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Advances in Bioresearch

Bio-Production and Characterization of Carotenoids from Marine Soil Bacterial Isolates and Exploring Its Sustainable Applications

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ABSTRACT
The present study is focusing on carotenoid pigment-producing bacteria isolated from marine soil. The morphological, cultural, and biochemical characteristics of the isolates were studied. These isolates were screened out based on their pigment production abilities on Zobell marine broth (ZMB) agar media plates and submerged fermentation. Pigment extraction was performed using methanol extraction method. The extracted and partially purified pigments were characterized and confirmed by TLC, UV-Visible spectrophotometry, and FT-IR spectroscopy analysis. Extracted pigments were applied for preparation in bio-balm, candle preparation and assessing its textile dyeing property. The optimization of pigment production was done based on parameters including pH, temperature, and incubation time. In addition, extracted pigment was analysed for antioxidant and antimicrobial activity. The obtained results showed potent antioxidant activity and antimicrobial activity which confers potent pharmaceutical applications.
Keywords: *Marine Bacteria, Carotenoids, Characterization, Optimization, Applications*

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INTRODUCTION

For many years multiple industries are using colors to make products visually attractive. Pigments, particularly synthetic ones, have dominated the whole market since their emergence two centuries ago because of their wide selection of roles in numerous industry sectors like textile, cosmetics, food, pharmaceuticals and many more due to notable features like low manufacturing costs, processability and desirable coloring attributes have contributed to the placing of synthetic pigments on the market [18]. Despite this, the use of synthetic colors has a negative impact on people and the environment due to their numerous hazardous effects [14]. Synthetic pigments made up of heavy metals and petroleum compounds which are toxic and unsafe for both environment and human health and has been reported to have cancer inducing, allergic, and organ damaging properties [24]. Over the recent decades, owing to global response, consumers have become more interested in green natural dyes due to their advantages over the dangerous synthetic pigments [14], including their improved biodegradability and environmental safety, as well as lower allergic reactions and toxicity [17]. To counter the negative effects of synthetic colorants, globally researchers have focused on production of pigments from alternative natural resources including plants and microbes. Naturally occurring pigments and colored compounds from plants, animals, algae, fungi, and bacteria are regarded as bio pigments and are in use since ancestral days for coloring substances [32]. Natural pigments obtained from plants and animals are usually limited, unstable, highly priced, and require more complex and tedious process for production and ethically unavailable [27]. In comparison, pigment obtained from microbes can be easily produced in sufficient amount, are cost effective and have a simpler

extraction and purification process [20]. Bacterial pigments are less complex to produce and reported to be secure for human use [1]. Furthermore, their extraction methods and scaling up processes are more economical. Furthermore, pigments are secondary metabolites produced by living creatures that help the cell in a variety of ways, that involve photosynthesis, UV protection, defense against opposing species, and even energy-storing molecules. Pigment producing bacteria are good competitors in current color industry because of their less complex growing, adaptability to temperature and pH changes, strain and pigment verities and environment friendly [28].The marine microorganisms are noted for maintaining and regulating the bio-geochemical cycle in the ocean environment, and the quorum-sensing of the microbial community in the ocean to maintain such a biogeochemical cycle extraordinarily [33]. Currently, the marine bacteria are being explored for their production of clinically and industrially important secondary metabolites; the pigments produced by marine bacteria as a result of quorum sensing are of current interest due to their anti-microbial, anti-cancer, photo protective, anti-parasitic, and immunosuppressive activities [22]. Marine organisms are well-known for the natural pigment production viz carotenoids, flxirubin, xanthomonadine, prodigiosin, violacein and anthracene [15]. Carotenoids are the widest spread naturally occurring yellow, orange, and red pigments due to their relatively simple biosynthetic pathway in bacteria. The huge international market for carotenoids has been met mainly by synthetic carotenoids and however due to the possible toxicity natural carotenoids have become increasingly attractive [35]. Hence, it may be assumed that incidence of such bacterial strains which produces pigment with consistency is of research interest, and if bacterial strains are obtained from an unusual or comparatively less exploited habitat it increases more study interest. The present study was focused on the screening and characterizing bacterial pigment producing isolates from marine soil, production and characterization of carotenoid pigments and the commercial applications of the extracted carotenoids.

MATERIAL AND METHODS

Sample collection and isolation

Soil samples were collected from different coastal regions of Bhavnagar, Gujarat. Bacteria were isolated from marine soil samples by standard serial dilution method with dilution up to 10^{-6}. For sample preparation, 1 g of marine soil was diluted in 10 mL sterile distilled water. A total of 100 µL were spread on Zobell marine broth (ZMB) agar medium and incubated at 28 ◦C for 24-48 h. The colonies showing yellow to orange pigment production were then selected and purified and pure isolates were preserved on ZMB agar slant at 4 ◦C until further studies.

Characterization of pigmented bacterial isolates

The isolates were streak on ZMB agar plate and incubated for 24-48 h and characters such as size, shape, margin, colour, opacity, elevation, and texture were studied. Morphological characters studied using Gram's staining test. Biochemical characteristics were studied using Methyl Red test, Voges-Proskauer test, Citrate utilization test, Indole production test, Gelatin hydrolysis test, Lipid hydrolysis test, Catalase test and Dehydrogenase test.

Screening of pigmented bacterial isolates

Isolates were chosen based on their ability to secrete dark pigments in a short period of time on ZMB agar medium. The pigment production was further evaluated by growing the cells in liquid broth.

Salt tolerance activity:

Salt tolerance activity of the bacterial isolates were studied by supplementing ZMB agar plate with different NaCl concentration (3%, 5%, and 10%) and incubated at 28 ºC for 72 h.

Pigment production by submerged fermentation:

Inoculum Development*:* Active cultures were prepared by inoculating 1 mL suspension of selected strains into 100 mL Erlenmeyer flak containing 50 mL sterile ZMB media and incubated on shaker of 150 rpm at room temperature for 48 h.

Production media preparation*:* 99 mL freshly prepared ZMB media with pH 7.5 ± 0.2 taken in 250 mL Erlenmeyer flask were sterilized and inoculated with the activated culture as inoculum at 1% (v/v) level and incubated on shaker of 150 rpm at 28 ºC for 7 days.

Optimization of pigment production

Various parameters that influence growth of bacterium were optimized toward achieving maximal growth using ZMB medium. One Variable at A Time (OVAT) stepwise approach was applied firstly to determine the effect of pH, temperature, and incubation time on the production of pigment using ZMB media.

Effect of pH:

Initial pH of the medium that could support maximal pigment production was determined by adjusting the pH of 100 mL ZMB in 250 mL flask with 1% (v/v) of bacterial culture to various level (7,8,9) with either 1N HCl or 1N NaOH and determining the pigment production on shaker of 150 rpm at 28 ºC for 48 h.

Effect of temperature:
1% (v/v) inoculum of bacterial culture were inoculated in production medium with 8.0 pH and incubated at various temperatures such as 28 ºC, 37 ºC and room temperature which is changing from 34 ◦C to 40 ◦C on shaker at 150 rpm for 48 h. Pigment production and cell density was measured.
Effect of incubation time:
Production medium with 8.0 pH was inoculated with 1% (v/v) of selected isolates DC1 and DC9 and incubated on shaker at 37 ºC, 150 rpm for 48 h. Pigment production and cell density was measured.
Pigment production and extraction
The standard methanolic extraction method was followed with some modification. After 7 days of incubation, 100 mL of fermentation broth was harvested for pigment extraction. [39]. The colored methanolic supernatant was collected in pre weighed crucibles and allowed to dry in hot air oven at 50 ºC overnight. Pigment quantification was done by dry weight method [15].
Study of solubility of pigments:
Solvents like acetone, methanol, n-hexane, and ethyl acetate were scrutinized to extract the intercellular water-insoluble pigment [6].
Characterization of pigments
Thin Layer Chromatography:
Thin layer chromatographic separation of extracted pigment was carried out using pre coated TLC plates with silica gel G-60 F254 (Merck) as stationary phase using hexane: ethyl acetate (6:4, v/v) as a mobile phase. Retention factor (R_f) value was determined according to following formula [9]:

λ_{max} Determination:
λ_{max} is the wavelength at which the maximum light is absorbed by a solution. Molecules absorb the wavelength and when this absorbance is plotted as a function of wavelength, one obtains spectrum is simply a plot of absorbance vs wavelength. The extracted pigment was then analyzed by using UV-Vis spectrophotometer between the range of 400-800 nm. Methanol was used as blank. The absorbance value was plotted against respective wavelength, and finally, the λ_{max} was calculated [12].

$$Rf = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by solvents}}$$

Fourier Transform Infrared Spectroscopy (FT-IR):
FT-IR is a rapid and non-destructive technique used for the structural characterization of unknown samples. The FT-IR spectra of the sample were recorded on FT-IR instrument in order to characterize the presence of functional groups in extracted pigments. The dried extracted pigment was applied on the surface area of crystal and all the measurements were carried out in the range of 400-4000 cm^{-1} at a resolution of 4.0 cm^{-1} in FT-IR (Bruker, Alpha) [11].
Applications of bacterial pigments
Antioxidant activity:
The free radical scavenging activity of pigment extract is found based on scavenging stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. 1 mL of pigment sample was added to 2 mL of a 0.1 mM DPPH (0.004 %) solution prepared in methanol. Absorbance was read at 517 nm after 30 min incubation at room temperature in dark, and the percentage scavenging activity was calculated using the formula given below. DPPH solution without the test sample was used as a control [25].
[(Acontrol – Atest) / Acontrol] ×100 = DPPH scavenging effect (%)
Where, the absorbance of the control reaction = A control, and
The absorbance in the presence of the test sample = A test.
Antimicrobial activity:
Antibacterial activity
Antibacterial assay was carried out by the well diffusion technique for each pigment along with a hexane and methanol as a control. Wells with 0.5 cm diameter is made on sterile Mueller Hinton agar plate. Pathogenic bacteria namely *Bacillus spp., Pseudomonas aeruginosa* and *Serratia marcescens* were swabbed on the surface of the agar and 100 µL of pigment solution were added into the wells. Plates were incubated at 37 ºC for 24 h. Antibacterial activity of the microbial pigments was determined by measuring the zone of bacterial growth inhibition around the well [23].
Antifungal activity
The degree of anti-fungal activity of extracted pigments was tested by agar well diffusion method [37] against *Penicillium spp*. fungal strain. Sterilized Potato Dextrose Agar (PDA) medium was swabbed with 1

× 10^6 spore suspension of the fungal isolate and punctured to make well of 0.5 cm diameter. The well was filled with 100 µL of extracted pigments. Methanol was loaded as a control. The zone of fungal growth inhibition was measured in mm after 48h of incubation at 28 °C [29].

Bio-lip balm:

The bio-lipstick was prepared using paraffin wax along with coconut oil and Vaseline were mixed in a ratio 10:10:10 (w/v/w) in a container. The container was kept in water bath and heated until the wax melts completely and all the ingredients were homogeneously mixed. The pigment extracts were added to it to impart color. The mixture was then poured into a container and allowed to cool [5].

Textile dyeing:

The extracted pigments from marine bacterial isolates were used for dyeing cambric cotton cloth and cotton strand. The samples were pre-mordant with 5% of ferrous sulphate and copper sulphate separately. Finally, piece of the cambric cotton cloth and cotton thread each weighing 1 g were dyed in 20 mL of pigment solution, dyeing time 1 h and incubated at 70-80 °C. After dyeing, washing of the samples were carried out using tap water and allowed to dry at room temperature. For the experiment, white cloth material was taken as a control [5].

Candle coloring:

In the bowl, 50 g of industrial transparent gel wax was taken in a bowl and melted in a water bath. A total of 2 mL of methanolic extract of pigments of 4 mg/mL concentration was added and thoroughly mixed. This was poured into the mold and left to cool at room temperature for two hours [15].

RESULTS AND DISCUSSION

Sample collection and isolation

Pure culture of different pigment producing bacterial strains which were isolated from marine soil sample, procured from the Department of Microbiology & Biotechnology, Gujarat University, Ahmedabad, India. They produce the dark orange, bright yellow, light orange, orange red and lemon yellow colored pigments. Total of 4 bacterial isolates producing yellow and orange pigment were obtained and selected for the further studies are named as shown in table 1.

Characterization of pigmented bacterial isolates

Cultural Characteristics:

The colony characteristics of pigment producing halophilic bacterial strains, DC1, DC9, DC-GY,and DC-BY (Fig.1) were observed on ZMB agar plate after 48 h of incubation at 28 ºC for the cultural identification. The results are shown in the Table 2.

Morphological characteristics:

Gram's staining of the halophilic pigmented bacteria was carried out to observe their gram's reaction and cell morphology (Fig 2). The results are shown in Table 3.

Biochemical characteristics:

The biochemical tests employ various media, which when inoculated with a particular species of bacteria, will follow a specific metabolic pathway to hydrolyze the substrate available to them. Some of the routine biochemical tests used for determining metabolic activities of bacteria done are shown in Table 4.

Screening of pigmented bacterial isolates

Salt (NaCl) tolerance activity of isolates:

Bacterial isolates were grown on ZMB agar media plates supplemented with 3%, 5%, and 10% NaCl concentration and let them grow at 28 °C for 72 h incubation. DC1, DC9, DC-GY and DC-BY shows maximum growth with pigment production at 3%. DC-GY and DC-BY shows moderate to good growth with pigment production at 5% and DC-BY shows poor to moderate growth at 10% NaCl concentration, which conclude that DC1, DC9 and DC-GY grows and produce pigment best at 3% NaCl, DC-BY grows and produce pigment best at 5% NaCl concentration. Results are shown in Table 5. 3% to 5% salt concentration for carotenoids production by marine bacteria has been reported [13].

Pigment production using submerged fermentation

Bacterial isolates were fermented in 150 mL broth for 7 days by using submerged fermentation.

Pigment Extraction

After 7 days of incubation of pigment production the fermentation broth was collected for pigment extraction. The methanol extraction method was used for pigment extraction from bacterial cell pellets. After extraction from DC1, DC9, DC-GY, and DC-BY isolates respectively dark orange, lemon yellow, golden yellow and bright yellow colored methanolic extract were collected as shown in figure 4.

Drying of Pigments:

Methanolic extract of pigments from DC1, DC9, DC-GY and DC-BY were collected in crucibles (Fig 5.) and kept overnight for drying in hot air oven at 50 °C. After drying, the pigments were collected in powder form

and the dry weight of the pigments are measured in mg/L. Amounts of dry weight are listed as shown in Table 6. 900 ± 70 mg/L carotenoids production by marine bacteria has been reported [19] and 390 mg/L β-Carotene production after 2 to 4 days has been reported [26].

Pigment solubility in solvent

All four of pigments from selected isolates shows good solubility in methanol. Pigments from DC1, DC9 and DC-GY shows maximum solubility in hexane, while pigment from DC-BY shows poor solubility in hexane. DC1 and DC9 pigments shows good solubility in acetone, while DC-GY and DC-BY pigments shows poor solubility in acetone. DC-BY pigment shows maximum solubility in ethyl acetate, while DC1, DC9 and DC-GY pigment shows poor solubility in ethyl acetate. All four of pigments shows poor solubility in distilled water as shown in Table 7.

Characterization of Pigments

Thin Layer Chromatography (TLC):

The TLC performed using solvent system containing hexane and ethyl acetate at 3:2, v/v ratio. DC1 pigment shows single orange spot and DC9, DC-GY, and DC-BY shows respectively single yellow spot on TLC plate shown in Fig 6. Rf value of pigments are respectively as shown in table 8. Rf values of carotenoids pigments are reported in the range of 0.92 to 0.34 [15], which matches with this study, confirming that all four of the pigments belongs to carotenoid family.

λ_{max} Determination:

The absorption spectra of methanolic extracts of DC1, DC9, DC-GY and DC-BY shows respective result interpreted from the absorption maxima graphs (Fig 7) and compiled in Table 9. Maximum absorbance in the range of 300-500 nm which is the characteristics of the carotenoid pigments [7]. Hence, it leads to conclusion that four of the pigments belongs to carotenoid family.

Fourier Transform Infrared Spectroscopy (FT-IR):

FT-IR spectra stretches are significant for containing the functional groups present in the molecules. The crude extract of DC1, DC9, DC-GY, and DC-BY pigments spectra stretches were interpreted from Fig 8 and compiled as shown in Table 10. Broad stretches at 3365.18, 3293.19, 3307.00 and 3199.87 for phenolic -OH and 2940.83, 2903.41, 2900.98 and 2920.97 -CH of alkenes were the characteristic feature of carotene. From the results dark orange, lemon yellow, golden yellow and bright yellow pigments were identified belonging to the carotenoid family. It indicates that extracted pigments are β-carotene or carotenoid derivatives [34].

OPTIMIZATION OF PIGMENT PRODUCTION

Effect of pH:

Studies conducted for optimization of pH indicated that the bacteria could produce pigments over a range of pH from 7 to 9. DC1, DC9 and DC-GY shows maximum pigment production at pH 7 and DC-BY shows maximum pigment production at pH 8 as shown in figure 9. pH 7 to 8 has been reported for optimized carotenoids production by bacterial isolates [40][21].

Effect of temperature:

A study conducted for optimization of piment production at different temperatures which are 28 ◦C, 37 ◦C and room temperature (RT) which is changing from 34 ◦C to 40 ◦C. DC1, DC9 and DC-BY shows maximum pigment production at 28 ◦C and DC-GY at 37 ◦C as shown in Figure 10. Optimized temperature for carotenoids production has been reported 28 ◦C for marine bacterial isolates [38].

Effect of incubation time:

Study conducted for optimization of pigment production at different incubation time, which are 0 h, 24 h, 48 h, 72 h, and 96 h. DC1, DC9, DC-GY and DC-BY shows poor pigment production at 48 h, moderate production at 72 h and maximum production at 96 h of incubation as represented in Fig 11. Optimized incubation time 96 h and more has been reported for carotenoids production by bacterial isolates [10].

APPLICATIONS OF MARINE BACTERIAL PIGMENTS

Antioxidant activity

The free radical scavenging activity of methanolic extract of pigment samples along with reference standard ascorbic acid was determined by DPPH assay. Stable DPPH has a blue color that disappears after reduction and converts into yellow, which is measured at 517 nm. The activity exhibited by DC1, DC9, DC-GY, and DC-BY pigments are 10.26 %, 39.63 %, 41.8 % and 36.21 % respectively (Table 11). From the assay it can be concluded that pigment extract from the marine bacterial isolates shows significant free radical scavenging activities. Bacterial isolates produced yellow pigment showed significant antioxidant activity in DPPH assay [3].

Antimicrobial activity

Antibacterial activity:

Antibacterial activity of the extracted pigments against human pathogens was detected by well diffusion method. The bacterial pathogens used are *Serratia marscences, Pseudomonas aeruginosa* and *Bacillus sp.* All four pigments show zone of inhibition against *Pseudomonas aeruginosa*, in which DC1 shows maximum inhibition of 11 mm diameter. DC-GY and DC-BY shows antibacterial activity against *Serratia marscences* in which DC-BY shows more activity giving 8.3 mm diameter. DC9 and DC-GY shows antibacterial activity against *Bacillus sp*. In which DC-GY shows more activity giving 6.0 mm diameter in Table 12. Orange pigment showed maximum zone of inhibition (11 mm) antimicrobial activity against *Pseudomonas aeruginosa* and yellow pigment found to inhibit the growth of both Gram-positive as well as Gram-negative bacteria and thus could be designated as broad-spectrum antimicrobial agent. So, it was concluded that the isolated bacteria could synthesize pigments that possessed antimicrobial activity against certain human pathogenic bacteria [36]. Yellow pigment from the *Streptomyces sp*., *Micrococcus luteus*, and *Flavobacterium sp.* Showed significant antimicrobial activity against bacterial pathogens [2].

Antifungal activity:

Pigment extracts from DC1 and DC-BY shows antifungal activity against *Penicillium sp.* Giving 30 mm and 6 mm of zone of inhibition respectively in Table 13. Antifungal activity of the yellow pigment from *Bacillus sp.* reported [6].

Bio-lip balm:

The formulated lip balm exposed suitable characteristics such as color, odor, and uniformity and solidified at the 37 ◦C temperature. It was noticed that there was no water formation or blooming after three days of observation when the lip balm was maintained at room temperature. The color formation of the lip balm was observed under room temperature after one month of incubation. The color formation of the product was stable under room temperature and odor characteristic of the lip balm remained stable throughout the 10 days of testing under all conditions evaluated. The visual aspect was considered uniform under the room temperature (Fig.12) [5].

Textile Dyeing:

Cotton textile materials were used to check the dyeing property of the extracted pigment. The textile materials were subjected to three consecutive normal water wash treatments and the orange, lemon yellow, golden yellow, and bright yellow pigments imparted were retentive for this textile material. In textile industries, the pigment extracted from biological source was used as an alternative to the synthetic colorants and which are safe and cost effective. A piece of cotton and cotton thread were used as textile materials to observe the coloring capacity of the hexane extracted pigments. Usually, any sort of dye requires a fixative or a mordant, which helps in the attachment of the dye to the material. In this study, it has been observed that the extracted pigment did not require any fixative to incorporate the colored texture to the textile materials. The colors were durable enough to withstand three regular water wash treatments in a row (Fig. 13) [5]. Similar fastness properties in textile materials using different shades of brown pigment isolated from *Streptomyces* sp. has been reported [41].

Candle preparation:

As shown in fig.14 when hot melted transparent gel mixed with extracted pigments of isolates, it was observed that the pigments mixed with the wax and colored the candle wax. Successful candle coloring using a prodigiosin red colored pigment from Serratia marcescens has been reported [30][15]. Extracted yellow pigments *from Micrococcus luteus* has been reported to be used in candle coloring [4].

Table 1. Marine bacterial isolates and colour of pigments

No.	Bacterial isolate	Pigment colour
1	DC 1	Dark orange
2	DC 9	Lemon yellow
3	DC-GY	Golden yellow
4	DC-BY	Bright yellow

Table 2. Colony characteristics of pigment producing bacteria

No.	Colony characteristics	DC 1	DC 9	DC-GY	DC-BY
1.	Size	Small	Medium	Small	Small
2.	Shape	Round	Round	Round	Round
3.	Margin	Entire	Entire	Entire	Entire
4.	Elevation	Convex	Flat	Convex	Raised
5.	Consistency	Smooth	Smooth	Moist	Moist
6.	Opacity	Opaque	Opaque	Opaque	Opaque
7.	Pigment	Dark orange	Lemon yellow	Golden yellow	Bright yellow

Table 3. Morphological characteristics of pigment producing bacteria

No.	Morphological characteristics	DC 1	DC 9	DC-GY	DC-BY
1.	Size	Small	Small	Small	Small
2.	Shape	Cocci	Cocci	Cocci	Cocci
3.	Arrangements	Single, cluster	Single, cluster	Single, cluster	Single, cluster
4.	Gram's reaction	Negative	Positive	Positive	Positive

Table 4. Biochemical test result of pigment producing bacteria

Sr. No.	Test	Result			
		DC1	DC9	DC-GY	DC-BY
1.	MR test	+	-	-	-
2.	VP test	-	-	+	-
3.	Indole test	-	-	-	-
4.	Citrate utilization test	-	-	-	-
5.	Lipid hydrolysis test	+	+	+	-
6.	Catalase test	+	+	-	+
7.	Dehydrogenase test	-	-	-	-
8.	Gelatinase test	+	+	+	+

Table 5. Screening of isolates on ZMB agar plate at different salt concentration

Where, - no growth, + Poor growth, ++ Moderate growth,

Sr. No.	**Isolate**	**Color**	**NaCl (%)**	**Incubation Time (h)**		
				24	48	72
1.	DC 1	Dark orange	3	+	+	+++
			5	-	-	+
			10	-	-	-
2.	DC 9	Lemon yellow	3	+	++	+++
			5	-	-	-
			10	-	-	-
3.	DC-GY	Golden yellow	3	++	+++	+++
			5	+	++	++
			10	-	-	-
4.	DC-BY	Bright yellow	3	++	++	+++
			5	+++	++	+++
			10	+	+	++

+++ Good growth

Table 6. Dry weight of pigments from isolates after production

No.	Bacterial isolate	Pigment	Pigment production
1.	DC1	Dark orange	960 mg/L
2.	DC9	Lemon yellow	845 mg/L
3.	DC-GY	Golden yellow	498 mg/L
4.	DC-BY	Bright yellow	436 mg/L

Table 7: Pigment solubility in different solvents

Pigment solubility in solvent	**Methanol**	**Hexane**	**Acetone**	**Ethyl acetate**	**Distilled water**
DC 1	**++**	**+++**	**++**	**+**	**+**
DC 9	**++**	**+++**	**++**	**+**	**+**
DC-GY	**++**	**+++**	**+**	**+**	**+**
DC-BY	**++**	**+**	**+**	**+++**	**+**

Where, - no solubility, +minimum solubility,
++ good solubility, +++ maximum solubility

Table 8. Rf values of pigments from isolates

No.	Pigment from isolates	Rf value
1.	DC1	0.90
2.	DC9	0.72
3.	DC-GY	0.78
4.	DC-BY	0.81

Table 9. Absorption maxima of pigments from isolates

No.	Pigment from isolates	Absorption maxima
1.	DC1	468 nm
2.	DC9	441 nm
3.	DC-GY	437 nm
4.	DC-BY	430 nm

Table 10. FT-IR spectra observation of pigments from isolates

Functional groups	DC1	DC9	DC-GY	DC-BY

C-O	1022.11	1057.25	1057.60	1080.58
CH_2 alkenes	1411.24	1401.24	1409.78	1405.00
C=C aromatic ring	1641.52	1648.65	1638.97	1631.96
C-H aromatic	2893.84	2848.25	2847.85	2858.98
-CH alkenes	2940.83	2903.41	2900.98	2920.97
-OH alcohol	3365.18	3292.19	3307.00	3199.87

Table 11. DPPH scavenging activity of pigments from isolates

Sr. No.	Pigments of isolates	Scavenging activity (%)
1.	DC 1	10.26
2.	DC 9	39.63
3.	DC-GY	41.8
4.	DC-BY	36.21

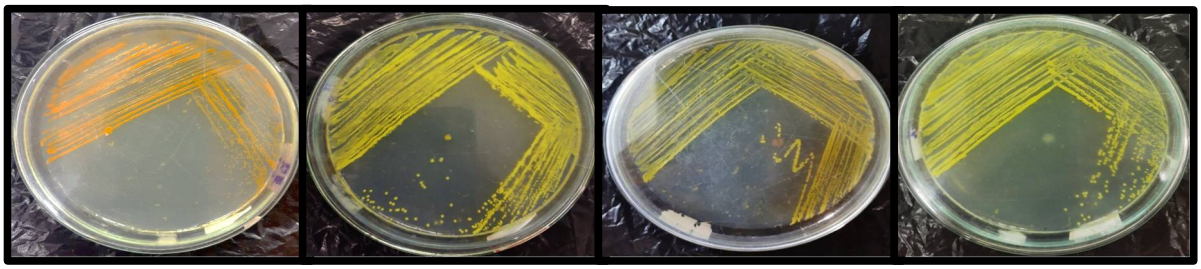
Table 12. Antibacterial activity of extracted pigments

Pigment	Test organism	Diameter of zone of inhibition in mm
DC 1 (Dark orange)	*Serratia marscences*	-
	Pseudomonas aeruginosa	11
	Bacillus sp.	-
DC 9 (Lemon yellow)	*Serratia marscences*	-
	Pseudomonas aeruginosa	2.5
	Bacillus sp.	0.5
DC-GY (Golden yellow)	*Serratia marscences*	6.00
	Pseudomonas aeruginosa	2.00
	Bacillus sp.	6.00
DC-BY (Bright yellow)	*Serratia marscences*	8.3
	Pseudomonas aeruginosa	2.00
	Bacillus sp.	-

Pigment	Test organism	Diameter of zone of inhibition in mm

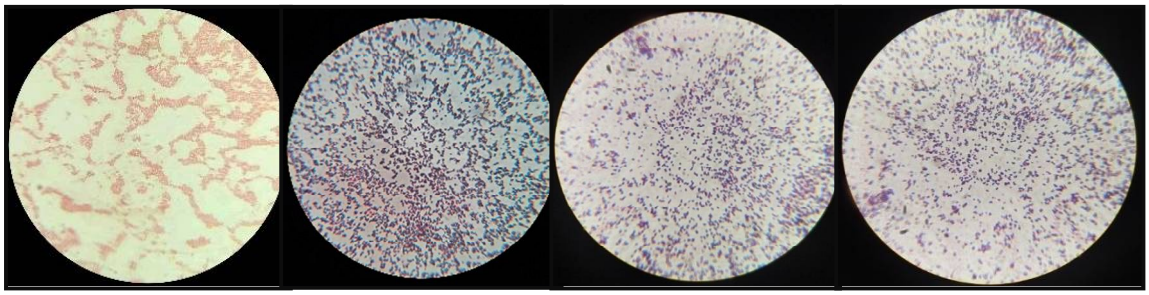
DC 1 (Dark orange)	*Penicillium sp.*	30
DC 9 (Lemon yellow)		-
DC-GY (Golden yellow)		-
DC-BY (Bright yellow)		6.00

Table 13. Zone of inhibition showing antifungal activity of pigment



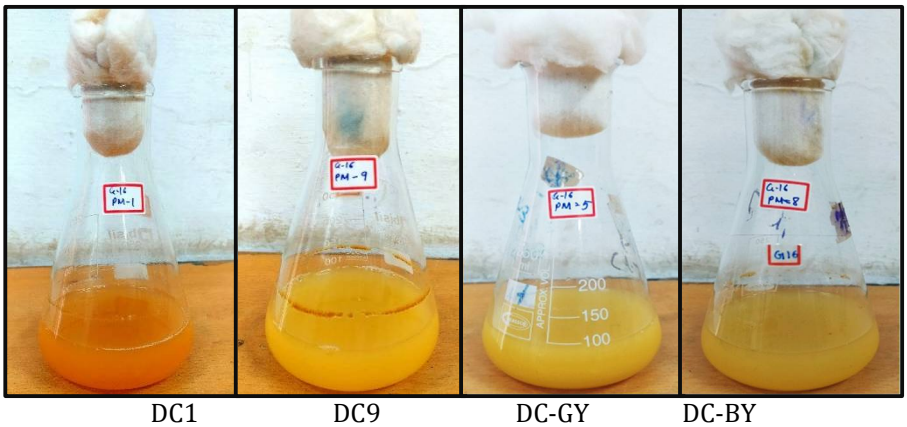
DC1 DC9 DC-GY DC-BY

Fig 1. Colony characteristics of isolates on ZMA media plates



DC1 DC9 DC-GY DC-BY

Fig 2. Morphological characteristics observed in microscope of pigment producing bacteria

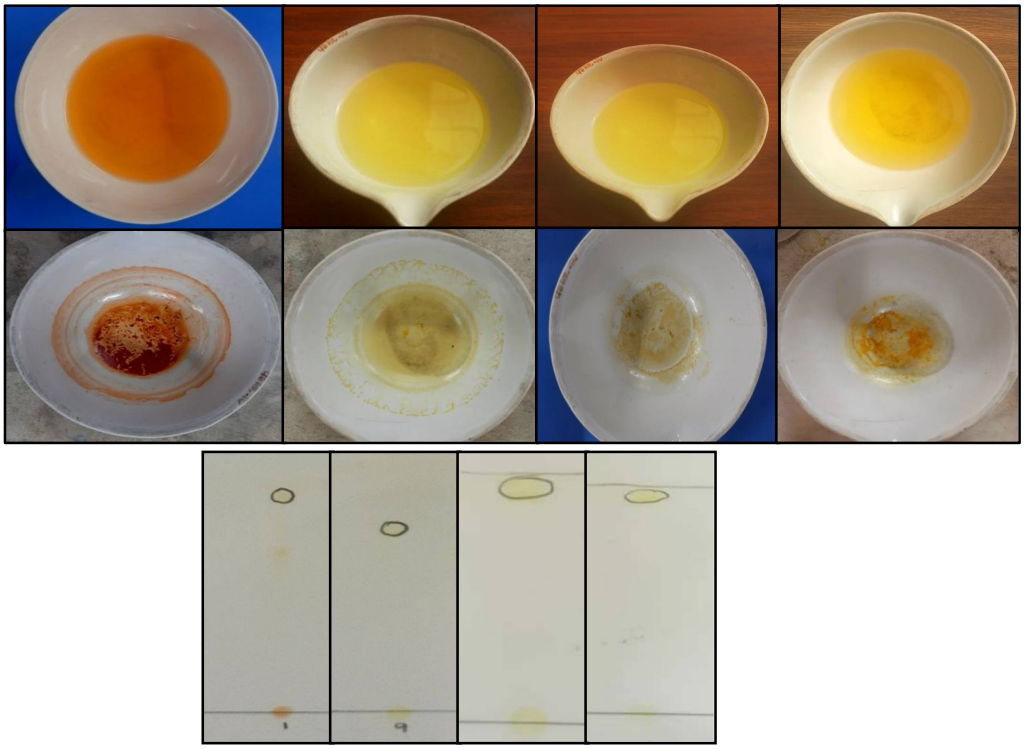


DC1 DC9 DC-GY DC-BY

Fig 3. Pigment production using submerged fermentation

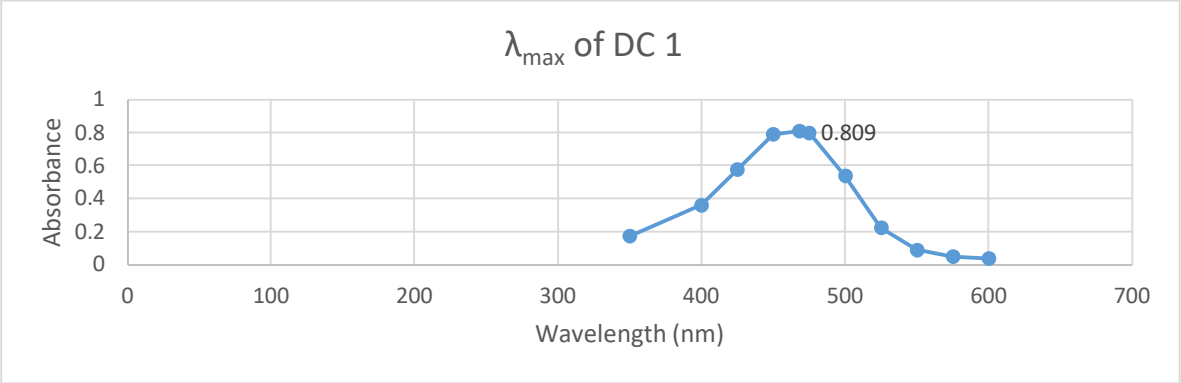
DC1 DC9 DC-BY DC-GY

Fig 4. Methanolic extracts of marine bacterial pigments



DC1 DC9 DC-GY DC-BY

Fig 6. TLC of pigments



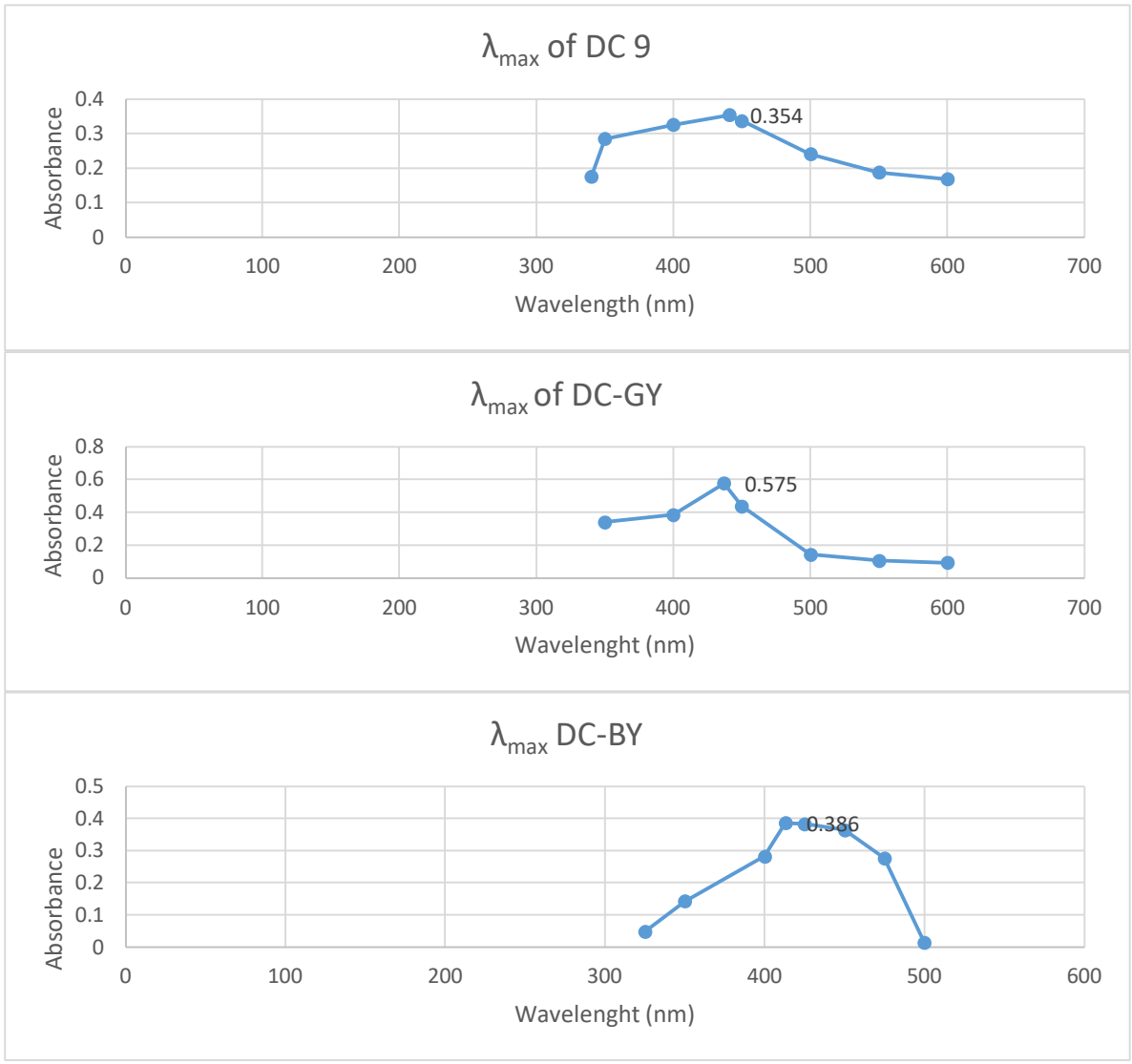
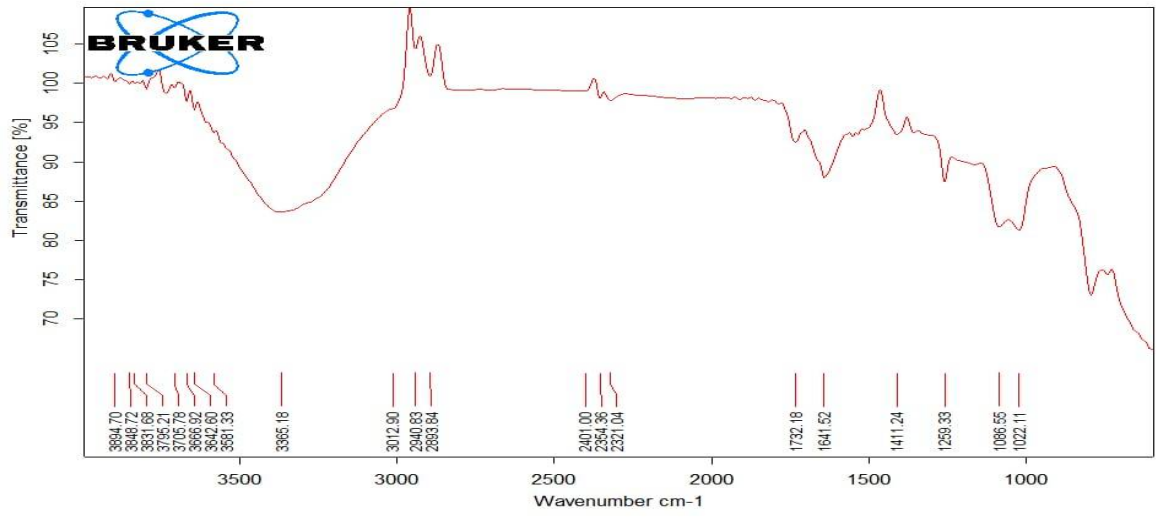
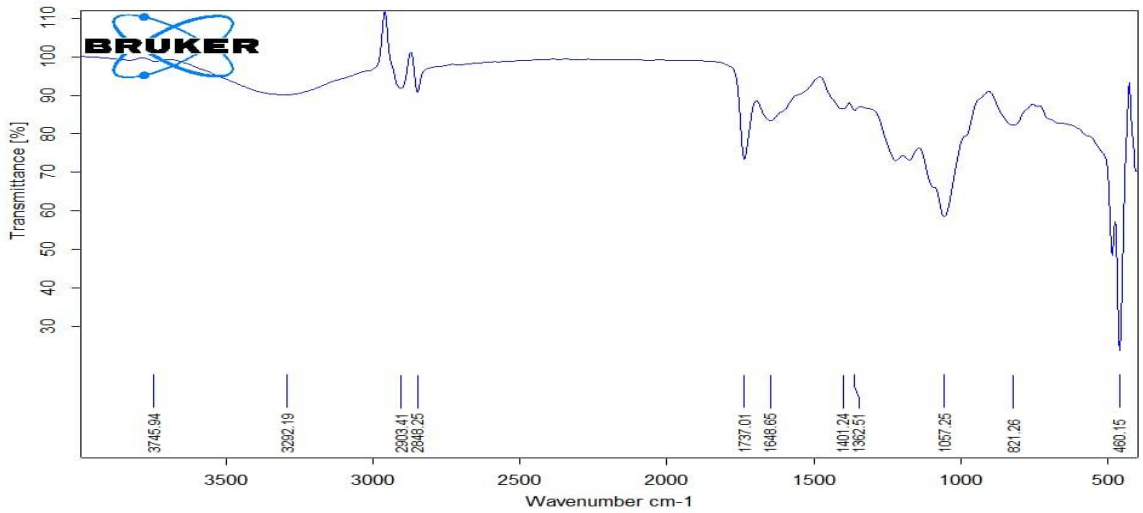


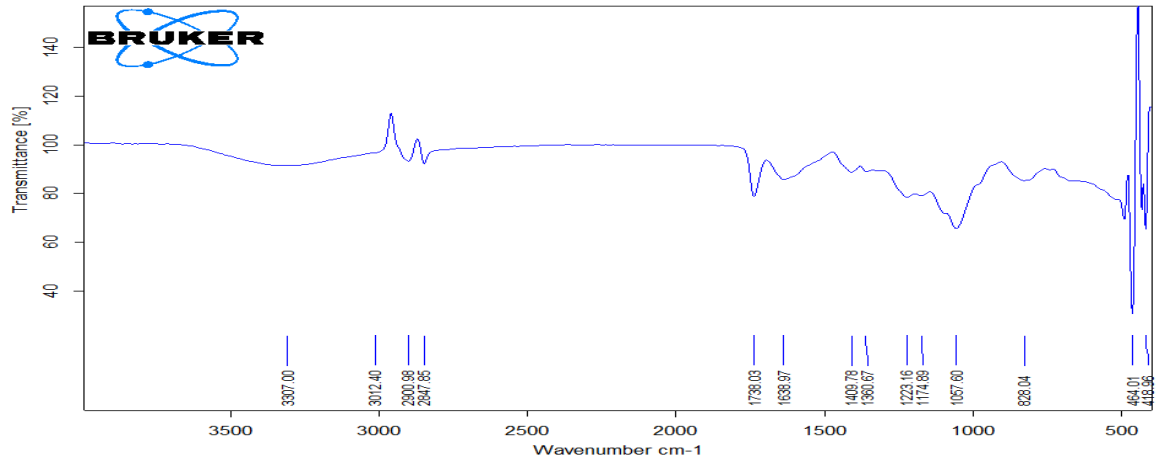
Fig 7. Maximum absorbance of pigment extract from bacterial isolates



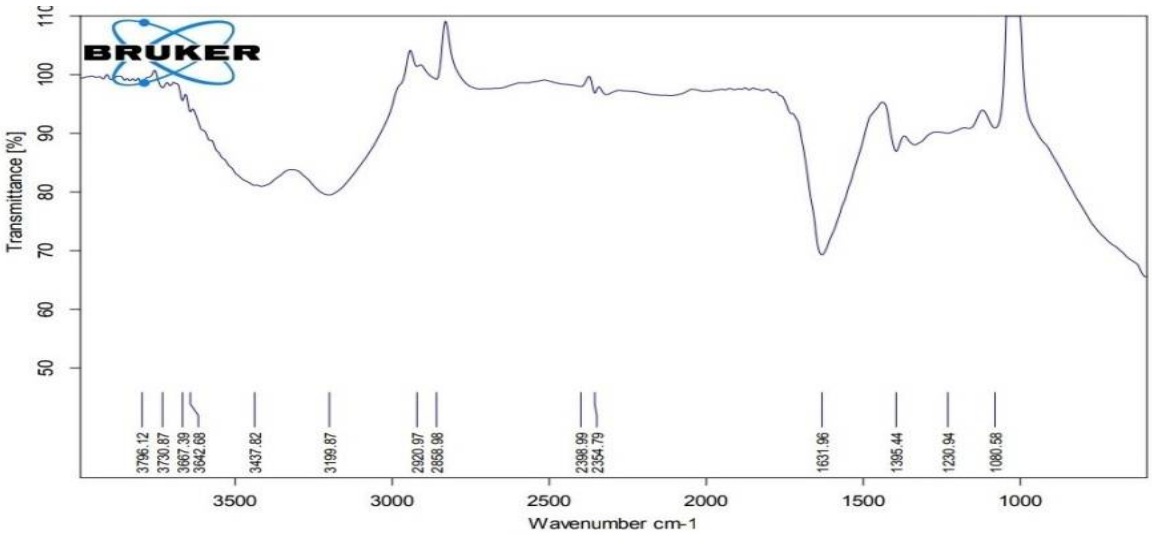
(A)



(B)



(C)



(D)

(A) DC1 (B) DC9 (C) DC-GY (D) DC-BY

Fig 8. FT-IR spectra stretches of pigments from isolates

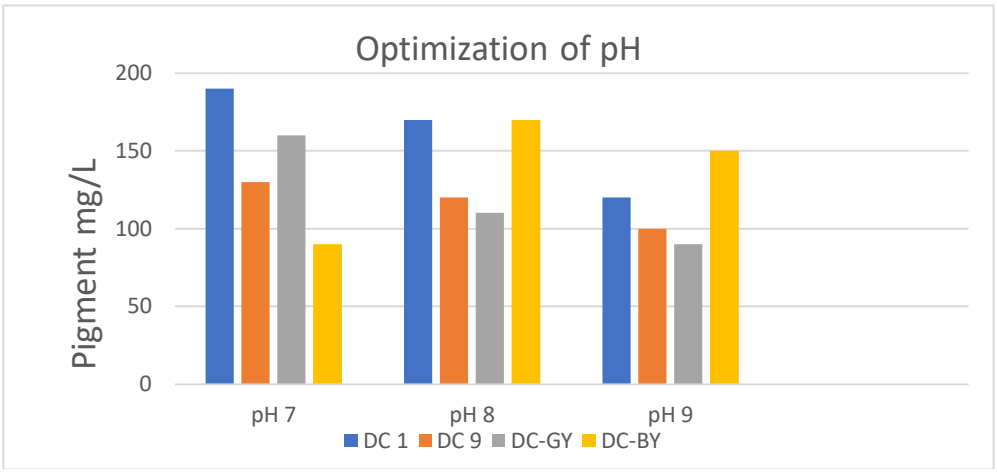


Fig 9. Optimization pH for pigment production

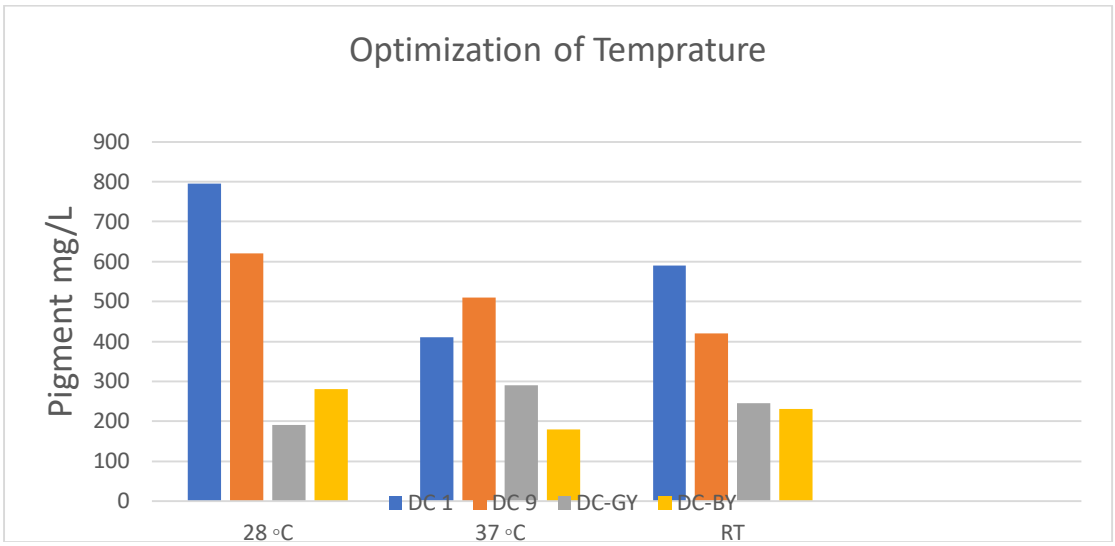
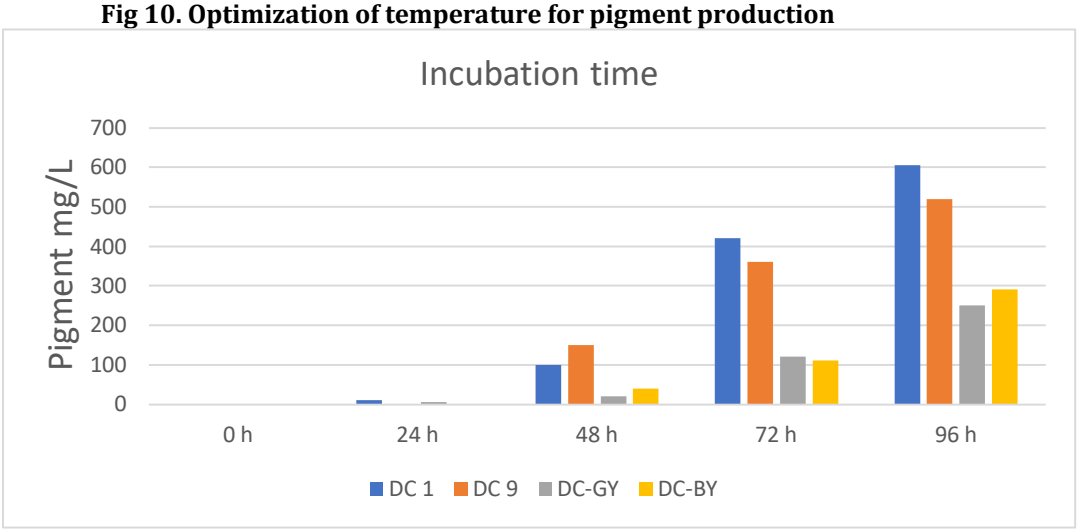
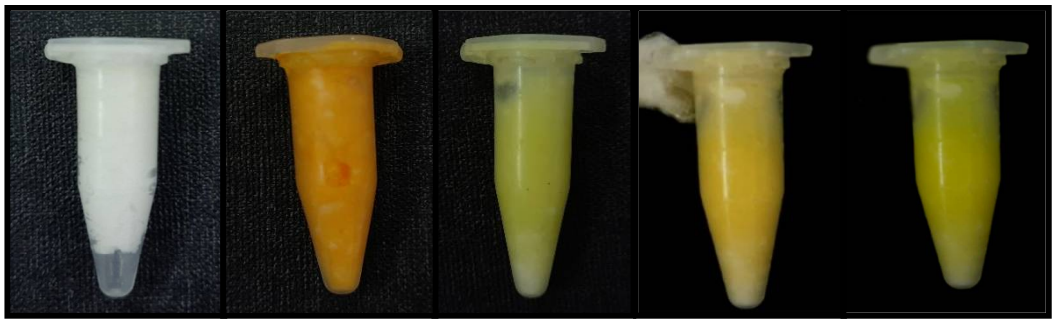


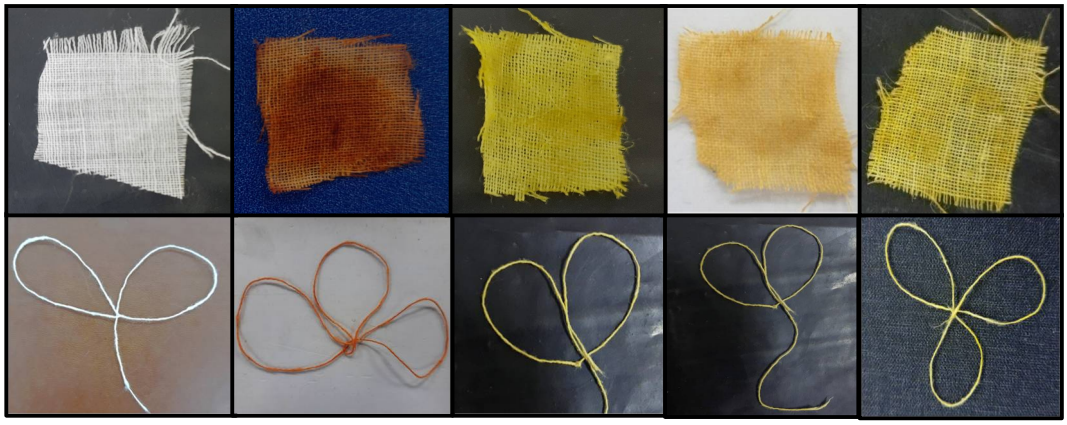
Fig 10. Optimization of temperature for pigment production





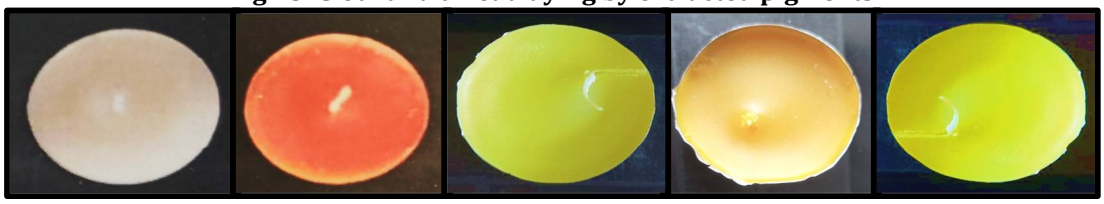
Control DC1 DC9 DC-GY DC-BY

Fig 12. Bio-lip balm preparation using pigment extract



Control DC1 DC9 DC-GY DC-BY

Fig 13. Cloth and thread dying by extracted pigments



Control DC1 DC9 DC-GY DC-BY

Fig 14. Application of pigments in candle preparation

CONCLUSION

Pigment producing marine bacterial cultures were procured from Department of Microbiology and Biotechnology, Gujarat University, Ahmedabad, India which were isolated from the coastal region of Gujarat. Dark orange, lemon yellow, golden yellow and bright yellow colored pigment producing bacterial isolates named DC1, DC9, DC-GY and DC-BY respectively were selected based on their pigment's visual high intensity, rapid growth, easy extraction with methanol as solvent and their high production. Salt tolerance activity of four of these cultures were studied using 3%, 5% and 10% NaCl concentration, from that they were found to be moderately halophilic. Submerged fermentation method was used for pigment production. The methanol extraction method was used mainly for pigment extraction. After quantifying the pigment production from DC1, DC9, DC-GY and DC-BY by dry weight method, the production was found to be 960 mg/L, 845 mg/L, 498 mg/L and 436 mg/L respectively. Pigment solubility in different solvent has been studied and it has been found that pigments from DC1, DC9 and DC-GY shows maximum solubility in hexane, while DC-BY shows maximum solubility in ethyl acetate. Characterization of extracted pigment by TLC, λ_{max} determination and FT-IR concluded that dark orange, lemon-yellow, golden yellow and bright yellow pigments from DC1, DC9, DC-GY and DC-BY are β-carotene or its derivatives. The study of optimization of pigment production concludes that at pH 7 to 8, 28 °C to 37 °C temperature and 72 h to 96 h incubation time parameters gives maximum pigment production for selected isolates. Pigment can be used as a crucibles factor in pharmaceutical industry. DPPH analysis of the pigments showed potential free radical scavenging activity. A study was conducted to determine the antimicrobial activity of the pigment against human pathogen, all four of them showed zone of inhibition against pathogens used for testing.

Hence, they contain the potential to be used in pharmacology. Pigments has been applied as coloring agent in bio-lip balm, textile material and candles and hence, they have the potential to be used in cosmetics, textiles, and other aesthetic industries.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interests to disclose.

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