CHAPTER-3

3.0. ISOLATION AND QUANTIFICATION OF COMMERCIALLY VALUABLE PRODUCT FROM HALOPHILIC BACTERIA AND PHYTOPLANKTON

3.1. INTRODUCTION

Various halophilic and halotolerant microbes inhabiting the salt pans are yet to be fully explored as potential producers of pharmaceutically significant molecules. Few reports are available on their antimicrobial potential in India. Goa’s traditional salt industry is said to have been a major supplier of salt to the country and an exporter to some foreign countries since the 10th century. But since 2002, only about 16 salt pans are in use for the commercial production of natural salt. Salinity in these ponds ranges from 10 to 400 psu during the peak salt-manufacturing season i.e. between November to May (Tonima and Kerkar, 2011).

Halophiles can be found in areas where the concentration of salt is five times greater than that salt concentration of the ocean, such as the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and in evaporation ponds. Miteva et al. (2004) successfully isolated nearly 800 bacterial strains and classified them into different groups based on morphology, amplified rDNA restriction analysis (ARDRA) patterns followed by phylogenetic and physiological characterization. Halophilic microorganisms have several biotechnological applications like β-carotene production of fermented foods. In
recent years, uses of halophilic microorganisms have significantly increased. Many enzymes, stabilizers and valuable compounds from halophiles may present advantages for the development of biotechnological production processes (Aneela Roohi et al., 2012).

Although salts are required for all life forms, halophiles are distinguished by their requirement of hypersaline conditions for growth. They may be classified according to their salt requirement: slight halophiles grow optimally at 2–5% NaCl; moderate halophiles grow optimally at 5-20% NaCl; and extreme halophiles grow optimally above 20-30% NaCl. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with requirement or tolerance for salts sometimes depending on environmental and nutritional factors (Galinski, 1993).

Carotenoids (compounds of an isoprenoid nature) detected in *Agromyces ramosus* (Collins and Bradbury, 1991) and two *Leifsonia sensulato* species, (Reddy et al., 2003), are phylogenetically close to *Rhodoglobus*. Some *Alteromonass* pecies show pigmentation from lemon - yellow to violet. Most members of the *Microbacteriaceae* family exhibit a yellow, orange or red color of varying intensity and tint, the color being determined by isoprenoids (carotenoids), quinones, prodigiosin and anthracyclinone derivatives, phenazines, and other heterocyclic compounds. Isoprenoids are synthesized via the nonmevalonate or mevalonate pathways, which are inhibited by fosmidomycin and mevinolin, respectively (Lichtenthaler, 2000; Kuzuyama, 2002). The suppression of pigmentation by these inhibitors at concentrations lower than
those inhibitory to growth may serve as an indication (along with the results of chemical analysis) that the pigmentation is due to isoprenoids. A red pigment and a yellow pigment were also reported in *B. megaterium* KM spores (Ellar and Postgate, 1974). Spores of *Bacillus megaterium* QM B1551 contain a red pigment that is associated with the spore membranes (Swerdlow and Setlow, 1984). Microbial mats are often comprised of consortia of prokaryotic and eukaryotic phototrophic microorganisms and thus contain complex mixtures of lipophilic pigments (Stal et al., 1984).

In the plant world the carotenoids are present in forms ranging from bacteria to the highest plants. Similarly, in the animal kingdom we find these compounds in many forms of both invertebrates and vertebrates (White, 1939). Some common types of pigments are:

**Carotenes:** Most species of the halophilic Archaea (family *Halobacteriaceae*) are colored pink-red due to a high content of carotenoid pigments in their cell membrane. It appeared to be red or pink due to a wide variety of isoprenoid compounds (phytoene, phytofluene, lycopene, and β-carotene) produced by this prokaryote (Rodriguez-Valera et al., 1979).

**Violacein:** The violet pigment violacein is an indole derivative, predominantly isolated from bacteria of the genus *Chromobacterium* that inhabit the soil and water of tropical and subtropical areas. Use of violacein as a chemical defense against eukaryotic predators has also been investigated (Rettori and Duran, 1998).
**Prodigiosin:** For several decades, prodigiosin has been known to be a natural compound showing a broad range of cytotoxic activity and is also produced by *Vibrio psychroerythrus* (Aoust and Gerber, 1974).

**Quinones:** Quinones are additional colored compounds with an aromatic ring structure that have been isolated from marine environment. Quinone derivatives range in color from yellow to red, exhibit antiviral, antiinfective, antimicrobial, insecticidal, and anticancer activities, and have many commercial applications as natural and artificial dyes and pigments (Margalith, 1992).

**Tambjamines:** It has long been noticed that marine bacteria have the ability to prevent biofouling. Recently, this yellow pigment was isolated from *P. tunicate* and was identified as a new member of the tambjamine class of compounds (Franks et al., 2005).

**Melanin:** *Vibrio cholerae, Shewanella colwelliana,* and *Alteromonas nigrifaciens* were some of the first marine bacterial strains described to produce melanin or melanin like pigments. The pigment synthesized by *Vibrio cholera* was reported to be a type of allomelanin derived from homogentisic acid (Fuqua and Weiner, 1993).

**Other Pigmented Compounds:** Scytonemin, a yellow green pigment isolated from aquatic cyanobacteria, forms when the bacteria are exposed to sunlight. It protects bacteria by preventing about 85–90% of all UV-light from entering through the cell membrane (Proteau et al., 1998).
Low pigment productivity is one of the main issues facing researchers, and some solutions have already been reported (Giri et al., 2004). Tonima Kamat and Savita Kerkar (2011) reported that marine salt pans are important ecological niches which inhabit halobacteria. These bacteria tolerate and thrive in salt concentrations ranging from 0.5 to more than 5 M in which only very few other organisms are able to survive.

Gargi et al. (2011) reported that various synthetic coloring agents have the potential of carcinogenicity and/or teratogenicity. Natural colors, extracted from fruits, vegetables, seed roots and microorganisms and often called “biocolors” due to their biological origin have proved to be safe and edible coloring agents. The pigment was extracted by solvent extraction method using methanol. The pigment extracts were analyzed by scanning the absorbance with a UV-Vis spectrophotometer and peaks were obtained at 437 nm, indicating that the pigment is a possible carotenoid (Gargi et al., 2011).

Ahmad (2012) reported that bacteria produce pigments for various reasons and it plays an important role. Some bacteria such as cyanobacteria have phycobilin pigments to carry out photosynthesis. Other example for pigment-producing bacterial strains includes *Serratia marcescens* that produces prodigiosin, *Streptomyces coelicolor* (Prodigiosin and actinorhodin), *Chromobacterium violaceum* (violacein) and *Thialkalivibrio versutus* (natronochrome and chloronatronochrome). Pigment produced by the bacteria can be isolated using solvent extraction. These pigments can be further purified and characterized for physical and chemical characteristics using various
instrumental based analytical techniques such as TLC, UV–VIS Spectroscopy, FTIR, ESI–MS, NMR, HPLC and Gel Permeation Chromatography (Ahmad, 2012).

Brock et al. (1976) described a method which enables the individual quantification of chlorophyll a and bacteriochlorophyll c when the two are present in a mixture. The method involves the use of thin-layer chromatography to first separate the pigments, followed by the standard spectrophotometric assays of the isolated chlorophylls. The method is readily adaptable to field studies, rapid, and relatively inexpensive (Brock et al., 1976).

The first description of a unicellular biflagellate red coloured algae living in concentrated brines was given in 1838 by Dunal, who reported occurrence of the organism we know today as *Dunaliella salina* in the salterns of Motpellier, on the Mediterranean coast of France (Oren, 2005).

The taxonomy of Dunaliella species has, in the past, been disputed (Massyuk, 1973; Ginzburg, 1987; Preisig, 1992). Three major genera of Dunaliella capable of growth at high salinities are now gradually accepted. *Dunaliella salina* appears red when cultured at high salinities; *Dunaliella parva* appears yellowish and has a lower carotenoid content, whereas *Dunaliella viridis* remains green even at elevated salinity. *Dunaliella tertiolecta* is mesohaline and stays green even at salinities above its optimum for growth (Borowitzka and Borowitzka, 1988).
*Dunaliella* species belong to the phylum Chlorophyta, order Volvocales and family Polyblepharidaceae, and are unicellular, photosynthetic and motile biflagellate microalgae morphologically distinguished by the lack of a rigid cell wall (Ben-Amotz and Avron, 1987).

*Dunaliella* cells are ovoid, spherical, pyriform, fusiform or ellipsoid with size varying from 5 to 25 μm in length and from 3 to 13μm in width. The cells also contain a single cup-shaped chloroplast which mostly has a central pyrenoid surrounded by starch granules (Ben-Amotz, 1980). Besides chlorophylls a and b, the members of Dunaliella contain valuable carotenoid pigments such as α and β carotene, violaxanthin, neoxanthin, zeaxanthin and lutein. Dunaliella cells have other organelles typical of the green algae: membrane-bound nucleus, mitochondria, vacuoles, Golgi apparatus and an eyespot (Ben-Amotz and Avron, 1989).

In addition to this exceptional halotolerance of most *Dunaliella* species, *Dunaliella acidophila* can grow in very acidic environment (pH 0-1). *Dunaliella salina* can tolerate high light intensities. Furthermore, *Dunaliella* is more tolerant to fuel oil contamination compared with other planktonic algae (Brown and Borowitzka, 1979). Thus, these organisms are unique in their abilities to adapt to some of the most severe conditions of the global habitats.

Nowadays, we know that *Dunaliella salina* is the best commercial source of natural β-carotene among all organisms in the world (Borowitzka, 1995). This alga accumulates large amounts of β-carotene as droplets in the chloroplast to prevent chlorophyll photo-damage, when culture conditions include high light
intensities, high temperature, high salinity and deficiency of nutrient (Ben-Amotz and Avron, 1987; Ben-Amotz and Shaish, 1992). The $\beta$-carotene contents of up to 14% of dry weight have been reported for *Dunaliella salina*. Mass cultivation of *Dunaliella salina* for $\beta$-carotene production has being accomplished in several countries including Australia, USA and China (Borowitzka and Borowitzka, 1989; Borowitzka, 1999). Pilot-scale projects have also being attempted in Spain, Kuwait, Chile, Iran, etc. (Borowitzka, 1990; Garcia-Gonzalez *et al*., 2003; Hosseini Tafreshi and Shariati, 2006). Some other aspects of *Dunaliella* biotechnology have also been reviewed elsewhere (Del Campo *et al*., 2007; Raja *et al*., 2007; Ye *et al*., 2008).

Recently, microalgae have been identified as a major natural source of biologically significant micromolecules such as carotenoids, phycocolloids, long chain polyunsaturated fatty acids etc. The Phycobiliproteins improve the efficiency of light energy utilization of plants and carotenoids shield them against solar radiation and associate effects.

In crystallizer ponds, the unicellular green alga Dunaliella is the sole primary producer that lives in association with dense communities of heterotrophic halophilic Archaea that color the brines red. This basic pattern is common to all saltern systems, inspite of local variations in climate and nutrient availability (Oren, 2009).
Dunaliella salina, which is often found in dense populations together with heterotrophic red Archaea and the red-orange Salini factor, which all contain different carotenoid pigments as well as retinal proteins that may also contribute to the pink-red color of the brines (Oren, 2009).

Dunaliella salina is a unique species of alga that has a high content of natural carotenoids, especially β-Carotene, antioxidants and essential vitamins. This algal species has served and still as the major source for the extraction of β-Carotene for commercial applications (Bosma and Wijffels, 2003; Leon et al., 2003; Ye et al., 2008). Carotenoids act as secondary pigments in photosynthetic organisms, and as antioxidants and provitamin factors of non-photosynthetic organisms, their antioxidant and colorant properties make them useful for the therapeutic, dietetic and industrial applications of carotenoids (Leon et al., 2003).

Dunaliella natural β-carotene productions are widely distributed in many different markets under three different categories: β-carotene extracts, Dunaliella powder for human use and dried Dunaliella for feed use (Ben-Amotz, 2004). Today, the price of extracted and purified natural β-carotene is much higher than that of synthetic β-carotene. The price difference reflects that the consumers prefer the natural products from the synthetic (Caswell and Ziberman, 2000).

Commercial production of Dunaliella salina as a source of β-carotene became the major microalgal industry when culture and production facilities were established by Western Biotechnology Ltd. and Betatene Ltd. in Australia in 1986 (Borowitzka, 1999). These were soon followed by other commercial plants.
in China, Israel, United States (US), as well as other plants in Chile, Iran and Japan (Borowitzka, 1999; Ben-Amotz, 2004; Gomez and Gonzalez, 2004).

**Objectives**

The present study was undertaken

- To isolate, purify and characterize the colourful halophilic bacterial strains to find a new source of pigment.

- To study the growth of isolated halophilic bacteria under different chemical and physical parameters like carbon, nitrogen, metal ions, inorganic nitrogen, different pH and salinity.

- To isolate and analyze the pigment from the bacterial culture.

- To isolate and identify the *Dunaliella salina* from the selected saltpan water samples.

- To estimate the amount of carotenoid from *Dunaliella salina*.

- To study the effect of salinity on carotenoid production in *Dunaliella salina*.
3.2. MATERIALS AND METHODS

3.2.1. Collection of sample

The water samples were collected from the selected Saltpans of Kanyakumari, Thoothukudi and Ramnad district, (Kovalam, Puthalam, Swamythoppu, Thoothukudi and Ramnad). The samples were collected by aseptically in sterile bottle and transported to the lab for further study.

3.2.2. Isolation of halophilic bacteria

The collected samples were diluted by serial dilution technique. From the serially diluted samples ($10^{-4}$ to $10^{-6}$), 0.1 ml was aseptically transferred and spread plated on halophilic agar medium. The plates were incubated at $37^\circ C$ for 10-15 days, after incubation period, the colonies were picked up and transferred to halophilic agar plate for the purpose of pure culture and then the isolates were streaked in slants and were incubated at $39^\circ C$ until growth was observed and then the slants were stored at $4^\circ C$ for further studies.
3.2.3. Identification of the isolates

The isolated bacterial strains were identified by the detailed procedure described by Holt et al., (1994). The detailed procedure has been given the previous chapter page no. 64-67.

3.2.4. Screening for enzyme production

The halophilic bacterial strain was tested for the following enzyme producing ability.

3.2.4.1. Screening of protease activity

The production of protease enzyme was determined by the isolated bacterial strain which was central streaked in skim milk agar plates and incubated the plates at 37ºC for 48 hours for formation of zone around the culture. It indicates positive for enzyme production, if the zone formation fails it is negative for enzyme activity.

3.2.4.2. Screening extracellular amylase activity

The amylase enzyme activity was determined by screening assay, the bacterial culture was streaked on starch agar medium and incubated the plates at 37ºC for 3 days. After incubation period the plates were poured with KI solution. A clear zone around the culture indicates positive for amylase production.

3.2.4.3. Screening of extracellular cellulase activity

Carboxy methyl cellulase activity of the culture was screened in a solid medium containing carboxy methyl cellulose (CMC) 5g l⁻¹, NaNO₃ 1gl⁻¹, KH₂PO₄ 2gl⁻¹, Kcl 1gl⁻¹, MgSo₄ 0.5gl⁻¹, yeast extract 0.5gl⁻¹, glucose 1gl⁻¹, Agar 17g l⁻¹,
and 10% and 20% salts for moderate and extreme halophiles respectively. After incubation at 37°C for 7 days, the plates were zoned with 0.1% Congo red solution. The clear zone around the colony indicated cellulose activity.

3.2.5. Determination of bacterial growth curve

Each of the isolated strains was grown in 100ml halophilic broth in a 250 ml Erlenmeyer flask and incubated on a rotary shaker at 120 rpm for 12 days at 25 ºC the bacterial growth was measured every 24 hours. Aliquots of the culture were used to measure growth in terms of optical density (OD) at 600 nm with a spectrophotometer. The experiment was carried out in triplicate.

3.2.6. Optimization of culture conditions

The culture conditions of *Shewanella* sp., *Halobacillus* sp., *Halobacterium* sp., *Halococcus* sp. and *Salinococcus* sp. were altered by changing the carbon, vitamin, organic nitrogen sources, pH and salinity in the isolation medium.

i. Growth on different carbon source

In order to optimize the media for the maximum production of pigment, the media was additionally added 2% of different carbon sources like glucose, fructose, maltose, maninitol and sorbitol. The isolated bacterial strains were inoculated in to the halophilic broth in different Erlenmeyer flask. The flasks were incubated in rotary shaker at 120 rpm for 12 days at 25°C. Aliquots of culture were used to measure growth in terms of optical density (OD) at 600 nm by a spectrophotometer.
iii. Growth on different vitamin source

In order to optimize the media for the maximum production of pigment, the media was additionally added 2% of two different vitamin sources like Vitamin B and Vitamin C and used for the study of growth standardization. The isolated bacterial strains were inoculated into the halophilic broth in different Erlenmeyer flasks. The flasks were incubated in a rotary shaker at 120 rpm for 12 days at 25°C. Aliquots of culture were used to measure growth in terms of optical density (OD) at 600 nm by a spectrophotometer. The experiments were carried out in triplicate.

v. Growth on different organic nitrogen source

In order to optimize the media for the maximum production of pigment, the media was additionally added 2% of four different Organic nitrogen sources like tryptone, beef extract, yeast extract and meat extract and were used for the study of growth standardization. The isolated bacterial strain was inoculated into the halophilic broth in different Erlenmeyer flasks. The flasks were incubated in a rotary shaker at 120 rpm for 12 days at 25°C. Aliquots of culture were used to measure growth in terms of optical density (OD) at 600 nm by a spectrophotometer. The experiments were carried out in triplicate.

vi. Growth on different pH

The isolated strains were grown in different pH such as 6, 6.5, 7, 7.5 and 8 separately in halophilic broth. The flasks were incubated in a rotary shaker at 120 rpm for 12 days at 25°C. Aliquots of culture were used to measure growth in
terms of optical density (OD) at 600 nm by a spectrophotometer. The experiments were carried out in triplicate.

vii. Growth on different salinity

The isolated strains were grown in different salinities such as 15, 20, 25, 30 and 35 separately in halophilic broth. The flasks were incubated in rotary shaker at 120 rpm for 12 days at 25°C. Aliquots of culture were used to measure growth in terms of optical density (OD) at 600 nm by a spectrophotometer. The experiments were carried out in triplicate.

3.2.7. Extraction of pigment from bacterial culture

A pure culture of bacterium from the halophilic agar plate were transferred in to Erlenmeyer flask containing 50ml of halophilic broth and incubated. The 24 hour old culture was used for the extraction. Each of the isolated strain was inoculated in 1000 ml halophilic broth in Erlenmeyer flask and incubated on a rotary shaker at 120 rpm for 20 days at 27°C. When the culture reached a stationary phase, the cells were harvested by centrifugation (4000 rpm for 5 minutes). Equal volume of methanol was added to the pellet and centrifuged at 10,000 rpm for 10 minutes. The pigment was then extracted with methanol by repeated centrifugation until the cell debris turned colorless. The pigment extracts
were analyzed by scanning the absorbance in the wavelength region of 400-600 nm using the spectrophotometer.

3.2.8. Spectroscopic analysis (A MAX)

Spectroscopic analysis (technocomp 8500) was made for the quantitative analysis of carotenoids. The absorption maxima (λ_max) were determined with the wave length range of 350 to 500 nm and graphs were plotted to determine the maximum absorbency. The values standard carotenoid absorption data for common food carotenoids by Rodriguez and Amaya (2001).

3.2.9. Purification of pigment by thin layer chromatography

The clean dry glass plates were placed over a flat surface. Silica gel slurry was prepared with distilled water in the ratio 1:1(w/v). Pour the prepared slurry over the glass plate at thickness of 0.25 mm uniformly from one end to the other using applicator. Leave the plate to dry at room temperature for 15 to 20 minutes. Then the plates were kept in oven at 100 to 110°C for 1 to 2 hours.

![Compounds separated through thin layer chromatography](image)

Compounds separated through thin layer chromatography
The sample was dropped at the center of glass plate and allows it to dry. The solvent used in the experiment are in the ratio 2:1 (benzene and acetone) and 98% methanol. The sample loaded silica gel plate was placed in the solvent chromatographic chamber for separation of the compounds. Then the plate was removed from the chamber and subjected with iodine crystals for developing the colored spots. Then the plates were viewed under UV-transilluminater and the distance moved by the solvent and solutes were measured. RF values of the samples were calculated.

\[ R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}} \]

### 3.2.10. Quantitative estimation of protein

**Reagents**

**NaOH (0.1 N)**

By dissolving 400mg of NaOH in 100 ml distilled water, 0.1 N NaOH solutions were prepared.

**NaOH (1 N)**

For the preparation of 1 N NaOH solution 4g of NaOH was dissolved in 100 ml of distilled water.

**Solution A**

Solution A was prepared by dissolving 2 g of sodium carbonate in 100 ml of 0.1 N NaOH.
**Solution B**

Solution B was prepared by dissolving 500mg of copper sulphate in 1% sodium potassium tartarate (1 g of sodium potassium tartarate in 100 ml distilled water).

**Solution C**

Solution C was prepared by mixing 50 ml of solution A with 1 ml of solution B.

**Folin phenol reagent**

Folin phenol reagent was prepared by mixing 1ml of folin phenol with 1 ml distilled water.

**Blank**

For blank, 0.5ml of 1N NaOH, 5 ml of solution C and 0.5 ml of folin phenol reagent were taken.

**Procedure**

0.5 ml culture supernatant was taken in a test tube and it was dissolved with 1N NaOH and made up to 5 ml. From this, 0.5 ml was taken and then 5 ml of the solution C was added and kept for 20 minutes. Finally 0.5 ml of folin reagent was added and the intensity of colour developed was read at 660 nm in a spectrophotometer.

**Calculation**

\[
\text{Protein present in supernatant} = \frac{\text{OD of the sample} \times \text{concentration of the sample}}{\text{OD of the standard}}
\]
3.2.11. Enrichment of samples

The water sample was centrifuged at 15000 rpm for 10min by using laboratory centrifuge. The sediments were taken in a 250 ml conical flask and added with 200 ml of algal working solution (Conway medium). Each sample was divided into two equal portion of sediment was enriched with Conway medium as a working solution.

3.2.11.1. Culture of samples

The enriched samples were kept in a stock culture room and provided with 2000 lux white fluorescent light without aeration. The samples were placed on a shaker under low motion. The conical flasks were observed every day for colour development. The development of colour was considered as an indicator for growth of algae.

3.2.11.2. Isolation of algae cell

From each enriched sample, a little sample was pipetted out on a slide and kept in microscope for identification. Based on observation, different microalgae present in a sample were isolated by specially made micro tubes and placed in 1ml transparent tube containing sterilized culture media. These transparent tubes were kept in a shaker illuminated with white fluorescent light for inducing the growth of isolated algae cells.

3.2.11.3. Identification of isolated cell

The algal cells were subjected for identification after the colour development. The cells were pipetted out on a microscopic slide and observed
under 100 x magnifications. The cells were photographed and referred with the microalgal identification key obtained from the following keys


### 3.2.11.4. Maintenance of stock culture

The sterile seawater after cooling was poured into clean conical flask and the required nutrients were added. Conway’s medium was added to enrich the water.

This is the ideal medium to maintain the stock culture of all the phytoflagellates (marine microalgae). About 10 to 20% of the inoculums in growing phase were transferred into culture flasks and placed in front of the tube lights (1000 Lux). Within 4 to 5 days, the culture reached log phase and from 8 to 12th days, the maximum exponential phase was achieved.

### 3.2.11.5. Composition of walne’s medium (Conway medium)

In the present study the algae was enriched with Conway or Walne’s medium, its compositions are as follows,
Macronutrients (Solution ‘A’)

Potassium nitrate - 100gm
Sodium di hydrogen orthophosphate - 20gm
EDTA, sodium salt - 45gm
Boric acid - 33.48gm
Ferric chloride - 1.3 gm
Manganese chloride - 0.36gm

All the above compounds were dissolved in one litre of distilled water for preparing stock solution ‘A’ stored in a cool place to avoid evaporation.

Micronutrients (Solution ‘B’)

Zinc chloride - 4.2 gm
Copper sulphate - 4.0 gm
Cobaltchloride - 4.0 gm
Ammonium molybdate - 1.8 gm

The above compounds were dissolved in one litre distilled water for preparing stock solution ‘B’ stored in a cool place to avoid evaporation.

Vitamins (Solution ‘C’)

Thiamine (vitamin B\textsubscript{1}) - 200mg
Cyanocobalamine (vitamin B\textsubscript{12}) - 10mg

These vitamins were dissolved in 100ml of water for preparing stock solution, ‘C’ stored in a refrigerator.

Solution A, B and C were prepared in separate reagent bottles. For the preparation of working solution, 1 ml of solution ‘A’, 0.5ml of solution ‘B’ and 0.1 ml of solution ‘C’ were individually taken and added into 1 litre of filtered and sterilized sea water.
3.2.11.6. Algal cell counting

The platform of the improved Neubauer chambers total ruled area is 9 sqmm. It consists of central heavy ruled area is 1 sq.mm and 4 others square the same size in each corner. The central area was divided into 16 squares and the corner squares are further divided in to 16 squares.

First all samples were treated with formalin, in order to kill the cells and then it was stirred well and then one drop of sample was taken in a sterilized pipette. After placing the coverslip on the Haemocytometer, The pipetted sample was introduced on to the counting grid of the haemocytometer gently. The sample was spread uniformly between the chamber and coverslip to form a thin film and the cultured algal cells are uniformly distributed on the counting chamber.

The numbers of cells were counted under the microscope and then total cell count in 1ml of culture medium was calculated by using the following formula:

\[
\text{Total cell count} = \frac{\text{No. of cell counted}}{\text{No. of square counted in particular type}} \times 10000 \times \text{Total No. of squares} \times 10000
\]

After 3-4 days the microalgae reached the exponential phase of growth and all the culture colour changed to dark in colour.

3.2.11.7. Harvest of algal cell

The algal cells were harvested from the cultured flaks by centrifugation at 3000 rpm for 10 minutes. The cells were repeatedly washed with distilled water. The resultant algal pellets were stored at 5 degree Celsius for further analysis.
3.2.12. Extraction and estimation of total carotenoids

The amount of carotenoids in the experimental algal culture was estimated spectrophotometrically as follows.

Algal culture was centrifuged at 5000 rpm for 5 minutes and then the algal cells were ground with acetone. This mixture was again centrifuged at 3000 rpm and the pellets were collected and centrifuged at 10000 rpm for 15 minute. Then the supernatant was collected in aneppendroff tubes and the amount of carotenoid present was measured by taking optical density at 456 nm.

Total carotenoid in the sample was then estimated by using the formula given below.

\[
C = \frac{D \times V \times F(10)}{2500}
\]

Where

- \( C \) = Carotenoid content
- \( D \) = Optional density
- \( V \) = Dilution factor if any
- \( 2500 \) = Middle median absorption co-efficient

3.2.13. Stock culture preparation

*Dunaliella salina* was subcultured in 15 test tubes containing walne’s medium. The test tubes were incubated for 7 days till getting a good population of algal cells. The test tubes which showed good growth characteristics were transferred to 200 ml conical flasks containing the culture medium. Finally the culture flasks were placed in front of the tube lights of 1000 lux. The temperature ranged from 28-33°C.
3.2.14. Mass culture of *Dunaliella salina*

10 litre glass tanks were used for mass culture of *Dunaliella salina*. The tank was washed with soap and thoroughly rinsed with fresh water to remove the soap solution.

In open culture system, 10 litre glass tank was chosen for the biomass cultivation and inoculum of 100ml/lit medium was initially used, and nearly 5 litres was maintained in the tank till harvesting. The tank was kept in good light source and covered with transparent polythene paper. Purity of the culture was checked daily from 0\textsuperscript{th} to day of harvest (Huang et al., 2010).

3.2.15. Effect of salinity on carotenoid production on *Dunaliella salina*

In order to understand the impact of salinity on carotenoid production in *Dunaliella salina*, the following study was designed. For this study, four 250ml conical flask were taken and marked as A, B, C, and D. Each flask was having 200ml of sterilized and cooled sea water with enough amount of working solution. The flasks were inoculated with *Dunaliella sp.* which was isolated from the saltpan. During this period, the flasks were experimented at different salinities such as 50, 100, 150 and 200ppt. The algae were allowed to grow in the respective flasks for a period of ten days. At the end of the tenth day, the algal samples were harvested and tested for total carotenoid content.
3.3. RESULTS

3.3.1. Isolation of halophilic bacterium

Totally seven different types of colonies such as orange, light brown, light yellow, dark yellow, sky blue, greenish yellow and red colonies were appeared in halophilic agar plates. The colonies grown well after 15 days from the date of plating. Orange and yellow colonies were predominantly observed in the Puthalam saltpan. Light yellow and light brown colonies were predominantly observed in Swamythoppu saltpan. Also the greenish yellow, red and sky blue colonies were predominantly observed in the selected saltpan.

3.3.2. Identification of the isolates

The isolated colonies were subjected to biochemical characterization and identified by Bergey’s manual (1994). The identified bacterial species were *Shewanella* sp., *Halobacillus* sp., *Halobacterium* sp., *Halococcus* sp. and *Salinococcus* sp. (Table 3.1).

3.3.3 Enzyme activity and screening

3.3.3.1. Screening for protease activity

From the isolated bacterial strains, the selected strains were screened for proteolytic activity on skim milk agar. Among the five tested strains, *Halobacillus* sp. produced protease enzyme and they formed clear zone and others did not produce any zone formation (Table 3.2).
3.3.3.2. Screening for amylase activity

Among the five, Halobacterium sp., Halococcus sp., and Salinococcus sp. showed amylase activity, whereas the other species such as Shewanella sp. and Halobacillus sp. did not show amylase activity (Table 3.3).

3.3.3.3. Screening for cellulase activity

From the isolated bacterial strains, the selected strains were screened for cellulase producing ability on carboxy methyl cellulase agar. As there were no zone formations due to hydrolysis of cellulose, it was concluded that no one of the five strains was identified as cellulase producer.

3.3.4.1. Growth characterization of halobacterium species

i. Growth on different carbon sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different carbon sources such as glucose, sucrose, maltose, sorbitol and manintol. Among the tested carbon sources, the sucrose supplemented medium had the maximum growth (1.517) followed by manintol (1.448), glucose (1.342), sorbitol (1.136) and maltose (1.038) (Figure 3.1).

ii. Growth on different vitamin sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different vitamin sources such as vitamin B and vitamin C. In this study vitamin B (1.801) had a positive effect on growth and vitamin C (1.007) had a negative effect on growth (Figure 3.2).
iii. Growth on different organic nitrogen sources (OD value)

The halophilic strain was inoculated into halophilic broth with different inorganic nitrogen sources such as yeast extract, meat extract, beef extract and tryptone. Among the organic nitrogen sources, growth was high in yeast extract (1.801) followed by meat extract (1.608), beef extract (1.548) and tryptone (1.098) (Figure 3.3).

iv. Growth on different pH (OD value)

The pH of the culture medium has been reported to play a key role in pigment synthesis. The *Halobacterium* species was cultivated at different pH (6, 6.5, 7, 7.5, and 8) in shake flask cultures. The maximum growth (0.691) was noticed in the culture medium of pH 7.0. The minimum growth (0.401) was noticed in pH 8 and intermediate growth of 0.591, 0.523 and 0.439 were noticed in pH 7.5, 6.5 and 6 respectively (Figure 3.4).

v. Growth on different salinity (OD value)

The halophilic strain was inoculated into halophilic broth of different salinities such as 15, 20, 25, 30, and 35 ppt. Among the tested salinities, 20ppt had the maximum growth (1.151) at 4th days of incubation (Figure 3.5).

3.3.4.2. Growth characterization of *Halobacillus* species

i. Growth on different carbon sources (OD value)

The halophilic strain was inoculated into halophilic broth of different carbon sources such as glucose, fructose, maltose, sorbitol, and manintol. Among the tested carbon source, growth was higher (1.906) in sorbitol followed by
manintol (1.735), and fructose (1.694). The other carbon sources like maltose (1.147) and glucose (1.015) gave poor growth (Figure 3.6).

ii. Growth on different vitamin sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different vitamin sources such as vitamin B and vitamin C. Among the studied vitamins, vitamin B inoculated medium was suitable for the maximum (1.665) growth of selected strain (Figure 3.7).

iii. Growth on different organic nitrogen sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different inorganic nitrogen sources such as yeast extract, meat extract, beef extract and tryptone. Among the organic nitrogen sources, growth was maximum (2.142) in beef extract followed by yeast extract (1.996), meat extract (1.841) and tryptone (1.688) (Figure 3.8).

iv. Growth on different pH (OD value)

The pH of the culture medium has been reported to play a key role in pigment synthesis. The *Halobacillus* species was cultured in different pH (6, 6.5, 7, 7.5, and 8). The maximum growth (1.478) was noticed in pH 7.0. The minimum growth (1.194) was noticed in pH 6.5 and the medium growth was found in pH 6.0, (1.346) pH 8.0 (1.335) and pH 7.5 (1.229) (Figure 3.9).

v. Growth on different salinity (OD value)

The halophilic strain was inoculated in to halophilic broth with different salinities such as 15, 20, 25, 30, and 35 ppt. Among the tested salinities, the
maximum growth 1.538 was obtained in 25ppt salinity followed by 1.171, 1.084, 1.063 and 0.436 in 20, 15, 30 and 35ppt respectively (Figure 3.10).

3.3.4.3. Growth characterization of *Halococcus* species

i. Growth on different carbon sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different carbon sources such as glucose, sucrose, maltose, sorbitol, and manintol. Among the tested carbon sources, the maximum growth (2.218) was observed in glucose and the similar effect were noticed in maltose (2.216), fructose (2.051), while in the others, sorbitol (1.954) and manintol (1.899) evoked a poor growth (Figure 3.11).

ii. Growth on different vitamin sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different vitamin sources such as vitamin B and vitamin C. Among the vitamin sources, vitamin C showed the maximum growth (2.051) and followed by vitamin B (1.187) (Figure 3.12).

iii. Growth on different organic nitrogen sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different organic nitrogen sources such as yeast extract, meat extract, beef extract and tryptone. Among the tested organic nitrogen sources, beef extract had maximum growth (1.689) followed by yeast extract (1.660), meat extract (1.641) and tryptone (1.538) (Figure 3.14).
iv. Growth on different pH (OD value)

When the *Halococcus* species was grown at different pH(6, 6.5, 7, 7.5, and 8) in shake flask cultures, the maximum growth (1.390) was noticed in pH 6.0 and followed by pH 8.0 (1.352), 6.5 (1.278), 7.5 (1.194) and 7.0 (1.117) (Figure 3.14).

v. Growth on different salinity (OD value)

The halophilic strain was inoculated in to halophilic broth with different salinities such as 15, 20, 25, 30, and 35. Among the tested salinities, the maximum growth (1.333) was observed in 20ppt and followed by 1.167, 1.104, 1.053 and 1.049 in 25, 15, 30 and 35ppt respectively (Figure 3.15).

3.3.4.4. Growth characterization of *Salinococcus* species

i. Growth on different carbon sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different carbon sources such as glucose, sucrose, maltose, sorbitol, and manintol. Among the tested carbon sources, the maximum growth of 1.253 was observed in fructose and followed by 0.989 and 0.896 in glucose and sorbitol respectively. While the others, manintol and maltose gave poor growth like 0.802 and 0.673 respectively (Figure 3.16).

ii. Growth on different vitamin sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different vitamin sources such as vitamin B and vitamin C. Among the tested vitamins, vitamin B showed the maximum growth (2.081) and followed by vitamin C (1.651) (Figure 3.17).
iii. Growth on different organic nitrogen sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different organic nitrogen sources such as yeast extract, meat extract, beef extract and tryptone. Among the organic nitrogen sources, growth was high (1.581) in yeast extract followed by tryptone (1.501) meat extract (1.393), beef extract (1.352) and tryptone (1.098) (Figure 3.18).

iv. Growth on different pH (OD value)

The *Salinococcus* sp. was cultivated in different pH (6, 6.5, 7, 7.5, and 8) in shake flask cultures. The maximum growth (1.528) was noticed in pH 7.0. The minimum growth (0.702) was noticed in pH 8.0 and an intermediate growth of 1.283, 1.103 and 0.954 were noticed in pH 7.5, 6.5 and pH 6 respectively (Figure 3.19).

v. Growth on different salinity (OD value)

The halophilic strain was inoculated in to halophilic broth having different salinities such as 15, 20, 25, 30, and 35 ppt. Among the tested salinities, the maximum growth (1.501) was observed in 15ppt followed by 1.386, 1.218, 1.111 and 1.085 in 25, 20, 30 and 35ppt respectively (Figure 3.20).

3.3.4.5. Growth characterization of *Shewanella* species

i. Growth on different carbon sources (OD value)

The halophilic strain was inoculated in to halophilic broth with different carbon sources such as glucose, sucrose, maltose, sorbitol, and manintol. Among the tested carbon sources, growth was high (1.901) in glucose followed by 1.667,
1.501 in fructose and sorbitol respectively while the others maltose (1.483), and manintol (1.208) gave poor growth (Figure 3.21).

**ii. Growth on different vitamin sources (OD value)**

The halophilic strain was inoculated in to halophilic broth with different vitamin sources such as vitamin B and vitamin C. Among the vitamins, vitamin B showed the maximum growth (1.519) and followed by vitamin C (1.111) (Figure 3.22).

**iii. Growth on different organic nitrogen sources (OD value)**

The halophilic strain was inoculated in to halophilic broth with different inorganic nitrogen sources such as yeast extract, meat extract, beef extract and tryptone. Among the organic nitrogen sources, growth was high (1.832) in meat extract followed by beef extract (1.583) yeast extract (1.492) and tryptone (1.302) (Figure 3.23).

**iv. Growth on different pH (OD value)**

The *Shewanella* sp. was cultivated in different pH (6, 6.5, 7, 7.5, and 8) in shake flask cultures. The maximum growth (1.780) was noticed in pH 7.0. The minimum growth (0.856) was noticed in pH 6 and intermediate growth of 1.704, 1.382 and 1.293 were noticed in pH 6.5, 7.5 and 8 respectively (Figure 3.24).

**v. Growth on different salinity (OD value)**

The halophilic strain was inoculated in to halophilic broth with different salinities such as 15, 20, 25, 30, and 35ppt. Among the tested salinities, growth was high (1.429) in 25ppt followed by 1.045, 0.891, 0.841 and 0.824 in 20, 15, 30 and 35ppt salinity respectively (Figure 3.25).
3.3.5. $\lambda_{\text{max}}$ analysis

The extracted pigments were analyzed and identified by $\lambda_{\text{max}}$ analysis. The pigments were phytofluene, Auroxanthin, croctein, Echinone, Phytotene and $\beta$-carotene 5, 8-epoxide (Figure 3.26 to 3.30).

3.3.6. Thin layer chromatography

The TLC analysis of pigment isolated from the selected isolates showed the spot at Rf values. Among the observed Rf values in various bacteria, the maximum Rf value was recorded in *Halococcus* sp. (1.51 cm) and the minimum Rf value was recorded in *Shewanella* sp. (0.50 cm) (Figure 3.31).

3.3.7. Estimation of protein

The protein was tested in various bacteria. Among the bacterial species, the maximum amount of protein was recorded in *Halococcus* sp. (362.5 mg/ml) and followed by *Salinococcus* sp. (362.5), *Halobacterium* sp. (312.5), *Halobacillus* sp. (233.13) and *Shewanella* sp. (197.42 mg/ml) respectively (Figure 3.32).

3.3.8. Effect of salinity on *Dunaliella salina*

The *Dunaliella* species grown in different salinities such as 50, 100, 150 and 200pp had an average total carotenoid content of 0.796, 0.996, 1.529 and 2.198µg/ml respectively (Figure 3.33).
3.4. DISCUSSION

The present study was carried out to isolate the halophilic bacteria and algae and to optimize the media for the maximum production of biomass and pigments. The halophilic bacteria are commonly found in natural environment containing significant amount of NaCl such as inland salt lakes and evaporated sea shore, pools as well as environment such as curing brines and saline soils (Prior, 1978). Halophiles are categorized as slight, moderate or extreme, by the extent of their tolerance towards various salt concentrations (Prasun Patel et al., 2010).

Natural colours (pigments) are generally extracted from fruits, vegetables, roots and microorganisms and are often called biocolours because of their biological origin (Pattnaik et al., 1997). There is an increasing demand for natural colours in the food, pharmaceuticals, cosmetics, textiles and in printing dye industry. Microbial pigments are a promising alternative to other colour additives extracted from vegetables or animals because they are considered natural. The production of pigments by bacteria has attracted attention from the beginning of the study of bacteriology, possibly because; the presence of pigment is one of the characteristics of microorganisms which is not readily observed. In recent years special emphasis has been placed on the chemical nature of pigments and their probable roles in the metabolism of bacterial cells (Ben Sobin and Grantl Stahly, 1941).

Research on carotenoids in plants and animals has resulted in the development of techniques whereby these compounds can be separated and
identified. Because of these ease of obtaining large amount of material, considerable knowledge of the carotenoids in plants has been gained as evidenced by such monographs as those of Palmer (1922) and Strain (1938). The investigation of the carotenoids present in bacteria has lagged, possibly because of the greater difficulties of obtaining sufficient material.

The presence of carotenoids in bacteria was demonstrated by Zopf (1889) who designated this group of bacterial pigments as lipochromes, because of their solubility in the fat solvents. Later Kliger (1914) and Krainsky (1914) demonstrated the wide spread presence of carotenoid pigments in different species of bacteria.

Halophilic bacteria were originally of interest because of the striking changes that caused in the landscape by imparting various shades of red to natural salterns spoiled food and discoloured hides. Halophiles produce a variety of red colour to the environment in which they are found. Due to their specific role as immune modulators and prophylactic action against cancers, these protein molecules gain importance in recent research activities. The biotechnological roles of bacterial pigments are only poorly understood.

The present work was aimed to adapt some of the methods used for the separation and identification of carotenoid pigments and the determination of absorption spectrum maxima of the carotenoid pigments present in the bacteria. The work also aimed to optimize the culture medium for the growth of halophilic strain. The pigmented bacterial strains were isolated in halophilic agar medium which support the growth of halophilic bacteria only. Zobell (1946) pointed out
that bacteria which display a requirement for sea water or its equivalent when initially isolated should be considered marine bacteria. Apparent properties of sea water such as potassium, magnesium, and calcium were supplied by halophilic agar for the better growth of halophilic bacteria.

The bacterial strains were identified as *Shewanella* sp., *Halobacillus* sp., *Halobacterium* sp., *Halococcus* sp. and *Salinococcus* sp. based on Bergey’s manual. Lewis and Corpe (1964) identified the red pigmented rod shaped bacteria and confirmed morphologically and physiologically as *Serratia* sp. in reference with Bergey’s manual.

In the present work, only one of the isolates showed protease activity. The protease screening was done in skim milk agar, while calorimetric assays were not used. Halophilic bacteria have been isolated from different marine and hypersaline environments. These organisms have been shown to produce a wide array of hydrolytic enzymes including proteases, amylases, xylanases, cellulases as well as lipases and DNAse. These enzymes are commonly applied in the production of fermented food and food supplements, in animal feed, laundry detergents and textile industries (Mathabatha, 2010). The present work identifies bacteria having protease and amylase activity. None of the isolates showed cellulase activity. There are interesting opportunities for research in determining the effects of environmental factors on the kinds and amounts of the carotenoid pigments produced by the bacteria.
In the present investigation, the halophilic bacterial growth was optimized through various physico-chemical parameters. Five halophilic strains such as *Shewanella* sp., *Halobacillus* sp., *Halobacterium* sp., *Halococcus* sp. and *Salinococcus* sp. were inoculated into halophilic broth with different carbon, nitrogen, vitamin, metal ions, organic nitrogen sources, pH and salinity. Most strain have better growth was observed in glucose as carbon source. And among the nitrogen source, NaCl offered maximum growth; among the vitamin sources vitamin C offered optimum growth; yeast extract as organic nitrogen source; MgSO$_4$ as metal ion source. The optimum pH for the growth of those halophilic strains was pH 7 and among the tested salinities, 20 and 25 ppt salinities had maximum growth.

The extraction of carotenoids from plant material may be accomplished by the use of any one of many of the fat solvents. This was observed to be not true for the bacterial carotenoids. It was discovered that the extraction of the carotenoids from the bacterial cells could be accomplished best in the presence of a small amount of water miscible fat solvent. Methyl alcohol proved to be well suited for this purpose (Ben Sobin and Grantl Stahly, 1941).

In the present work, among the five species tested, *Halobacterium* species only showed positive effect for the growth when optimized with 2% sucrose as carbon source. None of other species showed positive effect. Khanafari *et al.* (2010) reported that sucrose had a significant decreasing effect in growth curve. The sucrose of 2% might have supported the organic carbon bioavailable for the
growth of the *Halobacterium* sp. This may be the reason that the growth of the species was maximum from the original medium.

The culture media added with organic nitrogen had the better growth when compared to others. Chong *et al.* (2002) also reported that yeast extract enhanced the growth and pigmentation in *Paecilomyces sinclarii*. This might be due to the positive role of yeast extract to support higher mycelial growth which enhances the pigment production.

Among the halophilic medium optimized with different pH such as 6, 6.5, 7, 7.5 and 8, the growth was better in pH 7 in all the species. Khanafari *et al.* (2010) reported that the better growth of *Halorubrum sodomence* was also seen in pH 7.2 to 7.5. It seems that the optimum pH for the best growth is pH 7-8.

Bhaskar *et al.* (2010) characterized carotenoids from selected strains of *Streptomyces* sp. of *Tendania anhelan, Epinephelus dicanthus* and *Cyprinus corpio*. The interaction of microbes particularly *Streptomyces* sp. in the gut of fishes in two environmental biotopes and their ability of producing carotenoids were assessed. The UV spectrum, TLC and HPLC results showed the biosynthesis of carotenoids in various strains of *Streptomyces* sp. clearly indicate the presence of phytoene, phytoflune, α-carotene, β carotene and β- isorenieratene.

In the present study, the isolated halobacteria produced red, light yellow, dark yellow, orange, greenish yellow, dark brown and sky blue coloured pigments. These pigments were extracted using methanol and centrifugation. Carotenoidal estimation was analyzed by two different ways such as UV-spectral
analysis and thin layer chromatography. The pigments identified using UV-Vis scan spectrofiguremeter analysis were β-carotene, 5, 8-epoxide (λ max 407 nm), phytofluene (261 nm) and echinenone (461 nm).

The extraction of pigment was achieved by various methods like solvent extraction, centrifugation etc. Asker and Ohta (1999) extracted the pigment from the bacterial culture by centrifugation methods.

In this work, methanol was used as main extraction solvent, based on literature. Successful separation of carotenoids is dependent on the selection of proper solvent. The choice of solvent depends largely upon the particular group of carotenoids to be separated. Methanol and ethanol were found to be a good extraction solvents used in chlorophyll extraction from micro algal biomass. Both of them showed to be better than acetone, which is not very efficient in the extraction and quantification of pigments from autotrophic cell cultures.

The samples extracted with the methanol were scanned between 300 to 465 nm, using UV-Vis scan spectrofiguremeter and it showed individual peaks. The pigments were identified as phytofluene, Auroxanthin, croctein, echinenon, phytotene and β-carotene 5, 8, epoxide.

Turko et al. (2005) reported that pigmented microbacterium strains viz. yellow orange and red were found to have absorption spectra typical of C₄₀ carotenoids. These compounds were identified by using a combination UV-VIS spectral data and HPLC retention time. Polar organic solvents such as acetone and methanol have been extensively used for the extraction of carotenoids from bacterial culture. Carotenoids absorbs maxima at 3 wavelengths (533nm, 408 nm,
341 nm) resulting in three peak spectra which is characteristics of carotenoid pigment. The polyene, chromophores of carotenoid which absorb light in 400-556 nm range provide the basics of yellow to red colour and their ability to quench singlet oxygen (Umeno et al., 2005).

In the present study, pigments were identified in varying wavelengths of 300 to 460 nm. This change in absorbance is mainly due to the type of extracting solvent.

Although several new techniques have been used for the analysis of carotenoids of various sources, literature describing analysis of carotenoids using thin layer chromatography in detail is limited (Sharma, 2000). Thin layer chromatography is comparably easy to use and is employed for the separation and qualitative and quantitative analysis of carotenoids in food and nonfood product. Thin layer chromatography is widely used for food analyses to found composition, adulteration, contamination and degraded products of carbohydrate, protein, vitamin and lipids. The technique is often used for the isolation of individual classes of molecules present, because thin layer chromatography is often used for the isolation of individual classes of molecules present, because, thin layer chromatography is rapid, effective and inexpensive. Before the use of silica it is necessary to neutralize the effect of acidity, to avoid epoxide furanoxide rearrangement of carotenoid and chlorophyll phaeophytinization (Rodriguez and Amaya, 2001). Early TLC method used silica as stationary phase and non-polar solvent as mobile phase for separation of carotenoids. In the
present study TLC analysis confirmed identification of pigments from the bacterial culture in relation with the Rf value.

In the present work, the pigments were purified by thin layer chromatography using methanol as mobile phase. From the extracted compounds the maximum Rf value was given by the pigment from *Halococcus* sp. having the Rf value of 1.510 and the minimum Rf value was given by *Shewanella* sp. having the Rf value of 0.50. Khanafari *et al.* (2010) reported that Rf values of red, yellow and orange extracted pigments were 0.434, 0.333, and 0.263 respectively. This change in Rf value when compared to Khanafari *et al.* (2010) might be due to strain specificity and the type of extraction used.

The present study was undertaken to isolate, purify and characterize the halophilic bacterial strains because the biocolour has application in many industries. This study was also carried out for the optimization of culture media and extraction and purification of pigments and the determination of absorption spectrum maxima of pigments present in bacteria. The isolated bacteria were identified using Bergey’s manual. Optimization of the culture medium was done for the maximum production of biomass. The pigments were extracted using methanol as extractant. Individual peaks were obtained between 300 to 470 nm when the methanolic extracts of pigment were analyzed by scanning the absorbance with UV Vis spectrophotometer indicating the pigments were possibly phytofuene, Auroxanthin, croctein, echinenon, phytotene and β-carotene 5,8, epoxide.
*Dunaliella* species has the exceptional ability of synthesizing and accumulating high amounts of β-carotene. When maintained under growth limiting conditions (Ben Amotz *et al*., 1982; Borowitzka *et al*.; 1984; Borowitzka, 1988). The present study was an attempt to check the effect of salinity on the total carotenoid production by *Dunaliella salina*.

The unicellular microalgae *Dunaliella salina* accumulates massive amounts of β-carotene. The β-carotene synthesis increases with the unbalanced physiological condition of cell, created due to various stress factors. A normal cell has a condition of physiological balance. Under stress conditions, this balance is disturbed, such as formation of excessive free radicals. In order to protect and continue growth, the cells are known to generate additional β-carotene under stressed conditions (Boussiba and Vohshank, 1991).

It has been previously described by many works like Semenenko and Abdullayev (1980), Ben-Amotz and Avron (1990), Borowitzka *et al*. (1984), Ramazanov *et al*., (1988), Celekli and Donmez (2006), Fazeli *et al*. (2006) and Borowitzka and Siva (2007), that environmental factors played most effective roles on the level of carotenoid production by *Dunaliella* sp. The highest production of carotenoid is observed in high salinity, high temperature and high light intensity (Rad *et al*., 2011).

The result of the present study proved that the total carotenoid production by the species of *Dunaliella* was maximum (2.198 µg/ml) in the salinity of 200ppt. The total carotenoid production was minimum (0.7859 µg/ml) in the salinity of 50ppt. It was observed that the productivity of total carotenoids was
maximum at a higher salinity of 200ppt, and as the salinity increased, the productivity of carotenoids also increased. Salinity stress was found to be directly proportional to the total carotenoid production exhibited by *Dunaliella*. The results of the present study is in accordance with the results of Rad *et al.* (2011), who studied the effects of different salt concentrations on carotenoid production and reported that though the cell growth rate at higher salinity were significantly lesser than in lower salinity, the carotenoid production rate per cell at higher salinities were significantly higher. These results are in accordance with those reported by Ben-Amotz and Averon (1982), Gomez *et al.* (2003) and Fazeli *et al.* (2005). It has been reported by many workers that salinity stress tend to increase the carotenoid productivity by *Dunaliella* on a cellular basis. (Mironyuk and Einor, 1968; Semenonko and Abdullayev, 1980; Ben-Amotz *et al.*, 1982; Borowitzka *et al.*, 1984; Ramazanov *et al.*, 1988; Celekli and Donmez, 2006; Fazeli *et al.*, 2006; Borowitzka and Siva, 2007; Jahnke and White, 2003) also reported that although high salinity favours carotenoid production by the microalgae (Rad *et al.*, 2011).
3.5. SUMMARY

The present work was carried out to isolate and identify the halophilic bacteria and algae from the saltpan and to isolate and estimate the pigment produced by them. The water samples were collected from the saltpan and the halophilic bacteria were isolated in the laboratory, the bacterial growths in relation with different physiological, biochemical parameters were optimized. Moreover the pigments produced by the bacteria were quantified. *Dunaliella salina* is one of the few species of microalgae which can be mass cultured outdoors, including semi intensive systems in hypersaline lakes. The reason for that lies in its wide range, which allows the use of hypersaline culture medium not suitable for the growth of other organisms. Only under extreme culture conditions such as high salinity, it is possible to minimize contamination of mass culture with opportunistic species or other organisms. Nowadays *Dunaliella salina* is one of the micro algal species more often used for mass culture due to its ability to accumulate β-carotene, a molecule which in this algae can represent up to 95% of total carotenoids and has become the most commercially demanded carotenoid. *Dunaliella salina* was cultured at different salt concentrations such as 50, 100, 150, and 200ppt for the isolation and estimation of carotenoid. The important results obtained in the study have been summarized here under.

- The bacteria isolated from the saltpan had orange, light yellow, dark yellow, light brown, sky blue, greenish yellow and red coloured pigment.
The bacteria identified based on morphological and biochemical characteristics were *Shewanella* sp., *Halobacillus* sp., *Halobacterium* sp., *Halococcus* sp. and *Salinococcus* sp.

The five carbon sources, such as glucose, fructose, sorbitol, manintol and maltose were used for the optimization of media. The *Halococcus* sp. and *Shewanella* sp. had the maximum growth on glucose where as the *Halobacillus* sp. had better growth on sorbitol. *Halobacterium* sp. had the maximum growth on sucrose and *Salinococcus* sp. had the maximum growth on fructose.

The two vitamin sources such as Vitamin B and Vitamin C were used for the optimization of bacteria. All the tested strain except *Halococcus* sp. showed better growth on Vitamin B while *Halococcus* sp. had the maximum growth on Vitamin C.

Beef extract, yeast extract, meat extract and tryptone were used for the optimization of the media as organic nitrogen sources. The growth of *Halococcus* sp., *Halobacillus* sp. and *Halobacterium* sp. was maximum in Beef extract while *Salinococcus* sp. showed maximum growth on yeast extract. Meat extract was preferred by *Shewanella* sp. when compared to others.

Among the different ranges of salinity tested, the salinity 25 was suitable for *Halobacillus* sp. and *Shewanella* sp. 20ppt was suitable for *Halobacterium* sp. and *Halococcus* sp. and 15ppt was suitable for the maximum growth of *Salinococcus* sp.
Among the tested pH, pH 6 was preferred by *Halococcus* sp. whereas pH 7 was preferred by all the other species.

*Dunaliella salina* was cultured in four different salinities such as 50, 100, 150 and 200ppt. Among the tested salinities, the maximum carotenoid production was noted at 200ppt.