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ISOLATION AND IDENTIFICATION OF PIGMENT PRODUCING BACTERIA FROM THE RATARGUL SWAMP FOREST SOIL

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Abstract

Pigments are one of the most significant secondary metabolites produced by microorganisms. The aim of the present study was to isolate and identify pigment-producing bacteria from the Ratargul Swamp Forest (RSF) soil, which is the one and only fresh water swamp forest of Bangladesh. Soil samples were randomly collected from 10 different quadrates (10 m x 10 m) of RSF. The pH values of the soil samples were found to be strongly acidic and ranged between 4.71 and 5.48. Bacterial load of the samples ranged from 1.33×10^5 to 1.93×10^8 cfu/g, 6.05×10^6 to 9.07×10^7 cfu/g and from 1.16×10^7 to 1.61×10^8 cfu/g on nutrient agar (NA), peptone yeast-extract glucose (PYG) agar and Luria-Bertani (LB) agar media, respectively. Interestingly, both the highest and lowest bacterial counts were observed on NA, which was 1.93×10^8 cfu/g and 1.33×10^5 cfu/g, respectively. The isolates were found to produce various pigments like yellow, red, dark orange and sweet pink during their colony developments. A total of 71 bacterial isolates were obtained of which 11 were subjected to further study. All the selected bacteria were found to be rod shaped. Out of the 11 isolates, 9 were Gram-positive and 2 were Gram-negative. Provisionally identified potential pigment producing eight bacterial isolates were identified by using molecular marker. Seven of them were matched with their conventional identification up to generic level but conventionally identified *Erwinia stewartii* was found to be as *Aeromonas sobria*. Among the 11 isolates, 8 could produce three different types of pigments namely red, yellow and dark orange during *in vitro* pigment production. The isolated pigment producing bacteria could be used for better biotechnological application.

Introduction

Both natural pigments and synthetic dyes have been extensively used in various fields of everyday life such as food, feed, textile, paper, printing ink, cosmetic and pharmaceuticals. Since color is an important attribute that determines the consumer's acceptance of food, color additives are essential in food industry. As a result, various synthetic food colors have been manufactured but many of them encompass various hazardous effects. Due to the toxicity of several artificial colorants, uses of natural additives are of increasing interest. Increasing consumer's awareness put string emphasis on the production of colors or natural colors extracted from fruits, vegetables, roots and microorganisms [(1)].

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Microbial pigment production is a recent phenomenon. When the microbial cells are used to produce the color the term refers to 'microbial pigments'[2]. These are one of the most significant secondary metabolites produced by microorganisms. The main role of these pigments is to protect the bacterial cells against injurious rays of light. It also helps them in their photosynthetic activities and this has been observed specially in case of the members of cyanobacteria. Pigments produced from natural sources are of worldwide interest and is gaining significance. This type of pigment is highly productive because it is highly versatile and prolific over other sources. Cheap substrates can be used for bulk production of pigments even sometimes it can grow on waste products. Thus, it also recycles the waste of the environment[3].

Bacterial fermentation is inherently faster and more productive compared to any other chemical process. Bacterial genes are easy to manipulate, easy for propagation and wide strain selection can also be done. So simple and fast culturing of bacterial cells allow continuous bioreactor operation that makes it more convenient for large scale production of pigments. Though microbial pigments are several times more expensive, they still can compete with synthetic dyes for being natural and safe. There is an increased push to reduce the production costs for microbial pigments by using low cost substrates or strain improvements, and in the near future, there may be a monopoly market for microbial pigments.

Textile industries remain the largest consumer of organic pigments and dyes, while faster growth is expected to occur in other industrial sector such as printing inks, paints, and coating agents [4]. Considering the increasing demand of microbial pigments and environmental hazards caused by synthetic colors, the present study was aimed to isolate and identify pigment producing bacteria from the Ratargul Swamp Forest soil.

Materials and Methods

Ten soil samples were randomly collected from ten different quadrates of Ratargul Swamp Forest (RSF). Samples were collected aseptically in sterile plastic bags. The samples were labeled properly and brought into the laboratory as soon as possible and pH was measured by a pH meter (ToA-DKK, HM-31P, Japan). Serial dilution technique[5] was followed using nutrient agar (NA)[6], peptone yeast extract glucose (PYG) agar[7] and Luria-Bertani (LB) agar[7] media for enumeration of aerobic heterotrophic bacteria associated with the collected RSF soil samples. Inoculated plates were inversely incubated at 37°C for 24 h in an incubator (Memmert GmbH + Co Kg 8540 Schwabach, Germany) followed by seven days of refrigeration at 4ºC for developing discrete pigmented bacterial colonies. Then, the colonies were counted, isolated and screened on the basis of visual estimation for further studies. The selected bacterial isolates were purified through streak plate technique (Fig. 1). Following standard manuals, Gram staining and essential biochemical tests were performed. Characterization and identification of the isolates were made through standard microbiological methods[8].

Molecular identification of potential ten isolates was conducted by amplifying ~600bp fragments of 16S rDNA followed by sequence analysis using NCBI-BLAST search (Fig. 2).

Amplification of DNA was done using the primer pair CC [F] 5'-CCAGACTCCTAC GGGA GGCAGC-3 ′ and CD [R] 5'-CTTGTGCGGGCCCCCGTCAATTC- 3′ designed by Rudi *et al.*[9]. Supernatant of heat-lysed cell suspension was used as the source of template DNA for PCR amplification. The amplified products were separated electrophoretically on 1% agarose gel. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system (Microdoc DI-HD, MUV21-254/365, Cleaver Scientific, UK). Sequencing of DNA was performed in an automated gene sequencer and sequences were analyzed through NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and rRNA BLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs.

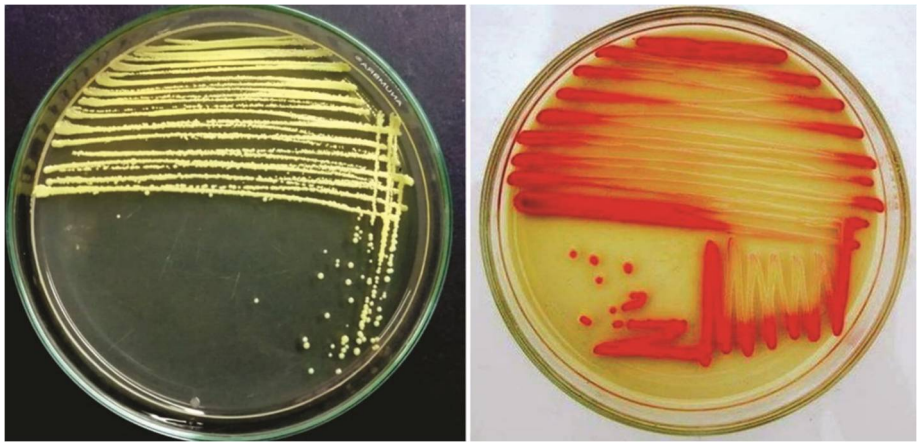

Fig. 1. Purification of pigmented bacterial isolate by streak dilution.

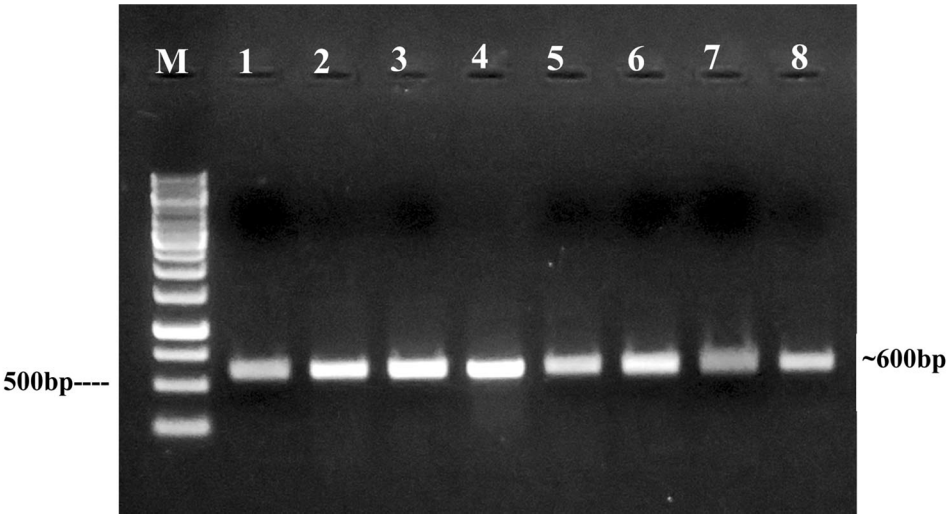

Fig. 2. PCR amplification of part of the 16S rRNA gene. Lane M is the 1.0 kb ladder and lanes 1-8 are representing 8 different bacterial isolates obtained from Ratargul Swamp Forest soil.

Results and Discussion

A total of ten soil samples were randomly collected from ten different quadrates of Ratargul Soil Forest. The bacterial load of different samples was shown in Table 1. The heterotrophic bacterial load of the collected samples ranged in between 1.33×10^5 to 1.93×10^8 cfu/g, 6.05×10^6 to 9.07×10^7 cfu/g and 1.16×10^7 to 1.61×10^8 cfu/g on NA, PYG agar and LB agar media, respectively. Both the highest and lowest bacterial count was observed on NA which was 1.93×10^8 cfu/g (Quadrate 1) and 1.33×10^5 cfu/g (Quadrate 3), respectively. The difference in bacterial count might be due to the difference between the physical and chemical properties of soil and biotic-abiotic stresses that might lead to pigment production of the bacteria present at that particular habitat as their defense mechanism. The indigenous organisms ecologically adapted to a particular habitat may be the cause of differences between isolated and referred organisms. In course of this adaptation their characteristics could be modified gradually[10]. The results revealed that pigment producing bacteria are randomly distributed in the soil in RSF.

Table 1. Heterotrophic bacterial load of the collected soil samples from the Ratargul Swamp Forest (RSF) soil.

Quadrate (Q) No.	Bacterial load (cfu/g) on different media		
	NA	PYG agar	LB agar
Q_1	1.33×10^5	2.20×10^7	1.61×10^8
Q_2	1.54×10^7	3.50×10^7	1.20×10^7
Q_3	**1.93×10^8**	1.57×10^7	1.41×10^7
Q_4	1.40×10^8	2.11×10^7	1.96×10^7
Q_5	9.92×10^7	5.23×10^7	2.19×10^7
Q_6	4.51×10^7	2.35×10^7	3.78×10^7
Q_7	3.34×10^7	9.07×10^7	1.37×10^7
Q_8	5.17×10^7	3.15×10^7	2.66×10^7
Q_9	5.23×10^7	1.22×10^7	1.16×10^7
Q_{10}	9.42×10^7	6.05×10^6	2.81×10^7
Average	**1.40×10^8**	**3.10×10^7**	**3.46×10^7**

NA = Nutrient Agar, PYG =Peptone Yeast extract Glucose, LB =Luria-Bertani

All the vibrant pigment producing bacterial isolates were subjected to qualitative screening on the basis of visual estimation. During this study, 71 pigment-producing bacteria were isolated from the different samples and finally 11 isolates were selected for conventional identification. Out of 11 isolates, 9 were Gram-positive rods and 2 were Gram-negative rods. This was in accordance with the findings of other workers[11] where they reported 4 Gram positive rod shaped pigment producing bacteria cultured from soil and air samples.

Table 2. Major biochemical tests and provisional identification of Gram positive bacteria isolated from Ratargul Swamp Forest (RSF) soil.

Isolates	Biochemical tests							Provisional Identification
	Catalase	VP	Starch	Casein	Citrate	Propionate	Nitrate	
S1/N/1	-	-	-	-	+	+	+	*Bacillus megaterium*
S2/L/1	+	+	-	+	-	-	+	*Microbacterium imperial*
S2/L/4	+	+	-	+	-	+	-	*Microbacterium laevaniformans*
S2/L/3	+	+	-	+	-	-	-	*Microbacterium imperial*
S2/L/7	+	+	-	+	-	+	-	*Microbacterium laevaniformans*
S3/L/2	+	-	-	+	-	+	-	*Microbacterium laevaniformans*
S4/P/3	-	-		-	+	+	-	*Bacillus megaterium*
S5/L/2	+	-	-	+	-	+	-	*Microbacterium laevaniformans*
S7/N/3	-	-	-	+	+	+	+	*Bacillus firmus*

VP =Voges-Proskauer,'+' = positive result '-' = negative result

Table 3. Major biochemical tests and provisional identification of Gram negative bacteria isolated from Ratargul Swamp Forest (RSF) soil.

Isolates	Biochemical tests							Provisional Identification
	Catalase	Oxidase	Urease	Indole	H_2S	Tyrosine	KOH	
S2/L/6	+	+	+	-	-	-	+	*Serratia marcescens*
S3/L/5	+	-	+	-	-	-	+	*Erwinia stewartii*

'+'= positive result '-' = negative result.

Results of some of the major biochemical tests for provisional identification are shown in Table 2 and 3. The isolated Gram-positive rods were provisionally identified as the different species of the two genera *Microbacterium* and *Bacillus.* In a recent study, Fatma *et al.*[12] reported a novel antioxidant pigment produced by a photochromogenic *Microbacterium oxydans* FJM1. Carotenoid production by a novel isolate of *Microbacterium paraoxydans* also reported by others[13]. In another study, Goswami and Bhowal[1] reported red pigment production from a novel strain of *Bacillus* species. On the other hand, 2 Gram-negative isolates were identified as *Serratia marcescens* and *Erwinia stewartii*. Sinha *et al.*[3] reported red and light orange pigment production by *Serratia* whereas Mohammadi *et al.*[14] reported pigment production by *Erwinia*.

Table 4. Molecular identification of some potential bacterial isolates from Ratargul Swamp Forest soil.

Isolate No.	Molecular identification				
	Scientific name	Strain	Max. coverage score	E value	Identity match (%)
S2/L/1	*Microbacterium* sp.	C-2 PWB-7	974	0.0	97.57%
S2/L/4	*Microbacterium oleivorans*	MV-19	789	0.0	91.53%
S2/L/6	*Serratia*sp.	Clone-48	1089	1e-23	87.61%
S2/L/3	*Microbacterium* sp.	HBUM 178211	989	0.0	98.08%
S2/L/7	*Microbacterium* sp.	M 24	989	0.0	98.40%
S3/L/2	*Microbacterium oleivorans*	MV 5	974	0.0	98.05%
S3/L/5	*Aeromonas sobria*	SB 16	1343	3e-20	97.01%
S5/L/2	*Microbacterium oleivorans*	UAC-76	664	0.0	87.82%

Identification of 8 potential pigment producing isolates was further confirmed through molecular analysis based on 16S rRNA gene sequencing. Following BLAST search analysis of the obtained sequences the isolates were identified as *Microbacterium* sp. C-2 PWB-7, *Microbacterium oleivorans* MV-19, *Serratia* sp. clone-48, *Microbacterium* sp. HBUM 178211, *Microbacterium* sp. M24, *Microbacterium oleivorans* MV5, *Aeromonas sobria* SB16, *Microbacterium oleivorans* UAC-76 (Table 4).

Table 5. Comparison between provisional and molecular identification of eight isolates obtained from Ratargul Swamp Forest Soil.

Isolate No.	Provisional Identification	Molecular Identification
S2/L/1	*Microbacterium imperiale*	*Microbacterium* sp. C-2PWB-7
S2/L/4	*Microbacterium laevaniformans*	*Microbacterium oleivorans* MV-19
S2/L/6	*Serratia marcescens*	*Serratia* sp. Clone-48
S2/L/3	*Microbacterium imperiale*	*Microbacterium* sp. HBUM 178211
S2/L/7	*Microbacterium laevaniformans*	*Microbacterium* sp. M 24
S3/L/2	*Microbacterium laevaniformans*	*Microbacterium oleivorans* MV 5
S3/L/5	*Erwinia stewartii*	*Aeromonas sobria* SB 16
S5/L/2	*Microbacterium laevaniformans*	*Microbacterium oleivorans* UAC-76

In this study, molecular identification of seven isolates were matched with their provisional identification up to generic level and only one isolate was found to be different. Comparison between provisional and molecular identification was shown in Table 5. Therefore, conventional identification of bacteria based on their morphology, physiological and biochemical profile was found to be valid to some extent in comparison to molecular identification.

The present study was found to be effective for the isolation of the pigment producing bacteria from RSF soil. Now a day's researchers are focusing on bio-color producing micro-organisms for replacing the demand of synthetic colors by it. The search for promising strains of pigment producers is a continuous process and development of efficient pigment producing bacteria is a prime need to control environmental hazards due to random use of synthetic non-biodegradable dyes. Thus, the present study deals with an approach of developing new sources of bio-colors from easily cultivable indigenous soil bacterial species that can be further exploited at larger scale.

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