Pigment isolation from halophilic bacteria and its application in textile dyeing.

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"ISOLATION OF PIGMENT FROM HALOPHILIC BACTERIA AND IT'S APPLICATION IN TEXTILE DYEING"** is based on the results of investigations carried out by me in the Biotechnology Discipline at the School of Biological Sciences and Biotechnology, Goa University, under the Supervision of **Prof. Savita Kerkar** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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This is to certify that the dissertation report "ISOLATION OF PIGMENT FROM HALOPHILIC BACTERIA AND IT'S APPLICATION IN TEXTILE DYEING" is a bonafide work carried out by Ms. Vaishnavi Prashantkumar sakte under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science Biotechnology in the Discipline Department of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

The main objective of this study is to extract pigments from halophilic bacteria, aim is to utilise pigments in textile dyeing. Bacterial pigments are natural, biodegradable, eco friendly in nature. These properties of the pigment make the ideal to use them as a textile dye. Isolation of the bacteria, extraction of the pigment, and dyeing of the textile is feasible, and process is ecofriendly compared to the dyeing with the synthetic dye.

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ABBREVIATIONS USED

Entity	Abbreviation
Absorbance of sample	A
Specific absorbance of the compound at a particular wavelength	$A^{1\%}$
Biological oxygen demand	BOD
Bacillus subtilis	*B. subtilis*
centimetre	cm
Copper sulphate	CuSO4
Chemical oxygen demand	COD
cyclin dependent kinase 4	cdk4
1,1-diphenyl-2-picrylhydrazyl	DPPH
N, N-dimethyl-p-phenylenediamine	DMPD
extreme halophilic agar	EHM
Escherichia coli	*E. coli*
ferrous sulphate	FeSO4
Grams	gm
High performance liquid chromatography	HPLC
human leukemia cell lines	HL60
Milli meter	mm
Milli liter	ml
nuclear factor erythroid 2-related factor 2	Nrf2
Sodium chloride	NaCl

NaCl tryptone yeast extract	NTYE
nutrient agar	NA
nanometer	nm
Sodium chloride	NaOH
Optical density	OD
phosphorylation of retinoblastoma	pRB
reactive oxygen species	ROS
Revolution per minute	rpm
Retention factor	Rf
synthetic phase	S phase
Staphylococcus aureus	*S. aureus*
Thin layer chromatography	TLC
Ultra violate	UV
X-ray crystallography	XRD
Zobell marine agar	ZMA

ABSTRACT

It is well recognized that halophilic bacteria are a rich supply of material with commercial potential. Halophilic bacteria known for their ability to thrive in high salt concentration have gained attention due to potential as natural sources of carotenoids for application in various industries including textile dyeing. The current study focuses on production of carotenoids by halophilic bacteria and evaluating their potential use as natural dye in textile dyeing processes. An attempt was made to isolate carotenoid producing bacteria from saltpan and extracting the carotenoid to utilize it as a natural dye in textile dying. Fabric dyed were cotton, polyester, satin, silk. Fixatives used were metal as well as bio mordants. This study focuses on the green dyeing of the textile.

Keywords: Halophilic bacteria, carotenoids, natural dye, textile dyeing, saltpan, Fixatives, metal, bio mordants

CHAPTER 1: INTRODUCTION

1.1 Background

Geek etymology for the word “halophile” suggest “salt loving”. These are extremophiles that live in water bodies with salinities five times higher than those of the ocean. Most halophiles fall into the category Archaea, together with certain eukaryotic organisms and bacteria. Halophiles are classified as intense, moderate, slight, or non-halophilic depending in how much they tolerate halogens. Extreme halophiles grow best in media containing at least 15% NaCl and they can even grow to salt saturation at about 32% of NaCl. They prefer 5 to 5.2 M (20-30%). The ideal growth condition for moderate halophiles are medium containing between 3 to 15% of NaCl or roughly 0.5 to 2.5M. (Rathakishnan, et al., 2022).

Among the most prevalent classes of pigment found in nature are carotenoids. Fruits and vegetables contain around 40 distinct carotenoids of which only some can be converted to vitamin A. Provitamin A carotenoids are those that are convertible. ascorbic acid, carotenoids, tocopherol, and tocotrienols all interact with free radicals particularly peroxyl radicals and singlet molecules oxygen to avert oxidation. Carotenoids including lycopene, beta-carotene, and several other oxycarotenoids decrease free radicals in lipid phase to perform antioxidant and immune boosting properties. Carotenoids have demonstrated the capacity to promote intercellular communication and have anticancer property. Despite being abundant in leafy plants, carotenoids can also be found in a variety of bacterial sources.

Structure and functionality

Carotenoids are ubiquitous in nature and more than 600 different types of carotenoids have been characterized, which consists of a C_{40} hydrocarbons backbone in case of carotenes. Functional groups present makes cyclic or acyclic xanthophylls. Based on the chemical structure carotenoids are classified into two groups: carotenes or carotenoids hydrocarbons that is composed of hydrogen and carbon only: and xanthophylls or oxygenated carotenoids which are oxygenated and may contain epoxy, carbonyl, hydroxyl, methoxy or carboxylic acid functional group. Lycopene and βcarotene are carotenes and lutein, canthaxanthin, zeaxanthin, violaxanthin, capsorubin and astaxanthin are xanthophyll carotenoids.

The most important carotenoids in terms of biotechnological use are astaxanthin (3,3’-dihydroxy-β,β’-carotene-4,4’-dione), β-carotene , canthaxanthin (β,β’-carotene-4-4’dione),β-cryptoxanthin (hydroxy-β-carotene),fucoxanthin, lutein, zeaxanthin, violaxanthin. (Rodrigo-Banos, 2015)

The core structural element of carotenoids is a polyene backbone that consists a series of conjugated C=C bonds. This feature is responsible for pigmentation property and antioxidant property.

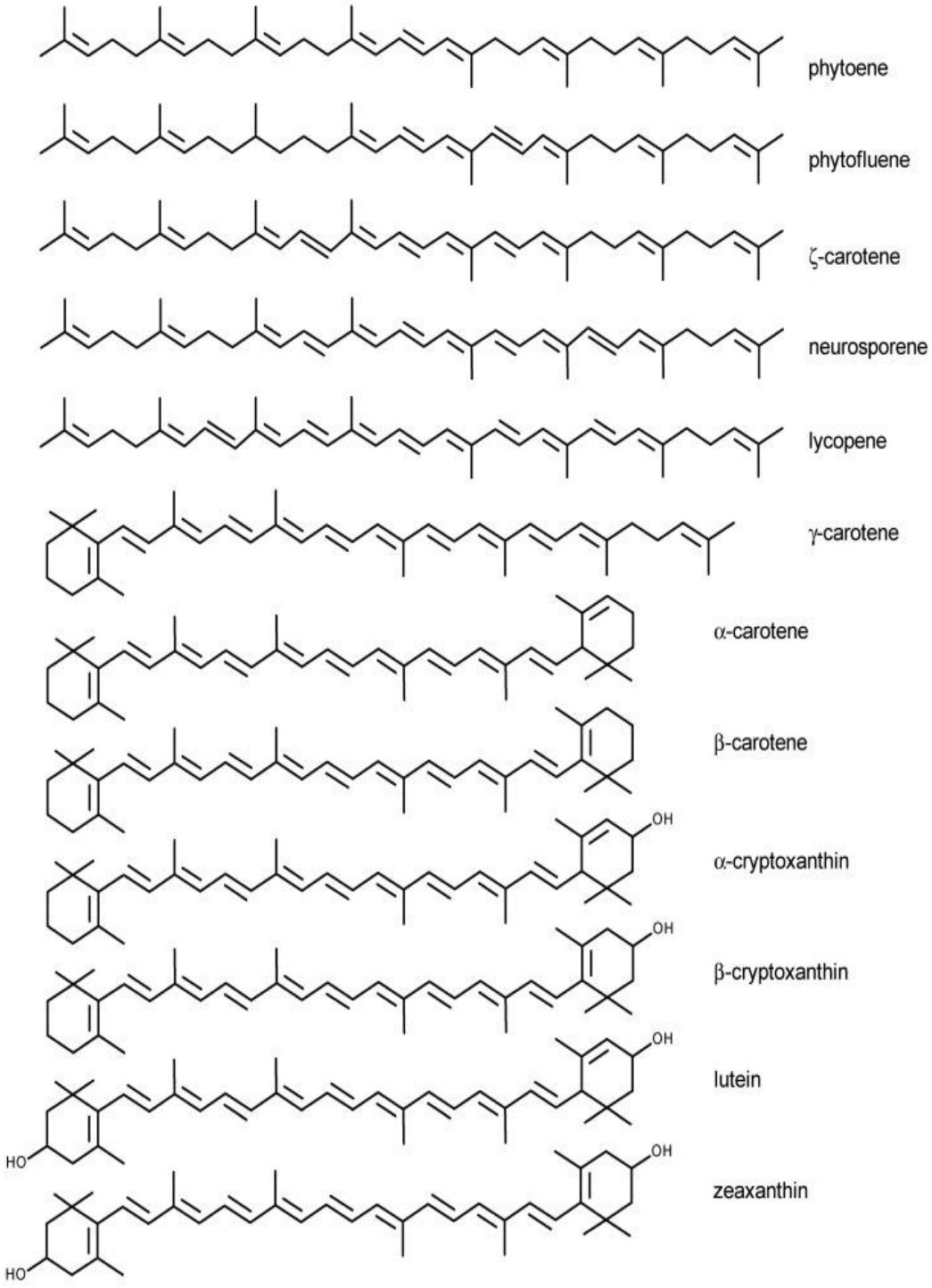


Fig. 1. Chemical structure of carotenoids (Fiedor, et al., 2014)

Antibacterial property

It was discovered that astaxanthin pigment was more significantly effective against every examined pathogen species, including *S.typhi* generated (20 mm) in diameter for the zone of inhibition, 24mm for *P. aeruginosa,* 18 mm for *B. subtilis* and 16mm for *S.aureus.*
According to (Neveen 2011), Penicillium purpurogenum's extracellular pigment was found to be more significantly efficient againstmicrobial species, including *Candida albicans*, *E. coli*, *P. aeruginosa, S. aureus*, and *B. subtilis*.
According to Sanjay's (2009) observations, pathogenic bacterial cells undergo lysis due to the antibacterial activity of xanthin pigment at a greater concentration of 400 µg ml-1.
The pigment extract of Sporobolomyces sp. was discovered to have a 2.8 inhibition zone, whichis strongly inhibiting of the bacterial pathogen *E.coli*, *P. aeruginosa and Enterococcus* (1.9 and 2.1cm).
Chloramphenicol, the common antibiotic, created inhibitory zones of 2.9, 2.0, 1.8, 2.8, and 2. 6 centimeters,correspondingly against Enterococcus sp., *S. aureus*, *S. faecalis*, *E. coli*, and *P. aeruginosa*.
Variations in cell wall composition can be the cause of the variation in susceptibility.
The variation in the cell wall architecture was cited as the cause (Singh et al., 2007).
A number of variables, including as charge density, lipopolysaccharide structure, and the lipid makeup of the cytoplasmic membrane in both Gram-positive and Gram-negative bacteria, may contribute to selective antibacterial action

Anticancer property

One of the main characteristics of cancer cells is a dysregulated cell cycle, in which the cells are unable to manage the pace of proliferation and the cell cycle. Evidence have shown that carotenoids interfere in the cell cycle of the tumour cells. β-carotene, one of the carotenoids, has been thought to have anticancer properties against human promyel ocytic leukemia (HL60) cells. At a concentration of 20 M beta carotene significantly decreased the viability of HL6O cells and arrested them by 39.4% in the G1 phase.Additionally, it reduced the production of cyclin A, the primary regulator of the G2/M phase progression, causing cell cycle arrest in human colon adenocarcinoma cell concentration ranging from 1 to 25 M.The G2/M phase arrest persisted for the course of the tr eatment, as evidenced by the accumulation of G2/M phase cells and a corresponding decrease

in the proportion of S phase cells.(Niranjana et al. 2015)
βcarotene increased the number of S and T cells in pituitary adenoma cells. According to studies, lycopene and apo-12′-lycopenal, Altered the normal cell cycle at different stages based on the exposure length, which considerably reduced the growth of prostate cancer cells (Holzapfel et al., 2013). According to a 2007 study by Ivanov et al., lycopene-induced cell cycle arrest in prostate cancer cells at the G0/G1 phase is caused by a reduction in the expression of cyclins D1 and E, cyclin dependent kinase 4 (cdk4), and phosphorylation of retinoblastoma (pRB). Lycopene was shown to stop the cell cycle in the G0/G1 phase of leukaemia cells.Neoxanthin suppressed DNA synthesis in the S phase of the cell cycle, which significantly slowed the growth of mouse embryonic mesenchymal (C3H10T1/2) cells (Chang).& Lin (1993). B-cryptoxanthin had an anti-proliferative effect in lung cancer cells by causing cell cycle arrest at the G0/G1 phase. This was linked to an increase in the expression of p21 protein and a decrease in the expression of cyclin D and E The anti-proliferative action of capsanthin and bixin in leukaemia (K562) cells was mediated via cyclin D1 down-regulation and p21 and nuclear factor erythroid 2-related factor 2 (Nrf2) up-regulation.it has been discovered that fucoxanthin causes cell cycles arrest in tumor cells, including those from neuroblastoma, hepatoma, leukaemia, colon, melanoma and prostrate cancer, at the G1, G2/M and G0 stages. (Lian et al. 2006)
According to a prior study, fucoxanthin causes a cell cycle arrest in gastric cancer cells during the G2/M phase by downregulating the expressions of cyclin B1 and survivin .Human neuro blastoma cells in the G0/G1 phase of the cell cycle were stopped from growing by fucoxanthi n(Yu et al. 2011)

Antioxidant property

Carotenoids are widely distributing across natural system. Recent studies have shown that the carotenoids can switch from antioxidant to pro-oxidant due to the oxygen concentration. The physical organization of the carotenoids affects its antioxidant property through interaction with Rfd89Leactive oxygen species. Carotenoids are very efficient physical and chemical quenchers of singlet as Wu/ell as potent scavengers of other reactive oxygen species. The increase in ROS level in the body results in oxidative stress. Carotenoids play a major role in ROS mediated disorders like cardiovascular disease, cancer, photosensitive or eye related disorder.

Dyeing with bacterial pigments: As a natural dye

Anciently, fabrics were dyed using colours from natural sources, until synthetic dyes were invented. Synthetic dyes are manufactured from petrochemical sources that involves hazardous chemical that is destroying the nature. Growing concern about the environment, manufacturers are shifting towards the natural dyes. Naturals dyes are non-substantive and applied on textiles by the help of mordants. Usually, metallic salts are used which have affinity for both dye and the fabric. The shade produced by natural dyes are usually soft, lustrous and soothing to the human eye. Natural dyes are renewable and biodegradable. (Akcakoca Kumbasar 2011)

Bacterial metabolites, or secondary metabolites, are by-products of bacterial growth and can possess antimicrobial, anticancer, antioxidative, and UV properties. These metabolites could be used as functional dyes for textile materials, offering a range of applications beyond their aesthetic qualities. Despite being insoluble in water, they form bonds with fabric. Dyes can enhance textile materials' antimicrobial and anticancer properties by binding without disrupting their antimicrobial or anticancer functions

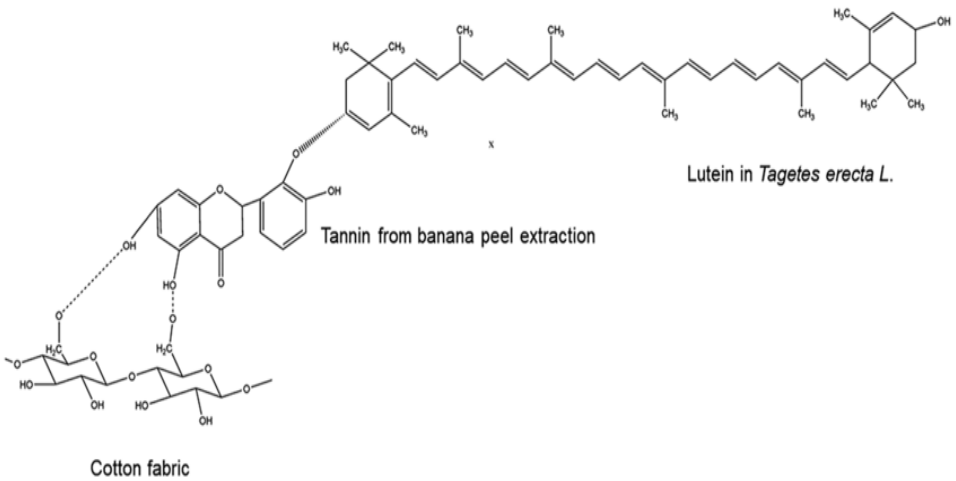
Bacteria, including Serratia, Streptomyces, and Pseudomonas, produce pigments or dyes that can color textile materials. The range of colours that these bacteria produce include pink, red-orange, yellow, blue, green. However, the extracted pigment's color may not always match the dyed fabric's color due to substrate nature and dyeing conditions like temperature, pH, and mordant use.(Kramar and Kostic 2022)

Natural dyes are either substantive or adjective, and most require a metal salt to create an affinity between the fiber and pigment. Mordants, derived from Latin mordere meaning "to bite," are chemicals that can be fixed on the fiber and combine with the dyestuff. They help produce faster shades by forming an insoluble compound of mordant and dyestuff. Other mordants include tannins and oils, which impart affinity for basic dyes and act as primary mordants for metallic salts. There are three types of mordants: metal salt, tannins, and oils.

Mordants have evolved from naturally occurring metal salts to metal salts of aluminium, chromium, iron, copper, and tin. Common mordants include alum, potassium dichromate, ferrous sulphate, copper sulphate, stannous chloride, and stannic chloride. Alum, originally a double sulphate of aluminium and potassium, crystallizes in octahedra with 24 molecules of water. It is not dissociated by boiling or diluting with water. Potassium dichromate, also known as red chromate or dichromate of potash, is produced by heating chrome iron ore with

lime and potash. It crystallizes in large orange triclinic prisms and is poisonous. Ferrous sulphate, also known as Copperas or green Vitriol, is prepared on large scale from iron pyrites and is widely used. Cupric sulphate, also known as blue vitriol or copper sulphate, is manufactured by roasting ores containing copper and dissolving them in sulphuric acid. Stannous chloride is prepared by dissolving tin in hot hydrochloric acid, resulting in monoclinic crystals known as tin crystals. Stannic chloride is prepared by oxidating stannous chloride with chlorine or potassium chlorate, making it an important mordant for cotton and silk.

Tannins, polyphenolic compounds with gelling properties, are found in various vegetables like oak galls, sumach, cutch, and barks. These tannins can bond color to plant-based fibers, providing a greater color range and successful results. Some tannins, like oak galls, can bind to fibers and remain clear, allowing dyes to saturate the fiber. However, some tannins can alter color, especially if dyes have yellow, pink, or brown tones.



Probable interaction in cotton fabric- banana peel biomordant-lutein complex.(Phromphen 2023)

1.2 AIM AND OBJECTIVE

AIM: isolation of carotenoids from halophilic bacteria and its application in dyeing of textile.

OBJECTIVE:

I. Isolation of pigment from halophilic bacteria

II. Extraction of the pigment

III. Application of the pigment in dyeing of textile.

1.3 HYPOTHESES

Carotenoids extracted from halophilic bacteria can serve as effective natural dyes for textile application, providing stable colour in terms of colour fastness and environmental stability.

Due to increasing risk of synthetic dye on environment textile industry are finding alternate ways of dyeing fabric. Using carotenoids from halophilic bacteria offer an eco-friendly alternative.

Carotenoids have been associated with antioxidant and antimicrobial properties. Textiles dyed with carotenoids may gain added functional benefit such as resistance to microbial infection.

1.4 SCOPE

The current study has been carried out to isolate pigments form halophilic bacteria, The more focus is on isolating carotenoid producing bacteria. Orange and pink pigment producing bacteria were grown in high salinity. Pigments extracted from these isolates were can be used for further characterisation TLC, uv-vis spectroscopy, FTIR, XRD, HPLC. Pigments have antioxidant, antimicrobial properties. Now a days bacterial pigments are being extensively used in textile industry as a natural dye. Synthetic dyes though they are effective but create harmful impact on environment. Alternative to that bacterial pigments are being used.

CHAPTER 2: LITERATURE REVIEW

Halophilic bacteria thrive in highly saline environments, giving a pink to red hue to the habitat. With the growing concern about the negative effects of synthetic food colorants, researchers have sought to find new sources of natural carotenoids such as canthaxanthin by isolating red halobacterium strains. The goal is to optimize carotenoid production conditions and identify new sources of natural pigments for use in food production.(Khanafari, Khavarinejad, and Mashinchian 2010)

Halophilic bacteria survive under high salt conditions such as solar salterns, salt lakes and salt mines. Recently many studies have been published on pigments from halophilic bacteria and its application in biotechnology-based industries. Studies have also demonstrated on exopolysaccharides, biopolymers, biosurfactants, compatible solutes, antioxidants, antimicrobial compounds and anti-tumour agents.

Hypersaline environments, characterized by high salinity, can also experience extreme temperatures, low oxygen, and high pH levels. These environments are categorized into two types: **Thalassohaline**, resulting from seawater condensation, and **Athalassohaline**, from the evaporation of inland water. The predominant microorganisms in hypersaline environments are moderately and extremely halophilic, with the latter group primarily represented by Halobacteriaceae. These microorganisms have potential biotechnological uses. Saltern ponds in these environments are often colorful due to pigmented microorganisms such as Dunaliella (rich in β-carotene), Haloarchaea (producing bacterioruberin), and halophilic bacteria like *Salinibacter ruber* (producing salinixanthin). The genus Halobacterium exhibits pigment variation based on the salt concentration in its environment. *Salinibacter ruber*, a halophilic bacterium, is commonly found in NaCl-saturated saltern crystallizer ponds alongside Halobacteriaceae members. *S. ruber* contains high levels of carotenoids in its membrane, particularly salinixanthin—a C-40 acyl glycoside carotenoid distinct from the C-50 bacterioruberins in haloarchaea. Both haloarchaea and extremely halophilic bacteria thrive in non-aseptic conditions due to high salt levels, which deter other microbial contamination.(De Lourdes Moreno et al. 2012)

Bacterioruberin is a pigment known for containing 13 pairs of conjugated double carbon bonds, providing effective free radical scavenging and singlet oxygen quenching capabilities. This pigment helps protect halobacteria from light-induced damage and offers resistance to oxidative DNA damage. Bacterioruberin plays crucial roles in maintaining membrane fluidity

and acting as a water barrier, enhancing bacterial survival in extreme environments.(Abbes et al. 2013)

Salinibacter ruber, an extremely halophilic eubacterium, exhibits light-induced inhibition of respiratory oxygen uptake rates similar to other species with retinal-based light-driven proton pumps or photosystems. The bacterium contains xanthorhodopsin, a retinal protein coupled with carotenoid salinixanthin, which functions as a light-harvesting antenna, increasing proton transport efficiency. This coupling enhances the flow of light quanta available for energizing proton transport. Xanthorhodopsin shares homology with bacteriorhodopsin, proteorhodopsin, and Gloeobacter violaceus rhodopsin. Salinibacter also contains genes for a halorhodopsin-like protein and two sensory rhodopsins, but only xanthorhodopsin expression has been detected so far. The study further explores the modulation of respiration rate mediated by xanthorhodopsin and compares it to similar phenomena in photosynthetic bacteria and an archaerhodopsin-containing archaeal species. Although the latter contains carotenoid bacterioruberin, it lacks the antenna function.(Oren 2013)

Salt stress can cause oxidative damage to lipids, proteins, and nucleic acids, affecting cell integrity. Detoxification is achieved through gene expression modulation using non-enzymatic antioxidant systems and enzymatic systems. These secondary metabolites from marine sources are exploited for various applications, including antioxidant, anticancer, antiviral, anti-inflammatory, antiarrhythmic, antiobese, antidiabetic, antiangiogenic, and antimalarial activities. Carotenoids, a class of bioactive metabolite produced by microorganisms, are potential antioxidants, anticancer, and anti-inflammatory agents. The ability of halophilic bacteria to grow at high salt concentrations opens up opportunities for discovering novel metabolites. In this study, seven halophilic bacteria from the salterns and mangrove forest of South India were investigated for the effect of salt stress on bacterial growth, production of pigmented secondary metabolites, and antioxidant activity(Subramanian and Gurunathan 2020)

The promising properties of carotenoids have led to increasing interest in discovering new natural carotenoids with practical health benefits, particularly in preventing human diseases. Carotenoids from terrestrial sources, such as beta-carotene and lycopene, have been studied as potential cancer preventive agents.(Rowles and Erdman 2020)

Carotenoids can be easily extracted from cells as NaCl concentrations below 15% cause cell lysis. These halophilic microorganisms offer a significant commercial alternative for carotenoid production from biological sources (Saini et al. 2022)

Carotenoids serve several important functions, including acting as food colorants, light energy absorbers, oxygen transporters, provitamin A sources, scavengers of active oxygen, antitumor agents, and enhancers of in vitro antibody production. some carotenoids can be converted into retinoids exhibiting vitamin A activity. They are also attracting interest in nutricosmetics as they have shown cosmetic benefits. (Meléndez-Martínez et al. 2022)

Industrial textile wastewater poses challenges due to unknown volumes and variations of many different colors, as well as low BOD/COD ratios that reduce biological decolorization efficacy. Around 10,000 dyes and pigments are used globally in the textile industry, with azodyes making up over half. These dyes have long shelf lives and are often non-biodegradable, stable against light, heat, and oxidizing chemicals, and compatible with other materials.(De Souza, Bonilla, and De Souza 2010)

Synthetic dyes, after their invention in the 19th century, became very popular in the textile industry due to their vibrant colors, affordability, and ease of use. The discovery of mauveine by William Henry Perkin in 1856 was a pivotal moment in the history of synthetic dyes, leading to their widespread adoption and replacing natural dyes. However, the use of synthetic dyes has significant environmental and health impacts. The effluent from dye industries often contaminates water sources, causing pollution and posing health risks to people. Synthetic dyes, derived from petrochemical compounds, are dominating the textile market due to their wide range of color pigments and consistent coloration. However, textile industries consume large amounts of water, making it difficult to treat hazardous wastewater. (Slama et al. 2021)

Research and development in bacterial pigment production focuses on finding low-cost and suitable growing mediums to improve the quality and efficiency of the process. Fermentation is considered a faster and more efficient method for producing pigments for industrial applications. Bacterial metabolites, or secondary metabolites, are by-products of bacterial growth and can possess antimicrobial, anticancer, antioxidative, and UV properties. These metabolites could be used as functional dyes for textile materials, offering a range of applications beyond their aesthetic qualities. Despite being insoluble in water, they form bonds with fibers. (Kramar and Kostic 2022)

Natural pigments from plants and microbes have advantages, but plant pigments face challenges like instability in light and heat, low water solubility, and limited availability. In contrast, microbial pigments are more stable, cost-effective, and adaptable, gaining interest for their rapid growth and resilience in changing conditions. Bacterial species like Flavobacterium, A. aurantiacum, and Micrococcus produce bio-pigments, with β-carotene from Blakeslea being the first approved food ingredient in 1995, meeting annual demand in food and textile sectors.(Rather et al. 2023)

CHAPTER 3: METHODOLOGY

3.1. SAMPLE COLLECTION

Salt sample from Curca saltpan was collected in a sterile container and immediately brought to the laboratory for further processing.

3.2. CULTURE GROWTH CONDITION

Sample collected was serially diluted and 0.1ml was plated on six different media including NaCl tryptone yeast extract, Zobell marine agar, extreme halophilic agar, nutrient agar, LB agar, MHM containing 50 % less concentration and 25% salt concentration, pH was adjusted to 7. Plates were incubated for 10-15 days at 30°c. The colonies were purified by streaking several times and purified cultures were stored on the slants.

3.3. OPTIMIZATION OF CULTURE CONDITION

Optimum condition for growth and carotenoid production was determined by inoculating the isolate in 50 ml Zobell marine broth (HIMEDIA) in 150 ml Erlenmeyer flask and incubating on a rotary shaker at 120 rpm for 15 days at 30°c. Growth and carotenoid production was determined spectrophotometrically at 600nm and 490nm respectively. The isolates were tested for NaCl concentration from 0% to 30%.

3.4. IDENTIFICATION OF THE HALOPHILIC ISOLATES

Various morphological (Gram staining), biochemical methods were used to identify and characterize the isolates.

3.4.1. MORPHOLOGICAL CHARACTERISATION

Gram staining of the isolate was done to identify the gram character of the bacteria.

3.4.2. BIOCHEMICAL CHARACTERISATION

a) **Screening for Amylase**

Screening of amylase producing halophilic bacteria was done by starch hydrolysis test on starch agar medium (gm/100ml): starch (1g), peptone (0.5 g), yeast extract (0.3 g), agar (2%),

NaCl (25%) according to the need of the bacteria isolated. The bacterial culture was streaked onto the plate and incubated at 30°c for 8 days. After incubation plates were flooded with grams iodine. Zone of clearance indicated positive test for amylase. (yadav, et al., 2021)

b) Lipid hydrolysis

Lipid hydrolysis of the bacteria was tested on tributyrin nutrient agar plates containing 1% of tributyrin (Himedia), 22% NaCl. Plates were incubated at 30°c for 10 days. Zone of clearance indicated positive test for lipase.

c) Proteolytic activity

Proteolytic activity of the bacteria was screened on gelatin agar plates containing 1g (g/100ml). flooding the plates with acidic mercuric chloride shows zone of clearance indicating positive result for proteolytic activity. (Rathakrishnan and Gopalan 2022)

d) IMViC

i. INDOLE PRODUCTION

SIM agar was prepared and added to the test tubes. Loopful of culture was streaked and incubated at 30°c for 48 hours. 5 Drops of kovacs reagent was added.

ii. METHYL RED TEST

Buffered peptone glucose broth was prepared and 50µl of culture was inoculated. Incubated at 30°c for 48hours. Methylred indicator was added. After 30min pinkcolor ring formation indicated positive result.

iii. Voges-Proskauer test

Tryptone broth was prepared and 50µl culture was inoculated. Incubated at 30°c for 48 hours. 5drops of kovacs reagent was added.

iv. CITRATE UTILISATION

Citrate utilisation test was performed according to). Simmons citrate agar (24.28/L), NaCl (according to the need of the bacteria). Test organism was streaked onto the slants and

incubated at 30°c for 8 days. Change in colour from green to deep blue indicates positive result. (de Figueroa et al. 2000)

e) HYDROGEN SULPHIDE TEST

SIM agar (HIMEDIA) was prepared and slants were made. Loopful of culture was streaked onto the slants and incubated at 30°c for 6 days. Black precipitate indicated positive result on addition of kovacs reagent.

f) CATALASE TEST

4-5 drops of 3% hydrogen peroxide were added on to the culture. The presence of bubbles displayed a positive test indicating the presence of enzyme catalase. If no gas is produced this is a negative reaction.

g) OXIDASE TEST

The oxidase test is used to determine if a bacterium produces cytochrome c oxidase. It uses disks impregnated with a reagent such as N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) or N, N-dimethyl-p-phenylenediamine (DMPD), which is also a redox indicator. The oxidised reagent forms the coloured compound indophenol blue. The cytochrome system is present in aerobic organism that are capable of using oxygen as the final hydrogen receptor. oxidase disc (HIMEDIA) was used to test for oxidase production. 50µL culture was added to the oxidase disc. After 3-5 min blue colour appears indicating positive result.

h) CARBOHYDRATE UTILISATION TEST:

sucrose, glucose, xylose, lactose, glucose, dextrose was made in stock and sterilised separately at 121°c for 3 min. 1% sterilised sugar was added separately to autoclaved peptone broth. 100µl culture was added to each test and incubated at 30°c for 48hours and analysed for acid production and carbohydrate utilisation.

3.4.3. CAROTENOID EXTRACTION AND ANALYSIS

Each of the isolated strain was grown in 100ml Zobell marine broth at 25% salt concentration in 250ml Erlenmeyer flask and incubated on a rotary shaker at 120 rpm for 12 days at 30°c. Culture was centrifuged at 9500 rpm for 15 min at 4°c, the supernatant was discarded and pallet was washed with distilled water. The pallet was dried and dry weight was taken. Dry

cells were suspended in absolute methanol and stored overnight at 4°c. Methanolic extract was used for uv-vis spectroscopy.

(a) **THIN LAYER CHROMATOGRAPHY:** Carotenoid samples were separated by thin layer chromatography. solvent used was chloroform: methanol: ethyl acetate (shikkandar, 2013) with ratio 9:1. Retention factor of each spot is calculated using the formula

Rf = distance travelled by solute/distance travelled by solute

(b) UV-VIS SPECTROSCOPIC ANALYSIS

Methanolic extract was used for uv-vis spectroscopic analysis at wavelength range 300-600 nm. The readings were calculated using equation as per (Reis-Mansur et al., 2019)

(A x volume (ml) x 10^4) / ($A^{1\%}$x sample weight (g))

3.4.4. ANTIOXIDANT ACTIVITY

- **DPPH ASSAY**

Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (shikkandar, 2013). The activity was performed with 3 different concentrations (50µg, 100µg, 150µg). 100µl of 0.1% methanolic DPPH was added to 2.9 ml sample. 100µl 0.1% DPPH was to 2.9 ml methanol which was taken as a control. Ascorbic acid was used as a standard. After 30 min incubation in the dark absorbance was taken at 517nm.

3.4.5 pH sensitivity of the pigment

pH of the pigment was adjusted from 3 to 10 using 1M HCl and NaOH. Colour of the pigment changes from orange to yellow.

3.4.6 DYEING OF THE FABRIC

a) FABRIC PRETREATMENT

For dyeing process, the fabric was pre-treated by scouring, this is done to increase the absorbency of the fabric. The fabric was washed with commercial detergent at 50°c for 30 minutes followed by washing with water and air drying.

b) DYEING WITH PIGMENT

For dyeing process, cotton, silk, polyester, satin was immersed in 20ml pigment extract and maintained at 80°c for 90 min with constant stirring to get equal dying of the fabric. After dying the fabric was air dried and used for further processing.

c) POST MORDANTING

Dye was fixed on to the fabric by using mordants. Mordants used were:

i. FERROUS SULPHATE AND COPPER SULPHATE:

5 grams of ferrous sulphate and copper sulphate was dissolved in 1 litre water. Fabric was soaked in the mordant for 40 min at 60°c. after mordanting wash the fabric with water to remove excess dye and air dry.

ii. BANANA PEEL EXTRACT:

10g of Banana peels were in 30ml water was maintained at 60°c for 90 min. The extract was filtered and used as a bio mordant. The dyed fabric was immersed in this solution for 30 min at 60°c and slowly the temperature was raised to 80°c for another 20 min. Fabric washed with water to remove excess mordant. Fabric was air dried.

iii. POMOGRANATE RIND EXTRACT:

25g pomegranate peels were dried in the oven at 40°c overnight. Powder was made of the dried peels and 10 grams was dissolved in 40% of 30 ml ethanol and kept at 60°c for 40min. incubated overnight at 4°c with 96% ethanol to precipitate the polyphenols. Next day the extract was filtered with Whattman filter paper. The dyed fabric was immersed in this solution at 60°c for 40min. washed with water and air dried.

3.4.7. FASTNESS TEST

- **WASH FASTNESS**

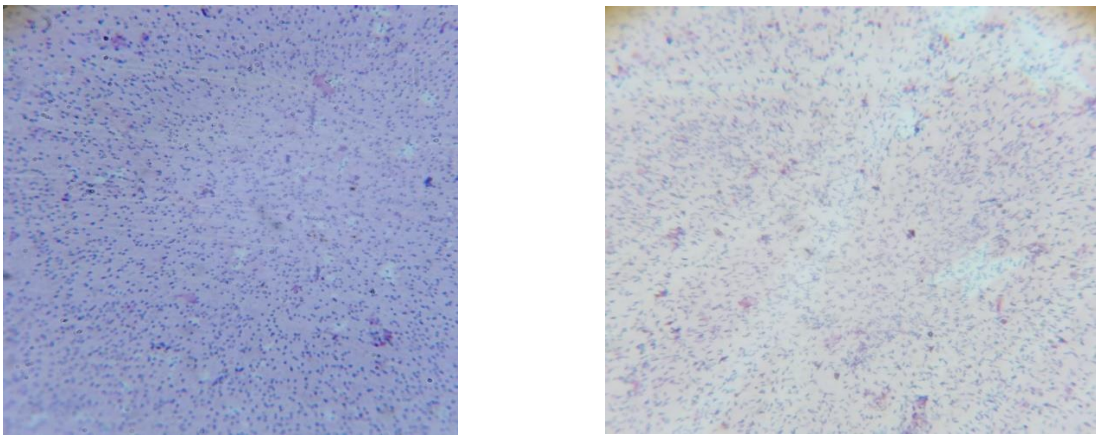
Dyed fabric was washed with cold water using non-ionic detergent. Air dried and colour of the fabric was analysed.

CHAPTER 4: ANALYSIS AND CONCLUSION

4.1. COLONY AND CELL MORPHOLOGY

The isolated colonies were circular, entire, convex. CS1 was translucent and CS2 was opaque.

CS1 isolate was found to be gram positive cocci and CS2 isolate was found to be gram positive rods.



(a) (b)

Fig.2. Gram staining. (a) CS1 isolate Gram positive cocci (b) CS2 isolate Gram positive rods

4.2.2. BIOCHEMICAL TESTS

isolates were tested for biochemical characteristics (Table 2).

a) **Catalase test:**

Both the strain was found to be catalase negative as they did not show any bubble formation on addition of H_2O_2.

b) **OXIDASE TEST:**

Both the isolates were found to be oxidase positive as the oxidase disc changed colour to blue.

c) AMYLASE TEST:

Both the isolates are negative for amylase activity.

d) GELATINASE TEST:

CS1 and CS2 isolate showed negative test for gelatinase.

e) LIPASE TEST:

Both the isolate showed positive test for lipase as there was appearance of iridescent sheen around the colonies.

f) HYDROGEN SULPHIDE TEST:

Both the strains were positive for hydrogen sulphide as black precipitate was seen.

g) IMViC TEST

1. INDOLE PRODUCTION:

Both the isolates showed negative test for indole production.

2. **Methyl red:** both the isolates showed negative test for methyl red. As there was no pink colour observed
3. **Voges Proskauer test:** both the isolates were negative for VP test as there was no colour change.

h) CARBOHYDRATE UTILIZATION

1. LACTOSE FERMENTATION:

CS1 and CS2 strain was positive for lactose fermentation. As CS2 showed gas production and acid production were as CS1 showed only acid production.

2. XYLOSE FERMENTATION:

CS1 was negative for xylose fermentation and CS2 was positive with only acid production and no gas production.

3. SUCROSE FERMENTATION:

Both the strains were positive for sucrose fermentation with acid production and gas production.

4. **GLUCOSE FERMENTATION:**

Both the isolates were positive for glucose fermentation with no gas production.

5. **DEXTROSE FERMENTATION:** Both the strains showed positive result for dextrose with acid as well as gas production

Table 1. Phenotypical characteristics of the isolates

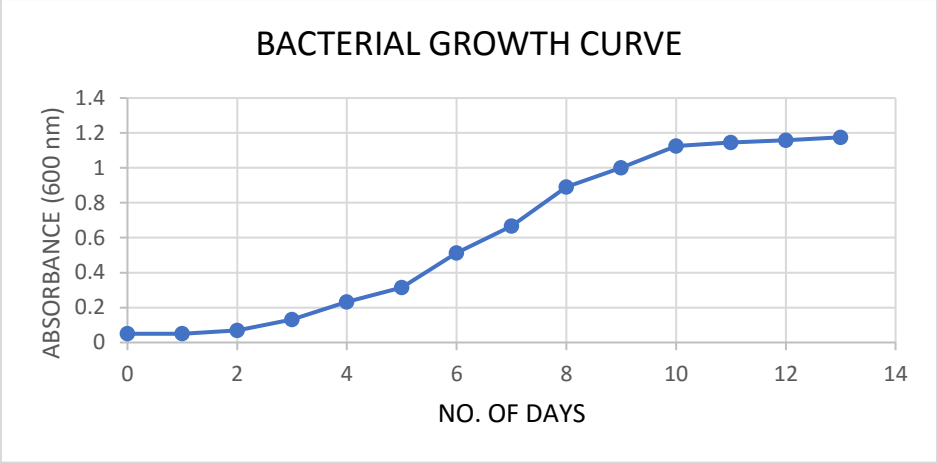
CHARACTERISTICS	**CS1**	**CS2**
COLONIAL MORPHOLOGY	Circular	Circular
Colony	Convex	Convex
Colony density	Translucent	Opaque
Pigmentation	Orange	Pink
Gram staining	+ ve	+ve
Cell shape	cocci	Rod

TABLE 2. Biochemical characteristics of the isolates

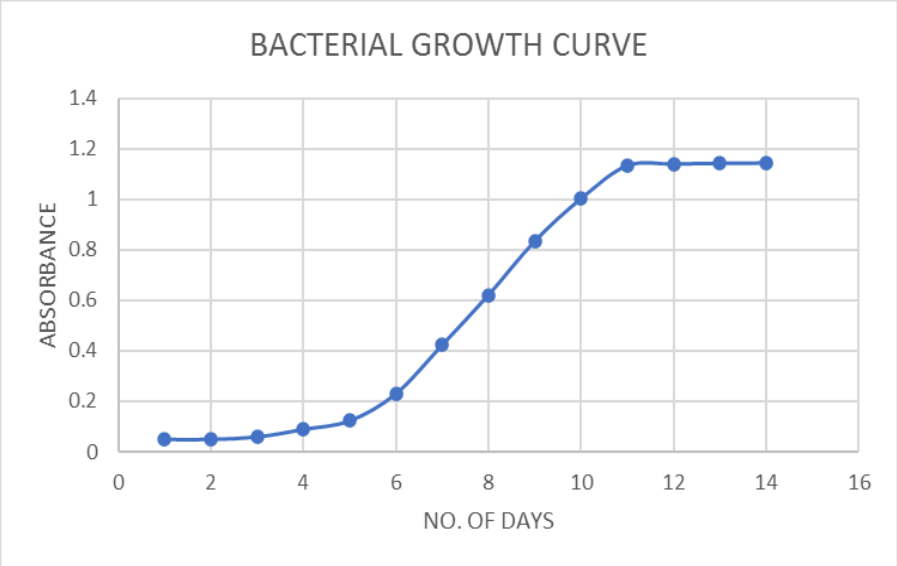
BIOCHEMICAL TEST	**CS1**	**CS2**
CATALASE	- ve	- ve

OXIDASE	+ve	+ve
AMYLASE	-ve	-ve
GELATINASE	-ve	-ve
LIPASE	+ve	+ve
HYDROGEN SULPHIDE	+ve	+ve
INDOLE PRODUCTION	-ve	-ve
METHYL RED TEST	-ve	-ve
VOGES PROSKAUER TEST	-ve	-ve
CITRATE UTILISATION	-ve	-ve
LACTOSE	+ve	+ve
XYLOSE	+ve	-ve
SUCROSE	+ve	+ve
GLUCOSE	+ve	+ve
DEXTROSE	+ve	+ve

4.2. GROWTH OPTIMISATION

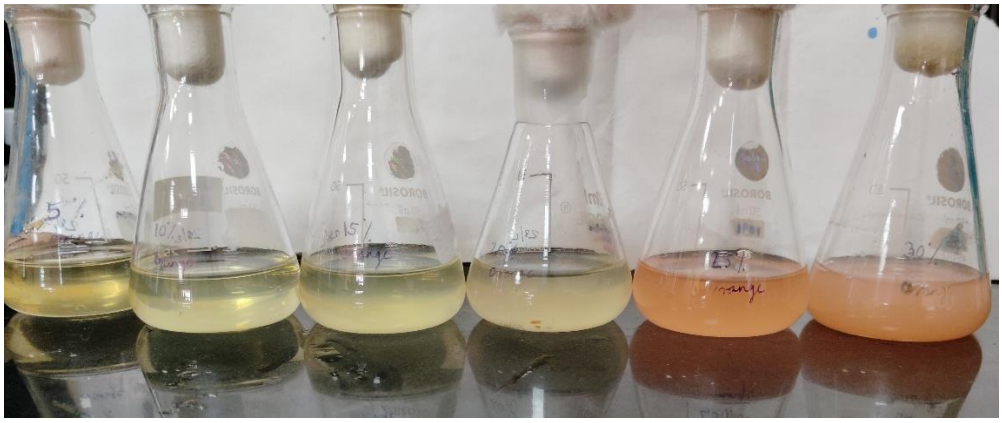


(a)



(b)

fig. 3 Growth curve of CS1 and CS2 isolate



(a)

(b)

Fig.4. Growth optimization at different salt concentration from 0%-30%. (a) growth and pigment production at 25% and 30% salt concentration by CS1 isolate. (b) growth and pigment production at 20%, 25%, 30% salt concentration by CS2 isolate.

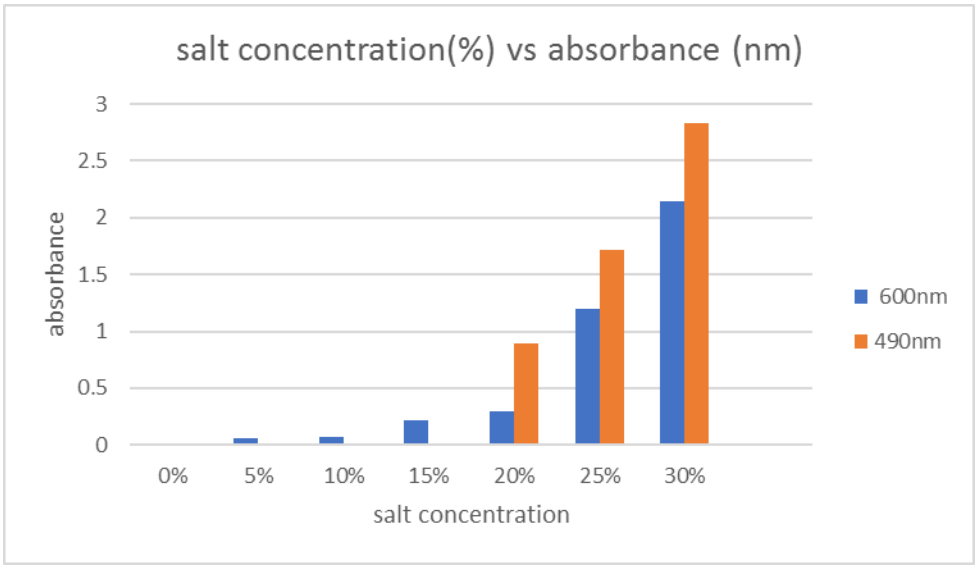


Fig. 4.1 cell density and pigmentation of CS1isolate at different salt concentration

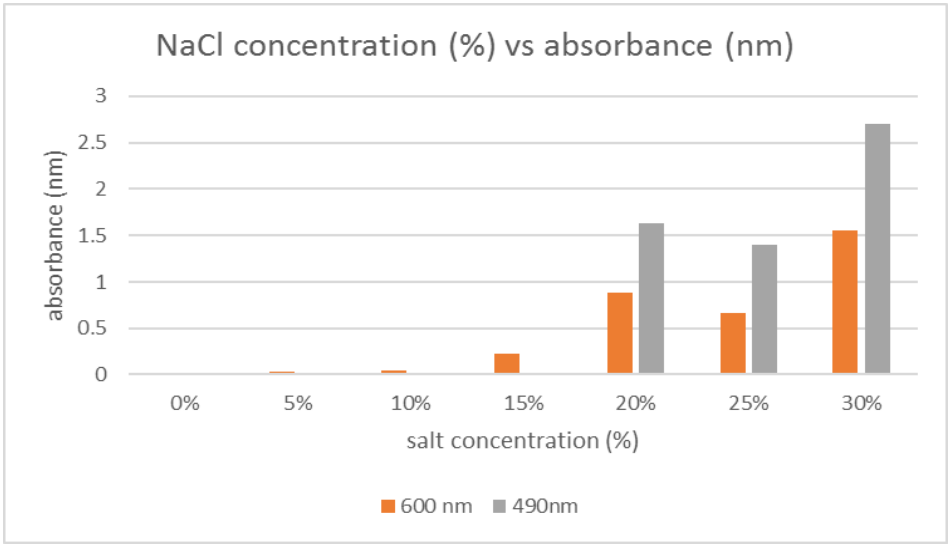


Fig.4.2 cell density and pigmentation of CS2 isolate at different salt concentration.

Table 3. Growth and biomass production at different salt concentration

ISOLATES	SALT CONCENTRATION	DRY WEIGHT (g/l) OF THE BIOMASS
CS1	25%	1.24g/l
	30%	2.4g/l
CS2	20%	1.75g/l
	25%	2.89g/l
	30%	3g/l

Table 4. Degree of pigmentation of pigments produced by CS1 and CS2 isolate

ISOLATE	GROWTH (OD 600)	PIGMENT (OD 490)	DEGREE OF PIGMENTATION ($OD_{490/600}$)
CS1	2.148	2.834	1.31
CS2	1.553	2.699	1.14

During summer in Goa the temperature increases to above 30°c, as a result the salt concentration increases in the saltpan making favourable conditions for the pigmented

halophilic bacteria to grow. Carotenoid producing bacteria were isolated from this environment. CS1 colony was orange pigmented and CS2 was pink pigmented. (fig.1,2).

The optimum time required by the isolates were 12-15days. Required around 20-25% salinity, temperature 30°c, pH 7. Growth condition was optimized at different salt concentrations, isolates showed highest growth at 30 % salinity which indicates that the isolated bacteria are extremely halophilic in nature. Growth did not occur below 15% salinity. It was observed that CS1 isolate had the highest value of the degree of pigmentation, indicating that it exhibited the highest pigment producing ability. (Asker, et al., 1999)

4.3. PIGMENT EXTRACTION AND CHARACTERIZATION

Fig 5. Extraction of pigments in methanol of CS1 and CS2 isolate

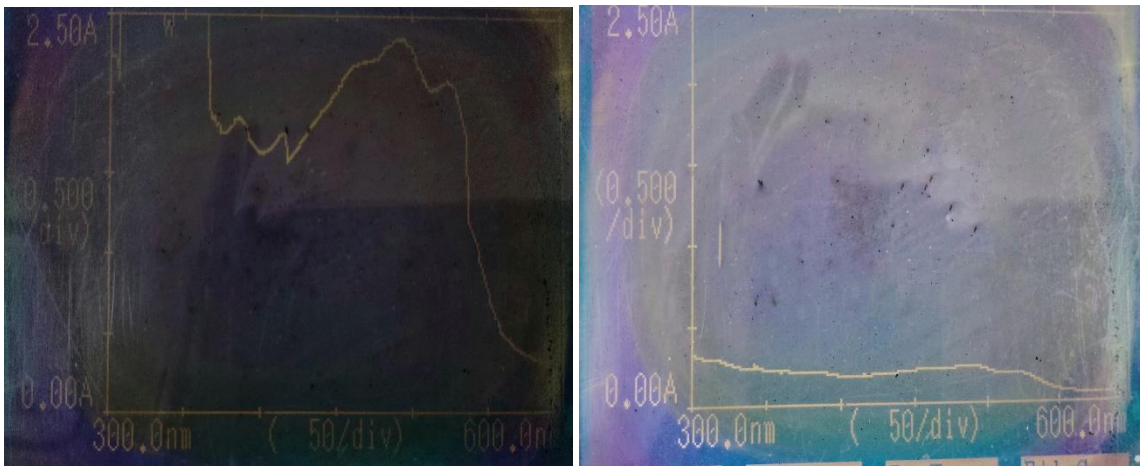
Cultures were grown at 25% salt concentration, centrifuged and pigments were extracted in absolute methanol and stored in dark. Gram positive bacteria have thick peptidoglycan layer. Methanol is a polar solvent which have the ability to penetrate into the cell wall, disrupt the membrane and solubilizes carotenoids. After extraction the mixture is centrifuged and stored at -20°c in the dark. Methanolic extract is used for quantification using uv -vis spectroscopy and separation is done using TLC.

4.3.1. THIN LAYER CHROMATOGRAPHY

Fig 6. Thin layer chromatography: separation in

Chloroform: methanol: ethylacetate

4.3.2 UV-VIS SPECTROSCOPY



(a) (b)

Fig 7. Absorption spectra of (a) Maximum peak at 490 nm pigment extracted from CS1 isolate (b) maximum peak at 488 nm of pigment extracted from CS2 isolate

The UV-VIS spectroscopy analysis was done in the range of 400-600nm (fig 7). The spectra are characterised by maximum peak at 495 and 488 nm. Pigment was separated in chloroform: methanol: ethyl acetate (10:10:10). The Rf value of pigment of CS1 strain is 0.76 and CS2 isolate is 0.69.

4.4. ANTIOXIDANT ACTIVITY

- **DPPH ASSAY**

carotenoids are known to have strong antioxidant property. Free radical scavenging property of the carotenoid was analysed by the DPPH assay. Antioxidant activity of carotenoid extracted from CS1 strain and CS2 strain was performed with three different concentration 50µg, 100µg, 150µg using DPPH assay.

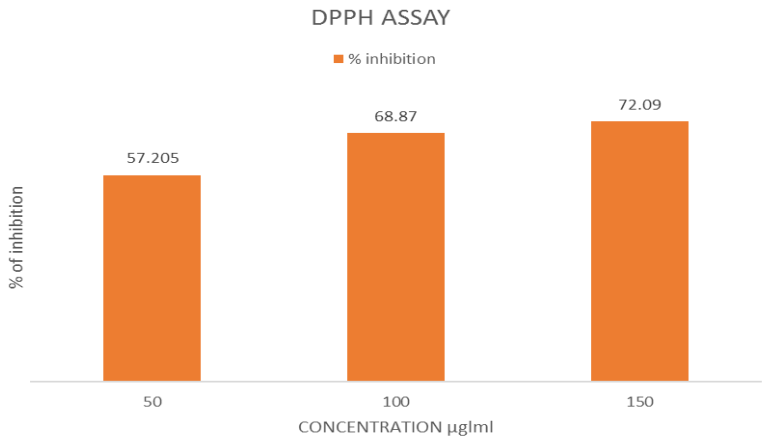


Fig.8.1 DPPH assay of pigment extracted from CS1 isolate

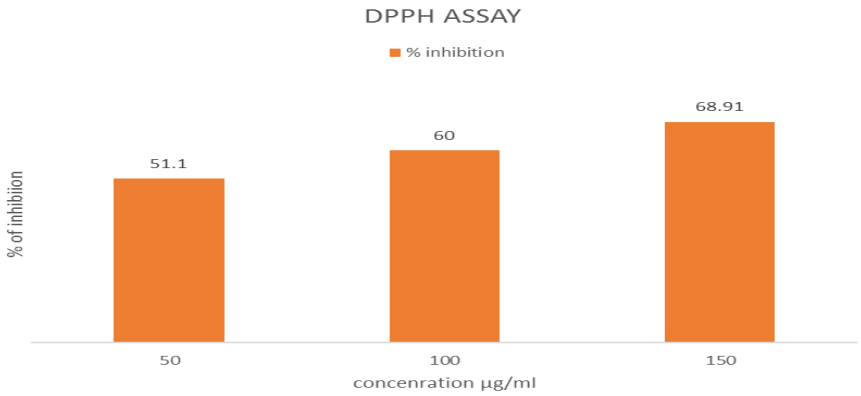
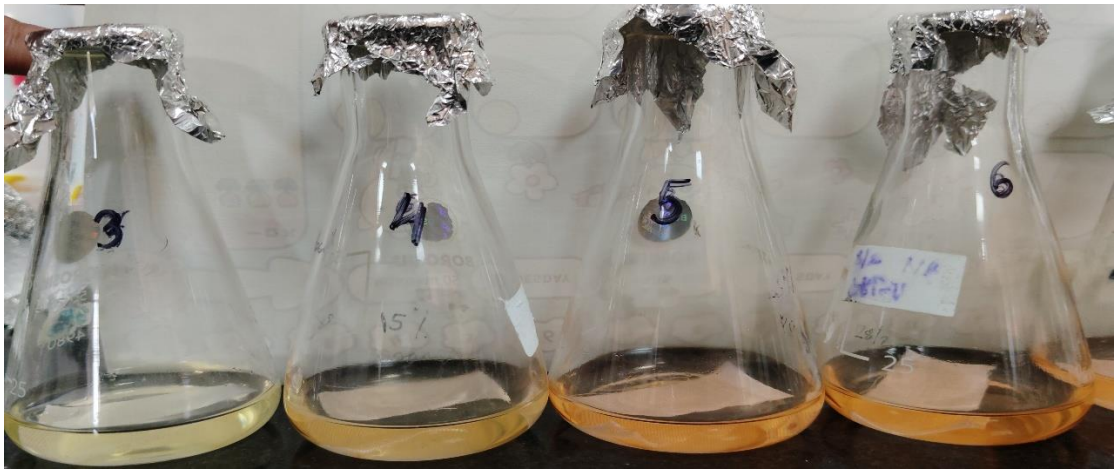


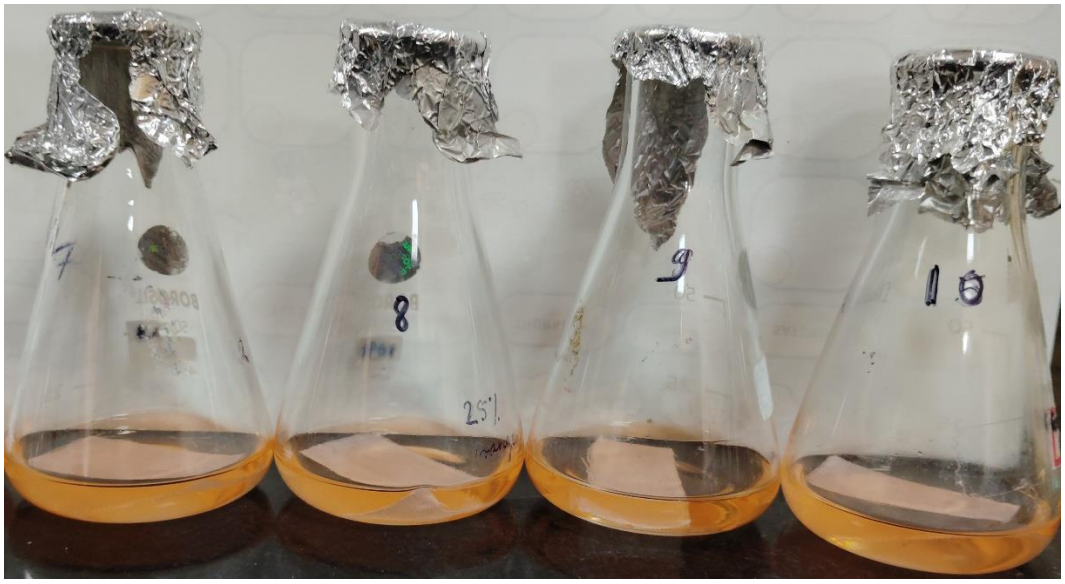
Fig 8.2 DPPH assay of pigment extracted from CS2 isolate

Percentage inhibition of CS1 strain increased with increase in concentration as 57%. 68% and 72%. The carotenoid extracted from CS2 strain showed considerable activity 56%, 61% and 68%. The results revealed strong ant oxidant property of the carotenoids according to (Sikkandar, 2013).

4.5. pH SENSITIVITY OF THE PIGMENT



(a)



(b)

Fig. 9 pH sensitivity of the pigment: pH of the pigment was adjusted from 3-10. (a) Change in colour from orange to yellow indicates that the pigment is sensitive to acidic pH. (b) As the pH increases pigment becomes darker.

4.6 DYEING OF THE FABRIC

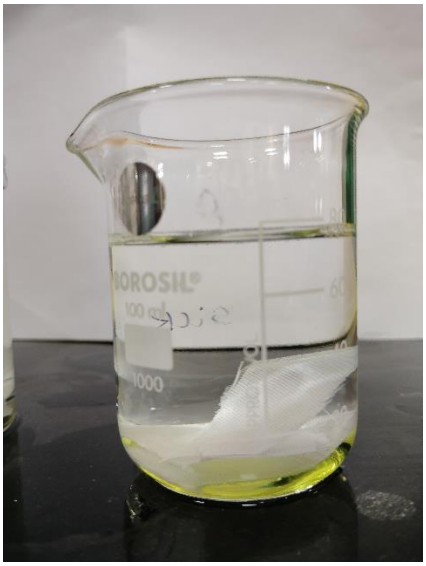


Fig 10.1 scouring: fabric is bleached by soaking in the detergent and $NaHCO_3$ to remove impurities.

Fig. 10.2 Fabric dipped in pigment extract at 80°c

4.7 BIO-MORDANTING:

4.7.1 Banana peel extract and pomegranate peel extract:

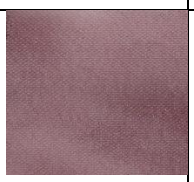
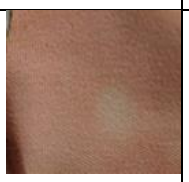
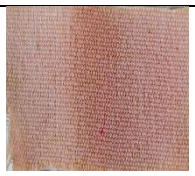
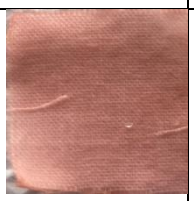
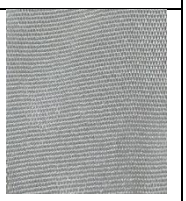
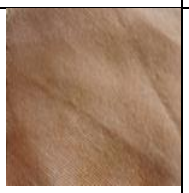
Fig 11.1 banana peel extract used as a biomordant.

(a) **(b)**

Fig 11.2 (a) powder of pomegranate peels. (b) extraction in ethanol.

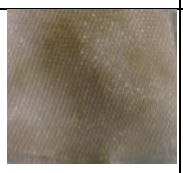
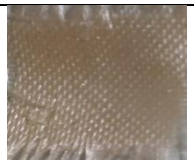
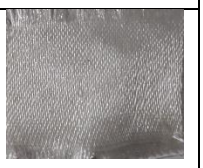
Banana peel extract and pomegranate peel extract contains tannin that forms bond with the fabric and pigment. They are biodegradable and have antioxidant property which makes them ideal mordant in textile dyeing.

Table 5. Fabric dyed without mordants and with banana peels extract, pomegranate rind extract as bio mordants and $FeSO_4$, $CuSO_4$ as metal mordants.

Fabric	Without mordant	Banana peel extract	Pomegranate rind	$FeSO_4$	$CuSO_4$
polyester					
Cotton					
silk					
satin					

4.8 WASH FASTNESS TEST

Table 6. wash fastness test

fabric	Without mordant	Banana peel extract	Pomegranate rind	$FeSO_4$	$CuSO_4$
polyester					
cotton					
Silk					
satin					

Pigment was used in dyeing of cotton, polyester, silk, satin (table 5) using banana peel extract, pomegranate peel extract as biomordants and FeSO4 and $CuSO_4$ as a metal mordant. Tannin present in banana peels and pomegranate rind extract acts as a fixative by fixing dye to the fabric. Among the mordants used banana peel extract showed the best dyeing of polyester after washing.

- **CONCLUSION**

The textiles are one amongst in the rapidly growing industries worldwide. Synthetic dyes utilised are hazardous to the environment and often difficult to treat and dispose. Based on the study an attempt was carried out to isolate pigment producing halophilic bacteria from saltpan. The pigment was dyed on cotton, silk, polyester, satin. Banana peel extract and pomegranate peel extract was used as a biomordant and ferrous sulphate and copper sulphate as metal mordants. Polyester showed good dyeing and wash fastness with banana peel extract.

- **FUTURE PROSPECTS**

1. More characterisation of the pigment is required like HPLC, FTIR, XRD.
2. Fastness property of the dye needs to be improved.

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