CAROTENOIDS FROM HALOPHILIC MICROORGANISMS AS FEED ADDITIVE FOR ENHANCING PIGMENTATION IN ORNAMENTAL FISH

A Dissertation for

Course code and Course Title: MID- Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

Microbiology

by

MS. POOJA VINAY PINGULKAR

Roll number: 22P0420012

ABC Id: 565842233215

PR number: 201906012

Under the supervision of

DR. BHAKTI SALGAONKAR

School of Biological Sciences and Biotechnology

M.Sc. Microbiology

Goa University

Date: **April 2024**

Microbiology Programme
School of Biological Sciences & Biotechnology
Goa University, Science Block E,
Taleigao Plateau, Goa - 403206

Seal of the school

Examined by:

COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Carotenoids from Halophilic microorganisms as feed additive for enhancing pigmentation in ornamental fish**" is a bonafide work carried out by Ms. Pooja Vinay Pingulkar under my supervision in partial fulfilment of the requirements for the award of the degree of **Master in Science in the Discipline Microbiology** at the School of Biological Sciences and Biotechnology, Goa University.

Date: 8/4/24

Dr. Bhakti B Salgaonkar
Assistant Professor
Microbiology programme

Prof. Bernard Rodrigues
Dean, School of Biological Sciences and Biotechnology

Date: 8/4/24

Place: Goa University

Dean of School of Biological Sciences
& Biotechnology
Goa University, Goa-403206

School stamp

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Carotenoids from Halophilic microorganisms as feed additive for enhancing pigmentation in ornamental fish**" is based on the results of investigation carried out by me in the M.Sc. Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Bhakti Salgaonkar 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.

I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Ms. Pooja Vinay Pingulkar
22P0420012
Master of Science in Microbiology
School of Biological Sciences and Biotechnology

Date: 28/04/2024
Place: Goa University

CAROTENOIDS FROM HALOPHILIC MICROORGANISMS AS FEED ADDITIVE FOR ENHANCING PIGMENTATION IN ORNAMENTAL FISH

A Dissertation for

Course code and Course Title: MID- Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

Microbiology

by

MS. POOJA VINAY PINGULKAR

Roll number: 22P0420012

ABC Id: 565842233215

PR number: 201906012

Under the supervision of

DR. BHAKTI SALGAONKAR

School of Biological Sciences and Biotechnology

M.Sc. Microbiology

Goa University

Date: **April 2024**

Examined by: Seal of the school

CAROTENOIDS FROM HALOPHILIC MICROORGANISMS AS FEED ADDITIVE FOR ENHANCING PIGMENTATION IN ORNAMENTAL FISH

A Dissertation for

Course code and Course Title: MID- Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

Microbiology

by

MS. POOJA VINAY PINGULKAR

Roll number: 22P0420012

Under the supervision of

DR. BHAKTI SALGAONKAR

School of Biological Sciences and Biotechnology

M.Sc. Microbiology

Goa University

Date: **April 2024**

Examined by: Seal of the school

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Carotenoids from Halophilic microorganisms as feed additive for enhancing pigmentation in ornamental fish**''is based on the results of investigation carried out by me in the M.Sc. Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Bhakti Salgaonkar 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.

I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Ms. Pooja Vinay Pingulkar
22P0420012
Master of Science in Microbiology
School of Biological Sciences and Biotechnology

Date:
Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Carotenoids from Halophilic microorganisms as feed additive for enhancing pigmentation in ornamental fish**" is a bonafide work carried out by Ms. Pooja Vinay Pingulkar under my supervision in partial fulfilment of the requirements for the award of the degree of **Master in Science in the Discipline Microbiology** at the School of Biological Sciences and Biotechnology, Goa University.

Date:

Dr. Bhakti B Salgaonkar
Assistant Professor
Microbiology programme

Prof. Bernard Rodrigues

Dean, School of Biological Sciences and Biotechnology

Date:

Place: Goa University

School stamp

Contents

Chapter		Title	Page No.
		Preface	i
		Acknowledgments	ii
		Tables and Figures	iv
		Abbreviations used	vii
		Abstract	ix
1.		Introduction	1-4
	1.1	Background	1-3
	1.2	Aim and Objectives	3
	1.3	Hypotheses / Research question	3
	1.4	Scope	4
2.		Literature Review	5-17
3.		Methodology	18-29
4.		Analysis and Conclusions	30-56
		References	57-65
		Appendix I: Media Used	66-67
		Appendix II: Stains and Reagents	68-69
		Appendix III: Workshop on Ornamental fish and Poster competition on waste management	70

Table of Contents

Chapter I: Introduction

1.1 Background....1
1.2 Aim and Objectives....3
1.3 Hypothesis / Research Questions....3
1.4 Scope....4

Chapter II: Literature Review

2.1 Ornamental Fish....5
2.1.1 Problems faced by Ornamental fish Industries....6
2.1.2 Aquarium fish feed in Market....6
2.2 Microbial carotenoids....8
2.2.1 Carotenoids....8
2.2.2 Halophilic Microorganisams producing Carotenoids....8
i. Salinibacter Ruber....9
ii. Halophilic Archaea belonging to family halobacteriaceae....10
iii. Dunaliella salina....13
2.2.3 Use of Carotenoids in Aquaculture....13
2.2.4 Effects of Microbial Carotenoids on fish health....16

Chapter III: Methodology

3.1 Sampling site and Sample collection....18
3.2 Isolation and purification of halophilic isolation....18
3.2.1 Media Preparation for isolation of halophile....18
3.2.2 Spread plate method....19
3.2.3 Isolation and purification of Halophilic microorganisms....19
3.3 Starter culture preparation....19
3.4 Polyphasic identification of isolates....20
3.4.1 Morphological Characteristics....20
(i) Colony characteristics....20
(ii) Cell characteristics: Phase contrast microscopy....20
3.4.2 Chemotaxonomic Characterization....20

(i) Antibiotic sensitivity……...20
(ii) Pigment Analysis.....21
(iii) Polar lipid Analysis.....21
(iv) Biochemical analysis
i. Carbohydrate utilization studies.....22
ii. Catalase test.....22
iii. Oxidase test.....23
(v) Screening for different hydrolytic enzymes.....23
i. Screening for Amylase activity.....23
ii. Screening for Protease activity.....23
iii. Screening for Gelatinase activity.....24
iv. Screening for Cellulase activity.....24
v. Screening for Xylanase activity.....24
vi. Screening for Lipase activity.....25
vii. Screening for Esterase activity.....25
viii. Screening for chitinase activity.....25
3.5 Parameter optimization for Carotinoids production.....25
3.5.1 NaCl concentration.....25
3.5.2 Substrate concentration…….....26
3.5.3 pH Range.....26
3.6 studies on the effects of halophilic culture on gold fish Experimental design.....26
3.6.1 Experimental Design.....26
3.6.2 Diets …….27
3.7 Proximate analysis.....28
3.7.1 Moisture Content.....28
3.7.2 Ash content.....28
3.7.3 Lipid content.....28
3.8 Analytical Methods.....29

Chapter IV: Analysis and conclusion

4.1 Sampling site and Sample collection.....30
4.2 Isolation and purification of halophilic isolation.....31
4.3 Polyphasic identification of isolates.....33
4.3.1 Morphological Characterization……..33

(i) Colony characterization....33
(ii) Cell characteristics....34
4.3.2 Chemotaxonomic Characterization....34
(i) Antibiotic sensitivity....34
(ii) Polar lipid Analysis....35
(iii) Biochemical analysis....37
(a) Carbohydrate utilization studies....37
(b) Catalase test....39
(c) Oxidase test....39
(iv) Screening for various hydrolytic enzymes....39
4.4 Optimization of physiochemical parameters....41
4.4.1 NaCl Concentartion....41
4.4.2 Substrate concentration.... 43
4.4.3 pH range....45
4.5 Studies on effects of halophilic cultures on gold fish....47
4.5.1 Aquarium setup....47
4.5.2 Experimental fish....48
4.5.3 Formulation of fish feed and Proximate analysis....50
4.5.4 Growth performance of fish fed experimental diets....52
4.5.5 Carotenoid content analysis....52
References
Appendix:
I) composition of media
II) Stains and reagents
III) Workshop on Ornamental fish and Poster competition on waste management

Preface

Fish used for ornamentation are known as living gems because of their array of vivid colors, shapes, and sizes. They are housed in aquarium systems, such as tanks, for both aesthetic and recreational purposes. Globally, keeping ornamental fish is a popular pastime. The issue here is that, in an artificial environment, their colour fades. The coloration is caused by carotenoids, which fish cannot synthesize, meaning it is de novo. Additionally, there is no decorative fish feed company in India. Furthermore, research indicates that up until now, feed additives containing carotenoids derived from halophilic microorganisms have only been utilized in combination with synthetic carotenoids and carotenoids derived from bacteria, algae, and fungi like yeast.

The sustainability, affordability, and efficacy of traditional sources of carotenoids, such as synthetic additions or those derived from plants and algae, are limited. Because they can survive in salty conditions, halophilic bacteria have become a viable substitute source of carotenoids. Due to their adaptation to harsh environments, these bacteria have special metabolic routes for producing carotenoids, which frequently result in pigments with exceptional intensity and purity. Moreover, their cultivation can be adapted to make use of seawater and non-arable land, which lessens the environmental impact of conventional carotenoid manufacturing techniques.

This introduction lays the groundwork for investigating the possibilities of halophilic bacteria' carotenoids as feed supplements for decorative fish. It illustrates the unique benefits provided by halophilic bacteria and highlights the need for sustainable substitutes for traditional carotenoids sources. This study is to assess the viability, effectiveness, and possible advantages of adding carotenoids from halophilic bacteria to ornamental fish diets by in-depth investigation and analysis. By doing this, it hopes to improve ornamental fish colours, lessen disease susceptibility, and promote sustainable aquaculture methods.

Acknowledgement

It is my pleasure to convey my gratitude to all those who supported and helped me in many ways during the completion of this dissertation.

I am grateful indebted to my guide **Dr. Bhakti Salgaonkar,** Assistant professor, microbiology programme, school of biological sciences and biotechnology, Goa University for her guidance, valuable suggestions, constructive criticism, patience, help and constant encouragement. Her scientific experience as well as effective writing skills and suggestions have influenced me throughout the successful completion of this research investigation.

I am thankful to **Dr. Lakshangy Charya**, programme director of microbiology programme, for providing the infrastructure.

I am thankful to **Prof. Sandeep Garg, Dr. Milind Naik, Dr. Trupti Asolkar, Dr. Judith Noronha** and **Dr. Lata Gawade**.

With the grate sense of gratitude, I would like to thank Research scholars **Mr. Tejas Naik** and **Ms. Gandisha Pawar** for their constant motivation and help throughout my dissertation period. I would also like to thank my friends **Ms. Tanaya Gawade** and **Mr. Sainil Naik** for their help.

My special thanks to **Mr. domingos Dias, Mr. Surendra Velip, Mr. Bhagwant Karpe, Mrs. Robertina Fernandes, Sneha Naik, Vandana Virnodkar, Lakshman Kerkar** and the non-teaching staff, microbiology programme for their assistance and timely support during the course of this study.

I thank **Assistant prof. Gandhita Kundaikar** from zoology department for her help.

I also thank Research scholar **Mr. Pritesh Khobrekar** from School of Chemical Sciences for his help with muffle furnace.

Lastly, I thank and dedicate my work to my **parents** who constantly encouraged and motivated me throughout my dissertation period, it wouldn't have been possible without their love and support.

Ms. Pooja Vinay Pingulkar

22P0420012

List of tables

Table	Title	Page no
Chapter II		
2.1.2	Fish feed manufacturing companies in India	**7**
2.2.3.a	Reports on synthetic carotenoids used in Ornamental fish feed	**15**
2.2.3.b	Reports on carotenoids from microbial source used in ornamental fish feed	**15**
2.2.3.c	Reports on carotenoids from microbial source in mom-ornamental fish feed	**16**
Chapter III		
3.6.2	Microbiology laboratory fish feed preparation	**27**
Chapter IV		
4.2	CFU/mL of colonies obtained on media plates	**31**
4.3.1	Colony morphology of isolates GUPPI AND GUPPII	**33**
4.3.2.a	Carbohydrate utilization study of isolate GUPPI and GUPPII	**38**
4.3.2.b	Hydrolytic enzyme activity shown by GUPPI and GUPPII isolate	**40**
4.5.3	Proximate composition of the Microbiology laboratory formulated feed	**51**
4.5.4	Growth performance indices and survival	**52**
4.5.5	Carotenoid content in scales of juvenile gold fish fed experimental diets	**53**

List of figures

Figure	Title	Page no
Chapter IV		
4.1	Sampling site and Sample collection	**30**
4.2	Growth of colonies after 5, 15 and 45 days of incubation on NT, NTYE, EHM, MHM plates inoculated with brine and sediment samples	**32**
4.3.1.a	Single colony picture of isolate (a) GUPPI and (b) GUPPII	**33**
4.3.1.b	Gram staining of isolates (a) GUPPI and (b) GUPPII observed under 100x	**34**
4.3.2.a	Antibiotic sensitivity of Isolate GUPPI (a, b)and isolate GUPPII (c, d), where (a and c) are controls and (b and d) are with Penicliine G	**34**
4.3.2.b	Glass vials containing polar lipid extract from GUPPI (a) and GUPPII (b) isolate	**35**
4.3.2.c	Thin layer chromatography of polar lipids of GUPPI and GUPPII isolate, left panel is of Glycolipids and right panel is of Phospholipids	**36**
4.3.2.d	Carbohydrate utilization test of isolate GUPPI (a-b) and GUPPII (c-d)	**37**
4.3.2.e	Screening of various hydrolytic enzymes for isolate (a) GUPPI and (b) GUPPII	**40**
4.4.1.a	Growth of GUPPII isolate in different NaCl concentrations	**42**
4.4.1.b	Carotenoid production by GUPPII isolate at different NaCl concentrations	**42**
4.4.2.a	Growth of GUPPI isolate in different glucose concentrations	**43**
4.4.2.b	Carotenoid production by GUPPI isolate at different glucose concentration	**43**
4.4.2.c	Growth of GUPPII isolate in different glucose concentrations	**44**
4.4.2.d	Carotenoid production by GUPPII isolate at different glucose concentration	**44**
4.4.3.a	Growth of GUPPII isolate in different pH range	**45**
4.4.3.b	Carotenoid production by GUPPII isolate in different pH range	**45**
4.4.3.c	Growth of GUPPI isolate in different pH range	**46**
4.4.3.d	Carotenoid production by GUPPI isolate in different pH range	**46**
4.5.1	Rectangular glass tanks used for the experiment	**47**
4.5.2.a	Experimental fish collection from Fisheries department of Central Coastal Agricultural Research Institute (ICAR), Ella, Goa.	**48**

4.5.2.b	Experimental fishes kept for acclimation	**49**
4.5.2.c	Labelling of tanks as Control, Commercial and Test	**49**
4.5.3.a	(a) Commercial fish feed for ornamental fish and the Microbiology laboratory formulated fish feed (b) control without culture and (c) with culture.	**50**
4.5.3.b	Proximate analysis of the Microbiology laboratory formulated fish feed without culture (Control) and with culture (Test).	**51**
4.5.5.a	Size and colour differences in juvenile gold fish fed experimental diets	**53**
4.5.5.b	Carotenoid content in scales of fish fed experimental diets	**54**

List of Abbreviations and Symbols

µl	Microliter
µg	Microgram
mL	Millilitre
mg	Milligram
g	Grams
%	Percentage
nm	Nanometer
°C	Degree Celsius
W/V	Weight / volume
g/L	Grams / litre
v/v	Volume / Volume
LC/MS	Liquid chromatography Mass spectrometry
Hrs	Hours
NTYE	NaCl Tryptone Yeast Extract
NT	NaCl Trisodium Citrate
RF	Retardation Factor
IU/m^{-1}	International Units per mL
KI	Potassium Iodide
CMC	Carboxymethyl cellulose
NH	Norberg-Hofstein
RT	Room Temperature
EHM	Extremely Halophilic Media
MHM	Moderately Halophilic media

pH	Potential of hydrogen
rpm	Revolutions per minute
min	Minutes
Sec	Seconds
α	Alpha
TLC	Thin Layer Chromatography
Ppm	Parts per million
WGP	Weight gain percentage
FCR	Feed conversion ratio

Abstract

Ornamental fishes, are referred as living jewels due to their different brilliant colours, shapes and sizes. They are kept in tank or other aquarium systems for their beauty as well as entertainment. Ornamental fish keeping is a hobby worldwide. There is an increase in the demand for purchasing attractive fish species for decorative purposes, their different characteristics and citing their alluring features and in recent decades, the global trade of ornamental fishes has rapidly increased. But the problem is that in non-natural habitat the fishes get faded and tend to lose their pigmentation. Pigment enhancement in this ornamental fish is due to the carotenoid supplement in fish feed and which is the major supplements that gives beautiful orange pigment. Fishes does not synthesis carotenoids on their own i.e *de novo*, they obtain carotenoid from the natural sources. Till now synthetic carotenoids and carotenoids from bacteria, fungi, algae and yeasts has been used and no report is available on use of carotenoids from Halophilic microorganisms. Pigments produced by these microorganisms include Salinixanthin produced by *Salinibacter ruber*, Bacterioruberin produced by Haloarchaea, β-carotene produced by *Dunaliella salina* etc. have beneficial effects such as enhanced pigmentation, enhanced disease resistance of ornamental fish if supplemented in their diet. Formulating fish feed with carotenoids derived from halophilic microorganisms could be a powerful way to improve the colour and general health of farmed fish. Carotenoids from halophilic microorganisms like β-carotene, astaxanthin, batericruberin can efficiently stimulate the natural hues that wild fish eat. In study fish feed was formulated and control and test were kept which were compared with commercial feed, Test contained culture GUPPII and in Control without culture. Proximate analysis of feed was done i.e moisture content, ash content and lipid content. Study done on parameter optimization for efficient carotenoid production by the two extremely Halophilic isolates revealed that the enzyme was active at NaCl concentrations (0-30%), glucose concentration (0-2%), pH range (5-9) with its maximum

activity at 15% NaCl, 2% glucose and pH 7. Morphological analysis, polar lipid analysis, carbohydrate utilization test and antibiotic sensitivity was carried out in order to determine similarities and differences between Bacteria and Archaea. Results revealed that the two isolates obtained from saltpans of shiroda, Maharashtra belonged to domain archaea. Thus, both isolates GUPPI and GUPPII were identified as “Haloarchaea”. Experiment was done on 18 Juvenile common gold fish (*Carassius auratus).* 3 tanks were maintained as Control, Commercial and Test. Average weight of fish was noted. Mortality was monitored and Carotenoid content in their skin and scales was recorded. It was found that the Test contains maximum carotenoid content then compared to Control and Commercial.

Keywords: Literature review, parameter optimization for carotenoid production, microbiology laboratory fish feed formulation, proximate analysis, carotenoid content analysis

Chapter I

Introduction

1. Introduction

1.1 Background

Ornamental fishes, are referred as living jewels due to their different brilliant colours, shapes and sizes. They are kept in tank or other aquarium systems for their beauty as well as entertainment. Ornamental fish keeping is a hobby worldwide. There is an increase in the demand for purchasing attractive fish species for decorative purposes, their different characteristics and citing their alluring features and in recent decades, the global trade of ornamental fishes has rapidly increased (Hoseinifar et al.2023). Approximately 90% of India's exports of freshwater ornamental fish are native species that are harvested in the wild (Silas et al. 2011). One of the 34 "hotspot" regions for diversity in globe is the West ghats of India. Out of the 299 freshwater fish species found in the West ghats, 154 are regarded as ornamental fish, with 116 of these species being native to the area. In 1991, ornamental fish export from India brought in approximately US$ 0.24 million, and in 2008, US$ 2.10 million. From 0.04% in 1991 to 0.15% in 2008, the proportion of ornamental fish exports to the total value of Indian fisheries exports has increased. Between 1991 and 2009, the global growth rate for ornamental fish export was 6.1% in terms of amount, 15.5% in terms of the quantity, and 8.1% in terms of the unit value decline. In terms of export amount, the India’s ornamental fish export showed a greater total growth of 14.4%, although the growth rate in term of quantity exported was lower at 12.1% and the growth rate in term of unit value was higher at around 2.1% (Rani et al. 2013). About 70% of all ornamental fish exports from India went to the following nations: Singapore, the United States of America, China, Hong Kong, Malaysia, and Japan. In terms of fish diversity, India is among the top 10 super diverse nations in the world. There are 3400 sps. of fish found in Asia. Approximately 60% of the fresh water and marine ornamental fish that are traded worldwide are produced in the Asia. Fresh water ornamental fish account for 90% of the trade, with marine fish making up the remaining portion. India ranked 31st in the world and

exported ornamental fish worth approximately US$ 1.06 million in 2016. For the sectors to flourish, technology for ornamental fish breeding, seed production, and culture in appropriate locations must be developed. But the problem is that in non-natural habitat the fishes get faded and tend to lose their pigmentation (Torrissen et al. 1989).

Pigment enhancement in this ornamental fish is due to the carotenoid supplement in fish feed and which is the major supplements that gives beautiful orange pigment. Fishes does not synthesis carotenoids on their own i.e *de novo*, they obtain carotenoid from the natural sources, i.e they obtain carotenoids from the diet they feed on such as plants and algae (Gomes et al., 2002). In artificial environment such as aquarium they require external source of carotenoids which are supplemented in their diet which is available in the market (Shahidi et al.,1998; Kalinowski et al.,2005)

And the feed for ornamental fish which is available in the market is coming from Philippines. Colour of these ornamental fishes is one of the major factors which determine the price of the of the fish in the world market (Lovell, R.T 1992).

Carotenoids are lipid-soluble pigments containing conjugated double bonds that and exhibit robust antioxidant activity. They serve as functional pigments due to their ability to impart vibrant colours to various organisms (Britton G. 1995). There are synthetic and natural carotenoids (Johnson, E. A. 1989). Carotenoids may be easily synthesized with low-cost ingredients and no extraction expenses when making synthetic pigments. Furthermore, a lot of synthetic pigments are made to meet consumer demand. Additionally, the market share of these artificial carotenoids has grown, and some customers find it more convenient and cost-effective to buy the right pigments. However, despite the fact that there are some advantages to directly adding synthetic pigments to diets, many consumers would rather use natural sources due to rising health consciousness, the toxicity of additives, the negative effects of synthetic products, the scarcity of synthetic pigments, and their high cost. The synthesis of synthetic carotenoids

is fraught with uncertainty and constraints (NOORI and Alirza, 2018); it uses both complicated organic solvents and petrochemical solvents, which might leave behind unwanted residues. Furthermore, only particular carotenoids, including astaxanthin and beta carotene, are present in manufactured goods. The producers of DSM in the Netherlands, BASF in France, and NHU in China synthesize a significant amount of astaxanthin, although the annual market value of astaxanthin synthesis exceeds $200 million. Additionally, the price at which astaxanthin is produced on a global scale increased from USD 447 million in 2014 to USD 600 million in 2018. On the other hand, the estimated cost of producing natural astaxanthin from Haematococcus using a theoretically planned plant in China is $718/kg astaxanthin, which is a significant amount of money. When it comes to the customer, using natural pigmentsm have more advantages than using refined pigments. This is due to the crude extracts meet nutritional requirements because they contain extra proteins, carbs, and fatty acids. Additionally, due to public interest in their potential health benefits and inexpensive cost, substitutes for natural sources of carotenoids have also been studied. Furthermore, according to certain research, the effects of synthetic and natural sources of carotenoids are comparable. For instance, it is discovered that the amount of carotenoid derived from natural sources was sufficient to provide the intended coloration at a threshold level that was appropriate for marketing (Rekha et al.,2022). Till now synthetic carotenoids and carotenoids from bacteria, fungi, algae and yeasts has been used and no report is available on use of carotenoids from Halophilic microorganisms.

1.2 Aim and objective

Aim

To enhance pigmentation in ornamental fish by supplementing carotenoids in their diet from halophilic microorganisms.

Objectives

i. Isolation of pigmented halophilic microorganisms from salt pan and its characterization
ii. Optimization of carotenoid production and formulation of fish feed
iii. Supplementation of carotenoids in diet of gold fish and checking its effect

1.3 Hypothesis /Research question

i. Enhanced Pigmentation: C50 carotenoids, derived from Halophlilic microorganisms, may offer enhanced/unique pigmentation properties.
ii. Reduced susceptibility to diseases: Carotenoids Contribute to improved health and immune function in cultivated fish potentially leading to enhanced disease resistance.

1.4 Scope

- Research and Innovation: Developments in feed formulation methods, ingredient technology, and nutritional research continue to spur innovation in the aquafeed sector. Making investments in research and development to provide innovative feed formulations that increase feed efficiency, support fish health and welfare, and improve environmental sustainability can help feed makers stand out in the market.
- Personalized feeding: As aquaculture systems advance in sophistication, prospects for customized feeding strategies may arise.

Chapter II

Literature Review

2. Literature Review

2.1 Ornamental fish

India's inland and coastal waters are home to a wide variety of ornamental fish, including approximately 400 types from marine habitats and over 195 native kinds from the country's West ghats and North-East regions. About 85% of every ornamental fish that India exports are wild kinds that are harvested from rivers in the country's northeast and south. These fish are the main exports from India. Fifty-five of the North-East Region's recorded fish species are valuable for ornamentation. Further, freshwater ornamental fish are only found in these regions and has a remarkable biodiversity. Among them, goby, barbs, eels, loaches, and catfish are prominent. Common in Indian waterways, brackishwater ornamental fish like Monodactylus argentus, M. sebae, and Scatophagus argus can be collected, bred, cultivated, and traded. Low salinity fishponds have been used to successfully breed the Pearl-spot, Orange and Indian Glassfish. In the Indo-Pacific area, aquatic ornamental fish are frequently harvested from coral reef environments. The aquatic aquarium trade has grown into a thriving multimillion dollar sector that provides opportunities for livelihood for those who depend on coral reef ecosystems. Clown fish, damsel fish, moorish idols, lion fish, parrot fish, box fish, trunk fish, marine angels, butterfly fish, cleaner wrasse, cardinal fish, sergeant fish, rabbit fish, squirrel fish, scorpion fish, seahorse etc are some possible marine ornamental fish sps. resources. Ninety percent of fresh water fish traded in ornamental fish in India are cultivated, with the other two percent coming from wild capture. Marine fish make up the remaining 10%, of which 2% are cultivated and 98% are caught. Few Indian ornamental fish breeders raise native fish, marine fish, or brackish water fish. The majority of fish breeders in India rase exotic species. Because goldfish are the most popular among hobbyists, the Indian ornamental fish industry is dominated by goldfish breeding.

2.1.1 Problems faced by ornamental fish industries

The ornamental fish industries in India is beset by a number of problems, both technical & financial. Absence of professional expertise, scientific knowledge about breeding, feed kinds, and feeding and health management are the major obstacles. Most company owners are ignorant of

that this emerging market has significantly lower capital requirements and less risk than the shrimp industry, yet it is far more profitable. Until far, India's export of this marine ornamental fish has not advanced significantly because of lack of infrastructure. Due to ignorance, the most of India's native ornamental fish are inexpensive and consumed as food in small cities and rural regions. In many way, the trade in Indian ornamental fish is poorly organized. Fish from India's northeastern states are the majority of the freshwater kinds that are currently exported as ornamental fish from India (Pandey, P. K., & Mandal, S. C 2017)

2.1.2 Aquarium fish feed in market

Plant or animal matter meant for ingestion by pet fish housed in aquariums or ponds is known as aquarium fish feed. Fish meals typically include the macronutrients, vitamins, and trace elements required to maintain the health of captive fish. Roughly 80% of hobbyists who keep fish only feed prepared diets, which are often made as flake, pellet, or tablet forms. Additionally, some fish meals include substances like beta carotene or sex hormones to artificially improve the colour of these ornamental fish (*Riehl et al., 1916).*

Market for this ornamental fish feed was estimated to be worth USD 2.9 billion in 2022. The market for ornamental fish feed is expected to increase at a annual compound growth rate of 8.60% between 2024 and 2032, from USD 3.2 billion in 2024 to USD 6.1 billion. The primary factors propelling the market expansion are the increasing millennial generation's desire for vibrant decorative fish for their aquariums and the advancements in aquarium designs.

Types of prepared feed:

- Processed Feed: items that are not alive and are either created by the aquarist or purchased previously prepared for fish use (Axelrod and Herbert R 1996)
- Live Feed: Live meals can be frozen, dried, or still alive, and they are based on small living organisms in their identifiable form. Food for live fish includes bloodworms, water fleas, sludge worms, earthworms, and feeder fish. Infusoria (Protozoa and other microbes), freshly hatched brine shrimp, and microworms are food sources for larvae and juvenile fish.

Table 2.1.2: Fish Feed Manufacturing companies in India

Sr. No.	**Company name**	**Place**
1.	Avanti Feeds Limited	Hyderabad, India
2.	Godrej Agrovet Limited (Godrej Industries Limited)	Mumbai, India
3.	Growel Feeds Pvt. Ltd.	Andra Pradesh, India
4.	IB Group	Rajnandgaon, Chhattisgarh, India
5.	UNO Feeds	Bhimavaram, India

2.2 Microbial carotenoids

2.2.1 Carotenoids

Carotenoids are organic pigments that are created by a variety of bacteria, archaea, fungus, and plants (*Nelson, David L.; Cox, Michael M. 2005)*. They are yellow, orange, and red in colour. Carotenoids are responsible for the distinctive colour found in daffodils, salmon, lobster, shrimp, tomatoes, canaries, parsnips, corn, pumpkins, and flamingos. The more than 1,100 known carotenoids are further divided into two classes: carotenes, which are just hydrocarbons and do not include oxygen, and xanthophylls, which are oxygen-containing compounds. Carotenoids absorb light with wavelengths between 400 to 550 nanometers, or green to violet. The compounds become intensely pigmented in shades of yellow, orange, or red as a result (*Yabuzaki, Junko (2017).*

2.2.2 Halophilic microorganisms producing carotenoids

Halophiles comprise a heterogeneous group of microorganisms that require salts for optimal growth. Even high salt concentration up to 4 M is required for some extremophilic species such those belonging the *Haloferacaceae* family, Archaea domain. The pigments produced by these halophilic organisms include phytoene, β-carotene, lycopene, derivatives of bacterioruberin, and salinixanthin . *Dunaliella salina* is one of the betterknown halophilic microorganisms in terms of carotenoids production. However, apart from that halophilic microalgae, only few studies have been carried out about production of carotenoids by halophiles and in most of the cases, the studies are focused on carotenoids isolation and characterisation by traditional biochemical procedures as those mentioned before.

Within the halophiles there is a family of particular interest in several fields of applications: micro-ecology, biotechnology and extreme metabolic adaptations. This is the case of the *Haloferacaceae* family (previously mentioned) grouping extreme halophilic archaea

inhabiting salty environments such as marshes or salty ponds from where NaCl is obtained for human consumption. The first study about carotenoid production by halophilic microorganisms from the *Haloferacaceae* family (previously called *Halobacteriaceae* family*)* were published in the latter half of the 1960s. During the last two decades of the last century, several research works demonstrated that some haloarchaeal species not only produce carotenoids but also produce them at high concentration. This fact makes possible to propose haloarchaea as a good natural source for carotenoids production at large scale by means of suitable bioprocess engineering tools, namely specifically designed bioreactors.

(i) Salinibacter ruber

A number of molecules having pigment were identified in this bacterium which are: salinixanthin - a novel carotenoid, xanthorhodopsin - a retinal-proton pump.

Salinixanthin, which is C40 carotenoid, is the primary carotenoid of *S. ruber*, accounting for about 96% of all carotenoids. Iso-C15:0 is present as an esterified fatty acid in it (Lutnæs *et al.*, 2002). Its greatest absorption in an organic solvent occurs at 478 nm, with a 506–510 nm shoulder. A method was presented for effectively removing salinixanthin from *Salinibacter*. Because salinixanthin is detected and measured by HPLC, and can also utilized as biomarker for description of microbial populations living in hypersaline conditions (Oren & Rodríguez-Valera, 2001).

Xanthorhodopsin (Mongodin *et al.*, 2005)—a proton pump powered by light that functions similarly to bacteriorhodopsin—was thoroughly examined. At 560nm highest absorption occurs. Proton uptake happens first in the xanthorhodopsin photocycle, which is more like the proteorhodopsin photocycle than the bacteriorhodopsin photocycle. Proton release happens at end of photocycle.

Moreover, xanthorhodopsin functions like a dual chromophore system, comprising a carotenoid and a retinal. When the pH variations in membrane vesicles caused by light were used to evaluate the active spectrum of xanthorhodopsin protons pumps or when the photo inhibition of respiration at various wavelengths was quantified. Salinixanthin functions as antenna pigment to gather light for xanthorhodopsin's proton pumping, and is located close to the retina.

Salinibacter's sensory rhodopsin I (SrSRI) was investigated as first eubacterial SRI identified as functional protein. This protein exhibits a delayed photocycle and an all-trans retinal. It controls both positive and negative phototaxis since it is a dual photoreceptor.

(ii) Halophilic archaea belonging to family halobacteriaceae

The severe halophilic organisms known as halophilic archaea are mostly classified under the Haloferacaceae family within the phylum Euryarchaeota of the Archaea domain. They have developed a number of coping mechanisms to survive in those circumstances: The surface of halophilic proteins is primarily composed of amino acidic residues; cells accumulate large intracellular concentrations of KCl to cope with high ionic strength or certain osmolytes, including 2-sulfotrehalose; cellular bilayers differ in composition and structure, among other factors. Owing to these modifications, haloarchaea are now a reliable and creative source of several compounds with great potential for use in biotechnology, including PHB and PHA, carotenoids, and enzymes active at high temperatures and ionic strengths.

In addition to helping with photoreactivation, this pigment shields the cells from harm caused by strong visible and ultraviolet radiation. It also contributes to the cell membrane's reinforcement. It was initially described using cells belonging to the Halobacterium species. The earliest reports on the general synthesis of C50 carotenoids and the effects of various chemical substances on this biosynthesis came from *Halobacterium cutirubrum*, a me2mber of

the Halobacteriaceae family. A few years later, it has been discovered that the synthesis of bacterioruberin is triggered by two factors: (i) low oxygen tension and high light intensity; and (ii) osmotic stress (iii) the presence of different compounds such as aniline. Additional C50 carotenoids, such as isopentenyldehydrorhodopin, bisanhydrobacterioruberin, and monoanhydrobacterioruberin, are also precursors to bacterioruberin (Edbeib, M. F. et al.,2016).

Bacterioruberin as an antioxidant compound: According to existing descriptions, the carotenoid content affects the oxygen reactive species' (ROS) ability to be scavenged. Conversely, the length of the carbon chain, the amount of conjugated double bond pairs, and the concentration of carotenoids all affect the antioxidant activity of carotenoids in general. As previously stated, bacterioruberin has thirteen pairs of conjugated double bonds as opposed to the nine pairs seen in β-carotene. Thus, as a radical scavenger, bacterioruberin is far superior to β-carotene. Its ability to shield cells from oxidative damage has been shown. Because of this crucial biological function, haloarchaea are able to resist oxidative DNA damage brought on by radiography, UV radiation, high doses of gamma radiation, and exposure to H2O2. They also manage to avoid being fatally injured under intense light. Thus far, it is evident that the carotenoids produced by halophilic bacteria have a greater potential for antioxidants than the carotenoids produced by other microbes, whether they are extremophilic or not.

Bacterioruberin regulates the membrane rigidity: having 4 hydroxyl substitutes in this dipolar C50 carotenoid, bacterioruberin has been suggested to act as a “rivet” in its membrane cells. This carotenoid has some effect on the fluidity of the membranes, acts like a barrier to water and facilitates permeability to oxygen and other molecules, so strains can thrive in hypersaline or low-temperature conditions (Saito, T. 1997).

The role of bacterioruberin in rhodopsin complexes: The retinal protein-carotenoid complex known as archaerhodopsin-2 is present in the claret membrane of *Halorubrum* sp. and other

species. It serves as a light-driven proton pump, which is crucial for the energy production of haloarchaea cells. It has been shown through crystallographic investigations that bacterioruberin binds to the voids that exist between the trimer-like subunits of the archaerhodopsin structure. Thus, structural support for the structure of archaerhodopsin is maintained by bacterioruberin. Additionally, in haloarchaea membranes like those from *Natronomonas pharaonis*, bacterioruberin is component of complex consisting of this carotenoid and halorhodopsin. A seven-transmembrane helix retinal protein called halorhodopsin functions as an inward light-driven Cl (−) pump (Jehlička, J. et al.,2013).

Bacterioruberin has potential applications as feed additive in aquaculture:

Significant Antioxidant qualities: Bacterioruberin's chemical structure confers significant antioxidant qualities. Because of its antioxidant properties, it can lessen the effects of oxidative stress in farmed fish that are brought on by things like handling stress, high stocking densities, and poor water quality. Bacterioruberin can enhance fish resistance and general health by lowering oxidative stress.

Enhanced Immune Response: Studies on fish have demonstrated the support of the immune system by antioxidants such as Bacterioruberin can help farmed fish respond better to illnesses by scavenging free radicals and decreasing oxidative damage to cells and tissues. This increases the fishes' resistance to infections and disease.

Enhanced Pigmentation: Bacterioruberin can help fish tissues become more pigmented, just like other carotenoids do. By adding bacterioruberin to fish feed, several fish species' colours can be improved, increasing their visual attractiveness to consumers. This is particularly important for animals that appreciate bright colours, such shrimp and salmonids.

Nutritional Advantages: Like other carotenoids, bacterioruberin provides farmed fish with nutritional advantages. It acts as a precursor to vitamin A, which is necessary for development, reproduction, and eyesight, among other physiological processes. Producers of fish feed for aquaculture can guarantee that their fish get enough of this crucial nutrient by adding bacterioruberin (Xie, W. et al.,2021).

(iii) Dunaliella salina

Green, single-cellular microalgae called *Dunaliella salina* are cultivated in saline conditions and are used in the food, energy, health, and pharmaceutical industries. Beta-carotene is the most important extraction product from *D. salina* (Geng et al., 2003; Lou et al., 2020; Monte et al., 2020). A useful biological compound, beta-carotene is a powerful antioxidant, a food pigment, and a precursor to vitamin A. In *D. salina,* beta-carotene can account for up to 14% (wt) of dry weight.

To increase beta-carotene levels, *Dunaliella salina* can be grown in a variety of nutrient- and environmental-rich environments. There are two common synthetic and biological processes used to create beta-carotene (Borowitzka and Siva, 2007). Nowadays, beta-carotene is created on an industrial scale using various synthetic techniques (Abu-rezq et al., 2010). As a carotenoid of the C40 type, beta-carotene consists of 40 carbon atoms. To create synthetic beta-carotene and the C40 chain, two or three smaller molecules can be joined in a number of ways. Microalgae are the primary source for the biosynthesis of beta-carotene.

2.2.3 Use of carotenoids in aquaculture

Carotenoids have been fed to salmonids, crustaceans, and other farmed species in the aquaculture sector, mostly as pigments to give these cultivated animals a desired hue. Customers erroneously associate a product's colour with its nutritional content, freshness,

health, and flavour. As a result, colour is an important quality factor that needs to be preserved and enhanced. Carotenoids may not only improve quality by boosting colour, but they may also improve consumers' perceptions of aquaculture goods given the growing body of knowledge on the benefits of carotenoids for human health.

Carotenoids appear to enhance certain production factors in farmed species in addition to their quality-enhancing qualities. However, their function as pigment has received the majority of attention in study to date. The enduring belief that hue is linked to enhanced flavour has been around for a while. Colour has far greater meaning for consumers when it comes to fish since they identify natural coloration with superior quality and health. For example, in farmed salmonids, muscle colour is thought to be the most significant quality criterion, right behind freshness. In addition to their rich flesh colouring, cultured red and yellow-skinned fish, like yellowtail, Japanese red sea bream, Australian snapper, and red porgy, are highly valued for their brilliant skin tones.

Skin colour is another crucial factor that influences market pricing and contributes significantly to the overall evaluation of ornamental fish. A bright and suitable hue is also linked to freshness and quality in crustaceans, such as shrimp, and the proper coloration must be maintained during storage, processing, and cooking. Bright yellow-orange sea urchin gonads are the most commercially valuable type in the industry, according to the production of marketable gonads. The colour of fish, birds, mammals, and economically significant invertebrates can be attributed to four primary types of pigments. These include carotenoids, pteridines, melanins, and porphyrins.

Vertebrates and many invertebrates get all of their black, grey, and brown colours from melanin, along with many of their red and yellow hues. Tyrosine metabolites combine to form the heterogeneous polymers known as melanins. Animals receive carotenoid pigments from

their meals, which are responsible for much of the vibrant red, yellow, and orange colours that are highly valued in aquaculture.

Table 2.2.3.a: Reports on synthetic carotenoids used in ornamental fish Feed

Sr. No	Fish	Carotenoids (synthetic)	Results
1.	*Oncorhynchys mykiss*	Astaxanthin	Improved skin pigmentation
2.	Larimichthys	Astaxanthin	Enhanced reddish coloration in ventral and dorsal skins
3.	Japanese ornamental carp	Carophyll	Pigmentation was improved
4.	Australian snapper	Astaxanthin	Gave higher pigment retention in skin
5.	Red porgy	Astaxanthin	Bright reddish colour in both dorsal and ventral area
6.	Giant tiger prawn	Astaxanthin	Improved pigmentation
7.	Tropical spiny lobster	Astaxanthin	Attained dark colour than that of control fishes
8.	Yellow cracker	Xanthophylls	Exhibited greater yellowness colour in ventral and dorsal skins

Table 2.2.3.b: Reports on carotenoids from microbial sources used in ornamental fish Feed

Sr..No	Fish	Carotenoid/source (microbial)	Results
1.	Koi carp	Biomass of *Rhodotorula paludigena* (yeast)	Enhanced pigmentation
2.	*Hemigrammus caudovittatus*	*Phormidium valderianum (cyanobacteria) –carotenoids*	Enhancement in carotenoid content
No reports available on the use of halophilic microorganisms			

Table 2.2.3.c: Reports on carotenoids from microbial sources used in non-ornamental fish Feed

Sr No:	Fish	Carotenoid/source (microbial)	Results
1.	Shrimps	*Spirulina spp*. (algae)- Beta- carotene, zeaxanthin *Dunaliella salina* (algae) - beta- carotene, alpha-carotene, zeaxanthin	Enhancement in fish skin colour
2.	Salmon and trout	*Paracoccus carotinifaciens* (bacteria) - astaxanthin	Carotenoid was bioavailable, efficient in colouring the flesh
3.	Salmonoid fish	*Phaffia rhodozyma* (yeast)- astaxanthin	Increase in carotenoid content of fish flesh

2.2.4 Effects of microbial carotenoids on fish health

Microbial carotenoids have various beneficial effects on fish:

Coloration: The vivid hues seen in a variety of fish species are caused by carotenoids. Fish must get these pigments from their food because they are unable to generate them on their own. Adding carotenoid-rich feed to fish in aquaculture can improve their pigmentation, which is typically desired for aesthetic reasons and can also be a sign of excellent health to prospective buyers.

Immune System Support: Fish with compromised immune systems may benefit from the antioxidant qualities of carotenoids. Carotenoids may enhance disease resistance and general health in aquaculture environments by scavenging free radicals and lowering oxidative stress.

Reproductive Health: It has been demonstrated that carotenoids affect fish reproductive success. They participate in various processes, including the formation of gametes, fertility, and embryos. Sufficient consumption of carotenoids can enhance the survivability of offspring and enhance reproductive efficiency.

Vision and Sensory Functions: Fish eyes and other sensory organs depend on carotenoids to stay healthy. In particular, they aid in the development and upkeep of visual pigments in the

retina, which are essential for healthy eyesight in animals that primarily depend on sight to seek prey or elude predators.

Growth and Development: Although carotenoids in fish may not be primarily involved in promoting development, their inclusion in the diet can indirectly aid in growth by improving general health and nutrient utilization. Fish in good health have a higher chance of growing quickly and maturing into marketable size.

Stress Response: It's possible that carotenoids help fish cope with the negative impacts of stress. Carotenoids can assist fish adapt to a variety of stressors seen in aquaculture, including handling, transportation, and changes in ambient conditions, by boosting the immune system and lowering oxidative damage.

Chapter III

Methodology

3. Methodology

3.1 Sampling site and sample collection

Halophiles are extremophilic organisms represented by archaea, bacteria, and eukaryotes that thrive in hypersaline environments like a salt pan, salt lakes, saline soils, marshes, etc. To isolate halophiles brine samples, sediment samples were collected from a salt pan located at Shiroda, Maharashtra having latitude 15.757253º and longitude 73.683079º. The samples were surface spread plated on various Halophilic media plates. And the physicochemical parameters of salt pan was checked like pH and temperature. Some of the sediment samples were collected in zip lock bag while the brine samples were collected in clean bottle and these samples were taken to lab for further studies (Quadri, et al., 2016).

3.2 Isolation and purification of the halophilic isolate

3.2.1 Preparation of media for isolation of halophile

The media used to isolate halophilic microorganisms is Extremely Halophilic Media (EHM) containing (g/L): NaCl-250; $MgSO_4.7H_2O$- 20; KCl-2; $CaCl_2.2H_2O$-0.36; NaBr-0.23; $NaHCO_3$ -0.06; $FeCl_3.6H_2O$-trace; Yeast extract-10; Peptone-5; agar-20, Moderately halophilic media (MHM) containing (g/L):NaCl-178; KCl-2; $MgSO_4.7H_2O$-1; $CaCl_2.6H_2O$-0.36; NaBr-0.23; $NaHCO_3$-0.06; Yeast extract-10; Peptone-5; $FeCl_3.6H_2O$-0.005; Agar-20, Norberg-Hofstein media(NH) containing (g/L): NaCl-200; $MgSO_4.6H_2O$-10; KCl-5; $CaCl_2.2H_2O$-0.2; Yeast extract-1, NaCl Tryptone Yeast Extract media (NTYE) containing (g/L): NaCl-200; $MgSO_4.7H_2O$-20; KCl-5; Yeast extract-3; Tryptone-5; Agar-20 and NaCl Trisodium Citrate (NT) containing (g/L): NaCl-200; $MgSO_4.6H_2O$-20; KCl-2 ; Yeast extract-10 ; Trisodium citrate-3 ; Agar-20. pH of media was adjusted to 7.5 using 5M NaOH solution (Schneegurt, 2012).

3.2.2 Isolation of halophilic microoragnisms

50µl of the brine sample and 10µl of sediment sample (1g in 10ml 15% NaCl) was surface spread plated on the EHM, MHM, NT, NTYE agar plate. The plates were then incubated at room temperature. After 30 days of incubation period various types of pigmented colonies were observed on the plates which initially appeared as non-pigmented transparent colonies (Sanders, 2012).

3.2.3 Purification of the halophilic microorganisms

Amongst the various colonies obtained on EHM, MHM, NT, NTYE media agar plates, the pigmented colonies were isolated using sterile nichrome loop and streaked on that respected media agar plates. The isolates were then purified by repeated streaking (quadrant streak) on EHM, MHM, NT, NTYE agar plates. The pure culture was maintained by periodically subculturing it after every 10-20 days (Vahed, et al., 2011).

3.3 Starter culture preparation

Starter culture was prepared by inoculating a loopful of the purified culture from EHM agar plate in 25ml of the sterile EHM broth contained in 100ml capacity Erlenmeyer flask and kept in shaker incubator at 190rpm (Yu, et al., 2022).

3.4 Polyphasic identification of the isolates

3.4.1 Morphological characterization

(i) Colony characteristics

The culture was Quadrant streaked on 15% EHM agar media [Appendix I (2)] to obtain isolated colonies. Colony shape, size, margin, elevation, opacity, pigmentation, consistency was determined (Sousa, et al., 2013).

(ii) Cell characteristics: Phase contrast microscopy

Gram staining was carried out by preparing smears of the 7days old culture on clean grease free slide, air dried and then heat fixed. Desalting of smear was done by adding 2% of acetic acid solution [Appendix II(8)]. Smear was then stained with primary stain crystal violet for 1min. Stain was then discarded and smear was flooded with Gram's iodine for 30sec. Then smear was rinsed with water and 70% ethanol was used as decolourizer for 30sec. At last safranine was used as counter stain for 1 min. Finally rinsed with water, air dried and examined under oil immersion objective (100x) of phase contrast microscope (AXIOM CL20). The smears were prepared using 15% NaCl solution [Appendix II(4)] (Emerson, et al., 1994).

3.4.2 Chemotaxonomic characterization

(i) Antibiotic sensitivity

15% NH media agar was prepared and autoclaved, to which separately autoclaved 2% glucose was added and then 100 IU/m^{-1} Penicillin G was added.

Culture was spot inoculated on media agar plates and plates were incubated at RT for 15 days. Control was kept.

(ii) Pigment analysis

- **UV Visible spectrophotometry**

Archaeal cells were harvested from 7 days old culture grown in 15% NH [Appendix I(1)] broth by centrifuging at 10,000rpm for 10min. Then cell pellet was suspended in methanol and the extraction process was carried out for 4 hours, the suspension was centrifuged at 10,000rpm for 10min. UV visible spectrophotometry scan of the supernatant was taken from 190-800nm (Sahli, et al., 2020 and Passos and Saraiva;2019)).

(iii) Polar lipid analysis

- **Growth of culture for lipids extraction:**

Cultures GUPPI and GUPPII were grown in EHM broth and pH of the media was adjusted to 7.5. and kept in shaker incubator.

- **Extraction of the lipids:**

Cell pellet was collected by centrifuging 50mL 7days old cultured media and suspended in 3.75 ml methanol: chloroform (2:1, v/v) and extraction was carried out for 4-6 hrs. The supernatant was collected by centrifuging at 10,000 rpm for 20 min, and the pellet was re-extracted with 4.75 ml methanol: chloroform: water (2:1:0.8v/v). 2.5 ml of chloroform and 2.5 ml of water was added by combining both the extracts (supernatants), for phase separation. Centrifuged at 10,000rpm for 20min, the chloroform phase containing lipids was collected in clean dry glass vial and then dried by evaporation.

- **Separation of the lipids:**

Then re-dissolved the lipids in 100µl of chloroform and then spotted on to silica gel plates using capillary tubes. The solvent system used for separating polar lipids contained, chloroform: methanol: acetic acid: water (104.9: 27.7: 12.3: 4.9, v/v).

- **Detection of the lipids:**

For Glycolipids detection- sprayed the plates with 0.5% of α – naphthol in 50% of methanol-water followed by 5% of H_2SO_4 in ethanol and the visualization of spots was done by heating TLC plate at 100ºC.

For Phospholipid detection- plates were kept in iodine chamber. Rf was calculated (Balkrishna, 2015)

(iv) Biochemical analysis

- **Carbohydrate utilisation studies**

In Carbohydrate utilization 10% stock of sugars was prepared, and autoclaved at 121ºC for 15min. Sugars used for carbohydrate utilization were D-glucose, sucrose, D-sorbitol, D-maltose, D-lactose, D-galactose, ribose, D-mannose, D-raffinose, L-arabinose, D-fructose, glycerol, trehalose, xylose. 500mL 15% NH (Norberg and Hoefstein) media [Appendix I(1)] was prepared consists of g/L: NaCl-200, KCl-5, yeast extract- 1, $MgSO_4.7H_2O$-10, $CaCl_2.2H_2O$-0.2. 0.01g of phenol red was added to the media, and media was distributed in the test tubes containing Durhams tubes and separately sterilized at 121ºC for 20min. Sugar stock was then added to the test tubes containing media. The culture was inoculated in tubes and tubes incubated at 37ºC for 15days. Growth was seen as turbidity and colour change from red to yellow with gas bubbles in Durhams tubes (Balkrishna, 2015 and Azhar et al., 2014).

- **Catalase test**

For catalase loopful of culture was taken on a clean grease free slide under sterile conditions and of 2-3 drops of hydrogen peroxide were added. Formation of effervescence indicates positive test for catalase (Reiner, 2010).

- **Oxidase test**

For oxidase a piece of filter paper was soaked in a sterile petri dish with the reagent solution 1% N, N, N′, N′-tetra-methyl-p-phenylenediamine dihydrochloride [Appendix II(12)]. Freshly grown culture was taken from the culture plate with help of a sterile loop and make smear was prepared on the treated filter paper.

Development of a deep purple-blue/blue colour indicates oxidase positive (Rods, 2014).

(v) Screening for various hydrolytic enzymes

The extracellular enzyme production by the isolate was screened by growing the culture on minimal medium, i.e., Norberg and Hoefstein (NH) medium, the media consists of g/L: NaCl-200, KCl-5, yeast extract- 1, $MgSO_4.7H_2O$-10, $CaCl_2.2H_2O$-0.2. The pH of media was adjusted to 7.5 by using 5M NaOH. Various substrates Were used as sole sources of carbon, and freshly grown culture from 15% NH broth was spot inoculated at centre of plates and allowed to dry for some time.

- **Amylase activity**

Screening of amylase activity was done by adding, 0.5% (w/v) of soluble starch as a substrate in NH media agar plate. Culture was spot inoculated at centre of plate and incubated for 15days, after incubation plates were flooded with 0.6% KI solution [Appendix II (1)), clear zone around the colony against deep blue background indicated the hydrolysis of starch.

- **Protease activity**

Screening of Proteolytic activity was done by adding 0.5% (w/v) skimmed milk as a substrate in NH medium agar plate. Culture was spot inoculated at centre of plate and incubated for 15

days. A clear zone around the culture growth against white background indicated positive test for protease.

- **Gelatinase activity**

Screening of Gelatinase activity was done by adding 0.5% (W/v) gelatine as a substrate in NH medium agar plate. Culture was spot inoculated at centre of plate and incubated for 15 days. After incubation plates were flooded with 15% mercuric chloride acidified with 20%(V/V) concentrated HCI [Appendix II (3)]. Clear zone around the colony against white background indicated positive test for gelatinase (Whaley et al., 1982)

- **Cellulase activity**

Screening of cellulose activity was done by adding 0.5% (w/v) of carboxy methyl cellulose (CMC) as substrate in NH medium agar plate.

Culture was spot inoculated at centre of the plate and incubated for 15days, after incubation plates were flooded with 0.3 % I_2 -0.6% KI solution solution [Appendix I (2). A clear zone around the growth of culture against brown background indicated positive test for cellulase activity.

- **Xylanase activity**

Screening of xylanase activity was done by adding 0.5% (w/v) Xylan beechwood in NH medium agar plate, culture was spot inoculated at centre of plate and incubated for 15days, after incubation plates were flooded with 0.3 % I_2 -0.6% KI solution [Appendix II (2)]. A clear zone around the growth of the culture against brown background indicated positive test for xylanase activity.

- **Lipase activity**

Screening of lipolytic activity was done by adding 0.5% (v/v) olive oil as a substrate in NH medium agar plate, culture was spot inoculated at the centre of plate and incubated for 15 days. A turbid zone around the growth of the culture against clear background indicated positive test for lipase.

- **Esterase activity**

Screening of esterase activity was done by adding 0.5% (v/v) tween 80 as a substrate in NH medium agar plate. Culture was spot inoculated at centre of the plate and incubated for 15days. A turbid zone of precipitation around the growth of culture against clear background indicated positive test for esterase activity.

- **Chitinase activity**

Screening of chitinase activity was done by adding 0.5% (w/v) colloidal chitin as substrate (appendix II) in NH medium agar plate. Culture was spot inoculated at the centre of plate and incubated for 15days after incubation plates were flooded with 0.3 % I_2 -0.6% KI solution [Appendix II (2)]. Clear zone around the growth of culture against brown background indicated positive test for chitinase activity.

3.5 Parameters optimization for carotenoid production

3.5.1 NaCl concentrations

NH media flasks containing various NaCl concentrations i.e 0%, 5%, 10%, 15%, 20%, 25% and 30% were prepared and 0.5% glucose added to all the flasks and flasks were inoculated with the culture and kept in shaker incubator. Similarly, NH medium agar plates with various

NaCl concentrations were prepared and the freshly grown culture was streaked (quadrant streak) on the media plates and incubated plates at RT. The NaCl concentration showing the best culture growth was determined as optimum NaCl concentration for growth and carotenoid production (Elevi et al.,2004).

3.5.2 Substrate concentrations

NH media agar plates with various substrate (glucose) concentrations i.e 0%, 0.5%, 1%, 1.5% and 2% were prepared, freshly grown culture was streaked (quadrant streak) on the media plates, then plates were covered properly and incubated at RT. The substrate concentration showing the best growth was determined as optimum substrate concentration for growth and carotenoid and production.

3.5.3 pH range

NH media flasks containing different pH i.e 5, 6, 7, 8, and 9 were prepared and 0.5% glucose was added to all flasks and the flasks were inoculated with the culture and kept in shaker incubator. The pH showing the best culture growth was determined as optimum pH for growth and carotenoid production (Singh et al., 2019).

3.6 Studies on the effects of halophilic cultures on gold fish

3.6.1 Experimental design

Total of 18 juvenile common gold fish *(Carassius auratus)* were maintained in 3 tanks, 6 in each tank also other parameters were checked like Temperature, pH, ammonia, nitrate, nitrite. Temperature was maintained at 26-27ºC, pH of 7-8 and ammonia, nitrate and nitrite of 0ppm. Each tank with a capacity of 50L.The tanks were cleaned after every 4 days.

Initial weight of all 18 fish was taken and accordingly they were fed 5% of their body mass. Final weight was taken at the time of slaughter (Torrissen, & Naevdal, 1984).

3.6.2 Diets

Diet 1- feed without carotenoids (Control)

Diet 2- feed with carotenoids (Test)

Diet 3- Commercial feed

Fish were fed twice daily. They were fed 5% of their body mass.

Table no 3.6.2: Microbiology laboratory fish feed preparation

Control (100%)	**Test (100%)**	**Commercial**
Soya bean	Soya bean	Fish meal
Fish meal	Fish meal	Shrimp product
Groundnut oil cake	Groundnut oil cake	Corn protein meal
Wheat flour	Wheat flour	Soyabean meal
Tapioca flour	Tapioca flour	Corn
Rice bran	Rice bran	Cassava pellet
Vegetable oil	Vegetable oil	Fish oil
Vitamins	Vitamins	Lecithin
Dried carrot powder	Dried carrot powder	Synbiotics
Dried spinach powder	Dried spinach powder	Vitamins and minerals
	Culture	Food colouring
		Antioxidants

All the ingredients were combined and then boiling water was added and stirred the mixture and allowed to soak for some time then the dough was kneaded and fish feed pellets were prepared. And oven dried and stored.

3.7 Proximate analysis

3.7.1 Moisture Content

Crucible was thoroughly washed and dried in oven. 2g of sample was placed in the pre-weighed crucible and kept in oven to dry at 105ºC for 5hrs. The crucible containing dried sample was then transferred to desiccator to get cool at room temperature before weighing again. This was repeated until constant weight was obtained. Then re-weighed the sample and the moisture was obtained by the differences in their weight (Lovell, 1975)

$$Moisture\ (\%) = \frac{weight\ of\ original\ sample - weight\ of\ dried\ sample}{weight\ of\ original\ sample} \times 100$$

3.7.2 Ash Content

The porcelain crucible was heated in oven at 105ºc for 2 hours and desiccator was used to cool the crucible till constant weight was obtained. Then weighed 2g of the sample in the crucible and kept in muffle furnace at 550ºC-600ºC for 4-6 hours. Temperature of muffle furnace was reduced to 105ºC and hold for 20 minutes. The crucible was cooled in desiccator then weighed again, ash weight was obtained by the differences in the weight (Maynard, 1970).

$$Ash\ content\ (\%) = \frac{weight\ of\ sample - weight\ of\ ash}{weight\ of\ sample} \times 100$$

3.7.3 Lipid content

Soxhlet extraction method was used to determine lipid content. 250 ml boiling flask was thoroughly washed and dried in an oven at 105ºC for 30 minutes. Weighed 2g of the dried

sample in a crucible. 200 ml of petroleum ether was filled in boiling flask and boiled at 50-60 ºC. Extraction thimble was plugged lightly with cotton wool and then the flask was placed in the extraction thimble to boil and the extraction was carried out for 6hrs. Thimble was removed carefully, and petroleum ether was collected at top of container and drained into another container for reuse. Rotary vacuum evaporator was used to remove the solvent and when no petroleum ether was left in the flask the flask was removed and kept in an oven overnight at 105ºC. Before weighing the flask it was transferred to a desiccator. Weight of flask was recorded containing lipid (Maynard, 1970).

$$Lipid\ (\%) = \frac{corrected\ weight\ of\ lipid}{weight\ of\ sample} \times 100$$

3.8 Analytical methods

Fish were visually judged to determine whether the pigmentation is commercially acceptable. At slaughtering, length and weight was recorded. Scale samples were collected and transferred to 2mL pre-weighed Eppendorf tubes. Samples were kept frozen (- 20°C) for 1-2 days before the carotenoid analysis is carried out. 1ml acetone was added to the sample. After 1 day of extraction in covered eppendorf tubes, at a temperature of 4- 5°C in the dark, until no more colours could be obtained. The solution was centrifuged at 10,000rpm for 10min and absorption of extract was measured at 190-800nm. The extinction coefficient, E= 2500, was used for calculating carotenoid content (Torrissen & Naevdal. 1984).

$$Total\ Carotenoids\ (\mu g/mL) = \frac{A\ x\ V\ (mL) x\ 10^6}{\varepsilon\ x\ 100}$$

Where, A is absorbance, V is total volume

Chapter IV

Analysis and Conclusions

4. Analysis and Conclusion

4.1 Sampling site and sample collection

The samples were collected on 14th May 2023 from a salt pan located at Shiroda village, Sindhudurg district of Maharashtra (having latitude 15.757253º and longitude 73.683079º) (Fig4.1: a and b). Samples collected were brine and sediment samples. The pH of the salt pan was found to be 7.5 and temperature was 45ºC.

Fig 4.1: (a-c) Sampling site of solar saltern of shiroda, Maharashtra, (d)Sample collection from the salt pan, (e) Tool used during the harvesting process and (f) Heap of salt stock.

4.2 Isolation and purification of the halophilic isolates

Samples collected from salt pan were plated on NT, NTYE, MHM and EHM media having 20% NaCl. Fifty microlitres (50µL) of brine sample and 10µL of sediment sample was surface spread plated and the plates were incubated at room temperature till growth was observed. After 15days of incubation White, yellow, cream and watery colonies were observed on the plates (Fig 4.2 e,f,g,h). Plates were further incubated, after 45 days of incubation red colour colonies started to appear on plates (Fig 4,2: i,j,k,l).

Table no 4.2: CFU/mL of the colonies obtained on media plates

CFU/mL			
Media	**5 days**	**15 days**	**45 days**
EHM B1 I	0	0	$3.4x10^2$
NTYE B1 I	0	0	$5.96x10^3$
NTYE S1 II	$8x10^2$	$4x10^2$	$1.57x10^4$
NTYE B1 II	0	0	$4.2x10^3$

On the basis of size, colour and pigmentation the isolated colonies were selected and streaked (quadrant streak) on that respected media to obtain pure cultures (Fig 4.2: m and n). it was found that the isolates grow best on NH media plates. Two halophilic isolates were obtained in pure form on NH media plates and were designated as (Fig 4.2: m) GUPPI and (Fig 4.2: n) GUPPII.

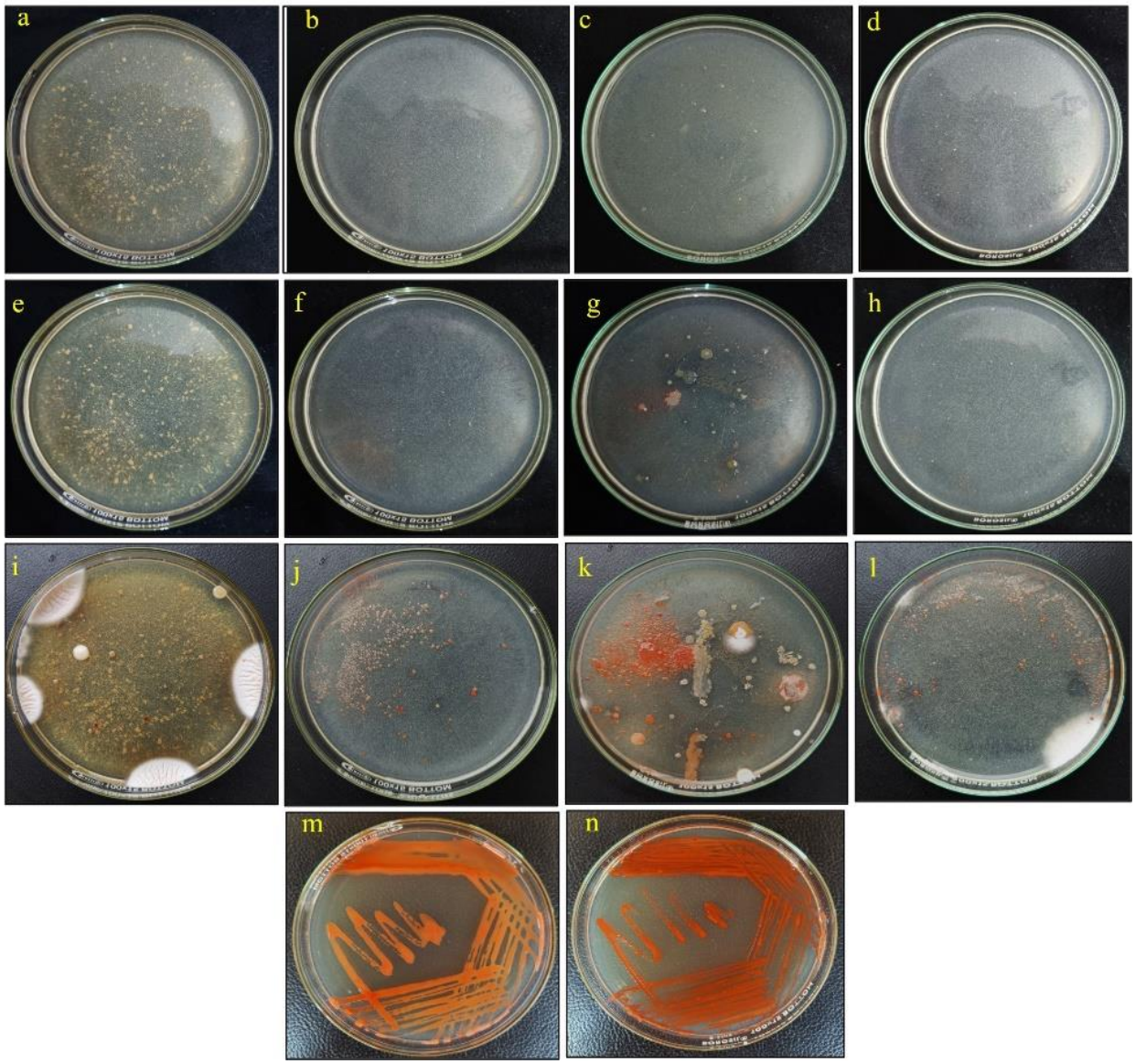


Fig 4.2: (a-d) Plates inoculated with sediment and brine sample after 5 days of incubation. Sample on NTYE and EHM media agar plates. (e-h) white, creamy, watery colonies obtained after 15days of incubation. (i-l) Red colonies started to appear after 45 days of incubation. (m and n) Pure cultures obtained after repeated streaking on NH medium (m) isolate GUPPI and (n) isolate GUPPII.

4.3 Polyphasic identification of isolates

4.3.1 Morphological Characterisation

(i) Colony Characteristics

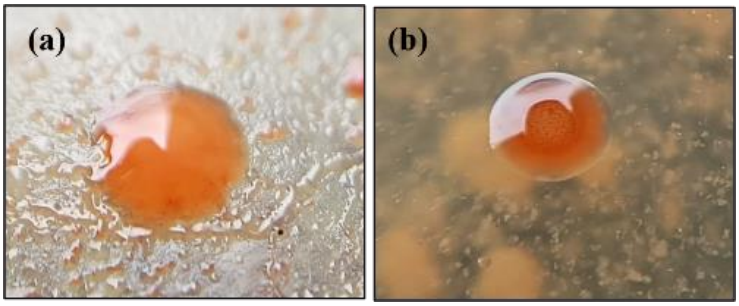


Fig 4.3.1.a: Single colony picture of isolate (a) GUPPI and (b) GUPPII

Table no 4.3.1. Colony morphology of isolates GUPPI and GUPPII

Characteristics	GUPPI	GUPPII
Shape	Circular	Circular
Size	1-3 mm	1-2 mm
Margin	Entire	Entire
Elevation	Convex	Convex
Opacity	Opaque	Opaque
Pigmentation	Orange	Red
Consistency	Butyrous	Butyrous
Cell morphology	Cocci	Cocci
Gram stain	Negative	Negative

(ii) Cell characteristics: Phase contrast microscopy

Gram stainined slides were observed under microscope at 100x magnification. Cocci like cells were visible for both the isolates. The isolates were found to be Gram negative.

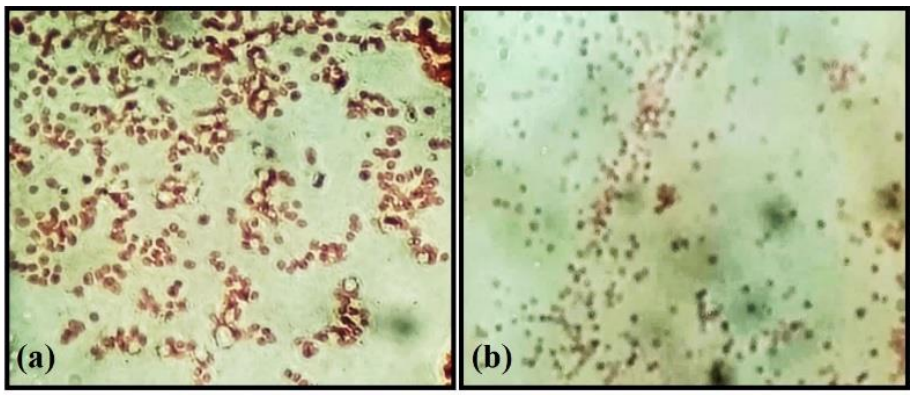


Fig 4.3.1.b: Gram staining of isolates (a) GUPPI and (b) GUPPII observed under 100x

4.3.2 Chemotaxonomic Characterisation

(i) Antibiotic sensitivity

Antibiotics are used to differentiate between Domain Bacteria and Archaea. Bacterial cell is made up of peptidoglycan containing N- acetylglucosamine and N- acetylmuramic acid which is sensitive to antibiotic Penicillin G, on the other hand Archaea lacks peptidoglycan and are therefore resistant to it.

The isolates GUPPI and GUPPII were able to grow on the media containing antibiotic Penicillin G, hence it belongs to Domain “Archaea”(fig 4.3.2.a).

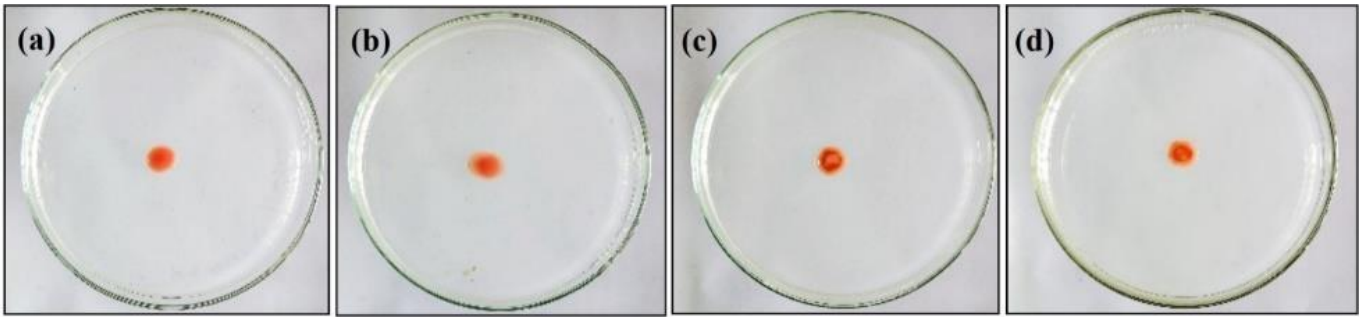


Fig 4.3.2.a: Antibiotic sensitivity of Isolate GUPPI (a, b)and isolate GUPPII (c, d), where (a and c) are controls and (b and d) are with Penicliine G.

(ii) Polar lipid analysis

The characteristic features of the members of the family Halobacteriacese, domain Archaea is their unique lipids. For identification and separation of these polar lipids TLC (thin layer chromatography) was used.

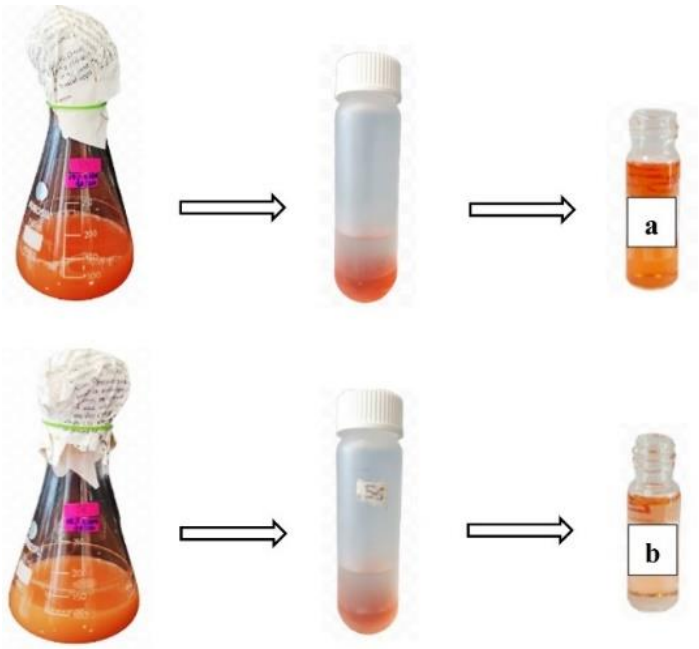


Fig 4.3.2.b: Glass vials containing polar lipid extract from GUPPI (a) and GUPPII (b) isolate

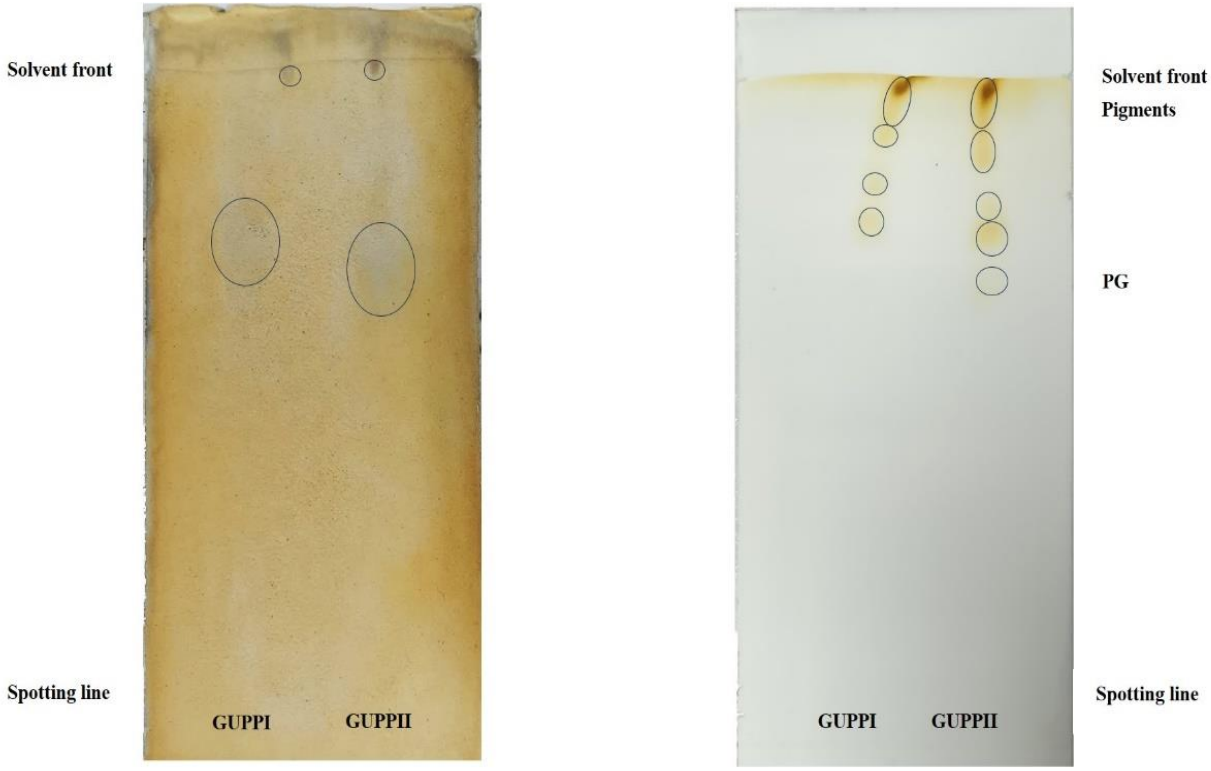


Fig 4.3.2.c: Thin layer chromatography of polar lipids of GUPPI and GUPPII isolate, left panel is of Glycolipids and right panel is of Phospholipids

Polar lipids were extracted from extremely Halophilic microorganisms and spotted on TLC plates.

Glycolipids appeared as distinct purple spots for isolates GUPPI and GUPPII (Fig 4.3.2.c: left panel), Phospholipids appeared as distinct yellow spots for isolates GUPPI and GUPPII (Fig 4.3.2.c: right panel). There were two distinct spots one with Rf of 0.66cm which is characteristic of phosphatidylglycerol (PG). Another spot with Rf of 0.98cm which are pigments of Haloarchaeal isolates (Balkrishna, 2015).

(iii) Biochemical analysis

- **Carbohydrate utilization studies**

For carbohydrate utilization 15 sugars were used: Raffinose, Mannitol, Sucrose, Glucose, Lactose, Mannose, Arabinose, Xylose, Ribose, Sorbitol, Trehalose, Maltose, Glycerol, Fructose and Galactose. NH media was used containing phenol red indicator and inverted Durhams tubes and pH of the media was adjusted to 7.5. After inoculation with the culture tubes were kept for 15days of incubation at room temperature. Colour change was noted, phenol red turns the media yellow indicating growth and acid production by the culture.

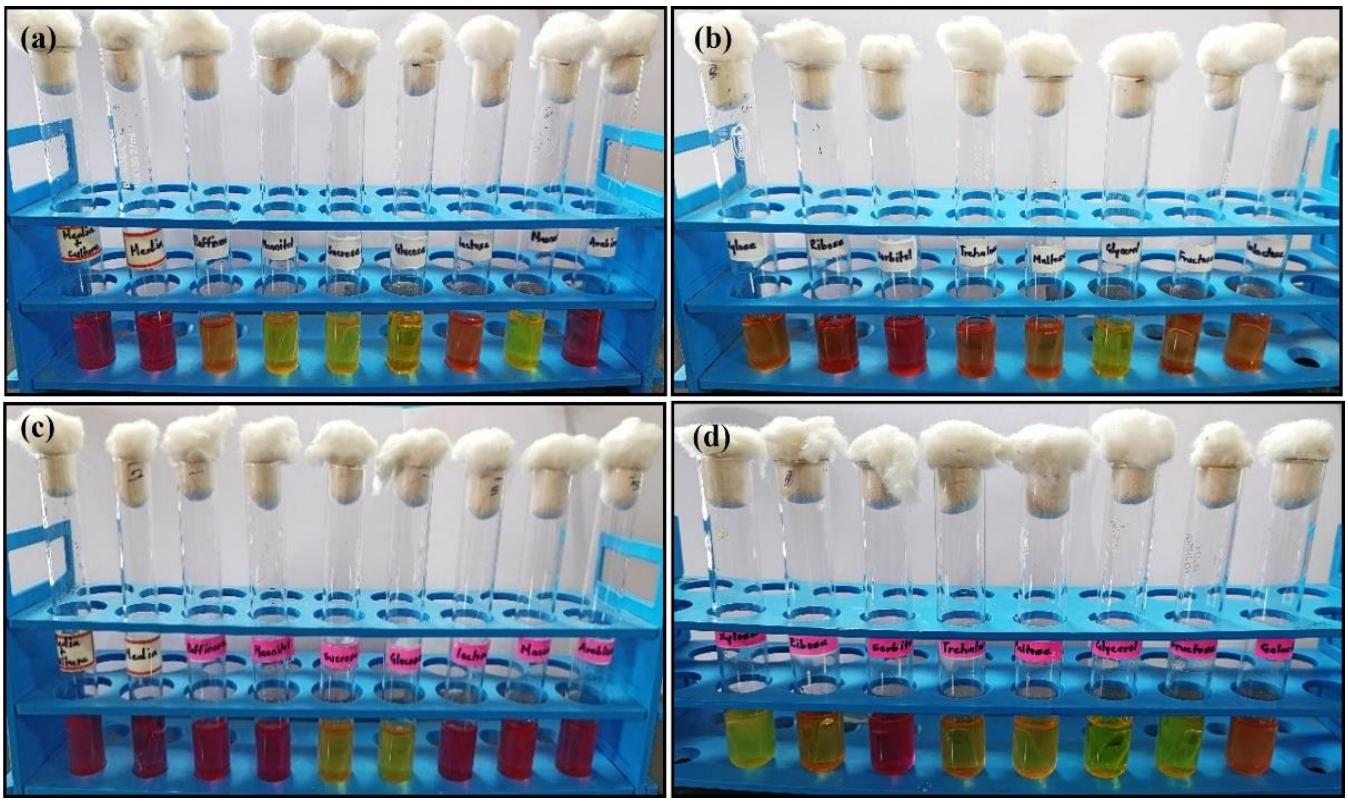


Fig 4.3.2.d: Carbohydrate utilization test of isolate GUPPI (a-b) and GUPPII (c-d)

Table no 4.3.2.a Carbohydrate utilisation study of isolate GUPPI and GUPPII

Sugars	**GUPPI**	**GUPPII**	***Halogeometricum pallidum***	***Haloferex massiliensis*** (Khelaifia et al.,2018)
Raffinose	+	-	-	+
Mannitol	+	-	-	+
Sucrose	+	+	-	+
Glucose	+	+	+	+
Lactose	+	-	-	+
Mannose	+	+	-	-
Arabinose	-	-	-	-
Xylose	+	+	-	+
Ribose	+	+	-	+
Sorbitol	+	-	-	+
Trehalose	+	+	-	+
Maltose	+	+	+	+
Glycerol	+	+	+	+
Fructose	+	+	+	+
galactose	+	+	+	+

Keys:

+ acid production

- no acid production

- **Catalase test**

Isolate GUPPI and GUPPII showed catalase positive. After addition of 2-3 drops of hydrogen peroxide on the slide containing culture smear it resulted in formation of effervescence which indicated positive test for catalase**.**

- **Oxidase test**

Isolate 54 showed oxidase positive. When smear was made of freshly grown culture on the treated filter paper with the reagent solution (1% N, N, N′, N′-tetra-methyl-p-phenylenediamine dihydrochloride). It resulted in development of a deep purple-blue/blue colour which indicated oxidase positive.

(iv) Screening for various hydrolytic enzymes

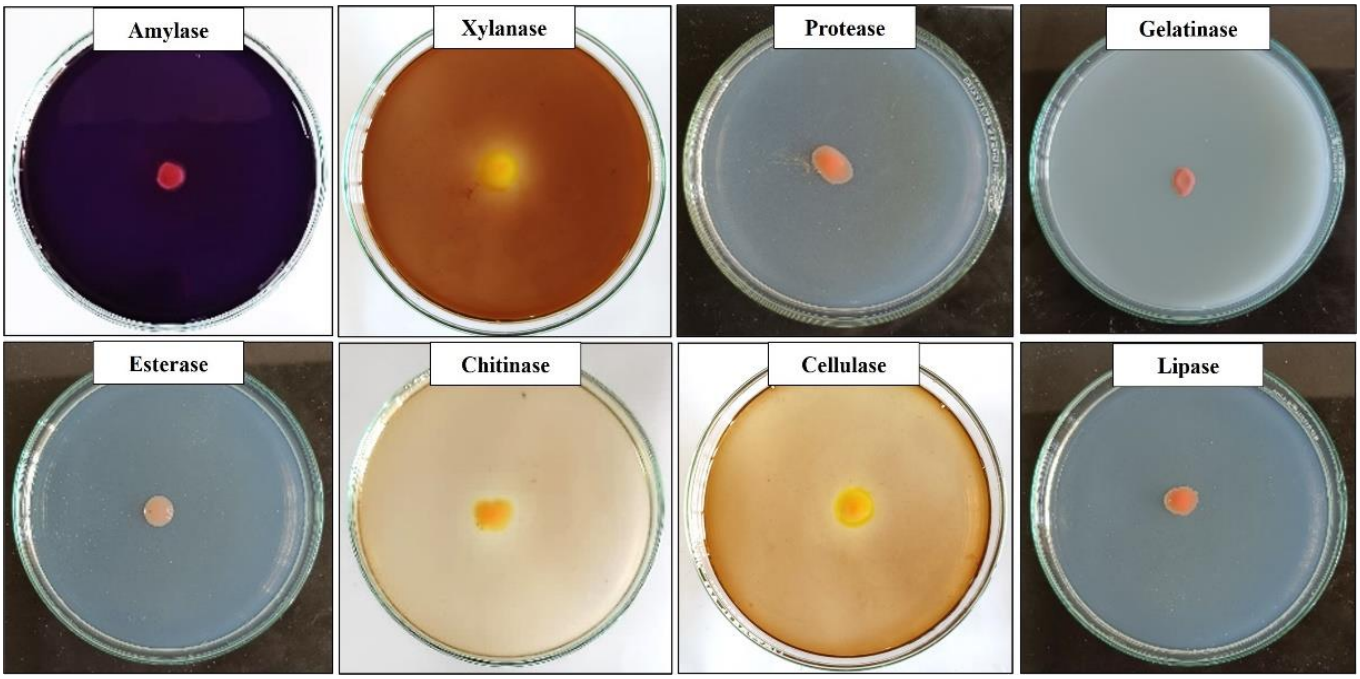
The culture GUPPI and GUPPII were exploited for their potential to produce various hydrolytic enzymes. The enzymes screened were amylase, xylanase, protease, gelatinase, esterase, chitinase, cellulase and lipase (Fig 4.3.2.e). Both isolates showed 3 enzymatic activities. Out of 8 enzymes screened, the isolated showed 3 enzymatic activities which includes Xylanase, Chitinase and Cellulase. The table no: 3 summarizes the results of various hydrolytic enzyme activities.

Table no 4.3.2 b. Hydrolytic enzyme activity shown by GUPPI and GUPPII isolate

Enzymes/Substrates	**GUPPI**	**GUPPII**
Amylase (starch)	-	-
Xylanase (xylan)	+	+
Protease (skim milk)	-	-
Gelatinase (gelatin)	-	-
Esterase (tween 80)	-	-
Chitinase (colloidal chitin)	+	+
Cellulase (CMC)	+	+
Lipase (olive oil)	-	-

Keys: + indicates enzymatic activity, - indicates no enzyme activity

(a)



(b)

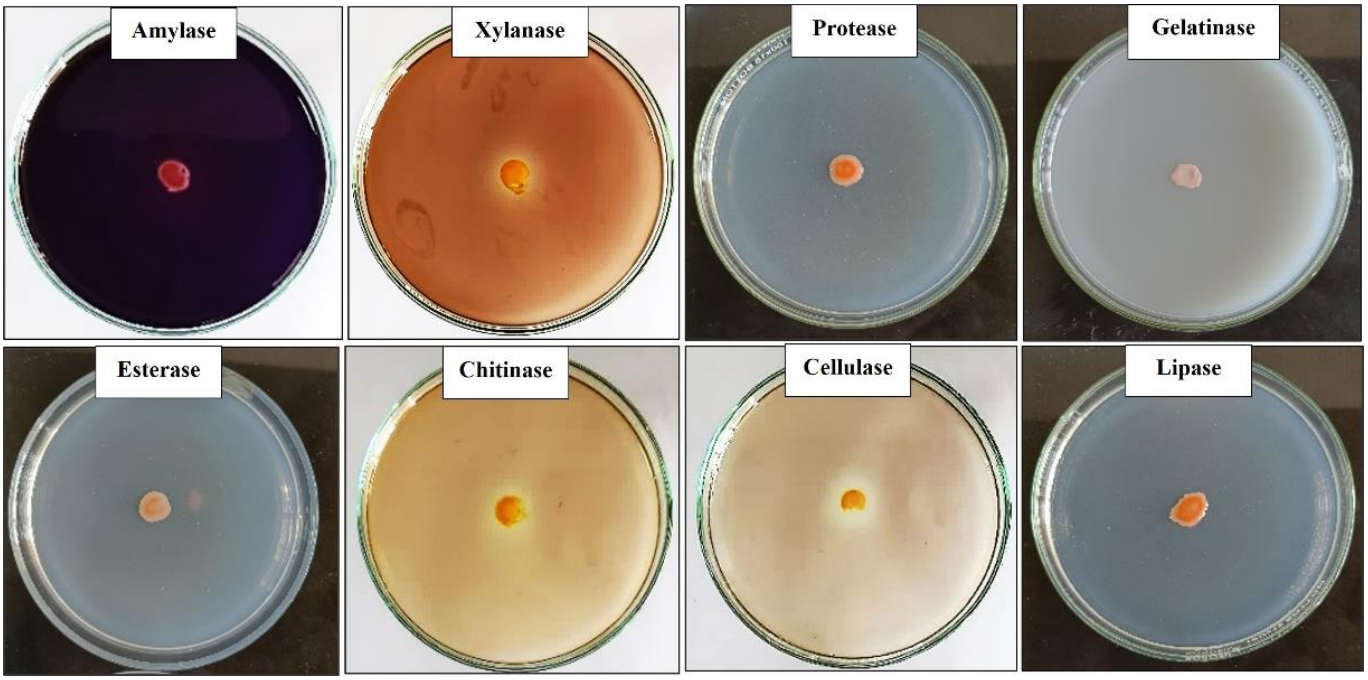


Fig 4.3.2.e: Screening of various hydrolytic enzymes for isolate (a) GUPPI and (b) GUPPII

4.4 Optimization of physiochemical parameters

The optimum growth conditions such as NaCl concentration, Substrate concentration and pH range for production of carotenoids was examined by using UV visible spectrophotometry.

4.4.1 NaCl concentration

The isolate GUPPII was grown in NH media containing different NaCl concentrations i.e., 0%, 5%, 10%, 15%, 20%, 25% and 30%. After 15 days of incubation it was observed that the growth (Fig 4.4.1.a) as well as the carotenoid production (Fig4.4.1.b) was maximum in media containing 15% NaCl concentration.

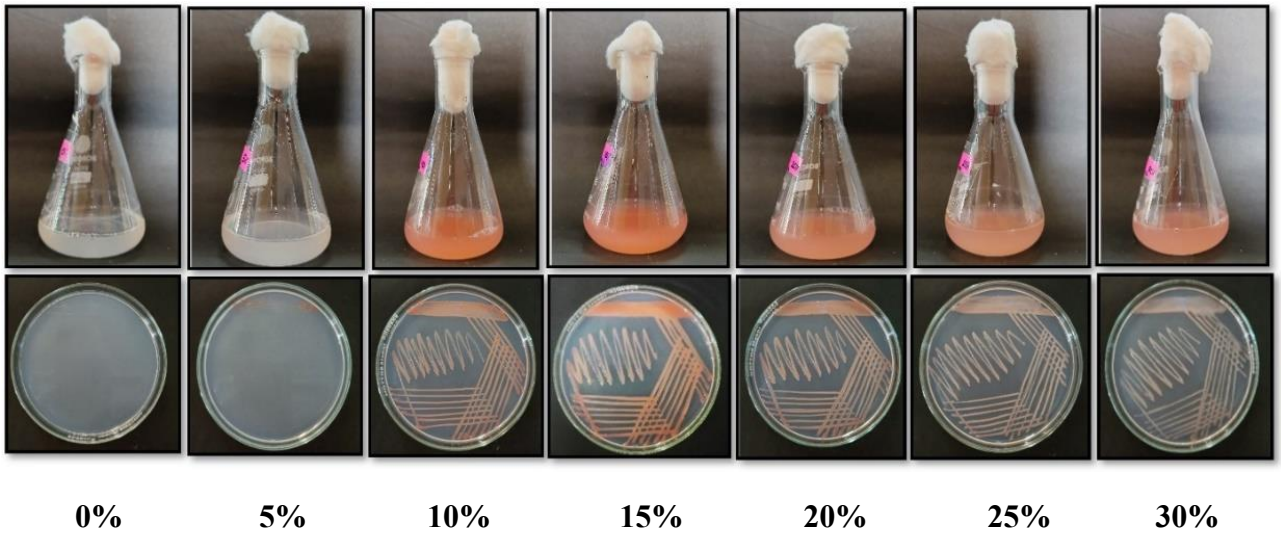


Fig 4.4.1.a: Growth of GUPPII isolate in different NaCl concentrations

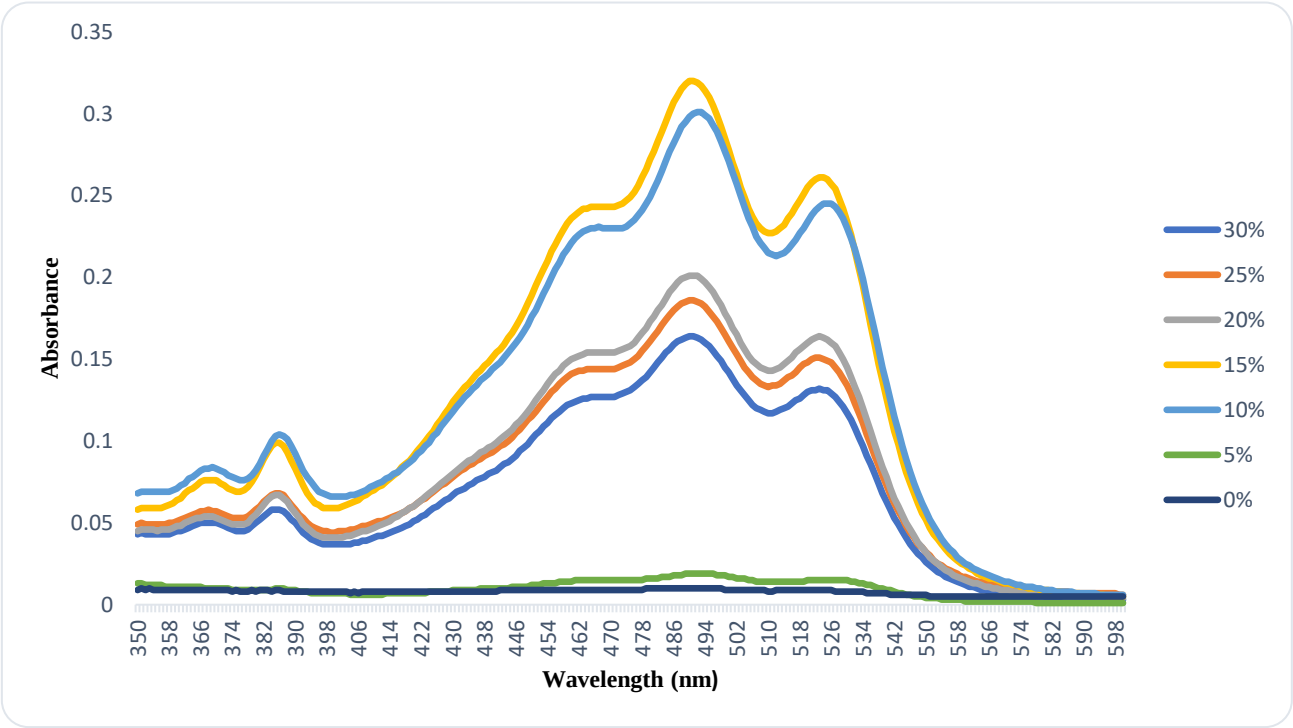


Fig 4.4.1.b: Carotenoid production by GUPPII isolate at different NaCl concentrations

4.4.2 Substrate concentration

The isolate GUPPI was grown in NH media containing different glucose concentrations i.e., 0%, 0.5% 1.0%, 1.5%, 2.0%. After 15 days of incubation it was observed that the growth (fig4.4.2.a) as well as the carotenoid production (fig 4.4.2.b) was maximum in media containing 2% glucose concentration

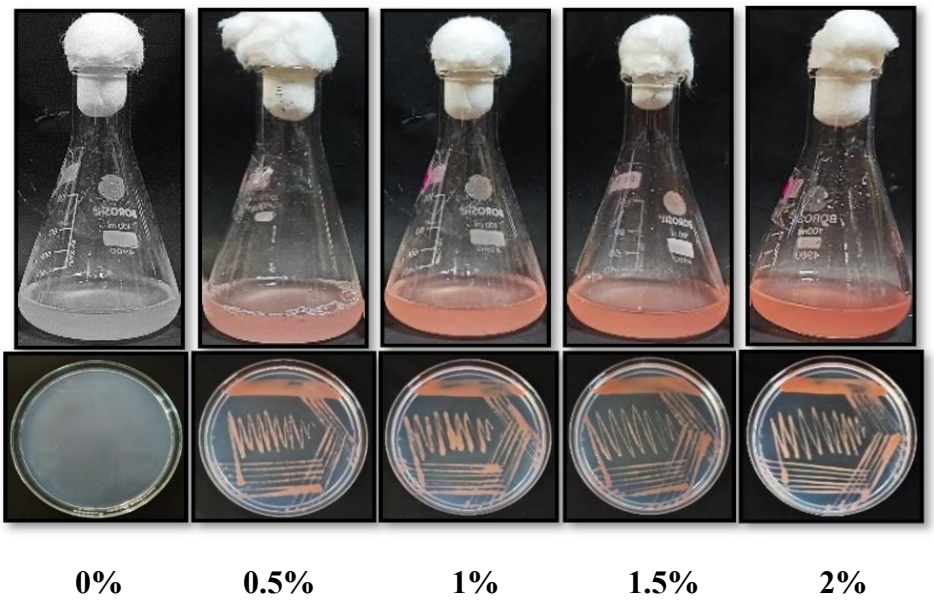


Fig 4.4.2.a: Growth of GUPPI isolate in different glucose concentrations

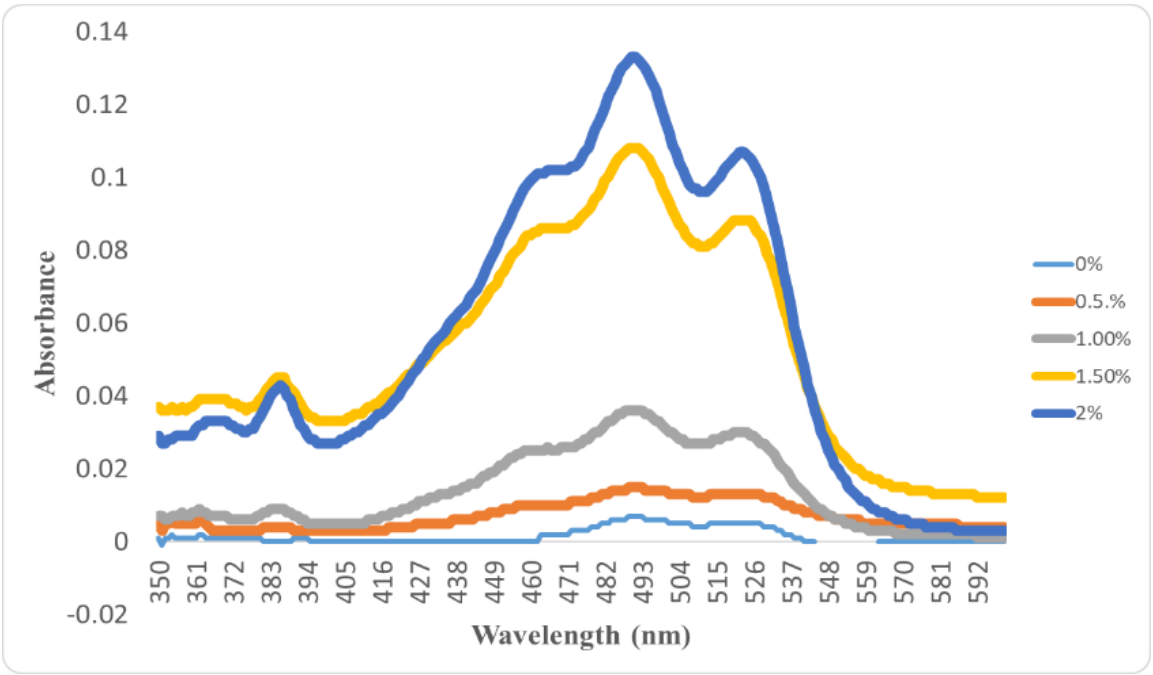


Fig 4.4.2.b: Carotenoid production by GUPPI isolate at different glucose concentration

The isolate GUPPII was grown in NH media containing different glucose concentrations i.e., 0%, 0.5% 1.0%, 1.5%, 2.0%. After 15 days of incubation it was observed that the growth (fig 4.4.2.c) as well as the carotenoid production (fig 4.4.2.d) was maximum in media containing 2% glucose concentration

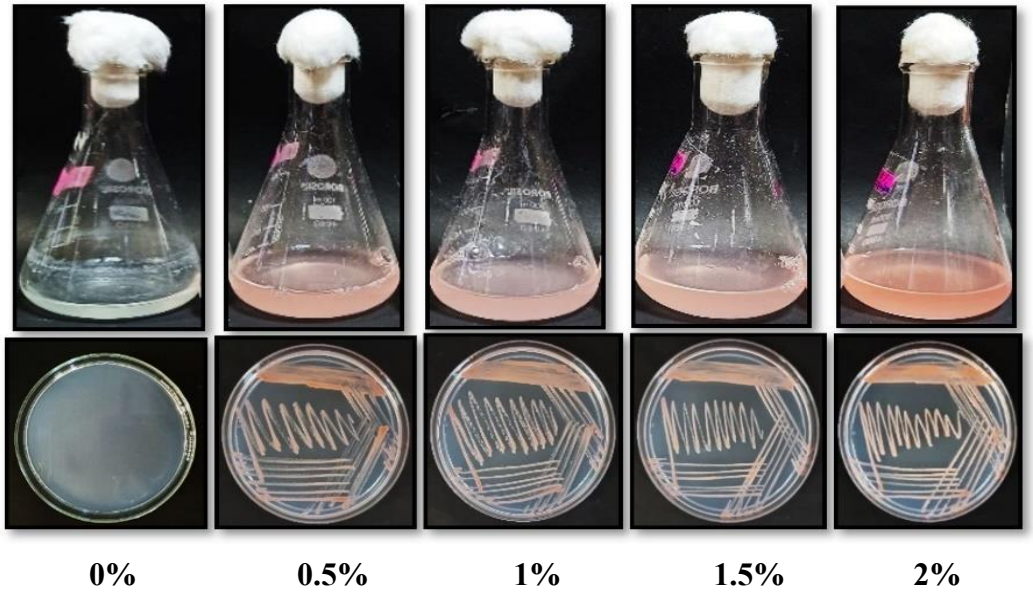


Fig 4.4.2.c: Growth of GUPPII isolate in different glucose concentrations

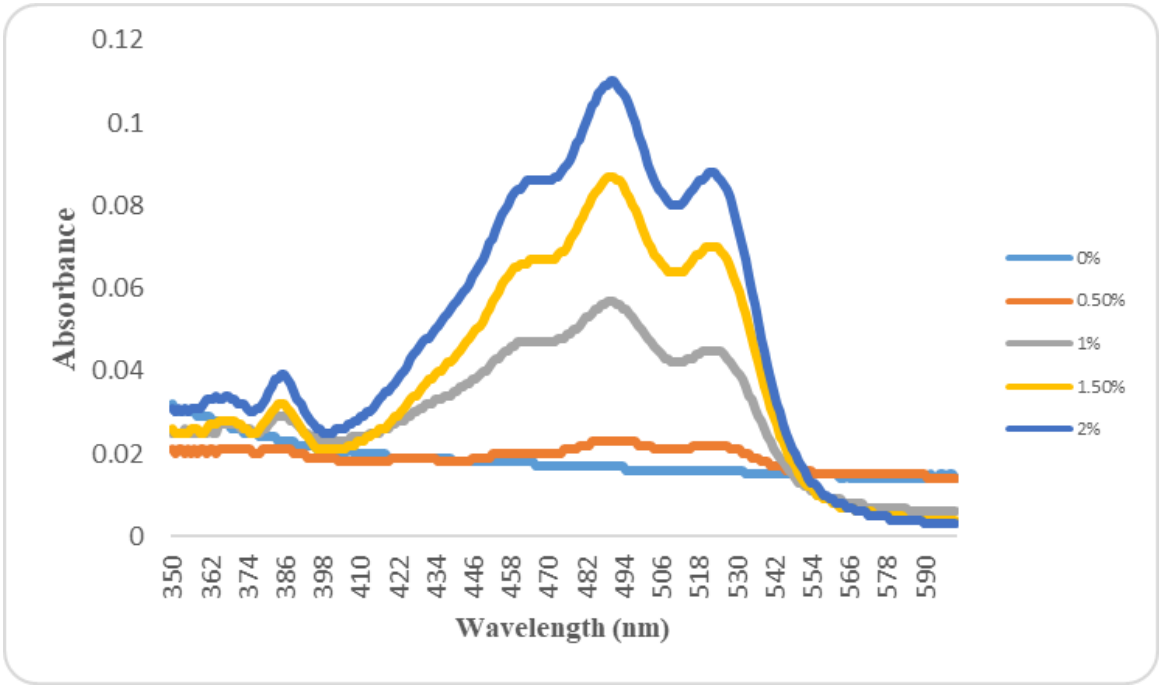


Fig 4.4.2.d: Carotenoid production by GUPPII isolate at different glucose concentration

4.4.3 pH Range

The isolate GUPPII was grown in NH media containing different pH range i.e. 5, 6, 7, 8 and 9. After 15 days of incubation it was observed that the growth (fig 4.4.3.a) as well as the carotenoid production (fig 4.4.3.b) was maximum in media with pH 7.

5 **6** **7** **8** **9**

Fig 4.4.3.a: Growth of GUPPII isolate in different pH range

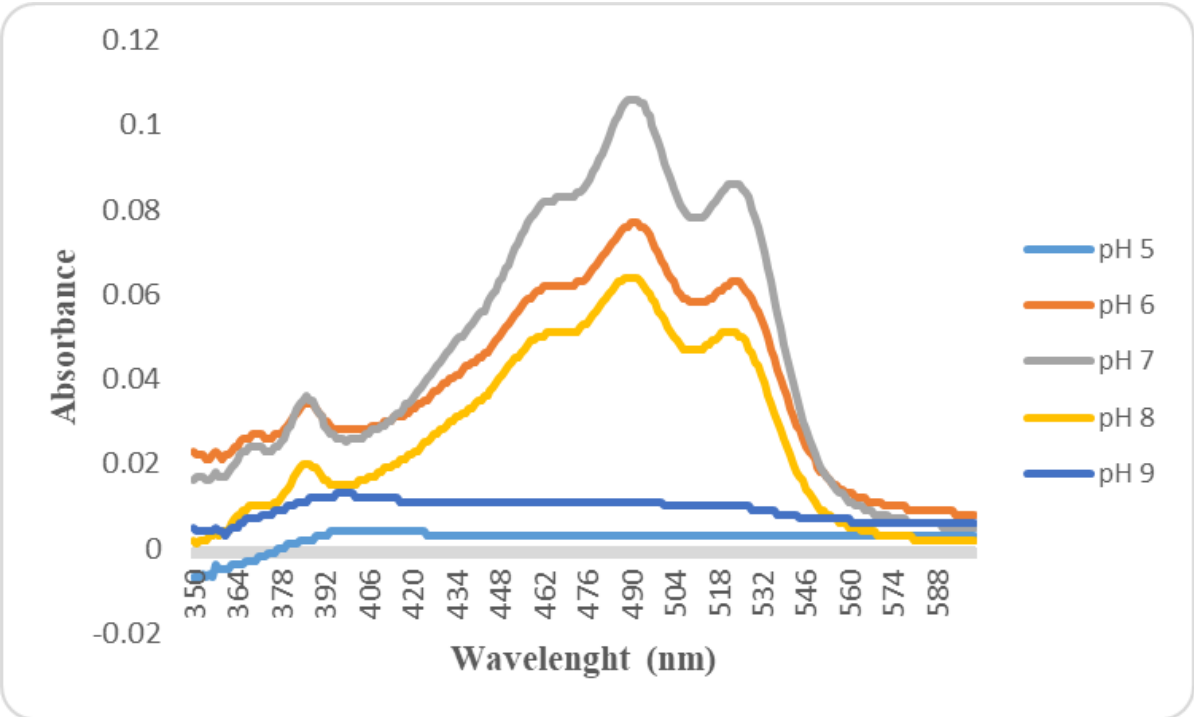


Fig 4.4.3.b: Carotenoid production by GUPPII isolate in different pH range

The isolate GUPPII was grown in NH media containing different pH range i.e. 5, 6, 7, 8 and 9. After 15 days of incubation it was observed that the growth (fig 4.4.3.c) as well as the carotenoid production (fig 4.4.3.d) was maximum in media with pH 7.

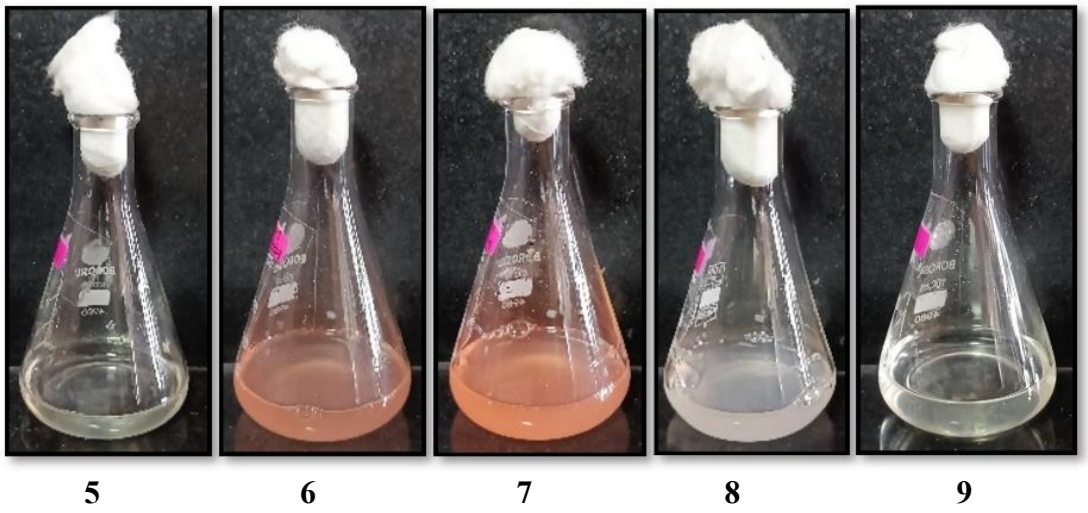


Fig 4.4.3.c: Growth of GUPPI isolate in different pH range

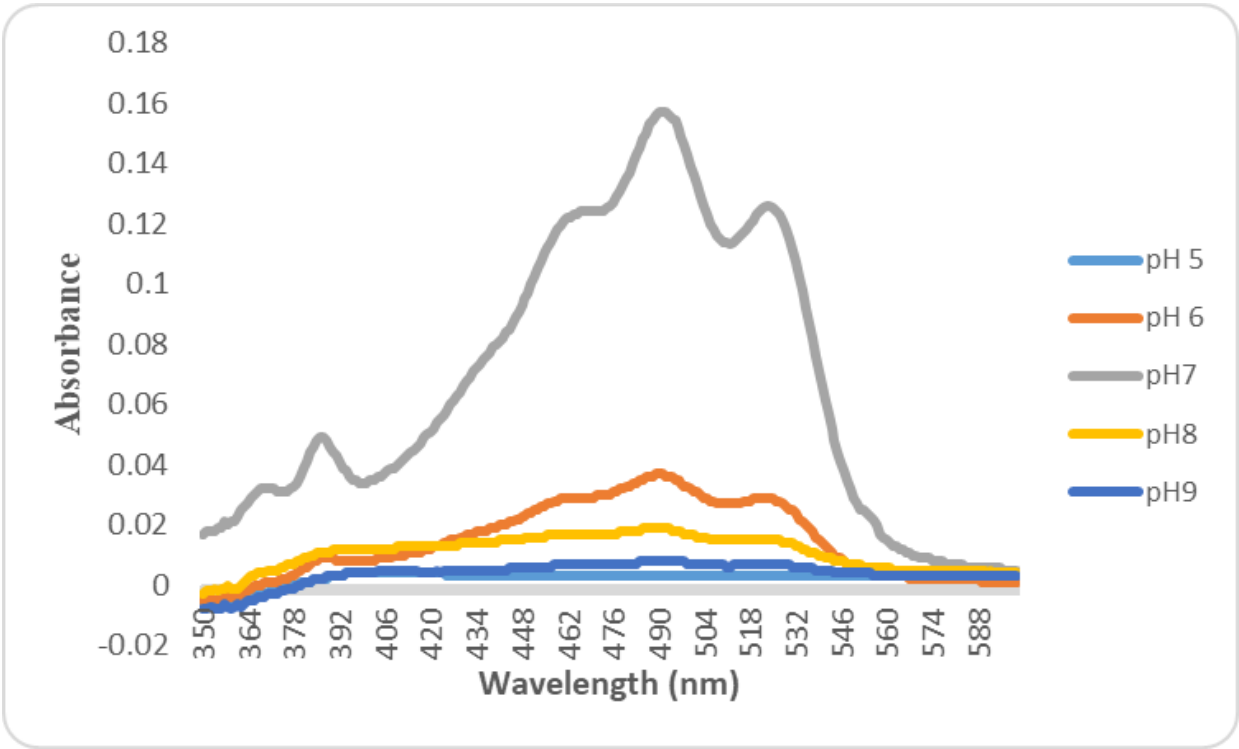


Fig 4.4.3.d: Carotenoid production by GUPPI isolate in different pH range

4.5 Studies on the effects of halophilic cultures on Gold fish

4.5.1 Aquarium setup

Essential factors for tank construction are the size and shape of the tank, thickness of the glass, volume of water and density of fish that it holds.

In this experiment the size of the tanks used was 60 x 30 x 30 (LxBxH), the thickness was 4mm, the water holding capacity of the tank was 50L.

After fabrication the tanks were filled with water to check if there is any leakage (fig 4.5.1).

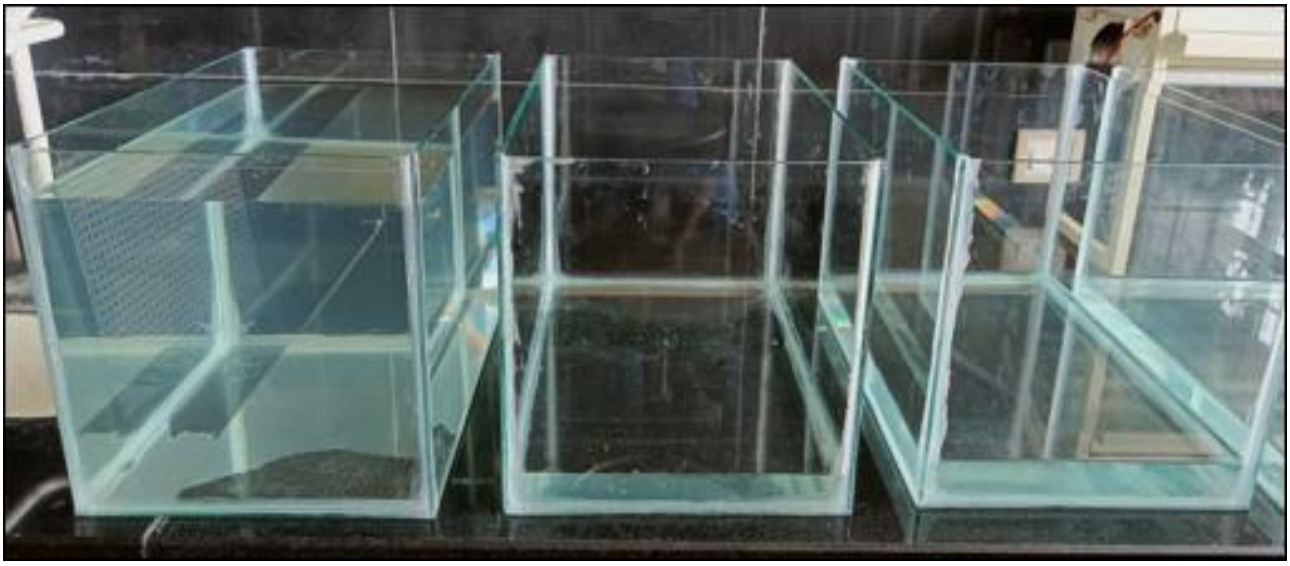


Fig 4.5.1: Rectangular glass tanks used for the experiment

The tanks were cleaned and washed properly and placed on thermocol sheets to prevent crashes, filter was installed in each tank and the tanks were filled with water. Before adding fishes to the tank de-chlorination of water was done. The de-chlorination method was followed by adding 1 drop of white-o-liquid per Gallon of water and the water was stored for a week. Also a spare tank was kept for storing water.

4.5.2 Experimental fish

Goldfish (*Carassius auratus*) is the most popular variety of ornamental fish. Besides body shape, fin shape, and size, an important characteristic affecting the market price of goldfish is body colour. Fishes were brought from Central Coastal Agricultural Research Institute (ICAR), from fisheries department on 15 March 2024, which is situated in Ella, Goa, India (having latitude 15.495672º and longitude 73.917084) (fig 4.5.2.a).

Fig 4.5.2.a: Experimental fsh collection from Fisheries department of Central Coastal Agricultural Research Institute (ICAR), Ella, Goa.

(i) Acclimation of fish to the tank

After reaching lab the fish bags were allowed to float in the aquarium for 10–15 minutes to acclimate them to the water temperature (fig 4.5.2.b).

Fig 4.5.2.b: Experimental fishes kept for acclimation

After acclimation the fishes were released from the bags. Three tanks were maintained and in each tank 6 fish were kept. And the tanks were labelled as Control, Commercial and Test as per the feed to be supplemented (fig 4.5.2.c).

Fig 4.5.2.c: Labelling of tanks as Control, Commercial and Test

(ii) Water parameters

Water parameters monitored were Temperature, pH, ammonia, nitrate and nitrite using API freshwater master test kit. All this parameter were checked weekly and the tanks were cleaned after every 4 days, 25% of water was changed and stored water was added. Temperature was maintained at 26-27ºC, pH of 7-8 and ammonia, nitrate and nitrite of 0ppm.

4.5.3 Formulation of fish feed and proximate analysis

Feed was prepared (b and c) by adding all the ingredients and a control was kept in which no culture was added, in test feed 7% culture was added for 100g, also commercial feed was used to compare the carotenoid content (fig 4.5.3.a).

Fig. 4.5.3.a: (a) Commercial fish feed for ornamental fish and the Microbiology laboratory formulated fish feed (b) control without culture and (c) with culture.

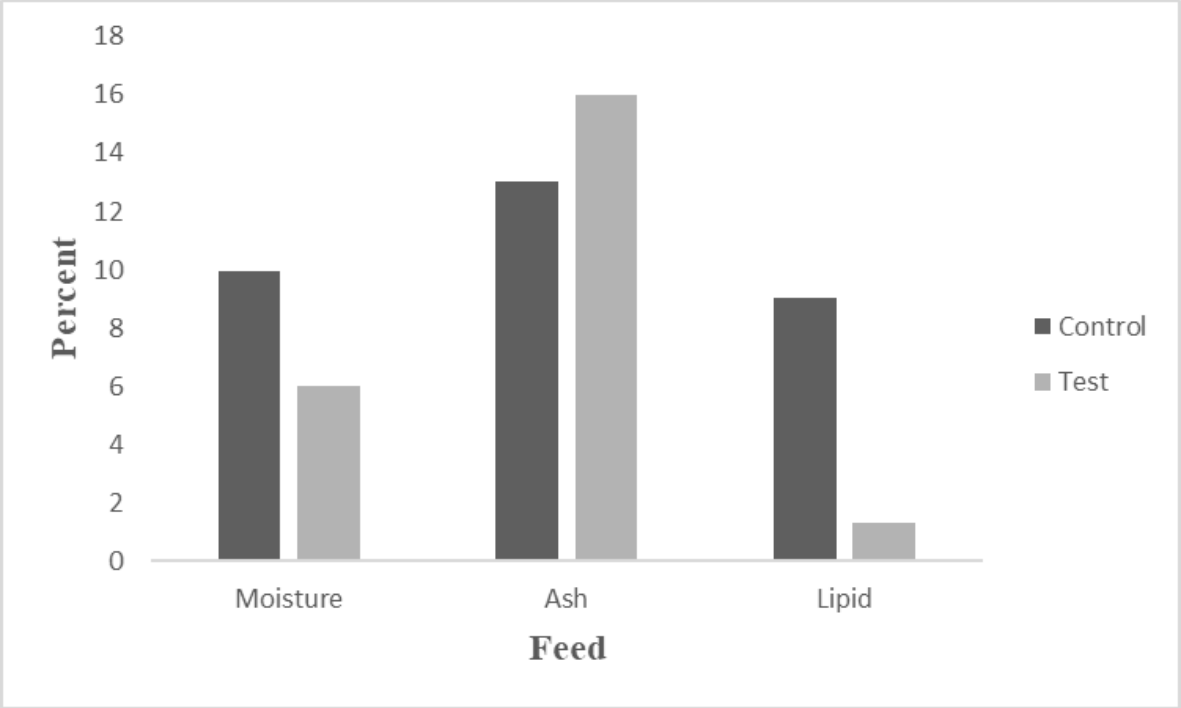


Fig 4.5.3.b: Proximate analysis of the Microbiology laboratory formulated fish feed without culture (Control) and with culture (Test).

Proximate analysis was done of the formulated feed and it was found that moisture content in control was 9.93% and in Test was 6%, ash content in control was 13% and in Test was 16%, Lipid content in Control was 9.03% and in Test was 1.33% (fig 4.5.3.b).

Table no 4.5.3: Proximate composition of the Microbiology laboratory formulated feed

Proximate composition	Control	Test
Moisture %	9.93	6
Ash %	13	16
Lipids %	9.03	1.33

4.5.4 Growth performance of fish fed formulated diet

The growth responses of Gold fish in terms of initial and final average weight is presented in (Table 4.5.4).

All fish grew normally, and no specific signs of disease were observed. All diets were accepted equally well by the fish. Fish weight increased at the end of the feeding trial. The carotenoid-supplemented diets did not appear to have any effect on goldfish growth rate.

Table 4.5.4: Growth performance indices and survival rate

	Dietary treatment		
	Control	**Test**	**Commercial**
Initial weight (g)	9.20	9.221	9.131
Final weight (g)	11.012	11.078	10.344
Weight gain(g)	1.811	1.857	1.631
Average daily weight gain(g)	0.064	0.066	0.058
WGP (%)	19.69	20.182	13.34
Length gain(cm)	1.1	1.5	0.9
Average daily length gain(cm)	0.039	0.053	0.032
FCR	0.152	0.176	1.183
Survival rate %	100	100	100

4.5.5 Carotenoid content analysis

After 4 weeks of feeding the gold fishes were killed by immersing them in ice slurry to obtain carotenoid content. The carotenoids were extracted from scales of the fish.

It was found that the fish fed formulated feed with culture contained more carotenoids than compared to fishes fed with formulated feed without culture and commercial feed.

The carotenoid content in fish fed with formulated feed containing culture was 6.60µg/g, fish fed with formulated feed without culture was 4.48µg/g and fish fed with commercial feed was 3.01µg/g (table no. 4.5.5)

It was also concluded that the lab formulated feed contained more carotenoids than commercial feed available in the market.

From this we can say that Carotenoids from Halophilic microorganisms can be used to enhance pigmentation in ornamental fish. And may also help to reduce susceptibility to disease.

Table 4.5.5: Carotenoid content in scales of juvenile gold fish fed experimental diets

Feed	**Carotenoid content in scales µg/g**
Commercial	3.01±0.64
Control	4.48±0.58
Test	6.60±0.29

A]1st week **Commercial** **Control** **Test**

B]4stweek

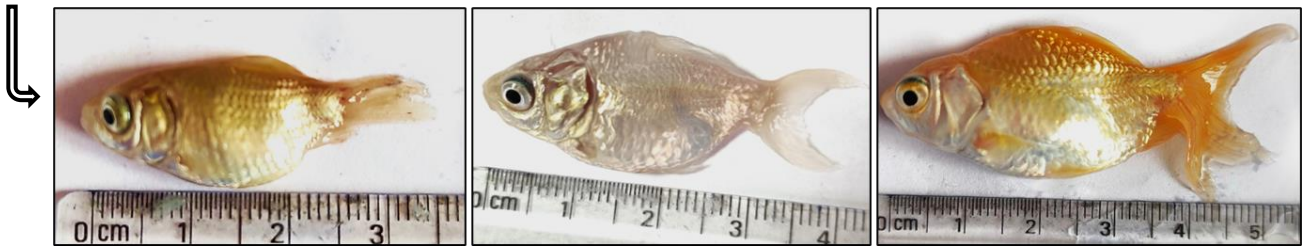


Fig 4.5.5.a: Size and colour differences in juvenile gold fish fed experimental diets.

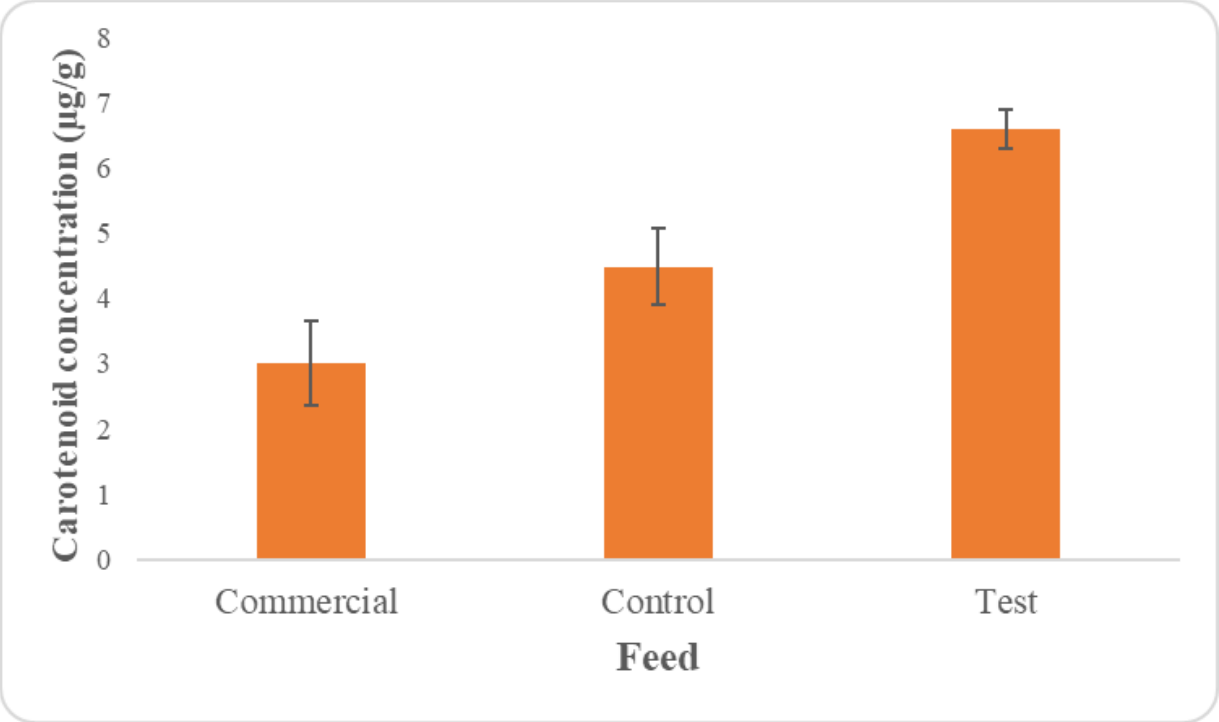


Fig 4.5.5.b: Carotenoid content in scales of fish fed experimental diets

Conclusion

In the present study, two halophilic cultures were isolated from the brine and sediment samples obtained from the Sali pan of shiroda, Maharashtra.

Both isolates were brightly pigmented and required 15% NaCl for their growth. The isolates were visible after 15 days of incubation.

Gram stain performed showed that the isolates are Gram negative.

These isolates showed presence of characteristic glycolipids and phospholipids when separated by thin layer chromatography (TLC).

When the isolates were spot inoculated on 15% NH media plates containing antibiotic Penicillin G, the isolates GUPPI and GUPPII were able to grow in presence of antibiotic indicating antibiotic resistant which is a typical feature of Archaea.

Carbohydrate utilization test was caried out using 15 sugars, Raffinose, Mannitol, Sucrose, Glucose, Lactose, Mannose, Arabinose, Xylose, Ribose, Sorbitol, Trehalose, Maltose, Glycerol, Fructose and Galactose. Isolates GUPPI isolate utilizes raffinose, mannitol, sucrose, glucose, lactose, mannose, xylose, ribose, sorbitol, trehalose, maltose, glycerol, fructose and galactose whereas isolate GUPPII utilizes sucrose, glucose, mannose, xylose, ribose, trehalose, maltose, glycerol, fructose and galactose

Catalase test was done by using hydrogen peroxide reagent, both isolates GUPPI and GUPPII were catalase positive which was indicated with formation of effervescence.

Oxidase test was carried out with 1% N, N, N′, N′-tetra-methyl-p-phenylenediamine dihydrochloride reagent, isolate GUPPI is oxidase positive which was indicated with deep purple colour.

Both isolates were screened for extracellular hydrolytic enzymes from which both cultures were producing Xylanase, Chitinase and Cellulase. none of the cultures were found to be producing Gelatinase, Protease, Amylase, Esterase, Lipase, Pectinase.

Different parameters like NaCl concentration, Glucose concentration and pH range were optimized for maximum growth and carotenoid production. And it was found that both the isolates grow best at 15% NaCl, 2% glucose and pH 7.

Formulated fish feed and supplemented with carotenoids by adding 7% of culture. Control, commercial and test was kept. In control contained no carotenoids and in Test carotenoids were added by adding culture. Proximate analysis was carried out of both Control and Test, moisture content, ash content and lipid content was determined.

Experiment was done on 18 juvenile common Gold fish (*Carassius auratus*), 3 tanks were maintained as Control, Commercial and Test each containing 6 fish. Initial weight was recorded of all fish and fed twice a day, feed added was 5% of their body mass. Final weight was recorded of all fish at the time of slaughter. Scales were removed and put for extraction and total carotenoid was obtained.

And it was found that fishes supplemented wish Test feed contained greater amounts of carotenoids then control and commercial feed, also it was concluded that the feed that was formulated contained more carotenoids than commercial.

References

REFERENCES:

1. Riehl, Rüdiger. Editor.; Baensch, HA (1996). Aquarium Atlas (5th ed.). Germany: Tetra Press. ISBN 3-88244-050-3. {{cite book}}: |first= has generic name (help)
2. Axelrod, Herbert R. (1996). Exotic Tropical Fishes. T.F.H. Publications. ISBN 0-87666-543-1.
3. Nelson, David L.; Cox, Michael M. (2005). Principles of Biochemistry (4th ed.). New York: W. H. Freeman. ISBN 0-7167-4339-6.
4. ^ Yabuzaki, Junko (2017-01-01). "Carotenoids Database: structures, chemical fingerprints and distribution among organisms". Database. **2017** (1). doi:10.1093/database/bax004. PMC 5574413. PMID 28365725.
5. Hoseinifar, S. H., Maradonna, F., Faheem, M., Harikrishnan, R., Devi, G., Ringø, E., ... & Carnevali, O. (2023). Sustainable ornamental fish aquaculture: the implication of microbial feed additives. Animals, 13(10), 1583.
6. Silas, E. G., Gopalakrishnan, A., Ramachandran, A., Anna Mercy, T. V., Sarkar, K., Pushpangadan, K. R., ... & Anikuttan, K. K. (2011). Guidelines for green certification of freshwater ornamental fish.
7. Rani, P., Immanuel, S., & Ranjan, N. (2014). Ornamental fish exports from India: Performance, competitiveness and determinants.
8. Torrissen, O. J., R. W. Hardy, and K. D. Shearer. 1989. "Pigmentation of salmonids—carotenoid deposition and metabolism." CRC Critical Reviews in Aquatic Sciences 1: 209–225.
9. Gomes, E., Dias, J., Silva, P., Valente, L., Empis, J., Gouveia, L., ... & Young, A. (2002). Utilization of natural and synthetic sources of carotenoids in the skin

pigmentation of gilthead seabream (Sparus aurata). European Food Research and Technology, 214, 287-293.

10. Shahidi, F., & Brown, J. A. (1998). Carotenoid pigments in seafoods and aquaculture. Critical Reveiws in Food Science, 38(1), 1-67
11. Lovell, R. T. (1992). Dietary enhancement of color in ornamental fish. Aquaculture Magazine, 18(5), 77-79.
12. Britton, G. (1995). Structure and properties of carotenoids in relation to function. The FASEB Journal, 9(15), 1551-1558.
13. Johnson, E. A. (1989). A pigment source in salmonid feed. Feed management, 40(12), 18-21.
14. NOORI, A., & Alireza, R. A. Z. I. (2017). Effects of dietary astaxanthin on the growth and skin and muscle pigmentation of sexually immature rainbow trout Oncorhynchus mykiss (Walbaum, 1792) (Teleostei: Salmonidae). Iranian Journal of Ichthyology, 4(4), 361-374
15. Rekha, R., Nimsi, K. A., Manjusha, K., & Sirajudheen, T. K. (2022). Marine yeast Rhodotorula paludigena VA 242 a pigment enhancing feed additive for the ornamental fish koi carp. Aquaculture and Fisheries.
16. Edbeib, M. F., Wahab, R. A., & Huyop, F. (2016). Halophiles: biology, adaptation, and their role in decontamination of hypersaline environments. World Journal of Microbiology and Biotechnology, 32, 1-23.
17. Saito, T., Miyabe, Y., Ide, H., & Yamamoto, O. (1997). Hydroxyl radical scavenging ability of bacterioruberin. Radiation Physics and Chemistry, 50(3), 267-269.
18. Jehlička, J., Edwards, H. G. M., & Oren, A. (2013). Bacterioruberin and salinixanthin carotenoids of extremely halophilic Archaea and Bacteria: a Raman spectroscopic

study. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 106, 99-103.

19. Xie, W., Ma, Y., Ren, B., Gao, M., & Sui, L. (2021). Artemia nauplii enriched with archaea Halorubrum increased survival and challenge tolerance of Litopenaeus vannamei postlarvae. Aquaculture, 533, 736087.
20. Alishahi, M., Karamifar, M., & Mesbah, M. (2015). Effects of astaxanthin and Dunaliella salina on skin carotenoids, growth performance and immune response of Astronotus ocellatus. Aquaculture international, 23, 1239-1248.
21. Pandey, P. K., & Mandal, S. C. (2017, May). Present status, challenges and scope of ornamental fish trade in India. In Conference: Aqua Aquaria India, At Mangalore (pp. 1-10).
22. Lutnæs BF, Oren A & Liaaen-Jensen S (2002) New C40-carotenoid acyl glycoside as principal carotenoid in Salinibacter ruber, an extremely halophilic eubacterium. J Nat Prod 65: 1340–1343
23. Oren A & Rodrıguez-Valera F (2001) The contribution of Salinibacter species to the red coloration of saltern crystallizer ponds. FEMS Microbiol Ecol 36: 123–130.
24. Hoseinifar, S. H., Maradonna, F., Faheem, M., Harikrishnan, R., Devi, G., Ringø, E., ... & Carnevali, O. (2023). Sustainable ornamental fish aquaculture: the implication of microbial feed additives. Animals, 13(10), 1583.
25. Silas, E. G., Gopalakrishnan, A., Ramachandran, A., Anna Mercy, T. V., Sarkar, K., Pushpangadan, K. R., ... & Anikuttan, K. K. (2011). Guidelines for green certification of freshwater ornamental fish.
26. Rani, P., Immanuel, S., & Ranjan, N. (2014). Ornamental fish exports from India: Performance, competitiveness and determinants.

27. Torrissen, O. J., R. W. Hardy, and K. D. Shearer. 1989. "Pigmentation of salmonids—carotenoid deposition and metabolism." CRC Critical Reviews in Aquatic Sciences 1: 209–225.
28. Gomes, E., Dias, J., Silva, P., Valente, L., Empis, J., Gouveia, L., ... & Young, A. (2002). Utilization of natural and synthetic sources of carotenoids in the skin pigmentation of gilthead seabream (Sparus aurata). European Food Research and Technology, 214, 287-293.
29. Shahidi, F., & Brown, J. A. (1998). Carotenoid pigments in seafoods and aquaculture. Critical Reveiws in Food Science, 38(1), 1-67
30. Lovell, R. T. (1992). Dietary enhancement of color in ornamental fish. Aquaculture Magazine, 18(5), 77-79.
31. Britton, G. (1995). Structure and properties of carotenoids in relation to function. The FASEB Journal, 9(15), 1551-1558.
32. Johnson, E. A. (1989). A pigment source in salmonid feed. Feed management, 40(12), 18-21.
33. NOORI, A., & Alireza, R. A. Z. I. (2017). Effects of dietary astaxanthin on the growth and skin and muscle pigmentation of sexually immature rainbow trout Oncorhynchus mykiss (Walbaum, 1792) (Teleostei: Salmonidae). Iranian Journal of Ichthyology, 4(4), 361-374
34. Rekha, R., Nimsi, K. A., Manjusha, K., & Sirajudheen, T. K. (2022). Marine yeast Rhodotorula paludigena VA 242 a pigment enhancing feed additive for the ornamental fish koi carp. Aquaculture and Fisheries.
35. Edbeib, M. F., Wahab, R. A., & Huyop, F. (2016). Halophiles: biology, adaptation, and their role in decontamination of hypersaline environments. World Journal of Microbiology and Biotechnology, 32, 1-23.

36. Saito, T., Miyabe, Y., Ide, H., & Yamamoto, O. (1997). Hydroxyl radical scavenging ability of bacterioruberin. Radiation Physics and Chemistry, 50(3), 267-269.

37. Jehlička, J., Edwards, H. G. M., & Oren, A. (2013). Bacterioruberin and salinixanthin carotenoids of extremely halophilic Archaea and Bacteria: a Raman spectroscopic study. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 106, 99-103.

38. Xie, W., Ma, Y., Ren, B., Gao, M., & Sui, L. (2021). Artemia nauplii enriched with archaea Halorubrum increased survival and challenge tolerance of Litopenaeus vannamei postlarvae. Aquaculture, 533, 736087.

39. Alishahi, M., Karamifar, M., & Mesbah, M. (2015). Effects of astaxanthin and Dunaliella salina on skin carotenoids, growth performance and immune response of Astronotus ocellatus. Aquaculture international, 23, 1239-1248.

40. Mongodin EF, Nelson KE, Daugherty S et al. (2005) The genome of Salinibacter ruber: convergence and gene exchange among hyperhalophilic bacteria and archaea. P Natl Acad Sci USA 102: 18147–18152

41. Geng, D., Wang, Y., Wang, P., Li, W., Sun, Y., 2003. Stable expression of hepatitis B surface antigen gene in Dunaliella salina (Chlorophyta). J. Appl. Phycol. 15, 451–456. https://doi.org/10.1023/B:JAPH.0000004298.89183.e5.

42. Lou, S., Zhu, X., Zeng, Z., Wang, H., Jia, B., Li, H., Hu, Z., 2020. Identification of microRNAs response to high light and salinity that involved in beta-carotene accumulation in microalga Dunaliella salina. Algal Res 48, 101925. https://doi.org/ 10.1016/j.algal.2020.101925.

43. Monte, J., Bernardo, J., Sa, ´ M., Parreira, C., Galinha, C.F., Costa, L., Casanovas, C., Brazinha, C., Crespo, J.G., 2020. Development of an integrated process of membrane

filtration for harvesting carotenoid-rich Dunaliella salina at laboratory and pilot scales. Separ. Purif. Technol. 233, 116021. https://doi.org/10.1016/j. seppur.2019.116021

44. Abu-Rezq, T. S., Al-Hooti, S., & Jacob, D. A. (2010). Optimum culture conditions required for the locally isolated Dunaliella salina. J. Algal Biomass Utln, 1(2), 12-19.
45. Borowitzka, M. A., & Siva, C. J. (2007). The taxonomy of the genus Dunaliella (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. Journal of Applied Phycology, 19, 567-590.
46. García-Chavarría, M., & Lara-Flores, M. (2013). The use of carotenoid in aquaculture. Research Journal of Fisheries and Hydrobiology, 8(2), 38-49.
47. Quadri, I., Hassani, I. I., l'Haridon, S., Chalopin, M., Hacène, H., & Jebbar, M. (2016). Characterization and antimicrobial potential of extremely halophilic archaea isolated from hypersaline environments of the Algerian Sahara. Microbiological Research, 186, 119-131.
48. Schneegurt, M. A. (2012). Media and conditions for the growth of halophilic and halotolerant bacteria and archaea. Advances in understanding the biology of halophilic microorganisms, 35-58.
49. Sanders, E. R. (2012). Aseptic laboratory techniques: plating methods. JoVE (Journal of Visualized Experiments), (63), e3064.
50. Vahed, S. Z., Forouhandeh, H., Hassanzadeh, S., Klenk, H. P., Hejazi, M. A., & Hejazi, M. S. (2011). Isolation and characterization of halophilic bacteria from Urmia Lake in Iran. Microbiology, 80, 834-841.
51. Yu, J., Lu, K., Dong, X., & Xie, W. (2022). Virgibacillus sp. SK37 and Staphylococcus nepalensis JS11 as potential starters to improve taste of shrimp paste. LWT, 154, 112657.

52. Sousa, A. M., Machado, I., Nicolau, A., & Pereira, M. O. (2013). Improvements on colony morphology identification towards bacterial profiling. Journal of microbiological methods, 95(3), 327-335.
53. Emerson, D., Chauhan, S., Oriel, P., Breznak, J.A., 1994. Haloferax sp. D1227, a halophilic Archaeon capable of growth on aromatic compounds. Archives of Microbiology 161, 445–452
54. Sahli, K., Gomri, M. A., Esclapez, J., Gómez-Villegas, P., Ghennai, O., Bonete, M. J., ... & Kharroub, K. (2020). Bioprospecting and characterization of pigmented halophilic archaeal strains from Algerian hypersaline environments with analysis of carotenoids produced by Halorubrum sp. BS2. Journal of basic microbiology, 60(7), 624-638.
55. Naziri, D., Hamidi, M., Hassanzadeh, S., Tarhriz, V., Zanjani, B. M., Nazemyieh, H., ... & Hejazi, M. S. (2014). Analysis of carotenoid production by Halorubrum sp. TBZ126; an extremely halophilic archeon from Urmia Lake. Advanced pharmaceutical bulletin, 4(1), 61.
56. Balkrishna, S. B. (2015). Synthesis of polyhydroxyalkanoates by halophilic archaea and bacteria and their osmoadaptation.
57. Reiner, K. (2010). Catalase test protocol. American society for microbiology, 1(1), 1-9.
58. Rods, G. P. (2014). UK standards for microbiology investigations. Public Health England.
59. Das, D., Kalra, I., Mani, K., Salgaonkar, B. B., & Braganca, J. M. (2019). Characterization of extremely halophilic archaeal isolates from Indian salt pans and their screening for production of hydrolytic enzymes. Environmental Sustainability, 2, 227-239

60. Whaley, D. N., Dowell Jr, V. R., Wanderlinder, L. M., & Lombard, G. L. (1982). Gelatin agar medium for detecting gelatinase production by anaerobic bacteria. Journal of Clinical Microbiology, 16(2), 224-229.
61. Sharma, N., Rathore, M., & Sharma, M. (2013). Microbial pectinase: sources, characterization and applications. Reviews in Environmental Science and Bio/Technology, 12(1), 45-60.
62. García-de Blas, E., Mateo, R., Viñuela, J., & Alonso-Álvarez, C. (2011). Identification of carotenoid pigments and their fatty acid esters in an avian integument combining HPLC–DAD and LC–MS analyses. Journal of Chromatography B, 879(5-6), 341-348.
63. Passos, M. L., & Saraiva, M. L. M. (2019). Detection in UV-visible spectrophotometry: Detectors, detection systems, and detection strategies. Measurement, 135, 896-904.
64. Azhar, M., Uniyal, V., Chauhan, N., & Rawat, D. S. (2014). Isolation and biochemical characterization of Halophiles from Sahastradhara region, Dehradun, India. Int. J. Curr. Microbial. App. Sci, 3, 753-760.
65. Elevi, R., Assa, P., Birbir, M., Ogan, A., & Oren, A. (2004). Characterization of extremely halophilic Archaea isolated from the Ayvalik Saltern, Turkey. World Journal of Microbiology and Biotechnology, 20, 719-725.
66. Singh, A., & Singh, A. K. (2018). Isolation, characterization and exploring biotechnological potential of halophilic archaea from salterns of western India. 3 Biotech, 8(1), 45.
67. Torrissen, O. J., & Naevdal, G. (1984). Pigmentation of salmonids—genetical variation in carotenoid deposition in rainbow trout. Aquaculture, 38(1), 59-66.
68. Lovell RT. Laboratory Manual for fish feed analysis and fish Nutrition study, Aubum University. 1975, 3-5.
69. Maynard J. Methods of Food Analysis, Academic Press, New York, NY. 1970, 163.

70. Naziri, D., Hamidi, M., Hassanzadeh, S., Tarhriz, V., Maleki Zanjani, B., Nazemyieh, H., Hejazi, M. A., & Hejazi, M. S. (2014). Analysis of Carotenoid Production by Halorubrum sp. TBZ126; an Extremely Halophilic Archeon from Urmia Lake. Advanced pharmaceutical bulletin, 4(1), 61–67. https://doi.org/10.5681/apb.2014.010

71. Khelaifia, S., Caputo, A., Andrieu, C., Cadoret, F., Armstrong, N., Michelle, C., ... & Raoult, D. (2018). Genome sequence and description of Haloferax massiliense sp. nov., a new halophilic archaeon isolated from the human gut. Extremophiles, 22, 485-498.

Appendix

Appendix I: Composition of media

1) NH media

Ingredients	g/L
NaCl	200
KCl	5.0
$MgSO_4.6H_2O$	10.0
$CaCl_2.2H_2O$	0.2
Yeast extract	1.0
Agar	20.0

2) EHM media

Ingredients	g/L
NaCl	200
KCl	2.0
$MgSO_4.7H_2O$	20.0
$CaCl_2.6H_2O$	0.36
Yeast extract	1.0
NaBr	0.23
$NaHCO_3$	0.06
Peptone	5.0
FeCl3.6H2O	0.005
Agar	20.0

3) NTYE media

Ingredients	g/L
NaCl	200
KCl	5.0
$MgSO_4.7H_2O$	20.0
Yeast extract	3.0
Tryptone	5.0
Agar	20.0

4) NT media

Ingredients	g/L
NaCl	200
KCl	2.0
$MgSO_4.7H_2O$	20.0
Yeast extract	10.0
Trisodium citrate	3.0
Agar	20.0

5) MHM media

Ingredients	g/L
NaCl	178
KCl	2.0
$MgSO_4.7H_2O$	1.0
$CaCl_2.6H_2O$	0.36
NaBr	0.23
$NaHCO_3$	0.06
Peptone	5.0
Yeast extract	10.0
FeCl3.6H2O	0.005
Agar	20.0

Note: media was sterilized by autoclaving at 15 lbs pressure (121ºC) for 15min

Appendix II: Stains and reagents

1) I_2

I_2	0.3%
KI	0.6%

2) Congo red (0.1%)

Congo red	0.1g
Distilled water	100mL

3) Mercuric chloride (15%)

Mercuric chloride	15g
Conc.HCL	20mL
Distilled water	80mL

4) NaCl (15%)

NaCl	15g
Distilled water	100mL

5) 5 M NaOH

NaOH pellets	10g
Distilled water	100mL

6) Gram's iodine

Gram' s iodine	1g
KI	1mL
Distilled water	300mL
(store in amber colour bottle)	

7) Decolourizer

Ethanol	70mL
Distilled water	30mL

8) Acetic acid (2%)

Acetic acid	2mL
Distilled water	98mL

9) Crystal violet

Crystal violet	2g
95% ethanol	20mL
1% ammonium oxalate	80mL

10) Safranine

Safranine	0.5g
Distilled water	100mL

11) Catalase test reagent

Hydrogen peroxide(30%)	10mL
Distilled water	90mL

12) Oxidase test reagent

N,N,N',N'-Tetramethyl-p-phenylene diamine dihydrochloride (1%)	1g
Distilled water	100mL

The reagent is light sensitive, so it was freshly prepared in a covered container just before use

Appendix III: Workshop on Ornamental fish and Poster competition on waste management

Workshop on

Ornamental Fish Keeping, Induced Breeding and Marine Entrepreneurship

Organized by

Biotechnology Discipline, School of Biological Sciences & Biotechnology, Goa University

Certificate

This is to certify that

Pooja Vinay Pingulkar

has actively participated in the workshop on "Ornamental fish keeping, induced breeding and marine entrepreneurship" at Biotechnology Program, School of Biological Sciences & Biotechnology, Goa University, held from 8-10 February 2024.

Convenor
Sr. Prof. Bernard Rodrigues
Dean, SBSB
Goa University

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa-403206

Organizing Secretary
Prof. Sanjeev Ghadi
Vice Dean- Academics, SBSB
Goa University

Dr. Sanjeev C. Ghadi
Professor in Biotechnology
School of Biological Sciences and Biotechnology
Goa University, Goa.

GOA UNIVERSITY

MICROBIOLOGY
SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY

Certificate of Participation

This is to certify that

Dr. / Mr. / Ms. POOJA PINGULKAR

participated / presented a poster at the one-day seminar on

Advances in Waste Management for Sustainable Future

held on 20th February 2024

Prof. Bernard Rodrigues
Convenor & Dean, SBSB

Dr. Trupti Asolkar
Organizing Secretary