Final Project Completion Report

Minor Research Project

entitled

"Development of Protocol for the production of bacterial pigments"

Submitted to

University Grant Commission
Western regional office (WRO)
Ganeshkhind, Pune. 411 007

Submitted by

Ms. V. M. Chaudhari

Department of Microbiology

PSGVP Mandal's, SIP Arts, GBP Science and STSKVS Commerce College, Shahada, Dist. Nandurbar.

(2019)

UNIVERSITY GRANTS COMMISSION
WESTERN REGIONAL OFFICE
GANESHKHIND, PUNE-411 007.

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Name and Address of the Principal Investigator : Prof. Ms. Varsha M. Chaudhari
   Dept. of Microbiology, P.S.G.V.P.Mandal's SIP Arts, GBP Science & STSKVS Commerce College, Shahada, PIN- 425 409, Dist. Nandurbar , (Maharashtra).

2. Name and Address of the Institution: P.S.G.V.P.Mandal's S. I.P. Arts, G.B.P. Science & S.T.K.V.S Commerce College, Shahada,PIN- 425 409, Dist. Nandurbar, (Maharashtra).

3. UGC Approval No and Date: F. 47-964/09 (WRO) dated 01-10-2009

4. Date of Implementation: 01.10.2009

5. Tenure of project 01-10-09- 31-03-13

6. Total grant allocated Rs. 1,30,000/-

7. Total Grant received Rs. 1,24,500/- (1st Install. -1,02,500/- & 2nd In. 22,000/-)

8. Final expenditure Rs. 1, 35,463/-

9. Title of the Project **"Development of protocol for the production of bacterial Pigments"**.

10. **Objectives of the project** Work was carried out on the basis of following objectives. (**Annexure** A)

    1) Isolation and screening of the pigment producing bacteria from soil.
    2) Characterization of isolated organisms.
    3) Extraction of pigments from isolates by various methods.
    4) Study of the applicability of the pigments.

11 Whether the objectives were achieved- Yes, The objectives of the study has been **successfully** completed. (**Annexure** B)

This study underlying the introduction of bacterial pigments was to find a suitable alternative for artificial synthetic colors, which could be used in pharmaceuticals, cosmetics or in food and textile manufacturing. In this study novel pigment producing

bacteria was isolated from distillery effluent and soil sample. The simplest method for bacterial pigment extraction was developed to overcome the higher price of natural pigments as compared to synthetic dyes. Applicability of pigments for growth promotion was studied which showed pigments can use as growth factors.

12. Achievements from the projects- The present study focuses on the microbial production of pigments- bio pigments as an eco-friendly alternative to the present day used environmentally hazardous synthetic colors. It is expected to contribute a lot to the field of environmental microbiology as an alternative approach in microbial production of natural eco-friendly and nontoxic, safe colors.

13. Summary of the findings- (**Annexure** C)

The present work focuses on the development of food pigments from natural sources. Though many natural colors are available, Microbial colorant plays significant role as food coloring agents, because of its flexibility in production and easy recovery. The effect of the pigment on the growth and germination of seeds were checked which revealed that as compared to control, germination was enhanced in the test where seeds coated with the pigment extract. This proved that the microbial pigment could exhibit growth stimulation in the germination of seeds. However, with development of a proper system and protocol this may reveal the expected details regarding the pigment and help in its identification. In this work emphasis is given on the isolation of pigment producing bacteria from soil and effluent source. Then Morphological, biochemical characterization of isolates was carried out. Applicability of the pigment in the growth promotion of seeds were checked which shows the pigment enhances seed germination. This project introduces natural colors in the food or pharmaceutical industry.

14. Contribution to the society- This project introduces natural dyes carotenoids a new pigments in the already existing list of pigments. This pigment introduces new antioxidizing agent in medicine.

15. Whether any Ph.D.enrollled /Produced out of the project- No

16. No. of publications out of the project- 02

9/3/19

(Ms.V.M.Chaudhari)
PRINCIPAL INVESTIGATOR

(Prof.Dr. R.S. Patil)
PRINCIPAL

PRINCIPAL
P.S.G.V.P. M'S, Arts, Science & Commerce Sr. College, SHAHADA
Dist.Nandurbar (Pin-425409)

Annexure A

Brief Objectives of the project

Introduction

Color is associated with many aspects of our life. For example, the main factors to evaluate food quality are color, flavor, and texture, but color can be considered the most important of them, because if it is not appealing consumers will not enjoy the flavor and texture of any given food. Color is added in the food to make it more attractive, appetizing, presentable and informing quality of food. Synthetic colorants are used recently in vigorous amount but there are number of disadvantages of synthetic colors. They are carcinogenic, terratogenic, tumor forming, cancerous. Hence use of synthetic colorants was banned and biocolours are used in the food, pharmaceutical, cosmetics and textiles.

In nature, a variety of microorganisms has exhibited wide diversity in pigment production and has spectacular applications in food, pharmaceutical and textile industries. Some of the more important natural pigments were the carotenoids, flavonoids (anthocyanins) and some tetrapyrroles (chlorophylls, phycobiliproteins).

Thus demand for natural color is increasing day by day as natural colors are better and safe for health when used in food products.

APPLICATIONS OF MICROBIAL PIGMENTS

1. Carotenoids comprise a major class of pigments molecules synthesized in a wide range of organisms including photosynthetic bacteria,fungi unicellular alga .Microorganisms due to their ease of manipulations provide an excellent system for large scale production of carotenoids.

2. IN FOODS ORIENTALS AND BREWERIES

Monascus pigment have been extensively used in asia for centures and are generally regarded as safe (GARS) as food colorant.

3. IN DYEING

Shirata et al (2000) found that the bluish purple pigment obtained from Janthinobacterium lividum was capable of dyeing not only natural fibers but also synthetic fibers.

4.**IN PHARMACEUTICALS**

Many caroenoids have been ascribed antioxidant roles (krensky 1993) in vitro it has been observed that canthaxanthin also has antioxidant capability (packer 1993).

5. **AS A VITAMIN**

The red colored pigment produced by Dunaliella salina is the best source of beta carotene tested by National Institute of Oceanography,Israel (Parrys Delta carotene).

6. AS A SIDEROPHORE AND BICONTROL AGENT

Meyer and Abdallah (1978) isolated a green fluorescent pigment from Pseudomonas species and found to be a siderophore. Siderophore have the capacity to chelate iron.

7. **OTHER USES**

De vrije et al (1998) studied the Monascus pigment for their suitability in nonfood applications like biodegradable coatings and plastics textiles cosmetics and packaging materials.

8. NOVEL APLICATIONS AND VALUE ADDITION TO PRODUCTS

Zeaxanthin a pigment produced by Flavobacterium multivorum could be used for the coloring of legs beaks skin fat flesh and egg –yolk of poultry (US patent No 05308759, 1994) The feeding studies of known amount of pigment from F. multivorum to poutry showed that the pigment is stable, pigments faster and 2-3 times more potent on a pure pigment basis.

In the view of this project work is carried out with the aim of isolation and identification of pigment producing organisms and study of applicability of their pigments.

The project work is done on the basis of the following points

I) Isolation and screening of the pigment producing bacteria from soil.

II) Characterization of isolated organisms.

III) Extraction of pigments from organisms.

IV) Study of the applicability of the pigment

Annexure B

Work done in the project and Objectives (results) achieved

1) Isolation of pigment producing organisms.

A) Soil sample collection-

Rhizospheric soil samples from different regions of Medicinal plant garden of PSGVPM'S Pharmacy college, soil from botanical plant garden of college, Soil from different fields of Shahada region located at Dongargaon region were collected in sterile polythene bags.

B) Effluent sample collection: - Effluent sample of Shri Satpuda Tapi Sahakari Sugar factory was collected in sterile screw cap bottle.

Screening and isolation:-

1gm of soil from each of these samples was enriched for 48 hours in sterile 100 ml Nutrient Broth, in sterile 100 ml potato dextrose broth, in 100 ml Luria Bertani broth. Enriched broths samples were diluted up to 10^{-5} and 0.1 ml of dilution was plated on sterile nutrient agar plate, sterile starch casein agar plate, sterile potato dextrose agar plate, sterile skim milk agar plate, sterile Luria Bertani agar plate and sterile yeast extract mannitol agar plate. Simultaneously loopful of enriched samples were streaked on each of the sterile medium agar plates. All the plates were incubated at 37^0 C up to 48 hrs.

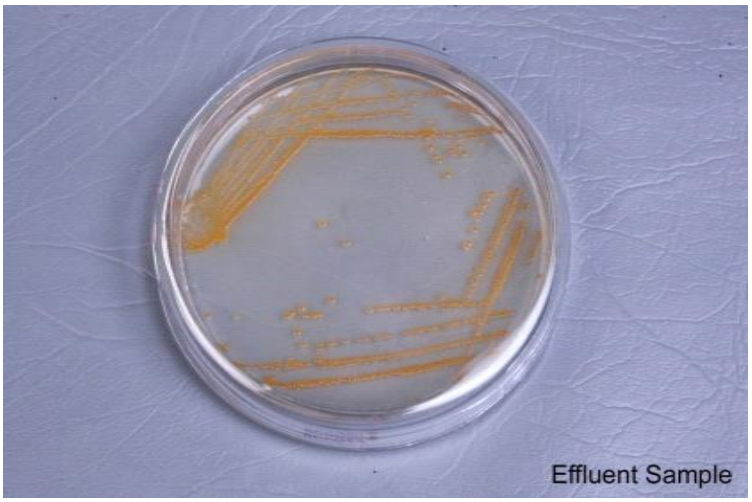
Growth of organisms from each of the sterile nutrient agar plate, sterile starch casein agar plate, sterile potato dextrose agar plate, sterile skim milk agar plate and sterile Luria Bertani agar plates were observed and best pigment producing organisms were selected. These were further purified from mixed population.

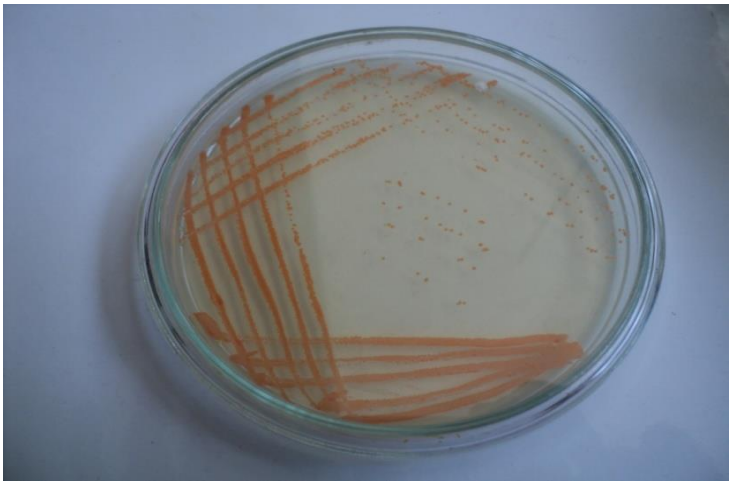
Prominent pigment producing bacteria which showed growth on sterile nutrient agar as well as on sterile yeast extract mannitol agar were selected for further studies.

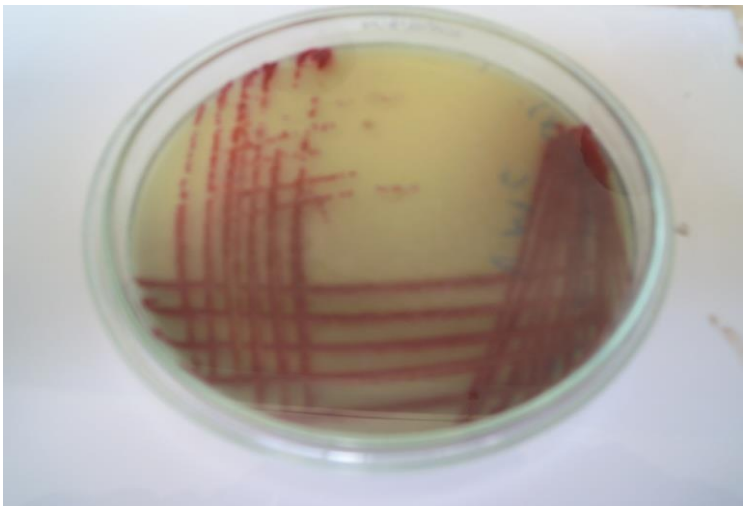
B) Effluent sample:-

Sample of distillery effluent was collected in sterile screw-cap bottles. Sample was serially diluted up to 10^{-6} dilution. From each 10^{-5} and 10^{-6} dilution 0.1ml of sample was transferred aseptically on the sterile nutrient agar plate, starch casein agar plate, sterile potato dextrose agar plate, sterile skim milk agar plate and sterile Luria Bertani agar Plate. All plates were incubated at 37^0 C for 48 hrs. Plates were observed after incubation and best pigment producing organisms were selected. Further purification was done on the same media. Purified organisms were maintained on sterile slants.

Prominent pigment producing bacteria were isolated from soil and effluent sample. The colonies have orange, Dark red, Green and Yellow and Turmeric yellow pigmentation







Isolated bacteria obtained from soil and effluent sample.

Isolation of Pigment Producing Bacteria

Sr. No.	Isolate Number.	Source	Medium	Color
1	Isolate 1	Effluent sample	Sterile Nutrient agar	Orange
2	Isolate 2	Soil sample	Sterile Yeast extract mannitol agar	Dark Red
3	Isolate 3	Soil sample	Sterile Nutrient agar	Green
4	Isolate 4	Soil sample	Sterile Nutrient agar	Orange
5	Isolate 5	Effluent sample	Sterile Nutrient agar	Yellow
6	Isolate 6	Soil sample	Sterile Nutrient agar	Turmeric Yellow.

Table 1:- Isolation of pigment producing bacteria.

Most prominent pigment producing two isolates were selected for further use.

2. Characterization of Isolated organism

Colony characteristics of isolate were studied and recorded.

S. N.	Size	Shape	Color	Margin	Opacity	Consist	Gm stain	Motility
1	.5 mm	Round	Orange	Uneven	Opaque	Moist	Gm+ cocci	Non m.
2	1.5 mm	Round	Orange	Irregular	Opaque	Moist	Gm - rods	Motile

Table 2:- Colony characteristics of different isolates.

Biochemical characteristics of isolates.

Isolates were identified on the basis of Biochemical characteristics from MTCC Chandigarh.

Biochemical characteristics of isolates were as follows.

Sr.No	Tests	Isolate 1	Isolate 2
1	ONPG	-	-
2	Lysine decarboxylase	+	+
3	Ornithine Decarboxylase	+	+
4	Urease	+	+
5	Phenylalanine Deamination	-	-
6	Nitrate reduction	-	-
7	H_2s production	-	-
8	Citrate utilization	+	+
9	Voges Proskauer's	-	-
10	Methyl red	+	+

11	Indole	+	+
12	Malonate	-	-
13	Esculin	-	-
14	Arabinose	-	-
15	Adonitol	-	-
16	Rhamnose	-	-
17	Cellobiose	-	-
18	Melibiose	-	-
19	Saccharose	-	-
20	Raffinose	-	-
21	Trehalose	-	-
22	Glucose	+	+
23	Lactose	+	+
24	Oxidase	-	+

Table 3:- biochemical characteristics of isolates.

On the basis of biochemical characteristics isolate was identified as a *Planococcus maritimus* and *Rhodococcus kropenstedii*.

Molecular identification of bacterial isolates based upon 16S rRNA sequence

16SrRNA sequences obtained from NCCS were submitted to GenBank and are available as **GenBank Accession Numbers JN873343.1** ***Planococcus maritimus strain*** (www.ncbi.nlm.nih.gov/ Blast). The sequences were blasted into Nucleotide Blast Tool' of 'National Center for Biotechnology Information' (available at www.ncbi.nlm.nih.gov/ Blast) for nucleotide homology. The maximum homology report (Taxonomy Blast Report) identified a high nucleotide homology of the 16SrRNA (99% maximum identity in 100 % query coverage) with 16SrDNA/ 16SrRNA. From the analysis of the generated taxonomy report of the 16SrDNA gene sequence, this bacterial strain with highest score of (2628), and lowest E-value (0.0) was identified to be *Planococcus maritimus* . However the bacterial strain under study showed a maximum of 99 per cent homology with the previously reported sequences. This established that the bacterial isolate was identified as *Planococcus maritimus* is a **novel strain** that has not been reported earlier.

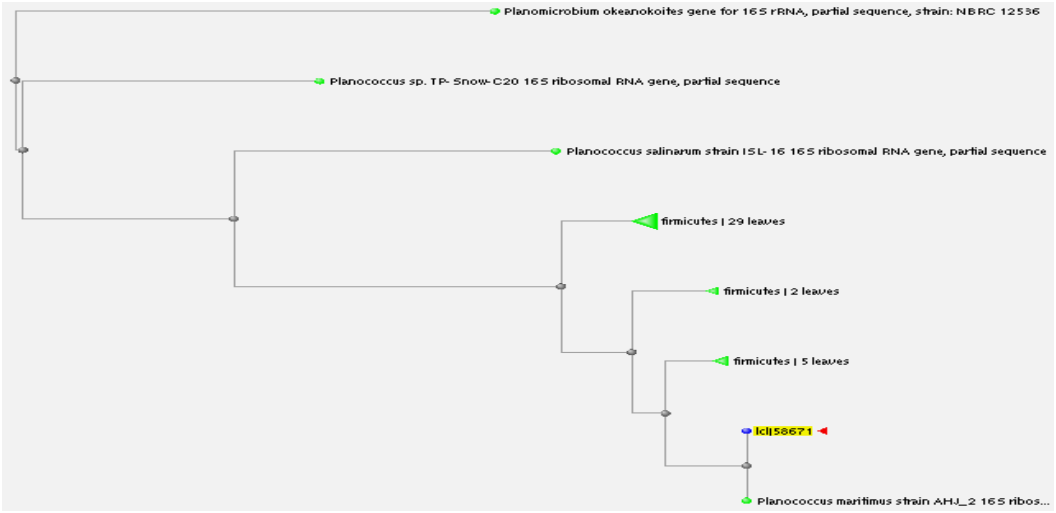


Figure 1: Neighbour-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the phylum Firmicutes

Production of Pigment-

Pigment production was carried out in 500ml Erlenmeyer flask containing 100ml of nutrient medium. 0.1 ml of inoculums was added in the broth and flask was incubated at rotary shaker at 120 rpm for 48 hrs.

In order to determine the Resistance or Sensitivity to particular antibiotics,

Antibiotic Sensitivity Test

Antimicrobial Susceptibility test of isolates was performed. Sterile Muller Hinton Agar (M173) plates were inoculated with 0.1ml of freshly prepared culture suspension of isolate 1 and isolate 2.Using aseptic techniques antibiotic discs were applied .Plates were incubated at 37°c for 2-3 days. Zones showing the complete inhibition was measured and the diameter of the zone were recorded.

Antimicrobial tests of *planococcus maritimus*

Extraction of pigment-

Protocol was design for the maximum extraction of pigments.

This is very important objective as it is necessary that maximum amount of pigment should be extracted and recovered during the process. Numbers of references were collected for the extraction of pigments from bacteria, yeasts and fungi. These methods are as follows.

3. Extraction of pigments by various methods

1. 1gm of cells pellet was taken and washed three times with sterile saline and centrifuged. Cell pellet after centrifugation was suspended in 3 ml of pure ethanol. Mixture was vortex for 5min and centrifuge to extract the pigments. Procedure was repeated three times. Residual pigment was extracted using water bath at 45°C. After centrifugation supernatant was collected and analyzed by scanning absorption specifically at 800-190nm. Maximum absorption was measured. Solvent is allowed to evaporate and dry weight of pigment is determined.

2.1 gm of cells was rinsed from the surface of agar with distilled water. Washed 3 times with distilled water and washed cells were extracted with 50 ml absolute methanol. Suspension agitated at R.T. for 30 min the cell pellet remains after second extraction was colorless. Pooled methanol saponified for 30 min at 65°C with 6% w/v KOH. Absorption was measured. Solvent is allowed to evaporate and dry weight of pigment is determined.

3. In the next procedure 1gm of freeze dried culture hydrolyzed with 1N HCl in a water bath at 70°C for 1 and half hour The acid free cells were soaked overnight in acetone: methanol (1:1) solution .then pigment extracted with acetone and transferred to light petroleum (40-60°C) in a separating funnel and washed twice thoroughly with distilled water. Absorption of light petroleum was measured. Solvent is allowed to evaporate and dry weight of pigment is determined. Solvent is allowed to evaporate and dry weight of pigment is determined.

4. In the 1gm of dry cells 10 ml of Dichloromethane: methanol 50:50v/w was added. Mixture was vortex vigorously for 1 min and homogenized then centrifuged pellet re-extracted until complete extraction. Absorption was measured. Solvent is allowed to evaporate and dry weight of pigment is determined.

5. In the 1gm of cells 2ml of Dimethyl sulfoxide was added and cell mass stored for 30 min. Then 6ml of acetone was added homogenized and centrifuged for 3500rpm for 5min .The

pellet re-extracted until complete pigment extracts. Absorption was measured. Solvent is allowed to evaporate and dry weight of pigment is determined.

6.1gm of cells were harvested and the pigment were hydrolyzed overnight in a98% ethanol-60% KOH mixture (10:1) Finally the pigments were extracted in ether and identified by their absorption maxima Spectra were recorded on spectrophotometer. Solvent is allowed to evaporate and dry weight of pigment is determined.

7. In one method of extraction 50 ml of methanol was added to a 1 gram of moist bacterial cells and the placing of the containers in hot water to bring the methanol to the boiling point quickly. The pigments were extracted in few minutes. The solution was then cooled and the cells removed by centrifugation. Absorption was measured on spectrophotometer. Solvent is allowed to evaporate and dry weight of pigment is determined.

From the various methods most suitable method of methanol extraction with HPLC grade was found out.

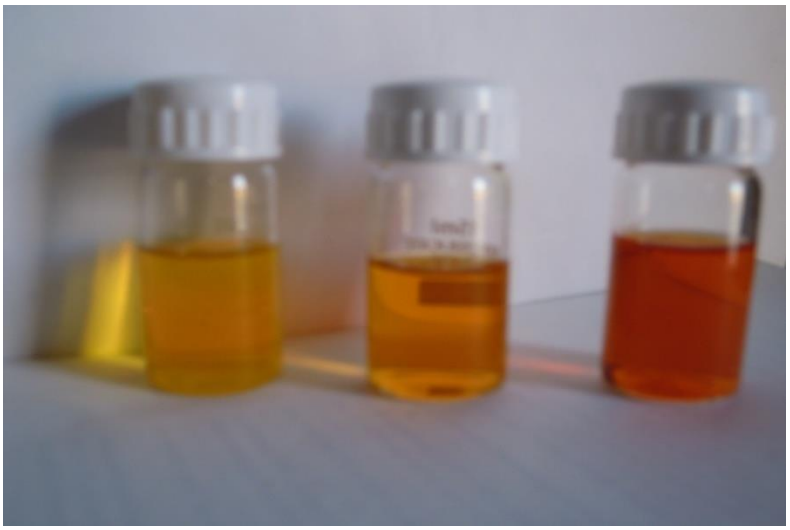
Results of extraction of pigment.

S.N.	Methods	λ max	Abs.	Total pigment yield. Mg\gm	Mean	Amount of residual colored biomass (mg)	Efficiency of Method
1	Method 1						
I		466	0.600	300		150	
II		465	0.623	325	314.0	100	Less efficient
III		466	0.602	315		190	
2	Method 2						
I		465	1.564	665		-----	
II		466	1.574	700	663.0	-----	Efficient
III		466	1.570	625		-----	
3	Method 3						
I		463	0.433	150		500	
II		464	0.432	110	146.6	435	Less efficient
III		465	0.434	180		400	
4	Method 4						
I		465	0.325	90		690	
II		466	0.327	120	103.3	565	Less Efficient
III		462	0.322	100		545	
5	Method 5						

I		461	0.328	0.15		700	
II		462	0.327	0.35	0.266	550	Less efficient
III		462	0.328	0.30		582	
6	Method 6						
I		460	0.430	190		500	
II		447	0.400	220	205.0	450	Less efficient
III		462	0.430	205		480	
7	Method 7						
I		465	1.599	715		-------	
II		466	1.580	702	702.3	--------	Most efficient
III		465	1.570	690		------	

Table 5:- Extraction of pigment by various methods in *planococcus*

Table 6:- Exraction of pigment in *Rhodococcus*



Extract of pigment in methanol

Drying of the pigment.

4. Applicability of the pigments.

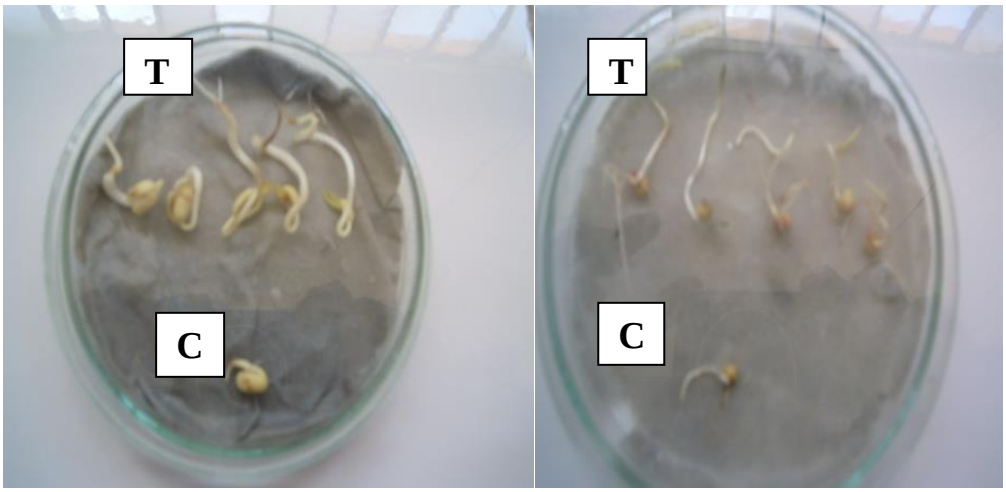
Seed germination assay

In order to check the applicability of the pigment with respect to effect on the germination of seeds, seed germination assay was performed. *Mung*, *math*, *gram* and *jowar* seeds were taken in order to check the effect of pigment on the growth and germination (these being the common productions in this area).

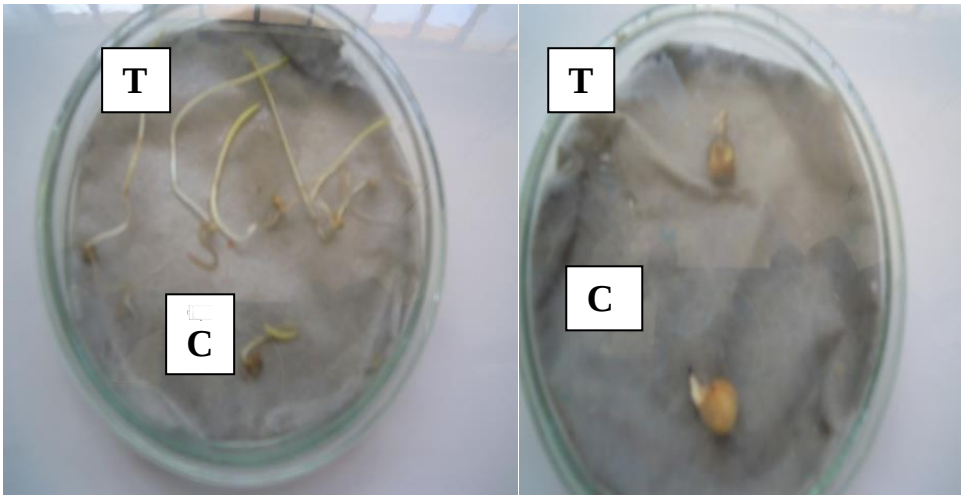
Method

The effect of pigment on the seed germination was checked. Surface sterilization of healthy seeds was carried out. Seeds of jowar, *mung*, *gram* and *math* were treated with 1% $CuSO_4$ solution. Then treated seeds were washed 3-4 times with sterile distilled water. After disinfection the seeds were dipped in 15% sucrose solution for 1 hour and soaked in 1 mgL^{-1} of pigment solution. Soaked seeds were dried in laminar air flow. These seeds were placed in sterile plate with moistened blotting paper. Un-soaked seeds were subjected for germination. Plates with the coated and uncoated seeds were incubated for 3-4 days. The effect of pigment on the germination was determined.

Result .



Pigment coated seeds of *mung* and *math* (growth greatly enhanced with better germination of seeds observable with pigment coated concentrate in test as compared to control).



Pigment coated seeds of *jowar* and *gram* (Growth quite stunted in test)

Pigment promotes the growth and enhances seed germination.

Pot germination assay.

Pigment coated seeds shows increase in growth as compare to unpigmented seeds.

Summary of the findings (Annexure C)

Most of the natural dyes are extracts from plants, plants products or produced by microorganisms, which are advantageous. Since the number of permitted synthetic colorants has decreased because of undesirable toxic effects including mutagen city and potential carcinogenicity interest focuses on the development of food pigments from natural sources. The present work focuses on the development of food pigments from natural sources. Though many natural colors are available, Microbial colorant plays significant role as food coloring agents, because of its flexibility in production and easy recovery. The effect of the pigment on the growth and germination of seeds were checked which revealed that as compared to control, germination was enhanced in the test where seeds coated with the pigment extract. This proved that the microbial pigment could exhibit growth stimulation in the germination of seeds. However, with development of a proper system and protocol this may reveal the expected details regarding the pigment and help in its identification. In this work emphasis is given on the isolation of pigment producing bacteria from soil and effluent source. Then Morphological, biochemical characterization of isolates was carried out. Applicability of the pigment in the growth promotion of seeds were checked which shows the pigment enhances seed germination. This project introduces natural colors in the food or pharmaceutical industry.

Most of the natural dyes are extracts from plants, plants products or produced by microorganisms, which are advantageous. Since the number of permitted synthetic colorants has decreased because of undesirable toxic effects including mutagen city and potential carcinogenicity interest focuses on the development of food pigments from natural sources. Though many natural colors are available, Microbial colorant plays significant role as food coloring agents, because of its flexibility in production and easy recovery.

In this work emphasis is given on the isolation of pigment producing bacteria from soil and effluent source. Then Morphological, biochemical characterization of isolates was carried out. Applicability of the pigment in the growth promotion of seeds were checked which shows the pigment enhances seed germination. This project introduces natural colors in the food or pharmaceutical industry.

Publications – Attached herewith PDF of Papers published.

References.

1. E.A.Johnson, W.A.Schroeder; *Advances in Biochem Eng. Biotechnolog*; **53,** 119 **(1995).**
2. E.Camera, A. Mastofrancesco, C. Fabbi, F.Daubrawa, M. Picardo, H.Sies, W.Stahl; Exp. Dermatol., **18**, 222- 231 **(2009)**
3. G.Fregova,E. Simova, K.Pavlova,D. Beshkova,D. Grogora; Biotechnol. Bioeng **44,** 888**(1994).**
4. G.Hussein, U.Sankawa, H.Goto, K.Mastumoto,H. Watanabe, J. Nat. Prod., **69**, 443-449 **(2006)**.
5. G.Sandmann, M.Albrecht, G.Schnurr, O.Knörzer P. Böger; Trends in Biotechnol; **17,** 233 **(1999).**
6. H.J.Nelis,A.P. De Leenheer, J. Appl.Bacteriol **70**,81 **(1991).**
7. J.Helmerson, J.Arlov, A. Larsson, S. Basu; Br. J. Natr., **101**, 1775-1782. **(2009).**
8. K.Wertz, P.B.Hunziker, N.Seifert, G.Riss, M.Neeb, G.Steiner, W.Hunziker,R.Goralezyk; J. Invest. Dermatol **124**, 428-434 (**2005**).
9. M.Careri, L.Elviri,A.J. Mangia; J. Chromatogr A; **854,** 233**(1999).**
10. M.Gurine, M.E Huntly,M. Olaizola, Trends Biotechnol., **21**, 210-216 **(2003)**.

11. N.R.Williamson, P.C.Fineran, T.Cristwood, S.R.Chawral, F.J.Leeper, G.P.C.Salmond; Future Microbial; 2, 605-618 **(2007)**.

12. R.P.Williams, C.L.Gott, S.H.M.Qadri,R.H.Scot; J. Bacteriol **106,** 438-443 **(1971).**

13. E.Camera, A. Mastofrancesco, C. Fabbi, F.Daubrawa, M. Picardo, H.Sies, W.Stahl; Exp. Dermatol., **18**, 222- 231 **(2009)**

14. M.Gurine, M.E Huntly,M. Olaizola, Trends Biotechnol., **21**, 210-216 **(2003)**.

15. J.Helmerson, J.Arlov, A. Larsson, S. Basu; Br. J. Natr., **101**, 1775-1782. **(2009)**

16. G.Hussein, U.Sankawa, H.Goto, K.Mastumoto,H. Watanabe, J. Nat. Prod., **69**, 443-449 **(2006)**.

17. K.Wertz, P.B.Hunziker, N.Seifert, G.Riss, M.Neeb, G.Steiner, W.Hunziker,R.Goralezyk; J. Invest. Dermatol **124**, 428-434 (**2005**).

18. N.R.Williamson, P.C.Fineran, T.Cristwood, S.R.Chawral, F.J.Leeper, G.P.C.Salmond; Future Microbial; 2, 605-618 **(2007)**.

19. R.P.Williams, C.L.Gott, S.H.M.Qadri,R.H.Scot; J. Bacteriol **106,** 438-443 **(1971).**

20. E.A.Johnson, W.A.Schroeder; Advances in Biochem Eng. Biotechnolog; **53,** 119 **(1995).**

21. G.Sandmann, M.Albrecht, G.Schnurr, O.Knörzer P. Böger; Trends in Biotechnol; **17,** 233 **(1999).**

22. G.Fregova,E. Simova, K.Pavlova,D. Beshkova,D. Grogora; Biotechnol. Bioeng **44,** 888**(1994).**

23. M.Careri, L.Elviri,A.J. Mangia; J. Chromatogr A; **854,** 233**(1999).**

24. H.J.Nelis,A.P. De Leenheer, J. Appl.Bacteriol **70**,81 **(1991).**

Journal of Scientific and Innovative Research 2013; 2 (6): 1044-1051
Available online at: www.jsirjournal.com

Research Article

ISSN 2320-4818
JSIR 2013; 2(6): 1044-1051

Received: 04-10-2013
Accepted: 31-12-2013

Varsha M.Chaudhari
Department of Microbiology, PSGVPM's SIPArt's, GBPatel Science, STSKVS Commerce College, Nandurbar, Maharashtra 425409, India

Correspondence:
Varsha M.Chaudhari
Department of Microbiology, PSGVPM's SIPArt's, GBPatel Science, STSKVS Commerce College, Nandurbar, Maharashtra 425409, India
Tel: +919850184264
E-mail:
mail.varsharaj_294@rediffmail.com

Optimization of the extraction parameters for the production of biopigment from the new isolate of distillery effluent

*Varsha M.Chaudhari**

Abstract

The present study has been carried out to investigate the effects of single factors such as temperature, extraction time, nature of solvent, and concentration of solvent on the contents of biopigments from the newly isolated organism *Planococcus maritimus* AHJ_2 isolated from distillery spent wash. On this basis, an L16 orthogonal multiple factors design of experiment was performed to determine the optimal conditions for the extraction of biopigments. The amount of pigments extracted reached its maximum value when extracted at 80ºC for 10 min by using 80% methanol with 2 times of extraction. Spectrophotometric, TLC and FTIR performed for the optimized extracts proved the presence of carotenoids.

Keywords: Carotenoids, Orthogonal experiments, Single-factor experiments, *Planococcus maritimus* AHJ_2.

Introduction

Pigments are colorants which have been widely used in food, cloth, painting, cosmetics, pharmaceuticals and plastics.[1] The currently used colorants are almost exclusively made from nonrenewable resources such as fossil oil. The production of the synthetic colorants is economically efficient and technically advanced with colors covering the whole color spectrum. However, synthetic colorants are facing the following challenges: dependence on non-renewable oil resources and sustainability of current operation, environmental toxicity, and human health concerns of some synthetic dyes.[2] In the food, cosmetics and pharmaceutical industries, due to the serious environment and safety problems caused by many artificial synthetic pigments research has focused on processes for the production of safe and natural pigments from natural resources. Thus, searching renewable and environmentally friendly resources for production of colorants is an urgent need.[3]

Biological pigments are natural and a better substitute to chemical dyes used in the industries and laboratories.[4] Nature produces many biocolorants from various resources including plants, animals, and microorganisms, which are possible alternatives to synthetic dyes and pigments currently employed.[5] One promising method is to produce biopigments by microbes because of their fast growth rate and the feasibility of bioprocess development.[6] Microorganisms produce a large variety of stable pigments such as carotenoids, flavonoids, quinones, and rubramines, and the fermentation has higher yields in pigments and lower residues compared to the use of plants & animals.[7]

Thus, biosynthesis of dyes and pigments via fermentation processes has attracted more attention in recent years.[8] Among the natural pigments produced by microbes reported so far, most research has focused on yellow and red pigment production, such as monascue produced by Meniscus sp.[9], carotenoid from Phaffia rhodozyma[10], Micrococcus roseus[11], Brevibacterium linens[12] and Bradyrhizobium sp. [13], and xanthomonadin from *Xanthomonas campestris* pv. [14]

Numbers of methods are available for extraction of pigments from various sources. Pigment extractions from microbial sources are generally carried out employing organic solvents. A wide range of polar and non-polar solvents either individually, sequentially or in combination have been employed for optimal extraction of pigments from algae, fungi and bacteria with and without pretreatment to biomass. Ultrasonic and hydrochloric acid assisted extractions, as well as supercritical CO_2 extraction have also been attempted for carotenoids extraction from microbial source. These studies revealed that the method of extraction and solvent to be employed depends on the nature of pigment as well as biomass produced by the typical microbial source. But there is a need to develop a suitable extraction method with maximum pigment yield from microbial sources, which can be applied on a commercial scale. Hence, present studies were conducted to determine the optimum conditions to extract pigment from *Planococcus maritimus* AHJ_2 in order to explore a proper process to utilize the pigment in the food as well as in pharmaceutical industry.

Materials and methods

All chemicals and culture media used for the growth of pigment producing bacteria were purchased from Hi-Media laboratories (Bombay, India). Double glass distilled water was used for the experimentation. Solvents used for the extraction of pigments were of HPLC grade and purchased from S.D Fine chemicals (Bombay, India).

Instruments

The instruments used in this study were UV/Visible spectrophotometer (UV Mini 1240 Shimadzu, Japan), commercial heavy duty shaker (REMI), cyclomixer and water bath (REMI).

Collection of distillery spent wash

Distillery spent wash from Shri Satpuda Tapi Sahakari sugar factory and distillery section, Purushottamnagar, Shahada was collected in sterile 500 ml Erlenmeyer flasks. Analysis of the sample was carried out in the research laboratory of Department of Microbiology, PSGVPM'S 'ASC' College, Shahada.

Microorganism

Culture of *Planococcus maritimus* AHJ_2 was isolated on Luria- Bertani agar from distillery spent wash of Shri Satpuda Tapi Sahakari sugar factory and distillery section. Isolate was purified and identified from Institute of Microbial Technology (IMTECH), Chandigarh, India. It was maintained on slants of Luria- Bertani Agar (LB) medium at 40^0 C and sub cultured after every 30 days.

Preparation of inoculum

Inoculum of *Planococcus maritimus* AHJ_2 was prepared from single colony of actively growing culture in sterile saline. 100 µl of suspension was inoculated in 500 ml Erlenmeyer flask containing 100 ml sterile Luria Bertani broth (casein enzyme hydrolysate $10gl^{-1}$; yeast extract, $5gl^{-1}$; NaCl $10gl^{-1}$; pH 7±0.5) Flask was incubated on rotary shaker at 120 rpm for 48 h. 100 µl of inoculum from this flask was used for further study.

Extraction of biopigment

The main factor that affects the extraction of pigments is the nature of the solvent, temperature, extraction time and amount of solvents. In the present study optimum extraction conditions were determined by orthogonal design of experiments i.e., three levels and four different parameters. R value (Range analysis) was carried out to investigate the effect of each factor in the extraction process of biopigments. Further as the carotenoids are thermolabile and sensitive to temperature; the extraction method was modified to obtain maximum yield.

Spectrophotometric analysis

Spectrophotometric analysis and estimation of the pigment was carried out at 466 nm by UV –VIS spectrophotometer.

Experimental design

Basically, experimental design used in the present study comprised of single-factor experimental design, which was used for screening of extraction process and orthogonal design that was as a multiple factors for the optimization of extraction process.[15]

Single factor experiment

Preliminary experiment was carried out to determine the effect of different variables on the extraction of biopigments.

1. Solvent Type

Extractions of biopigment using various different types of solvents like acetone, methanol, ethanol, dichloromethane and dimethyl sulphoxide (DMSO) was carried out. Each solvent was used at fixed condition of temperature and a fixed extraction time of 10 min. The best solvent type was chosen based on the highest value of total pigment content express as a dry weight in mg /100 g cell mass.

2. Solvent concentration

The solvent concentration was investigated using the best solvent with concentration ranging from 60% to 100% (v/v). Various concentrations of solvent were prepared in the double distilled water ranges from 60% to 100% (v/v). The best solvent concentration was selected based on the highest value of total pigment present as a dry weight in mg/100 g cell mass.

3. Extraction Temperature

In order to determine the effect of extraction temperature, extraction was executed by using the best solvent composition at fixed time, under various temperatures, which were 70, 80, 90 and 100^0C. The extraction procedures were repeated. The best extraction temperature was chosen based on the highest value of pigment (mg/ 100 g cell mass).

4. Extraction Time

The impact of extraction time on the yield of biopigment was varied from 5, 10, 15, 20, min. Extraction was accomplished by applying the best solvent composition at optimum temperature. The extraction procedures were repeated. The best extraction time was chosen according to the highest value of pigment (mg / 100 g cell mass).

Multiple factor design

Development and Optimization of pigment extraction using L16 orthogonal design

The orthogonal experiment was designed based on the single factor experiment. The parameters and the orthogonal design of the experiment for the extraction of biopigment were given in the Table 1 and 2. The results were made in the form of range analysis. The results were shown in Table 3 & 4.

Factors for the extraction of biopigment

Table 1: Orthogonal Experiment of the extraction

	A	**B**	**C**
Levels	**Temperature (^{0} C)**	**Extraction Time (min)**	**Solvent (%)**
1	60	5	60
2	70	10	70
3	80	15	80
4	90	20	90

Table 2: L16 Orthogonal design of experiment

No.	**A**	**B**	**C**
1	1	1	2
2	1	2	3
3	1	3	4
4	1	4	1
5	2	1	2

6	2	2	3
7	2	3	4
8	2	4	1
9	3	1	2
10	3	2	3
11	3	3	4
12	3	4	1
13	4	1	2
14	4	2	3
15	4	3	4
16	4	4	1

Results and discussions

Effect of different solvent type on the extraction of biopigment

The result in Figure 1 revealed that the maximum amount of pigment content was observed when extracted in methanol. Considering one of the aims of this work is to propose a suitable solvent for extracting the pigment. Among various solvents like acetone, ethanol, methanol, dimethyl sulphoxide (DMSO), dichloromethane (DCM) etc., methanol and ethanol were selected as a right choice because it is environmentally benign and relatively safe to human health. Methanol was found to be more effective solvent for the extraction; it interacts with the pigments probably through non-covalent interactions and promotes a rapid diffusion of the pigment into the solution.

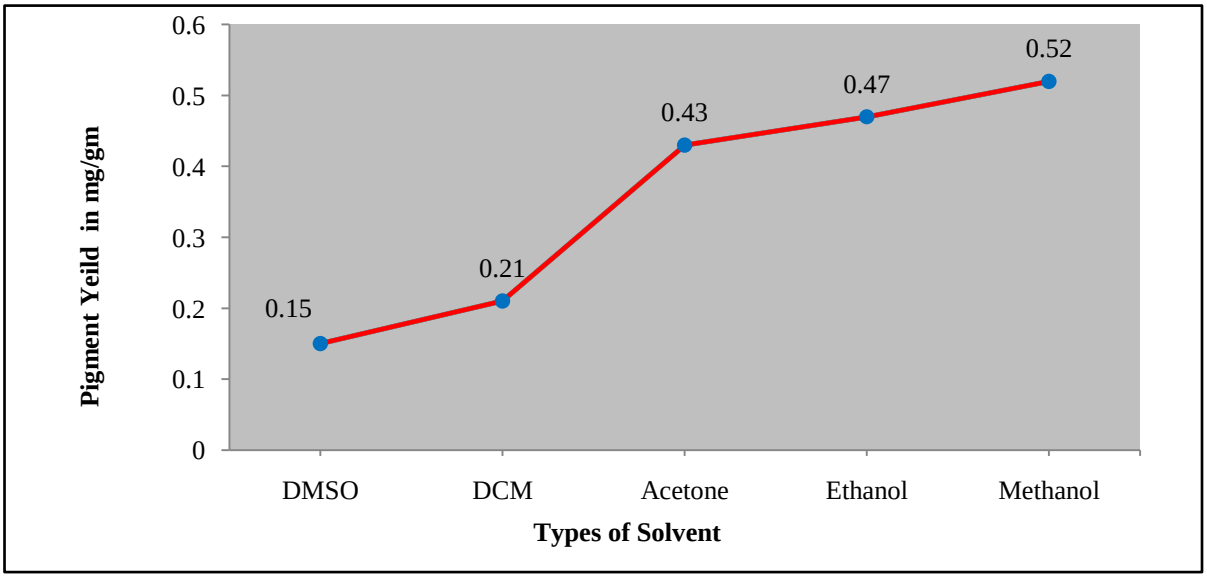


Figure 1: Effect of different solvent type on the extraction of pigment

Effect of concentration of solvent on the extraction of biopigment

It observed from the Figure 2 that the content of pigment extracts, increases with the concentration of methanol i.e., 60% to 100%. Various concentration of methanol prepared in distilled water used exhibited different effect in the content of pigment. Change in the fluid polarity had diverse effect on the solubility enhancement of the pigment but optimal methanol concentration for extraction of pigment was found to be 80 %.

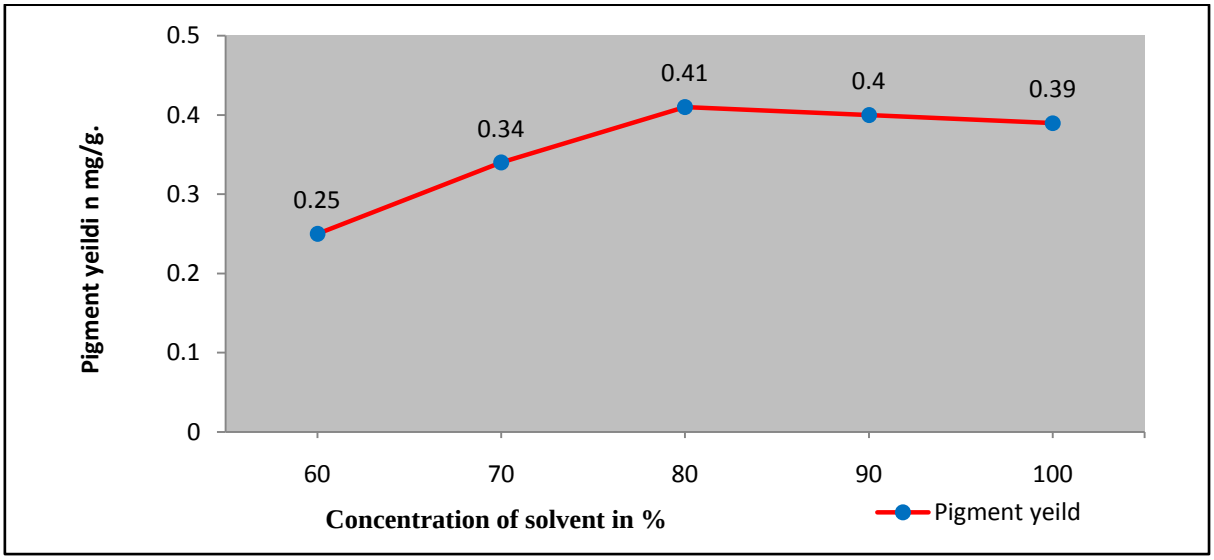


Figure 2: Effect of the methanol concentration on the extraction of biopigment

Effect of extraction time on the content of biopigment

The results of the Figure 3 showed that the contents of pigment extracted for 10 min reached maxima and prolonged time for extraction may not yield an increased content.

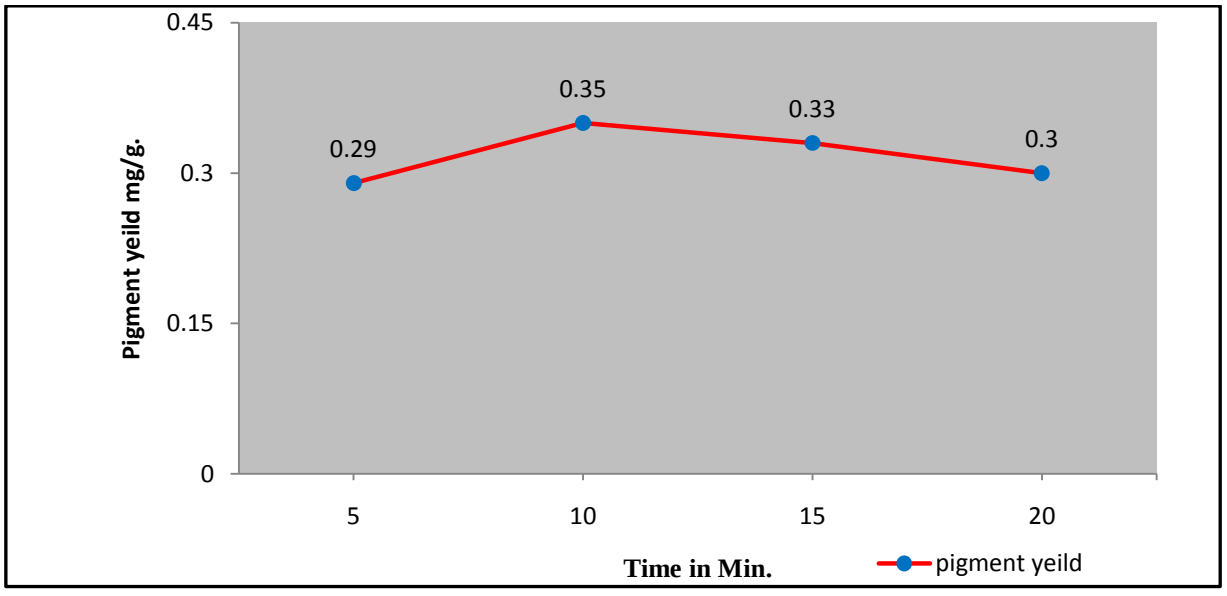


Figure 3: Effect of extraction time on the production of biopigment

Effect of temperature on the extraction of biopigment

Figure 4 showed the contents of pigment tended to increase gradually with a rise in the temperature range from 70ºC to 100ºC. The contents of pigments gradually increased with a rise in the temperature in a range of 70ºC to 100ºC with a 10ºC temperature interval. It may be probable that at higher temperature causes the diffusion of pigment more quickly from cell to extracting agent. Temperature's effect on extraction is dual. On one hand, higher temperature can accelerate the solvent flow and thus increase the increase the extraction of pigment.

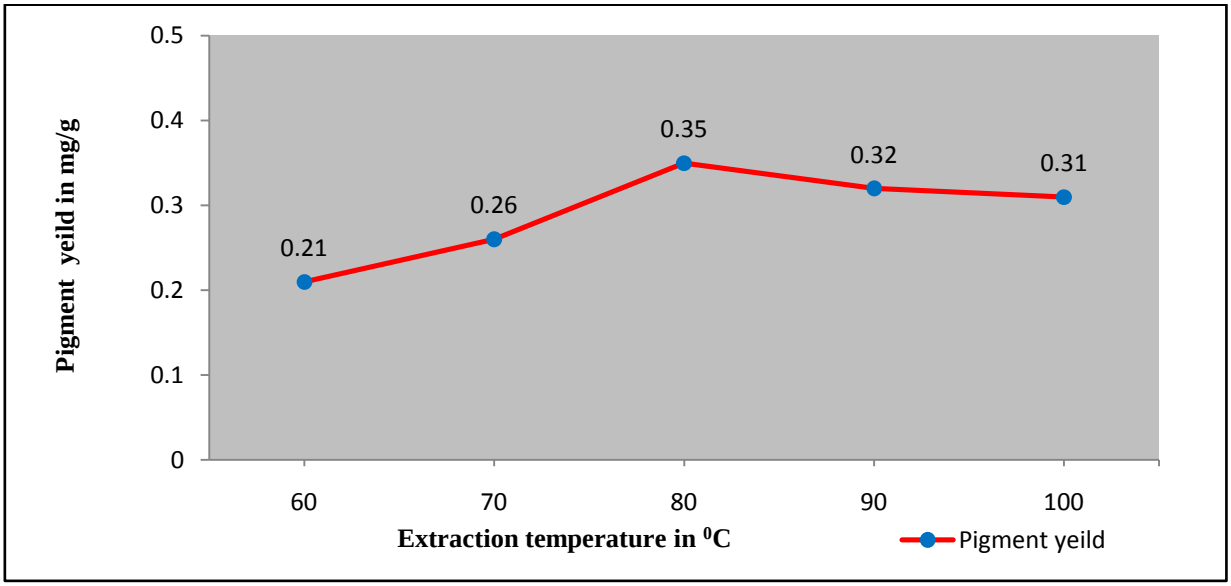


Figure 4: Effect of extraction temperature on the production of biopigment

Multiple factor experiment

Optimization of biopigment extractions using L16 orthogonal design

The parameters and the orthogonal design of experiment for the extraction of pigments were given in the Table 3. The results were made in the form of range analysis. The order of the effect of factors on pigment extraction was A>C>B. The concentration and nature of the solvent had the greatest effect on the extraction procedure. An equivalent effect was observed in the temperature. The other factors such as time of extraction and number of extractions did not play a vital role in extracting the pigment to a higher yield. The optimum extraction conditions obtained from the range analysis were A3B2C3. It means that 80°C, 10 min of extraction duration, and 80% methanol concentration.

Table 3: Yield of biopigment and range analysis

No.	A	B	C	Pigment Yield mg\g
1	1	1	2	1.92
2	1	2	3	2.54
3	1	3	4	3.10
4	1	4	1	2.64
5	2	1	2	3.84
6	2	2	3	4.57
7	2	3	4	3.21
8	2	4	1	2.95
9	3	1	2	3.05
10	3	2	3	5.04
11	3	3	4	3.74
12	3	4	1	3.01
13	4	1	2	2.36
14	4	2	3	1.92
15	4	3	4	2.60
16	4	4	1	1.90

K1	2.55	2.79	2.63	
K2	3.64	3.51	2.8	
K3	3.71	3.16	3.52	
K4	2.19	2.62	3.16	
k_1	0.63	0.69	0.65	
k_2	0.91	0.87	0.69	
k_3	0.92	0.79	0.88	
k_4	0.54	0.65	0.79	
R	0.38	0.22	0.23	

TLC and Spectrophotometric results

The results of TLC and Spectrophotometric revealed the presence of carotenoids by absorption at 466 nm by spectrophotometer UV mini Shimadzu.

Conclusion

In conclusion, the extraction conditions for biopigments were optimized to find that the extraction temperature 80ºC, 10min of extraction duration, 80% methanol and 2 times of extraction were the optimal conditions. Moreover, temperature was found to be a significant factor that affects the extraction procedure. The Spectrophotometric, TLC and HPLC results of the optimized extracts were found to contained carotenoids compounds.

Acknowledgements

The authors are greatly thankful for the financial support by UGC WRO Pune, India, for carrying out the above research work.

References

1. Yuan Lu., Liyan Wang., Yuan Xue., Chong Zhiang., Xin-Hui Xing., Kai Lou. *Et al*. Production of violet pigment by a newly isolated psychrotrophic bacterium from a glacier in Xinjiang, China. Bioche. Engi. Journal. 2009; 43: 135–141.

2. Farzaneh Alihosseini, Kou-San Ju, Jozsef Lango, Bruce D., Hammock Gang Sun., Antibacterial Colorants: Characterization of Prodiginines and Their Applications on Textile Materials. Biotechnol. Prog. 2008; 24: 742-747.

3. Cho Y.J., Park J.P., Hwang H.J., Kim S.W., Choi J.W., Yun J.W., Production of red pigment by submerged culture of *Paecilomyces sinclairii*. Lett. Appl. Microbiol. 2002; 3:195–202.

4. Palanichamy V., Aachhari Hundet., Bhaskar Mitra., Reddy N., Optimization of cultivation parameters for growth and pigment Production by streptomyces spp. isolated from marine sediment and rhizosphere soil. Int.J.of Plant, Animal and Envir.Sci. 2011; 1(3):158-170.

5. Mapari S. A. S., Nielsen K.F., Larsen T.O., Frisvad J.C., Meyer A.S., Thrane U., Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants. Curr. Opin. Biotechno. 2005; 16 (2): 109-238.

6. Zhao, D.H., Lu L., Qin H.L., Studies on the properties of a microbial blue pigment. Food Ferment. Ind. 1998; 5: 21–24.

7. Dura´n, N., Teixeira M.F.S., R.De. Conti. Esposito E., Ecological-friendly pigments from fungi. Crit. ReV. Food Sci. Nutr.42, 2002; 1:53-66.

8. Hobson D. K., Wales D.S., Green colorants. J. Soc. Dyers Colour. 1998; 114: 42-44.

9. Yongsmith B., Krairak S., Chaisrisook C., Fermentation of yellow pigments by cassava starch utilizing Monascus spp., Biotechnol. Sustainable Util. Biol. Resour.Trop. 1998; 12: 235–244.

10. Vazquez M., Santos V., Parajo J.C., Effect of the carbon source on the carotenoids profiles of *Phaffia rhodozyma* strains. J Ind. Microbiol. Biotechnol. 1997; 19: 263–268.

11. Chattopadhyay M.K., Jagannadham M.V., Vairamani M., Shivaji S., Carotenoid pigments of an Antarctic psychrotrophic bacterium *Micrococcus roseus*: temperature dependent biosynthesis, structure and interaction with synthetic membranes. Biochem. Biophys. Res. Commun. 1997; 239: 85–90.

12. Guyomarc'h F., Binet A., Dufossé L.,Production of carotenoids by *Brevibacterium linens*: variation among strains, kinetic aspects and HPLC profiles. J. Ind. Microbiol. Biotechnol. 2000; 24: 64–70.

13. Lorquin J. F., Molouba B.L., Dreyfus, Identification of the carotenoid pigment canthaxanthin from photosynthetic Bradyrhizobium strains. Appl. Environ. Microbiol. 1997; 3: 1151–1154.

14. Poplawsky A.R., Urban S.C., Chun W., Biological role of Xanthomonadin pigments in *Xanthomonas campestris* pv. Campestris. Appl. Environ. Microbiol. 2000; 9: 5123–5127.

15. Bohua W., Liping L., Lei L., Weiping C., Optimization of β-carotene production by a newly isolated *Serratia marcescens* strain. Electronic Journal of Biotech.2012; 6:1-8.

ISSN : 0974 - 7435 Volume 7 Issue 5

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 7(5), 2013 [194-200]

Isolation, identification and characterization of novel pigment producing bacteria from distillery spent wash

Varsha M.Chaudhari, Arpana H.Jobanputra*

Department of Microbiology, PSGVPM's S.I.Patil Arts, G.B.Patel Science and S.T.S.K.V.S Commerce College, Shahada, District Nandurbar, Maharashtra, 425409 (INDIA)

E-mail : arpana_j12@rediffmail.com

ABSTRACT

An unidentified orange bacterial strain isolated from distillery spent wash when characterized for morphological, microscopic, biochemical and molecular features (16SrRNA sequencing) was identified as *Planococcus maritimus AHJ_2*. Small orange colonies measuring 2-3 mm in diameter on Luria Bertani agar medium was the striking feature noted in the organism. The bacterium could grow over a wide range of media, pH (3.0-11.0) and temperature (20-37°C) but optimal growth and pigmentation was observed in LB medium at pH 7.0 at 37°C containing 0.5 percent NaCl. Spectrophotometric, FTIR and HPLC analysis of pigments revealed the presence of carotenoid type of pigment.

KEYWORDS

Orange bacterium;
16S rRNA identification;
Growth;
Pigmentation;
pH;
Temperature.

INTRODUCTION

As against widespread use of synthetic dyes not known to be environment friendly, demand for natural pigments for coloring fabrics, foods/feeds, cosmetics and printing inks are increasing[1]. A number of natural pigments produced by plants contribute to enhanced immune system and reduced risk of degenerative diseases, such as cancer, cardiovascular diseases, macular degeneration, cataract and acting as anti-aging agents[2-5]. Of the various natural pigments, microbial pigments prodigiosin and violacein are types of red and violet bacterial pigments that have found application in medical areas due to their activities as immunosuppressive, anticancer, antibacterial and antifungal agents[6,7]. Carotenoids are currently produced for use as food colorants, nutritional supplements, cosmetics or health purposes[8]. In addition to their pigmenting abilities, carotenoids may function as antioxidants by quenching photosensitizers, interacting with singlet oxygen, and scavenging peroxy radicals[9]. The species of the various taxonomic groups' bacteria, fungi and yeasts are efficient natural producers of carotenoids. Facing the growing economic significance of carotenoids, much interest has been devoted to new supplies of this type of pigment[10,11]. The fermentation conditions, such as cultivation temperature, NaCl, pH[12], play important roles in the carotenoids forming activity of microorganisms as well as composition ratio of carotenoids.

In the view of the significance of biopigments, the present work in this paper deals with the characterization of the orange pigmented bacteria both for its iden-

tification and production of an intense orange carotenoid pigment.

EXPERIMENTAL SECTION

Materials

All chemicals and culture media used for the growth of pigment producing bacteria were purchased from Hi-Media laboratories (Bombay, India). Double glass distilled water was used for the experimentation. Solvents used for the extraction of pigments were of HPLC grade and purchased from Bombay, India.

Instruments

The instruments used in this study were UV/Visible spectrophotometer (UV Mini 1240 Shimadzu, Japan), commercial heavy duty shaker (REMI), cyclomixer and water bath (REMI).

Collection of distillery spent wash

Distillery spent wash from Shri Satpuda Tapi Sahakari sugar factory and distillery section, Purushottamnagar, Shahada was collected in sterile 500 mL Erlenmeyer flasks. Analysis of the sample was carried out in the research laboratory of Department of Microbiology, PSGVPM'S ASC College, Shahada.

METHODS

Enrichment of sample

1 mL of effluent sample was enriched in sterile 100 mL Nutrient Broth, Potato Dextrose Broth, and Luria Bertani broth each. All flasks were incubated for 48 hours on rotary shaker at 100 rpm (REMI, India Ltd.)

Screening and isolation

Enriched broth samples were diluted up to 10^{-5} and 0.1 ml of dilution was plated on sterile nutrient agar plate, sterile starch casein agar plate, sterile potato dextrose agar plate and sterile Luria Bertani agar plate. All the plates were incubated at 37^0 C up to 48 hours. Growth of organisms from each of the sterile nutrient agar plate, sterile starch casein agar plate, sterile potato dextrose agar plate, sterile Luria Bertani agar plates were observed and best pigment producing organisms were selected. These were further purified from mixed population. Six pigment producing bacteria were isolated from effluent samples. Out of six isolates, most prominent pigment producing bacteria grown on sterile nutrient agar as well as on sterile Luria Bertani agar were selected. A pure culture of the isolate was maintained on sterile LB agar (casein enzyme hydrolysate $10gL^{-1}$; yeast extract, $5gL^{-1}$; NaCl $10gL^{-1}$; pH 7) slants followed by storage at 4°C as master culture and working stocks. When needed, culture was inconsistently derived from a master culture by streaking on LB agar in order to maintain its genetic stability. The bacterial isolate when cultured on this medium at 37±0.5°C formed intense orange colored colonies after incubation period of 3-4 days and used for further studies.

Identification of isolates

Colony morphology and cell characterization

The bacterial isolate was plated on Luria Bertani agar, allowed to grow at 37±5°C for 3-5 days and then studied for different cultural and cell morphological parameters, such as colony size, elevations, margin and colony pigmentation. Motility (hanging drop method) and Gram's reaction of the bacterial cells were performed using standard methods.

Biochemical characterization

Identification of isolate was done on the basis of morphological, cultural, biochemical and physiological characteristics from MTCC-Chandigarh. The isolates were cross identified on the basis of biochemical characters conducted routinely according to the protocols of 'Bergey's Manual' on 48 hours grown bacterial cultures. The pre-sterilized Hi-carbohydrate biochemical kit (KB 002 and KB 009, Hi Media, Mumbai, India) was used for biochemical identification of the isolate.

Molecular identification of bacterial isolate based upon 16SrRNA sequence

Molecular identification of the isolate by 16S rRNA sequencing was done at 'National Center for Cell Science', Ganeshkhind, Pune. The determined sequence of this 16SrRNA fragment was submitted to GenBank for GenBank Accession (www.ncbi.nlm.nih.gov/ Blast). This sequence was blasted into Nucleotide Blast Tool' of 'National Center for Biotechnology Information' (available at www.ncbi.nlm.nih.gov/ Blast) for nucle-

otide homology. The maximum homology report (Taxonomy Blast Report) was identified.

Pigment production

Growth and pigment production was carried out in 500 mL Erlenmeyer flask containing 100 ml of Luria Bertani broth inoculated with 1 mL of 24 hours culture suspensions. Flasks were incubated on rotary shaker at 120 rpm for 72 hours.

Extraction and spectrophotometric analysis of pigment

For the extraction of the pigment a screening programs involving various methods were accomplished and the method found most suitable was used further for all the investigations. Culture samples from each flask were centrifuged at 10000 X g for 15 minutes. In the harvested cells 50 mL of HPLC grade methanol was added to the moist cell mass. Mixture was vortex for 5 minutes and kept for incubation up to 6 hours. All the pigments were extracted in methanol and colorless cell debris were removed by centrifugation. UV visible scanning spectra of the methanol extract containing pigment were recorded between 200 and 800 nm on UV visible spectrophotometer (UV Mini 1240 Shimadzu, Japan). The absorption maxima were thus determined.

Fourier transforms infrared analysis (FTIR)

FTIR analysis was carried out to detect the presence of functional groups in the extracted pigment. For pigment analysis, FTIR was considered as a complementary technique that could provide the molecular and structural information of organic and inorganic molecules present in the pigment.

Effect of medium pH on growth and pigmentation

In order to determine the optimum conditions for growth and pigment production, optimization of pH and salt concentrations were studied. In an experimental set up 500 ml Erlenmeyer flasks containing 100 ml of the growth medium LB broth was inoculated with a 100 µl of the inoculum. The pH of the growth medium was varied as 3, 5, 7, 9 and 11. The flasks were incubated on rotary shakers for 72 hours at 37°C. After incubation, the relative growth and extent of pigmentation was recorded spectrophotometrically. All the separate shake flask experiments were performed in triplicates and results were expressed as mean±SD.

Effect of incubation temperature

Effect of incubation temperature (20, 25, 30, 37, 45°C) on the bacterial cell growth and pigmentation was observed after growing the inoculated LB broth medium at different temperatures. After 72 hours of incubation period, the growth and pigmentation of the bacterial isolates was recorded spectrophotometrically.

RESULTS AND DISCUSSION

Colony morphology and cell characteristics

The colony morphology and cell characteristics of the bacterial isolates in Luria Bertani agar showed that within 3-5 days of incubation the bacterial isolate grew to form intense orange colonies (2-3 mm in diameter) having entire margin and smooth consistency (Photplate 1A). Gram staining of bacterial cells revealed the presence of Gram positive cocci.

Plate 1 : Growth and pigment production by the bacterial isolates in LB agar medium

Biochemical characteristics

The intense orange pigment forming isolate was identified on the basis of biochemical characters from Microbial Type Culture Collection, Chandigarh. The results of various biochemical tests are as depicted in TABLE 1. On the basis of biochemical characterizations, isolate was identified from MTCC, Chandigarh as a *Planococcus maritimus.*

Molecular identification of bacterial isolates based upon 16S rRNA sequence

16S rRNA sequences obtained from NCCS were

submitted to GenBank and are available as GenBank Accession Numbers JN873343.1 *Planococcus maritimus strain AHJ_2* (www.ncbi.nlm.nih.gov/Blast). The sequences were blasted into Nucleotide Blast Tool' of 'National Center for Biotechnology Information' (available at www.ncbi.nlm.nih.gov/Blast) for nucleotide homology. The maximum homology report (Taxonomy Blast Report) identified a high nucleotide homol-

Sr. No	Characteristics	Isolate 1
1	Configuration	Circular
2	Margin	Entire
3	Elevation	Raised
4	Surface	Moist
5	Pigment	Orange –Yellow
6	Opacity	Opaque
7	Gram's Reaction	+ve
8	Cell Shape	Coccus
9	Size mm	0.5-1
10	Spores	–
11	Endospores	–
12	Position	–
13	Shape	–
14	Sporangia	–
15	Motility	Motile
16	Anaerobic	–
17	Physiological tests	
18	Growth at temperature	
	4°C	–
	15°C	+
	25°C	+
	30°C	+
	37°C	+
	42°C	+
	55°C	–
19	Growth at pH	
	pH 5.0	+
	pH 7.0	+
	pH 8.0	+
	pH 9.0	+
	pH10.0	+
	pH 11.0	+
20	Growth on NaCl (%)	
	2.0	+
	4.0	+
	6.0	+
	8.0	+
	10.0	+
	11.0	(+)
	12.0	–
21	Biochemical Tests	
	Methyl Red Test	–
	Voges Poskauer test	-
	Casein hydrolysis	+
	Citrate	-
	Nitrate	-
	Arginine	-
	Ornithine	+
	Indole	-
	Gelatin Hydrolysis	-
	Starch hydrolysis	-
	Esculin hydrolysis	–
	Catalase test	+
	Oxidase test	–
	Growth on Mac-Conkeys	NLF
	Tween-20	-
	Tween-40	-
	Tween-60	-
	Tween-80	-
	Urea production	–
19	TSI Results	
	Butt	Yellow
	Slant	Yellow
	H_2S Production	-
20	Acid Production from	
	Trehalose	–
	Lactose	-
	Melibiose	-
	Adonitol	-
	Inositol	-
	Raffinose	+
	Cellobiose	+
	Maltose	+
	Xylose	-
	Sucrose	+
	Sorbitol	-
	Salicin	+
	Rhamnose	-
	Galactose	-
	Fructose	+

FULL PAPER

ogy of the 16S rRNA (99% maximum identity in 100 % query coverage) with 16S rDNA/ 16S rRNA. From the analysis of the generated taxonomy report of the 16S rDNA gene sequence, this bacterial strain with highest score of (2628), and lowest E-value (0.0) was identified to be *Planococcus maritimus AHJ_2*. However, the bacterial strain under study showed a maximum of 99 per cent homology with the previously reported sequences. This established that the bacterial isolate identified as *Planococcus maritimus AHJ_2* is a novel strain that has not been reported earlier.

Separation and type determination of nature of the pigment

The methanol extract of the pigment was subjected to partition between immiscible solvents to separate the type of pigment produced by the organism. Extraction was achieved in the ether phase in the separatory funnel. Presence of the pigment in the diethyl ether phase exhibits the presence of carotenoids or carotenols.

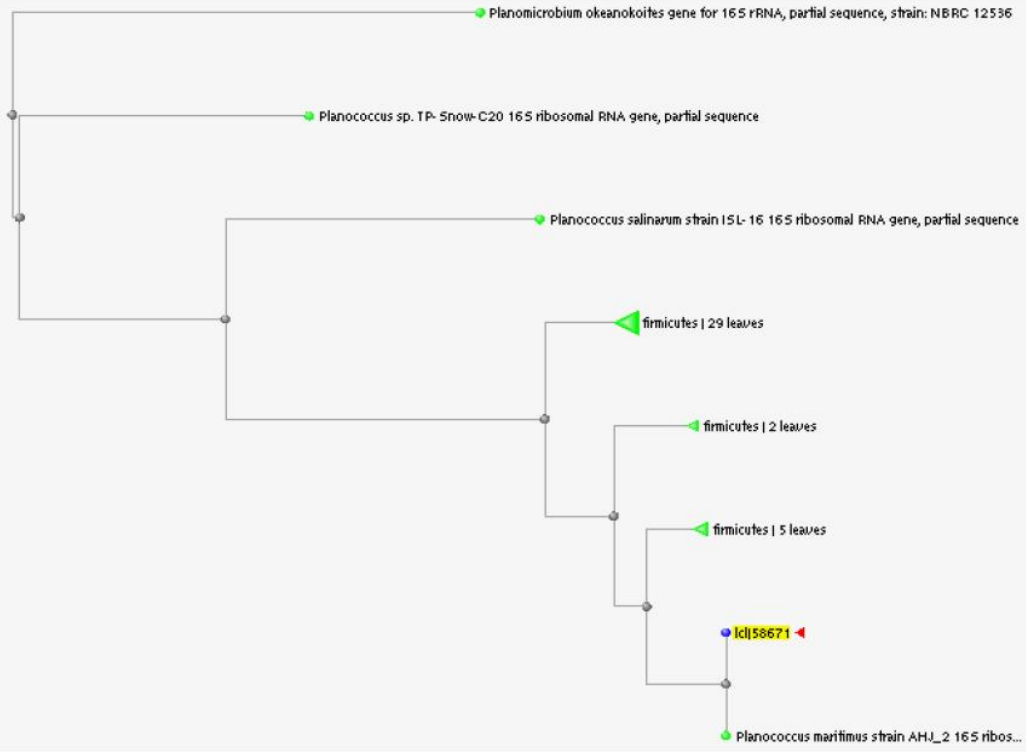


Figure 1: Neighbour-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the phylum firmicutes

UV-visible spectra absorption spectra of the pigment

UV-visible absorption spectra of carotenoids pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids. The UV-visible absorption maxima were 466 nm in methanol. This absorption spectrum of the pigment was characteristic of carotenoids (Figure 2).

Identification of pigment by IR analysis

One of the main identification tests for pigment is IR spectrum. Pigment exhibited bright spectral absorption lines, 466.79 cm^{-1} peak related to the hydroxy group

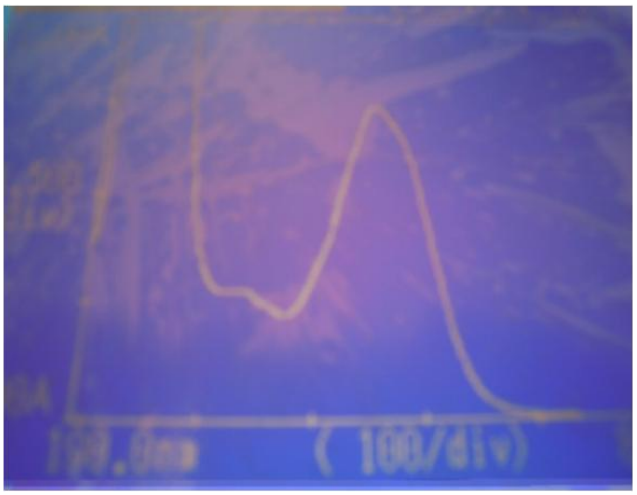


Figure 2 : Absorption spectrum of the pigment from *Planococcus maritimus AHJ_2*

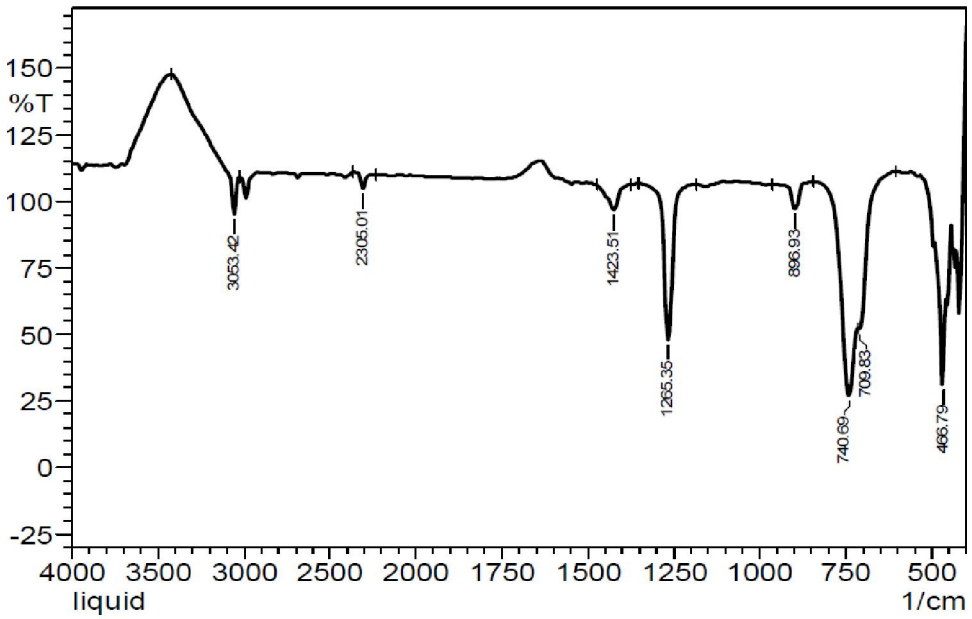


Figure 3 : FTIR analysis spectrum of the methanol extracted pigment

(OH), 709.83-740.69 cm^{-1} attribute to the O-H bend ing peaks, peak at 1265-1435 cm^{-1} related to the amino second group (NH), 2305-3053.42 cm^{-1} peaks related to amino group (NH) in pigment extracted from *Planococcus*. The IR spectral analysis of the methanol extracted pigment reveals the presence of carotenoids (Figure 3).

Effect of medium pH and incubation temperature on growth and pigmentation of the bacterial isolate

The effect of pH value of the growth medium on growth and pigment production of *Planococcus* wad studied. The isolate has shown remarkable ability to grow at pH values (3.0, 5.0, 7.0, 9.0 and 11.0) and produces orange and intense orange pigment over a wide range of medium pH. However, it showed its maximum growth and pigmentation efficiency at pH value of 7.0. This suggested that pigmentation was directly related with growth and that in spite of its capacity to grow over a wide pH range, the bacterial isolates was neutrophilic in its nature (Figure 4).

Temperature

In order to determine the optimum temperature, for the growth and pigmentation, the bacterial isolate was grown in LB broth and growth was observed at five different incubation temperatures (20, 25, 30, 37 and 40°C). All the incubation temperatures allowed the growth of

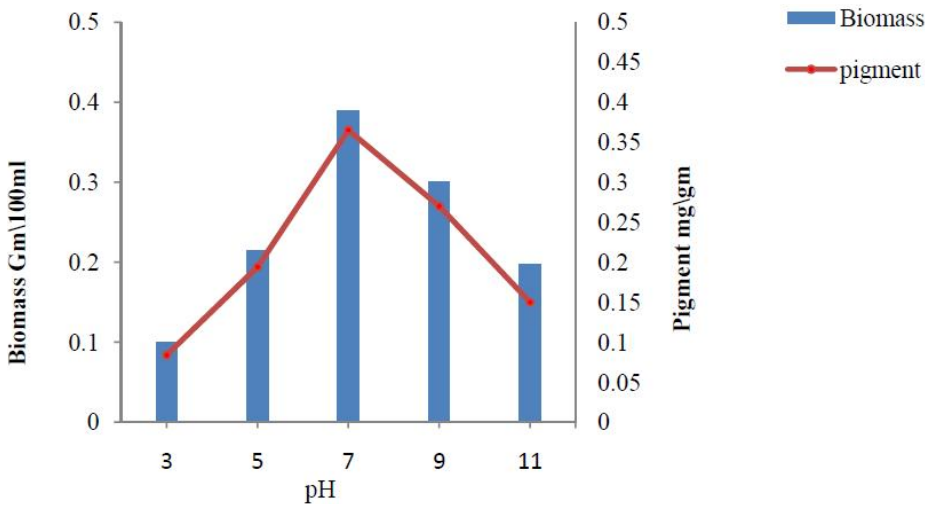


Figure 4 : Effect of pH on growth and pigment production

FULL PAPER

the bacterial isolate indicating its temperature stability over a mesophilic range. However, the optimum growth of the bacterial isolate was observed in the cultures incubated at 37°C, followed by 30°C (Figure 5).

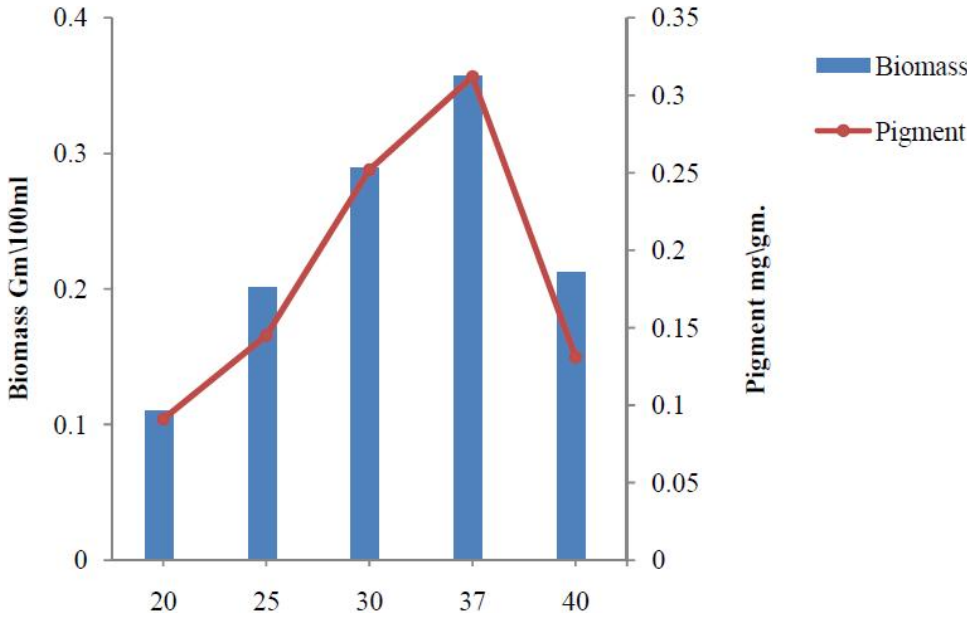


Figure 5 : Effect of temperature on growth and pigment production of *Planococccus*

CONCLUSIONS

Based upon various morphological, microscopic, biochemical and molecular parameters, an orange pigmented bacterial strain was isolated and identified as a novel strain of *Planococcus maritimus* AHJ_2. This strain could actively grow and was a potent producer of orange pigment in medium containing 0.5-1.0% NaCl and having initial pH of 6.0-7.0 and at an incubation temperature of 37°C. Preliminary investigations of the extracted pigment exhibited its close resemblance to carotenoids. Thereby studies related to its further analyses by sophisticated instrumentation and its applicability as food grade pigment are underway and are likely to yield encouraging results.

REFERENCES

[1] E.Camera, A.Mastofrancesco, C.Fabbi, F.Daubrawa, M.Picardo, H.Sies, W.Stahl; Exp.Dermatol., **18**, 222- 231 **(2009)**.
[2] M.Gurine, M.E.Huntly, M.Olaizola; Trends Biotechnol., **21**, 210-216 **(2003)**.
[3] J.Helmerson, J.Arlov, A.Larsson, S.Basu; Br.J.Natr., **101**, 1775-1782 **(2009)**.
[4] G.Hussein, U.Sankawa, H.Goto, K.Mastumoto, H.Watanabe; J.Nat.Prod., **69**, 443-449 **(2006)**.
[5] K.Wertz, P.B.Hunziker, N.Seifert, G.Riss, M.Neeb, G.Steiner, W.Hunziker, R.Goralezyk; J.Invest.Dermatol, **124**, 428-434 **(2005)**.
[6] N.R.Williamson, P.C.Fineran, T.Cristwood, S.R.Chawral, F.J.Leeper, G.P.C.Salmond; Future Microbial, **2**, 605-618 **(2007)**.
[7] R.P.Williams, C.L.Gott, S.H.M.Qadri, R.H.Scot; J.Bacteriol., **106**, 438-443 **(1971)**.
[8] E.A.Johnson, W.A.Schroeder; Advances in Biochem Eng.Biotechnolog, **53**, 119 **(1995)**.
[9] G.Sandmann, M.Albrecht, G.Schnurr, O.Knörzer, P.Böger; Trends in Biotechnol, **17**, 233 **(1999)**.
[10] G.Fregova, E.Simova, K.Pavlova, D.Beshkova, D.Grogora; Biotechnol.Bioeng., **44**, 888 **(1994)**.
[11] M.Careri, L.Elviri, A.J.Mangia; J.Chromatogr A., **854**, 233 **(1999)**.
[12] H.J.Nelis, A.P.De Leenheer; J.Appl.Bacteriol, **70**, 81 **(1991)**.