawil* biomass and synthetic β-carotene. The elevated levels of serum creatinine (1.4mg dL⁻¹) indicated the renal damage. In *D. bardawil* and synthetic β-carotene treated groups the serum creatinine levels after CCl₄ intoxication was found to be 0.4 and 0.7mg dL⁻¹ respectively. Hence the study indicated the possible benefit of *D. bardawil* biomass under experimentally induced disease conditions.
4.3. Conclusions

A series of experiments were carried out in order to develop a mass cultivation method for *D. bardawil*. The available literature suggests that *D. bardawil* can be successfully cultivated by optimizing conditions for nutrients, salinity, along with other culture conditions such as light and temperature. During our experiment it has been observed that *D. bardawil* has two distinct phases in its life cycle. The first being vegetative growth, which is highly efficient at 0.5M-1.0M NaCl concentration at indoor culture conditions. The next stage being carotenogenesis stage, which occurs at, high light and salt conditions, wherein the algae showed arrest in the growth rate and begin to accumulate carotenoids. In these studies, a maximum of 20g l\(^{-1}\) wet biomass was obtained on 30\(^{th}\) day at indoor culture condition. When exposed to high light (30-35 Klux) *D. bardawil* accumulates over 4% carotene content. This is on par with the published reports available for *Dunaliella* sp. Thus it has been possible to establish large scale cultivation methodology, which can be adapted at an industrial scale.

Different parameters were tested such as varied concentration of CO\(_2\) from different sources, NaCl levels during vegetative phase and its influence on the carotene production. An attempt has been made to identify the influence of metal ions in the form of micronutrients, which is expected to regulate carotene accumulation. In this study, Fe and Zn showed increase in carotene content without affecting the vegetative growth.

After successful mass cultivation, it was important to find an efficient method of harvesting, drying and storage conditions. It was demonstrated that online centrifugation is highly efficient for harvesting, followed by freeze drying and storage at ultra low temperature.

Based on the available literatures it is known that carotenogenesis is greatly affected by the quantity of light. It was speculated to be due to transcriptional regulation of key regulatory genes involved in the carotenogenesis pathway by light. Phytoene synthase, phytoene desaturase, lycopene cyclase and β-carotene hydroxylase were the candidate genes selected to study the role of light in its transcriptional activation. The cultures were subjected to different light intensity for a period of 5 days to accumulate carotenoids. The results indicated that high light induced up regulation of phytoene synthase, phytoene desaturase and lycopene cyclase genes. However the gene
involved in conversion β-carotene to lutein (β-carotene hydroxylase) did not show any significant change in transcript levels at different lights. Therefore, it was evident that light plays a major role in regulating carotenoid pathway.

The safety of *D. bardawil* biomass was assessed in albino rats by oral administration of *D. bardawil* biomass. Results indicated that at the given dose, *D. bardawil* biomass did not induce any treatment related observable toxic effects, when compared to control group of animals receiving normal diet. The result revealed that *D. bardawil* is safe and can be exploited as a potential health supplement after human trials.

*In vitro* and *in vivo* models were used to study the bioavailability of *D. bardawil* carotenoids. The *in vitro* study revealed that the percent bioavailability of β-carotene and lutein was 22 and 12% respectively after gastric and intestinal digestion. Further, the bioavailability of *D. bardawil* carotenoids and vitamin A conversion was studied in experimental rats using single oral dose and multiple doses (7 days), compared with the synthetic β-carotene treated group. The study revealed that maximum retinol conversion takes place within 4 hrs after single oral dosage. The β-carotene was present at detectable level in the serum after single oral dose of either *D. bardawil* biomass or synthetic β-carotene. The study also revealed that the accumulation of β-carotene in liver was observed at 8 hrs after oral dosage.

A high liver store of retinol and β-carotene was observed after 7day feeding trial. Enhanced accumulation of retinol and β-carotene in liver by 3-4 fold was observed in the experimental groups over the control group. The overall study indicated that, the liver retinol content (69-78%) and liver β-carotene (72-85%) was higher in both synthetic β-carotene and *D. bardawil* treated groups when compared to animals devoid of any carotene supplementation. *D. bardawil* biomass was efficient in terms of retinol formation and its accumulation in the liver. The bioaccessibility and bioavailability studies indicated that *D. bardawil* carotenoids are nearly effective in terms of carotenoid accumulation and retinol conversion in both serum and liver, when compared to synthetic β-carotene.

The protective effect of *D. bardawil* biomass was assessed using CCl₄ induced toxicity in rats. The study revealed that the carotene rich *D. bardawil* biomass ameliorated the toxic effects of CCl₄, indicating that it can be useful in scavenging free radicals. This study also reflected the beneficial attributes of *D. bardawil* and its
potential to modulate experimentally induced disease conditions during liver and kidney damage.

The investigation embodied in this thesis has largely addressed aspects of cultivation of indigenous strain of *D. bardawil*, nature of carotenoids, bioefficacy of *Dunaliella* biomass from the angle of utility as a source of carotenoid and safety aspects.