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Evaluation and standardization of cultural conditions for maximum pigment production by *Pseudomonas* sp. from hot water springs

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Abstract
Water samples were collected from hot water springs of Himachal Pradesh and two pigment producing bacterial isolates M1 (Yellow) and MS2 (Orange) were obtained. Cultural conditions, including best carbon and nitrogen sources, were optimized and both the bacterial isolates showed maximum growth and pigment production in Luria Bertani medium for an incubation period of 72 hrs at pH 7.0 and temperature 40°C. Glucose for M1 and maltose for MS2 at 0.5% were found to be the best carbon source for maximum pigment production. Peptone (0.5%) for M1 and potassium nitrate (0.5%) for MS2 as nitrogen sources was best for pigment production. The results of spectral analysis showed λ_{max} at 437 for M1 and 435 for MS2 indicating the presence of carotenoids qualitatively. Hot water springs are excellent natural source for isolation of pigment producing microorganisms. Bacterial isolates produced good yield of pigment under controlled cultural conditions. Obtaining pigments from natural sources is an environment friendly approach. Pigment production from natural sources is also helpful for humans in lowering allergic reactions and several vital diseases

Keywords: Pigment, hot water spring, characterization, *Pseudomonas* sp. optimization, carotenoids

Introduction
Color is one of the significant visual properties and is an important attribute of any article. The color determines the acceptance of a product and has paramount influence on human life. Many synthetic colors used in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing pose various hazardous effects like allergies, tumor, cancer and severe damages to the vital organs (Duran *et al*., 2002). Moreover, the effluent of synthetic dyes poses series threat to the environment. Consequently, many synthetic colors have been banned due to their toxicological problems. With the increasing awareness about the toxic effects of synthetic colors and consumer safety, there is an increasing interest in the development of colors from natural sources.

Natural colors are generally extracted from fruits, vegetables, roots and microorganisms which are often called as bio-colors due to their biological origin. The presence of bio pigments has been reported in almost all the microorganisms including bacteria, fungi, yeasts and algae. These microorganisms can produce variety of bio-pigments such as carotenoids, melanins, flavones, quinines, prodigiosin, and monascins (Jiang *et al*., 2005; Dofosse, 2006) [13, 7]. Bacteria are good source of pigments. Bacterial pigment production is one of the emerging fields of research to demonstrate its potential for various industrial applications (Venil and Lakshmanaperumalsamy, 2009) [29]. The advantages of pigment production from bacteria include easy and fast growth in cheap culture medium, independent of weather and faster fermentation for bulk production (Venil *et al*., 2013) [29].

The hot springs and geothermal vents are found in different parts of the world which contain several prokaryotes, especially adapted to grow in these environments. These microorganisms are often colored due to the presence of photosynthetic and carotenoids pigments. Due to high temperature of hot springs, microbes are classified as thermophiles. These thermophiles are adapted to survive in extreme conditions with higher reaction rates, higher solubility and stability of most chemicals. The biocolors are used in food, textile and pharmaceutical industries. The use of non-allergic, nontoxic and eco-friendly natural dyes in textiles has become a matter of significant importance due to increased environmental awareness. Hence, due to harmful effect of chemical dye on environmental pollution a number of countries have issued strict regulations so as to preserve our environment. As a consequence there is a revived

interest in the use of natural pigments and dyes, which could be subjected to biodegradation in the environment.

Materials and Methods

Collection and physico-chemical analysis of water samples

In total 20, water samples were collected in sterilized screw capped tubes from the two locations viz., Manikaran of Kullu district and Tattapani of Mandi district of Himachal Pradesh. Samples were evaluated for physico-chemical characteristic viz., pH and temperature during survey. The samples were brought to Microbiology Laboratory, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan for further research work.

Isolation and enumeration of pigment producing bacteria

Isolation of the pigment producing bacteria from the collected water and soil samples were done by using standard serial dilution and plate count technique (Subba Rao, 1999) on Nutrient Agar medium. Plates were incubated for pigment production.

Screening of pigment producing bacterial isolates

After isolation, the sharp pigment producing colonies were selected and purified using streak plate technique on the medium for sharpness and stability of color. The isolates were primarily examined according to their colony morphology and pigment color.

Optimization of cultural conditions for maximum pigment production

Standardization of medium for maximum pigment production

The selected pigment producing bacterial isolates were grown on different nutrient medium viz. Nutrient agar, Luria Bertani, Tryptic soya agar and Yeast mannitol agar and their effect was studied on growth and pigment production after incubation.

Optimization of incubation period, pH and temperature

The effect of various cultural conditions like different incubation period (viz., 24, 36, 48, 96 and 120h), pH (3 to 9) and incubation temperature (25ºC to 50ºC with the interval of 5ºC) on growth and pigment production was studied separately for both the isolates by bacterization in the selected medium. The growth as well as the pigment production was determined separately.

Effect of carbon and nitrogen sources

Effect of various carbon (sucrose, maltose, lactose, fructose and galactose) and nitrogen sources (ammonium sulphate, sodium nitrate, potassium nitrate, peptone and urea) were studied in the selected media and their effect was studied on growth and pigment production after incubation. The effect of different concentrations (0.1, 0.2 and 1.0) of selected carbon and nitrogen sources were also studied growth on growth and pigment production.

Spectrophotometric analysis of pigments

The pigments from both the bacterial isolates i.e. M1 and MS2 were analyzed spectrophotometrically in a UV-Visible spectrophotometer for determining the maximum absorbance. The scanning range was selected from 200-800 nm and absorbance at an interval of 50 nm was measured (Slater *et al*. 2003) [27].

Degree of pigmentation

Degree of pigmentation was calculated by dividing the optical density of pigment (437 nm) by optical density of growth (540nm) (Sasidharan *et al*. 2013) [25].

Yield of pigment

To measure the yield, the grown matter was removed from surface of the media by scratching with spatula, dried at 60ºC and weighed (Attri and Joshi, 2005) [2].

Identification and characterization of pigment producing bacterial isolates M1 and MS2

Morphological and taxonomic characterization

Taxonomic and morphological based identification of the selected hyper pigment producing bacterial isolates was done as per Bergey's Mannual of Systematic Bacteriology (Claus and Berkley, 1986).

Biochemical characterization

In order to determine the biochemical characterization of the selected bacterial isolates following biochemical tests were performed (Holt *et al*. 1994) [11].

Molecular characterization

The molecular identification of the selected bacterial isolates was done by 16S rDNA sequencing and phylogenetic analysis (Pace, 1997) [22].

Results

Physico-chemical analysis of water from two hot water springs of Himachal Pradesh.

The pH and temperature of 20 water samples, collected from two different hot water springs representing 10 samples from Manikaran and 10 from Tattapani were determined and the results for variation are presented in Tables 1 and 2.

Table 1: Variability in pH of water collected from two hot water springs of Himachal Pradesh

Sites	Hot water spring samples										F_{cal}	F_{tab}
	I	II	III	IV	V	VI	VII	VIII	IX	X	1.26933	3.178893
Manikaran(M)	5	5.3	6.1	5.8	6	5.6	5.8	4.3	4.6	4.5		
Tattapani (T)	4.2	4.4	4.6	4.8	5.9	5	5.1	4.7	6	4.9		

Table 2: Variability in temperature (ºC) of water collected from two hot water springs of Himachal Pradesh

Sites	Hot water spring samples										F_{cal}	F_{tab}
	I	II	III	IV	V	VI	VII	VIII	IX	X	1.560247	3.178893
Manikaran	105	76	104	60	59	100	101	95	94	95		
Tattapani	30	56	40	45	59	55	56	57	68	80		

Isolation, enumeration and screening of pigment producing bacterial isolates

Isolation was done from collected hot water samples and a total of 33 bacterial isolates were obtained which were then subjected to morphological studies. The results for variation in morphological characteristics i.e., form, elevation, margin and color were noticed and are presented in Table 3.

Out of the 33 bacterial isolates, 2 bacterial isolates viz., M1 and MS2 from Manikaran hot water spring were sharp pigment producers with yellow and orange pigmentation and were therefore selected for further studies (Fig. 1(a) and 1(b)).

Table 3: Morphological characteristic of bacterial isolates from two hot water springs

Site	Sample name	Morphotypes	Colony morphology				Viable count ($\times 10^4$cfu/ml)
			Form	Elevation	Margin	Color	
Manikaran	M1	M11	Circular	Flat	Entire	Yellow	38
(M)		M12	Punctiform	raised	Entire	Cream	45
	M2	M21	Circular	Flat	Entire	Cream	35
		M22	circular	flat	Entire	Light brown	64
	M3	M31	Circular	Raised	Entire	Pale yellow	45
	M4	M41	Irregular	Flat	Undulate	Cream	45
		M42	Circular	Raised	Entire	Pale yellow	59
	M5	M51	Irregular	Flat	Undulate	Light orange	36
	MS1	MS11	Punctiform	Raised	Entire	Cream	48
		MS12	Circular	Flat	Entire	Light brown	34
	MS2	MS21	Circular	Flat	Entire	Orange red	67
		MS22	Irregular	Flat	Entire	Cream	42
	MS3	MS31	Irregular	Flat	Undulate	Light brown	59
		MS32	Circular	Raised	Entire	Light yellow	47
	MS4	MS41	Irregular	Flat	Undulate	Cream	55
	MS5	MS51	Irregular	Flat	undulate	White	67
		MS52	Punctiform	Raised	Entire	Cream	44
Tattapani	T1	T11	Circular	Raised	Entire	Cream	55
(T)	T2	T21	Circular	Raised	Entire	Light orange	45
		T22	Circular	Convex	Entire	Cream	44
	T3	TS31	Circular	Raised	Entire	Cream	55
	T4	T41	Circular	Flat	Entire	Pale yellow	39
		T42	Circular	Raised	Entire	Cream	33
	T5	T51	Circular	Raised	Entire	White	60
		T52	Circular	Flat	entire	Light orange	55
	TS1	TS11	Circular	Flat	Entire	Cream	43
	TS2	TS21	Circular	Raised	Entire	Light orange	55
		TS22	Circular	Flat	Entire	Cream	43
	TS3	TS31	Circular	Raised	Entire	Pale yellow	66
	TS4	TS41	Circular	Raised	Entire	Light orange	51
		TS42	Circular	Flat	Entire	Cream	56
	TS5	TS51	Circular	Raised	Entire	White	32
		TS52	Circular	Convex	Entire	Cream	56

Identification of pigment producing bacterial isolates M1 and MS2

Morphological and biochemical characterization

The selected pigment producing bacterial isolates were marked on the basis of their morphological and biochemical characteristics and results are presented in Table 4. Both the bacterial isolates were gram negative, motile and rod shaped. Both the bacterial isolates utilized glucose and also showed positive results for catalase, oxidase and simmon's citrate tests.

Table 4: Morphological and biochemical characteristics of the selected pigment producing bacterial isolates

Morphological Characters		
Characteristics	M1	MS2
Gram's reaction	-	-
Colony morphology	Rod	Rod
Color	Yellow	Orange red
Motility	+	+
Biochemical Characters		
Catalase	+	+
Oxidase	+	+
Simmon's citrate	+	+
Growth with7.5 % NaCl	-	-
Mannose	-	-
Lactose	-	-
H2S production	-	-
Glucose	+	+
Methyl Red Test	-	-
Voges Proskaur Test	-	-
Indole Test	-	-
Urease Test	-	-

Molecular characterization of the selected isolates M1 and MS2

Amplification of isolated 16S rRNA gene was done by PCR. The results for *In-silico* analysis of entire 16S rRNA gene sequencing and phylogenetic analysis for the bacterial isolates i.e. M1 (NCBI Gen Bank accession No. KY940035) and MS2 (NCBI GenBank accession No.KY947105) showed that both the bacterial isolates were highly similar to *Pseudomonas* sp. (Fig. 2).

Spectrophotometric analysis of pigment

The pigments produced by both the bacterial isolates were analyzed by scanning the pigments within a range of 200-800 nm in spectrophotometer. The absorption maxima for both the bacterial isolates were recorded at 437 nm for M1 and 435 nm for MS2 isolate with broad shoulder at 437 nm for both the isolates. The results indicated that pigments belong to carotenoid family as carotenoids has absortion spectrum around 450nm (Fig. 3(a) and 3(b)).

Optimization of cultural conditions for maximum pigment production

Effect of different media on growth and pigment production

For the maximum pigment production by the bacterial isolates effect of different nutrient media viz., Nutrient Agar (NA), Luria Bertani (LB), Yeast Extract Mannitol Agar (YEMA) and Tryptic soya agar, was tested. Both the bacterial isolates showed maximum growth as well as pigment production on LB medium (Fig. 4(a) and 4 (b)).

Effect of incubation period on growth and pigment production

Incubation period is one of the important factors which affect the growth and product accumulation by microorganisms. Here effect of different incubation period was studied on growth and pigment production by both the bacterial isolates and results are presented in Fig. 5(a) and 5(b). Results showed that an incubation period of 72 hrs was best for the growth and pigment production. After reaching to maximum growth at 72 hrs the growth of pigment producing microorganisms started decline gradually on further incubation.

Effect of incubation temperature on growth and pigment production

Temperature of incubation is the main factor which depends on the type of microorganism. The production of microbial pigments is greatly affected by the temperature of incubation period. The optimum temperature was 40ºC for maximum pigment production by both the pigment producing bacterial isolates in the present study (Fig. 6(a) and 6(b)).

Effect of pH on the growth and pigment production by the selected bacterial isolates

The results on the effect of pH on growth and pigment production by the selected pigment producing bacterial isolates are presented and illustrated in Figure 7(a) and 7(b). The pigment production was observed maximum at pH 7 for both the bacterial isolates and minimum at pH 3. The results indicated that neutral to alkaline pH favored the pigment production.

Effect of carbon source and its concentration

Glucose for M1 and maltose for MS2 at a concentration of 0.5 was found to be the best carbon source for maximum growth and pigment production (Fig. 8(a) and 8(b); Fig. 9(a) and 9(b)). Glucose due to ease in substrate transportation is most suitable for growth of microorganisms.

Effect of nitrogen sources and its concentration.

The present studies have shown that both the strains utilized different nitrogen sources. The yellow color producing M1 strain utilized peptone and orange color producing MS2 utilized potassium nitrate as nitrogen source at a concentration of 0.5 percent (Fig. 10(a) and 10(b); Fig. 11(a) and 11(b)).

Discussion

In the present study two sharp pigment producing bacterial isolates M1 and MS2 with yellow and orange pigmentation were isolated from hot water springs of Himachal Pradesh. Both the bacterial isolates showed good potential for pigment production. Similar to our studies pigment producing microorganisms were isolated from air and soil on Nutrient Agar medium (Sasidharan *et.al.*, 2013; Goswami *et al.* (2010) [25, 10].

According to Bergey's manual both the bacterial isolates M1 and MS2 were *Pseudomonas* sp. (Holt *et al.* 1994) [11]. 16S rRNA gene analysis result also showed that both the bacterial isolates were *Pseudomonas* sp.16S rRNA gene analysis result showed that both the bacterial isolates were *Pseudomonas* sp. Mukhrerjee *et.al*. (2012) isolated a green pigment producing bacteria from Bakreshwar Hot Spring and 16S rRNA gene sequencing result indicated that the isolate showed 100 percent sequence alignment with *Pseudomonas aeruginosa* strain GIM 32.

Maximum absorption spectra of pigments produced by both isolates was observed at 435 nm and 437 nm which was similar to the absorption spectra of carotenoids. UV-VIS spectrophotometer analysis of yellow pigment from bacteria exhibited the absorption maxima at 437 nm. Goswami *et. al*. (2010) [10], Khanafari *et.al*. (2010) [16] and Sasidharan *et al*. (2013) [25] also suggested the absorption spectrum of carotenoids to be between 400 nm to 550nm.

Grossart *et al*. (2009) reported maximum pigment production using Luria Bertani medium. Isolates M1 and MS2 showed good growth in Lubria Bertani medium instead of other media.

Bacterial isolates showed good growth and pigment production for an incubation period of 72 hrs followed by a decrease after 96 hrs. Growth and pigment production was decreased on further incubation (Goswami *et al*., 2010) [10]. Bhat and Marar (2015) [4] also reported the orange pigment production by *Salinicoccus* sp. after 72 hrs of incubation period.

Temperature of incubation is the main factor which depends on the type of microorganism. The production of microbial pigments is greatly affected by the temperature of incubation period. In the present study a temperature of 40˚C was best for maximum growth and pigment production by bacterial isolates M1 and MS2 followed by 45˚C. A decrease in pigmentation and growth was observed after 45˚C. The cell biomass and pigment production increased with increasing temperature up to 40ºC and lessened sharply above 45ºC due to the denaturation of the enzymes system of microorganisms (Ibrahim, 2008). Sasidharan *et. al.* (2013) [25] and Goswami and Bhowal (2014) [9] also reported the growth of yellow and red pigment producing bacterial strains at 37˚C which stands at par of our results.

A neutral pH was best for maximum pigment production as after pH growth and pigment production started decline. This showed that acidic or alkaline pH has inhibitory effect on growth and pigment production of both the isolates. Various factors affecting the pigment production have been studied by Goswami *et al*. (2010) [10], Mukherjee *et al*. (2012) [21], Tarangini and Mishra (2014) [28] and Bhat and Marar (2015) [4]. They have reported that a pH of 7 was optimum for maximum pigment production.

The results have shown that glucose (0.5%) for M1 and maltose (1.0%) for MS2 (0.5%) were the best carbon sources for high pigment production. The high growth and pigmentation at given concentration and source may be due to the reason that the growth of pigment producing microorganism is affected by the type of carbon sources. Glucose due to ease in substrate transportation is most suitable for growth of microorganisms. Maximum yield of pigment and growth was found in medium having glucose and maltose as carbon sources which was also observed by Lee *et al*. (2011) [18], Pongrawee and Saisamorn (2011) [24] and Shahitha and Poornima (2012) [26].

The present studies have shown that both the strains utilized different nitrogen sources. The yellow color producing M1 strain utilized peptone and orange color producing MS2 utilized potassium nitrate as nitrogen source at a conc. of 0.5 %. These differences may be attributed to the ability of micro organism to use a particular nitrogen sources using specific pathways. Padmavathi and Tallapragada (2011) [23] also studied the effect of different nitrogen sources on the pigment production and growth. They reported that the bacterial strain was able to grow on peptone media. Highest yield of biomass and pigment was obtained by *Sarcina* sp. in medium supplemented with potassium nitrate as a nitrogen source (Joshi *et al.*, 2011) [2]. *Monascus* sp. showed the good growth and pigment production by utilizing peptone and ammonium as nitrogen source (Kumar *et al*., 2015) [17].

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