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Optimization of Pigment Production by a Novel *Bacills* sp. BBMRH Isolated from Cow Dung

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Cow dung has substantial potential for microbial diversity. On account of that an attempt was made to study the total aerobic heterotrophic bacteria of cow dung procured from local market of Bhubaneswar, Odisha. Out of 15 bacterial isolates, CD-5 showed deep red pigmentation in nutrient broth culture medium. The biochemical characterization of red colour pigment producing bacterial isolate resembled to the genus *Bacillus*. Moreover, significant pigment production was observed at pH 7.0 ±0.1 and temperature 34ºC. The extracted pigment showed maximum similarity with Rhodamine-6G as revealed from absorption spectrum. The potent bacterial isolate was identified by 16S rRNA gene sequencing and found to be Bacillus sp. with accession number KF175230. Then the bacteria was identified by 16S rRNA gene sequencing and found to be *Bacillus* sp. with accession number KF175230.

Key words: Pigmentation, *Bacillus*, 16S rRNA, Rhodamine-6G, Absorption.

Indiscriminate use of anthropogenic colorant possesses various effects in food, cosmetics and pharmaceutical industries. To minimize this, there is an urgent need to develop processes for intact pigment production from natural resources. Natural pigments can be obtained from two major sources such as plants and microorganisms[1]. Microorganisms produce various pigments like carotenoids, melanins, flavones, quinones and more specifically monascins, violacein or indigo[2]. Hence microbial pigment production is one of the emerging fields of research to demonstrate its potential for various industrial applications. This is due to stability, solubility than eukaryotic sources, rapid growth and productivity round the clock, microbial pigments are of industrial interest[3, 4]. Apart from the advantages of pigment production from bacteria include easy and fast growth in cheap raw materials, independence from weather conditions and colours of different shades[5]. Thus many bacterial isolates were reported to produce different colour pigments. Moreover, *B. subtilis* var. *niger* acquired its name from its black pigment[6], *B. megaterium* red pigment carotenoid associated with the membrane[7]. Similarly a mutant of *B. thuringiensis* producing melanin which was significantly resistant to UV radiation at 254 and 366nm than its non-pigmented parental strain, thereby increasing its insecticidal activity under field conditions[8].

Cow dung is a "gold mine" due to its wide applications in agricultural, environmental and pharmaceutical realm along with cheap and easily availability regarded as rich source of microbes. In India, there are an estimated over 250 million cattle and one third of the dung produced annually[9]. Ideally it is a mixture of dung and urine in a ratio of 3:1 (w/v) [10] Moreover, pigments produced by bacteria can be extracted using solvent extraction process for production of dyes and colour[11]. According to[11] pigments produced by bacteria can be extracted using solvent extraction process for production of dyes and colour. This interesting group of compounds occurs extensively

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throughout both the plant and the animal kingdoms [12]. The present research work is aimed at characterization and application of pigment produced by bacterial isolates of cow dung origin.

MATERIALS AND METHOD

Sampling and isolation of bacteria

Representative cow dung samples were collected form unit-1 market of Bhubaneswar, Odisha at an elevation of 45 meters with 20°15'48"N latitude and 85°49'57"E. The samples were collected in sterile plastic bag and were transported to the laboratory aseptically for further analysis. The samples were processed in the laboratory for isolation of aerobic heterotrophic bacteria using standard procedures of serial dilution and spread plating. Colonies of distinguished morphologies were individually picked and sub-cultured and preserved in NA slant at 4°C for further use.

Biochemical characterization of bacterial isolates

The morphological and physiological properties of the isolates were investigated on the basis of their colony characteristics on the Nutrient agar medium and Gram's reaction. After the microscopic examination the bacterial isolates were processed for identification by the standard prescribed biochemical tests, enzymatic and sugar utilization test required by Bergy's manual of determinative bacteriology[13] and PIB-Win software [14].

Optimization of growth parameters

Temperature

The temperature tolerance test was conducted to find out the optimum temperature for growth of the bacterial isolate. Selected bacterial cultures were revived in nutrient broth. 100ml of nutrient broth was taken in different conical flask and the temperature was varied from 28°C to 40°C, keeping constant pH at 7. 100µl of the overnight culture was dispensed into each conical flask and incubated at different temperature (28°C, 30°C, 32°C, 34°C, 36°C, 38°C and 40°C) for 24 hours. Then the optimum temperature was determined by taking O.D at 600nm.

pH

The pH tolerance test was conducted to find out the optimum pH for growth of the bacterial isolate. Selected bacterial cultures were revived in nutrient broth. 100 ml of nutrient broth was taken in different conical flask and the pH was adjusted from 4-10 with help of 1N HCl, 1N NaOH. 100µl of the overnight culture was dispensed into each conical flask and incubated at 34°C for 24 hours. Then the optimum pH was determined by taking O.D at 600nm.

Pigment production and extraction

The pigment extraction was carried out by minor modification of method described by [11]. The selected bacterial isolate (CD-5) was cultured in nutrient agar plate at optimum pH and temperature 7±0.1 and 34°C respectively. The pigmented bacterial cells biomass was collected in a 50ml centrifuge tube by scraping method. Then the cell biomass was tried for extraction of pigment using various organic solvents such as hexane, benzene, chloroform, methanol and ethanol. However, successful extraction of pigment was observed in the organic solvent ethanol with a purity of 99.7%, when centrifuged at 4ºC, 7500g for 17 minute and the supernatant was collected. Similarly the cell pellets were then again rinsed with ethanol followed by centrifugation to recover the residual crude pigment extract. Then the crude pigment was primarily identified by measuring O.D. at 200-800nm using an UV-visible spectrophotometer (UV 1601PC; Shimadzu).

Antibiotic sensitivity test

The selected bacterial isolate (CD-5) was screened for the antibiotic resistance by following disc diffusion method[15]. The isolate was exposed to most common antibiotics like Nystatin, Penicillin-G, Bacitracin, Ampicillin, Polymyxin-B, Optochin, Chloramphenicol, Streptomycin, Gentamicin and Amoxicillin to observe the effect of these antibiotics on the physiology of the pigment producing bacterial isolate.

Phylogenetic analysis of bacterial isolate

The bacterial genomic DNA was isolated by phenol-chloroform method. Then the 16S rDNA fragment was amplified using 16S rDNA bacterial forward and reverse universal primer. DNA sequencing was performed by Xcelris Labs Ltd., Ahmadabad, India. The sequence was aligned and analyzed to identify bacterium and its closest neighbors' using the NCBI web based BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST). The sequence of the 16S rDNA was submitted to NCBI database to obtain GenBank accession number. Closest known species were compared

with percentages of identity. Multiple alignments of the sequence were performed by CLUSTAL_W software [16]. Phylogenetic tree was constructed with the evolutionary distances using the neighbour-joining method. Tree topologies were evaluated by performing bootstrap analysis of 100 data sets with the MEGA 5.1 software[17].

RESULTS AND DISCUSSION

Isolation and screening of bacteria

Cow dung consists of water and undigested plant materials are rich in nutrients, which supports growth of various microbes. Thus in our research work, fifteen bacteria were isolated from fresh cow dung sample. All the bacterial isolates were Gram positive cocci except one CD-5 was Gram positive rod which showed red pigmentation (Fig. 1). The pigmented bacterial isolate colony morphology (Table 1) exhibited round shape, undulate and moist appearance with red in colour. Similar results were observed by [18]and [19] they isolated *Bacillus subtilis*, *Bacillus cereus* and *Bacillus licheniformics* from cow dung. Our research findings can be corroborated with [20], who isolated *Bacillus* sp. from waste material, capable of producing pigment. Moreover, the genus *Bacillus* is predominant in nature, capability of growing in inexpensive and structurally unrelated carbon sources and high growth rate in comparison to other bacteria [21].

Biochemical characterization of pigmented bacterial isolate

Furthermore, the isolate exhibited positive results in oxidase, catalase, indole, methyl red, esculin hydrolysis, nitrate reduction, ONPG test, arginine dehydrolase test, urease, gelatin liquefaction, casein hydrolysis and DNase test presented in table 2. In the oxidation and fermentation medium the isolate utilised all the sugars such as sucrose, fructose, melibiose, galactose, xylose, lactose, maltose, mannose, dextrose, rhamnose, sorbitol, dulcitol, raffinose, cellobiose, arabinose and mannitol used in the study. Moreover, utilization of various sugars by the isolate indicates its capability of growing in inexpensive and structurally unrelated carbon sources.

Table 1. Morphological characteristics of bacterial isolate (CD-5)

Sl.No.	Organism CD -5	Characteristics
1	Gram's stain	Positive
2	Size	Medium
3	Shape	Round
4	Colour	Red
5	Margin	Undulate
6	Surface	Wrinkled
7	Elevation	Low convex
8	Transparency	Opaque
9	Viscosity	Moist

Optimization of Temperature and pH

Temperature tolerance of the bacterial isolate revealed (Fig. 3) that; 34°C was optimum temperature for growth of the pigment producing bacterial isolate. Moreover, moderate growth was observed at 32°C and mild growth was observed at 36°C onwards. Similarly optimum pH of the bacterial isolate was found to be 7.0 and then growth was reduced at pH 8.0 onwards (Fig. 4). Similar results were also obtained by [22, 23], they observed optimum temperature and pH for growth of bacterial isolates are 37°C and 7.0 respectively. This might be a result of molecular adaptation of these bacterial isolates as well as increases the activity of enzyme for optimal growth.

Pigment extraction and characterization

Extraction of red coloured pigment from the selected bacterial isolate (CD-5) was carried out by ethanol as a solvent. Ethanol with purity 99.7% showed to be better than all the solvent

Table 2. Biochemical characteristic properties the bacterial isolate (CD-5)

Sl. No.	Biochemical tests	Results
1	Indole	Positive
2	Methyl red	Positive
3	Voges -Proskauer	Negative
4	Oxidase	Positive
5	Catalase	Positive
6	Nitrate reduction	Positive
7	Urease	Positive
8	Esculin hydrolysis	Positive
9	Casein hydrolysis	Positive
10	Casein hydrolysis	Positive
11	Arginine dehydrolase	Positive
12	DNase	Positive
13	ONPG	Positive

such as ethanol, methanol, hexane, benzene, chloroform and butanol used in the extraction process. In our study crude extract containing red pigment from the bacterial isolate gave maximum absorption spectra at 530nm (Fig. 6), which is comparable to Rhodamine 6G. This result can be corroborated with[11, 24, 25], they observed red coloured pigment production using *Serratia marcescens*.

Table 3. Antibiogram profile of the bacterial isolate (CD-5)

Sl. No.	Antibiotic used	Results
1	Nystatin	Sensitive
2	Penecillin-G	Sensitive
3	Bacitracin	Sensitive
4	Gentamicin	Resistance
5	Streptomycin	Resistance
6	Ampicillin	Sensitive
7	Polymixin-B	Sensitive
8	Chloramphenicol	Resistance
9	Optochin	Sensitive
10	Amoxycillin	Resistance

Antibiotic sensitivity test

The antibiogram profile showed (Table-3) that, the isolate CD-5 was sensitive to Nystatin, Penicillin-G, Bacitracin, Ampicillin, Polymixin-B, Optochin and found resistant to Chloramphenicol, Streptomycin, Gentamicin and amoxicillin respectively. Similar assessment was made by[26]; they evaluated antibiotic susceptibilities of five bacterial isolates of cow dung origin. The antibiotic resistant pattern indicates presence of plasmid in the selected pigment producing bacterial isolate.

Molecular characterization

The pigmented bacteria CD-5 was identified by polyphasic approach such as biochemical and molecular characterization. The CD-5 isolate's 16S rDNA sequences (1443-bp partial sequence) were compared and found to be belonging with *Bacillus* genera (NCBI GenBank accession No.KF175230). The phylogenetic tree showed (Fig. 5) that isolates (CD-5) had 99.7% homology with *Bacillus* sp. NCCP 758 (NCBI Gen Bank accession No.AB715355).

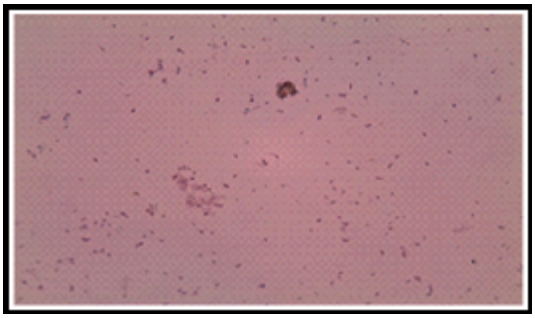

Fig. 1. Micrograph of the bacterial isolate (CD-5)

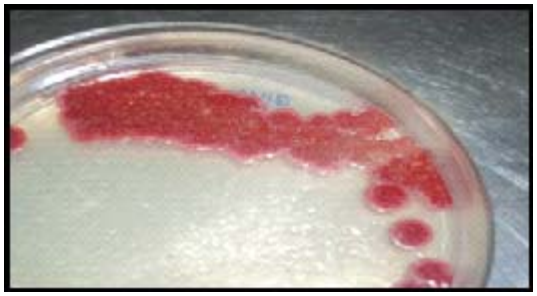

Fig. 2. Pigmented bacterial isolate (CD-5) grown on NA medium

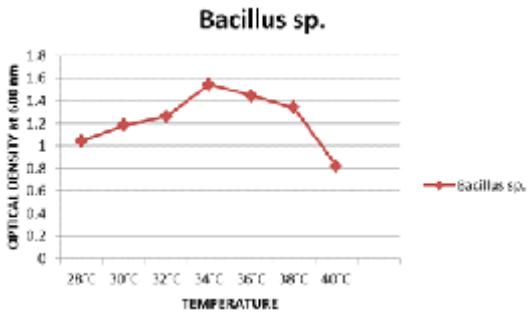

Fig. 3. Effect of temperature on growth of the bacterial isolate (CD5)

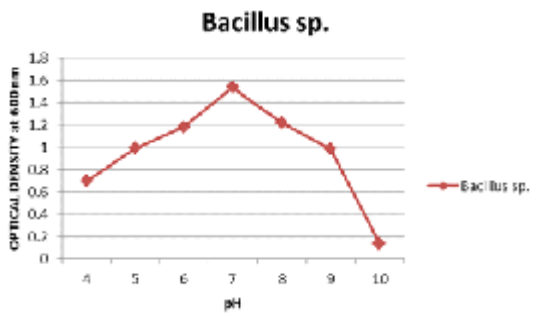

Fig. 4. Effect of pH on growth of the bacterial isolate (CD5)

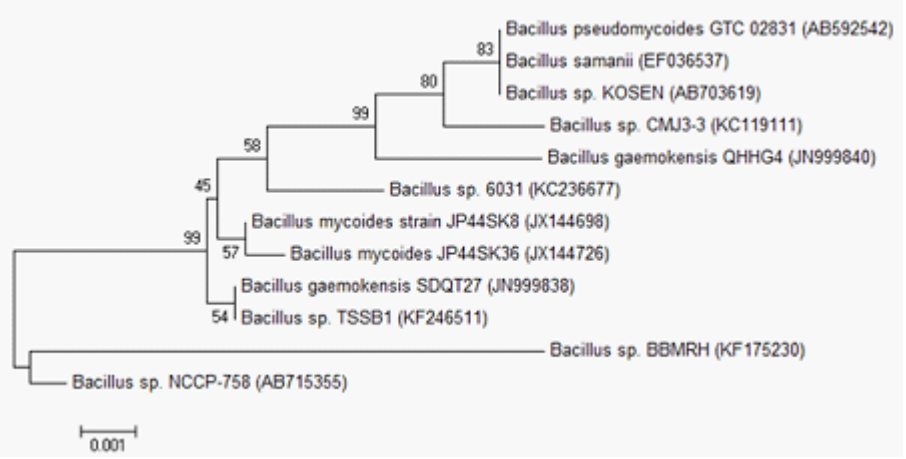

Fig. 5. Phylogenetic relationship of *Bacillus* BBMRH (KF175230)

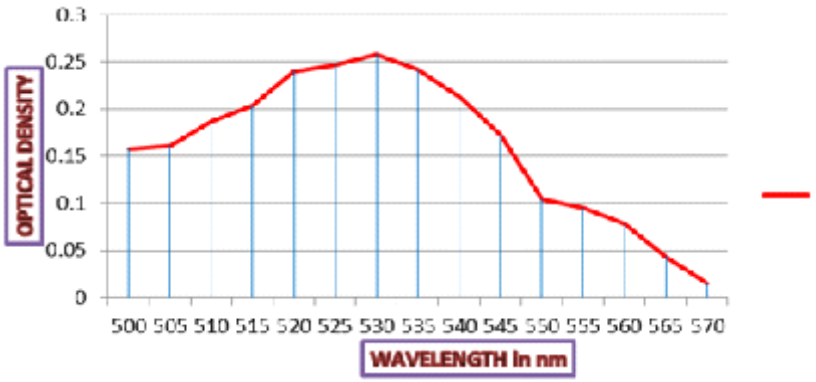

Fig. 6. Spectral analysis of red colour pigment extracted from *Bacillus* BBMRH (KF175230)

CONCLUSION

The isolate *Bacillus* sp. BBMRH possesses the potential for production of red pigment, which is comparable to Rhodamine 6G. It is pertinent to mention that *Bacillus* species are dominant bacteria in industry due to, they are growing in chief raw material and rapid growth rate leading to short fermentation cycle times for production of various industrial products. Thus, the red pigment produced by the bacteria is further characterized for precise industrial applications.

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