The Bioscan 11(3): 1541-1545, 2016
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

STANDARDIZATION OF BIO-PARAMETERS FOR PIGMENT PRODUCTION BY *PSEUDOMONAS* SP. BBMB ISOLATED FROM COW DUNG

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KEYWORDS
Cow dung
Pigmented bacteria
Pseudomonas sp.
Pyocyanin

Received on :
20.062016

Accepted on :
20.08.2016

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ABSTRACT
Cow dung possess abundant prospective for microbial diversity. A total 10 bacteria were isolated from representative cow dung samples collected from the local market, Bhubaneshwar, Odisha. Out of 10 isolates one showed deep green pigmentation in nutrient broth culture medium. The morpho-physiological characterization confirmed that the isolate belongs to the genus *Pseudomonas*. The pigment was characterised to be pyocyanin. Bio parameters were optimized for ideal crude pyocyanin production. It was observed, at pH 7.0 ±0.2 and temperature 36ºC, 8.77ì g/ml of pyocyanin was produced by the isolate. The pigmented bacteria was identified by 16Sµ DNA sequencing and found to be *Pseudomonas* sp., the sequence was assigned with the accession number KF965279.

INTRODUCTION

Colours are the vital constituents and probably the first characteristical properties observed by human senses (Pattanaik *et al*., 1997). Now-days commercial markets are reigned by synthetic colorants some of which are toxic, carcinogenic causing severe damage even to vital organs (Duran *et al*., 2002). This has ignited the zeal for development and application of eco-friendly and economical pigments from natural resources. The various sources of natural pigments are ores, microbes, insects, and plants. Microbes have immense potential to produce various pigments like carotenoids, monascins, violacien and flavins (Duffose, 2006). More specifically, bacteria has the potential to produce different pigments in presence of cheap raw material supplemented to the production medium, that can radically shrink the cost of industrial production (Joshi *et al*., 2003; Venil *et al*., 2009; Ahmad *et al*., 2012). Moreover the microbial pigments are stable, soluble than those derived from plant and animals (Jiang *et al*., 2005; Gunasekaran and Poorniammal, 2008). Deb and Madhugiri, (2012), used apple pomace as cheap carbon source for production of pigment by *Micrococcus flavus*. Cow dung has wide microbial diversity like *Bacillus, Pseudomonas, Lactobacillus, Azotobacter, Aspergillus, Tricoderma,* yeast (Swain *et al*., 2007; Punitha *et al*., 2010; Teo and Teoh, 2011; Rana *et al*., 2014) which has been used in environmental, agricultural and pharmaceutical sectors. Mondal *et al*. (2015) isolated pigment producing bacteria, *Bacillus* sp. BBMRH from cow dung. These natural pigments can be extracted using solvent extraction process for production of dyes and colour (Ahmed *et al*., 2012). In view of this the present research work is aimed at characterization and standardization of bio parameters for pigment production by bacterial isolate from cow dung origin.

MATERIALS AND METHODS

Sampling and isolation of bacteria

Cow dung sampling was carried out from local market of Bhubaneswar, Odisha. The samples were collected aseptically in sterile plastic bag and were transported to the laboratory for isolation of aerobic, heterotrophic, mesophilic bacteria using serial dilution and spread plate technique. Distinct morphological colonies were individually picked, sub-cultured and preserved in NA slant at 4ºC for further use.

Biochemical characterization of bacterial isolates

The morpho-physiological properties of bacterial isolates were studied 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 recommended biochemical test suggested by PIB-Win software (Bryant, 2004) and Bergy's manual of determinative bacteriology (Holt *et al*., 1994).

Antibiotic sensitivity test

The selected bacterial isolate (CD-7) was screened for the antibiotic resistance by following disc diffusion method Bauer

et al. (1966). The isolate was exposed to most common antibiotics like Furazolidone, Cephradine, Kanamycin, Clarithromycin, Penicillin-G, Fosfomycin, Doxycycline hydrochloride, Bacitracin, Sparfloxacin, Ampicillin, Ofloxacin, Ciprofloxacin, Amikacin, Cephadroxil, Amoxyclav, Amphotericin-B, Polymixin-B, Methicillin, Erythromycin, Cefaclor, Gentamicin, Amoxycillin, Nitrofurantion, Gatifloxacin, Streptomycin to observe the effect of these antibiotics on the physiology of the bacterial isolate.

Optimization of bio-parameters

Temperature

The endurance towards different temperature by bacterial isolate was analysed to find out the optimum temperature for its growth. Nutrient broth (100mL) was taken in different conical flask and the temperature was varied from 28ºC to 40ºC, keeping constant pH at 7. Then 100μL of the overnight culture was dispensed into each conical flask and incubated at varied temperature (28ºC, 30ºC, 32ºC, 34ºC, 36ºC, 38ºC and 40ºC) for 24 hours. Then the optimum temperature was determined by taking O.D at 600 nm (Ziayoddin *et al*., 2010).

pH

The optimum pH of the bacterial isolate was analysed by spectrophotometric method. Nutrient broth (100 ml) was taken in different conical flask and the pH was adjusted from 4-10 using 1N HCl, 1N NaOH. Day old (100μl) of the culture was dispensed into each conical flask and incubated at 36ºC for 24 hours. Then the optimum pH was determined by taking O.D at 600nm (Ziayoddin *et al*., 2010).

Phylogenetic analysis of bacterial isolate

The bacterial genomic DNA was isolated by phenol-chloroform method (Neumann *et al.*, 1992). Then the 16S rDNA fragment was amplified using forward and reverse universal primer (8F-AGAGTTTGATCCTGGCTCAG and 1492R-ACGGCTACCTTGTTACGACTT). DNA sequencing was performed by Xcelris Genomics, Ahmadabad, India. The sequence was aligned and analysed to identify bacterium and its closest neighbours using the NCBI web based BLAST programme. Closest known species were compared with percentages of identity. Multiple alignments of the sequence were performed by CLUSTAL_W software (Potter, 2008). Phylogenetic tree was constructed with the evolutionary distances using the Maximum Likelihood method.Tree topologies were evaluated by performing bootstrap analysis of 1000 data sets with the MEGA 6.0 software (Tamura *et al*., 2013). The sequence of the 16S rDNA was submitted to NCBI GenBank database (http://www.ncbi.nlm.nih.gov).

Pigment production, extraction and quantification

The bacterial isolate (CD-7) was cultured in nutrient broth at 7 ± 0.1 pH and temperature 36ºC respectively and was transferred to a 1000 mL separating flask and mixed with chloroform in the ratio of 1:2 (Broth: Chloroform) and shaken vigorously. This mixture was kept undisturbed for 10-15 minutes, for the pigment fraction in the broth to get extracted to the chloroform layer. The blue coloured chloroform layer formed in the separating flask was collected and stored in a 250 ml BOD bottle at 4ºC. Then primary identification of the crude pigment was carried out by wavelength spectrum scanning at 200-800nm. Further to confirm the presence of pyocyanin 0.2 N HCl was added to crude extract resulting change in colour of the pigment to deep pink. Pyocyanin was quantitatively assayed measuring the absorbance of pyocyanin in the acidic form at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per millilitre of supernatant, were determined by multiplying the optical density at 520 nm by 17.072. (El-Fouly *et al*., 2015).

RESULTS AND DISCUSSION

Isolation and screening of bacteria

Out of total 10 bacteria isolated from cow dung, nine were Gram positive rod and one CD-7 which was Gram negative rod (Fig. 1) producing green pigment (Fig. 2). The pigmented bacterial isolate colony morphology (Table 1) exhibited rod shape, smooth and moist appearance with green in colour. El-Fouly *et al*. (2015), where 63 isolates belonging to the genus *Pseudomonas* were isolated from different environmental sources including; soil, water and clinical specimens, out of them twenty were identified as *Pseudomonas aeruginosa* which corroborates with our findings. The pigment produced by the *Pseudomonas aeruginosa* was identified as pyocyanin.

Biochemical characterization of pigmented bacterial isolate

The isolate (CD-7) exhibited positive results in oxidase, methyl red, citrate utilization, nitrate reduction, esculin hydrolysis, lysine decarboxylase, arginine decarboxylase, amylase, gelatin liquefaction, growth in Mac-conkey agar, blood haemolysis and lipase (Table 2) . In the oxidation and fermentation medium the isolate utilised sugars such as glucose, fructose, mannose, dextrose, galactose, dulcitol and cellobiose. Utilization of various sugars by CD-7 isolate dictates its ability to grow in presence of cheap carbon sources.

Antibiotic sensitivity test

The antibiogram profile (Table 3) exhibited that the isolate CD-7 was sensitive to Gatifloxacin, Streptomycin, Kanamycin, Gentamicin, Polymixin-B, Ciprofloxacin, Amikacin, Fosfomycin, Ofloxacin and found resistant to Nitrofurantion, Clarithromycin, Bacitracin, Sparfloxacin, Ampicillin, Methicillin, Erythromycin, Doxycycline Hydrochloride, Amoxyclav, Amphotericin-B, Cephadroxil, Amoxycillin, Cefaclor, Penicillin-G, Cephradine, Furazolidone. Report of Sabir *et al*. (2014), depicts similar the resistance and sensitivity by *Pseudomonas aeruginosa* by disc-diffusion method.

Optimization of temperature and pH

Temperature optimization of the isolate revealed that the organism showed moderate growth at 34ºC and mild growth

Table 1: Morphological characteristics of *Pseudomonas* sp. BBMB

Sl.No.	Organism CD -7	Results(characteristics)
1	Size	Medium
2	Shape	Rod
3	Colour	Green
4	Margin	Swell
5	Surface	Smooth
6	Elevation	Low convex
7	Transparency	Opaque
8	Viscosity	Moist

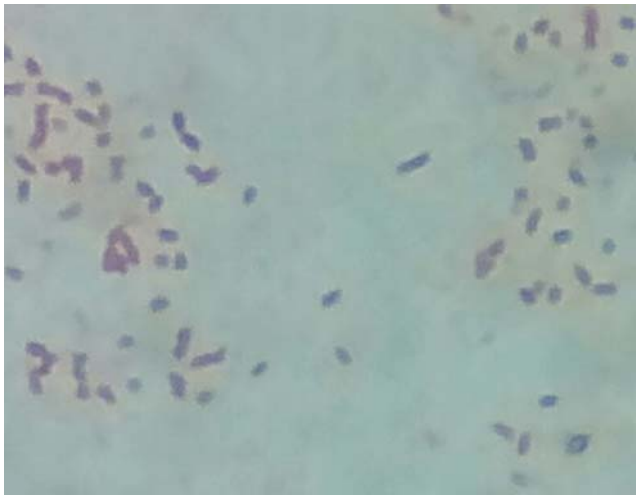

Figure 1: Gram's variability of *Pseudomonas* sp. BBMB

Figure 2 : Pigment production by *Pseudomonas* sp. BBMB

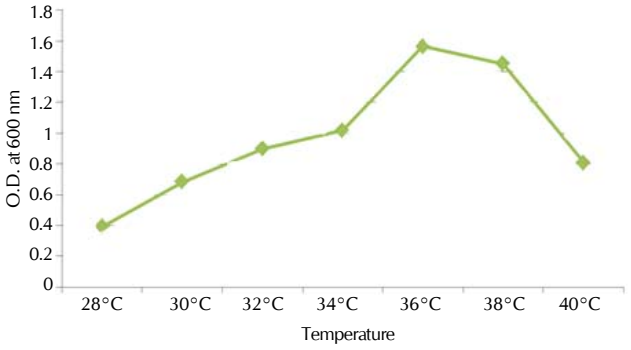

Figure 3 : Standardization of temperature for growth of *Pseudomonas* sp. BBMB

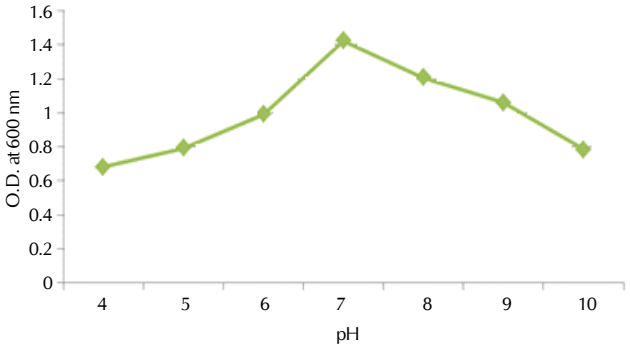

Figure 4 : Standardization of pH for growth of *Pseudomonas* sp. BBMB

Table 2 : Biochemical characterization of *Pseudomonas* sp. BBMB

Sl. No.	Biochemical tests	Results
1.	Catalase	Negative
2.	Oxidase	Positive
3.	Indole	Negative
4.	Methyl red	Positive
5.	Voges –Proskauer	Negative
6.	Citrate Utilization	Positive
7.	Nitrate Reduction	Positive
8.	Urease	Negative
9.	ONPG	Negative
10.	Esculin Hydrolysis	Positive
11.	Ornithine Decarboxylase	Negative
12.	Lysine Decarboxylase	Positive
13.	Arginine Decarboxylase	Positive
14.	Amylase	Positive
15.	Gelatin Liquefaction	Positive
16.	DNase	Negative
17.	Cellulase	Negative
18.	Lipase	Positive
19.	Protease	Negative
20.	Chitinase	Negative
21.	Blood Haemolysis	Positive
22.	Lecitninase	Negative
23.	Growth in MacConkey agar	Positive

at observed at 38ºC and above. It showed optimal growth with pigment production at 36ºC (Fig. 3). 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). Ziayoddin *et al*. (2010) determined the growth conditions of *Pseudomonas aeruginosa* ZSL-2 isolated from sea-shore. The bacterium was able to grow at temperature ranging from 20ºC to 40ºC and pH 5 to 11, with an optimal growth at 30ºC and pH 8 which corroborates with our findings.

Evolutionary analysis of *Pseudomonas* sp. BBMB

The pigmented bacteria CD-7 was identified by polyphasic approach such as biochemical and molecular characterization. The 16S rDNA sequences (1360-bp partial) revealed that the isolate CD-7 belongs to the *Pseudomonas* sp. with accession number KF965279 (Fig. 5).

Pigment extraction, characterization and quantification

Extraction of blue coloured pigment from the selected bacterial isolate (CD-7) was carried out with the organic solvent chloroform with 99.7% purity (Merck, India) was found better than all the solvents such as ethanol, methanol, hexane, benzene and butanol used in the extraction process. In the present finding the crude chloroform extract containing

Table 3: Antibiogram profile of *Pseudomonas* sp. BBMB

Sl No	Antibiotics	Zone of inhibition (mm)	Results Resistant	Sensitive
1.	Nitrofurantion	0	+	
2.	Gatifloxacin	20		+
3.	Streptomycin	12		+
4.	Furazolidone	0	+	
5.	Cephradine	0	+	
6.	Kanamycin	10		+
7.	Penicillin-G	0	+	
8.	Gentamicin	13		+
9.	Amoxycillin	0	+	
10.	Cefaclor	0	+	
11.	Polymixin-B	8		+
12.	Ciprofloxacin	22		+
13.	Amikacin	10		+
14.	Cephadroxil	0	+	
15.	Amoxyclav	0	+	
16.	Amphotericin-B	0	+	
17.	Doxycycline hydrochloride	0	+	
18.	Fosfomycin	25		+
19.	Clarithromycin	0	+	
20.	Bacitracin	0	+	
21.	Sparfloxacin	0	+	
22.	Ampicillin	0	+	
23.	Methicillin	0	+	
24.	Erythromycin	0	+	
25.	Ofloxacin	15		+

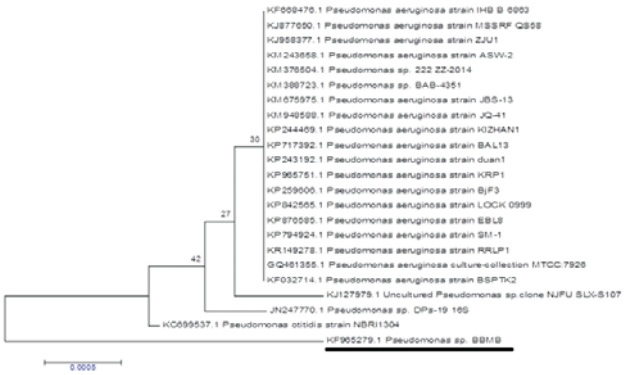

Figure 5: Phylogenetic relationship of *Pseudomonas* BBMB with other *Pseudomonas* species available in the NCBI data library

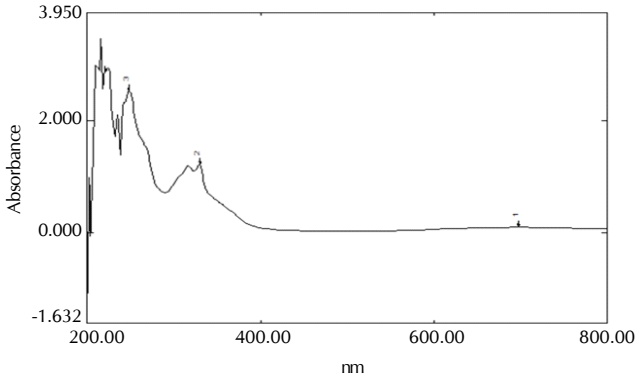

Figure 6 : UV-Vis spectrum of crude pyocyanin produced by *Pseudomonas* sp .BBMB

pigment from the bacterial isolate gave maximum absorption spectra at 248 nm (Fig. 6) which is comparable to pyocyanin. Crude pyocyanin was quantitatively assayed by measuring the absorbance of pyocyanin in the acidic form at 520 nm. Concentration of crude pyocyanin produced per millilitre of culture supernatant, was about 8.77ì g/mL it's the highest crude pyocyanin produced by *Pseudomonas aeruginosa* isolated from cow dung.

ACKNOWLEDGEMENT

We thankfully acknowledge UGC, Govt. of India for providing Rajiv Gandhi National Fellowship to Mr. S.K. Mondal. The authors have no conflict of interest to declare

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