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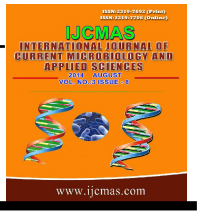

Original Research Article

Identification and Characterization of Extracellular Red Pigment Producing Bacteria Isolated from Soil

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Isolation, Characterization, *Bacillus* spp, Red Pigment, Antibacterial Activity

A B S T R A C T

Red pigment producing bacterium strain isolated from local soil when characterized for morphological, physiological and biochemical parameters was identified as a novel strain of *Bacillus* species. The isolated strain produced extracellular red pigment on nutrient agar medium. The optimum conditions for pigment production was determined which revealed the maximum production of pigment at 30-37^{0}C and at pH 7 after 7 days. The red color was extracted by acidified ethanol and the extract was analyzed by scanning the absorbance with a UV-VIS spectrophotometer and peak was obtained at 740 nm. The red pigment had inhibitory effect on both Gram positive and Gram negative bacteria and the isolated strain was resistant to different antibiotics.

Introduction

Both natural pigments and synthetic dyes have been extensively used in various fields of everyday life such as foods/feeds, textile, paper, printing inks cosmetic, pharmaceuticals, etc. (Tibor, 2007). Since color is an important attribute that determines the consumer's acceptance of foods, color additives are essential in food industry. As a result various synthetic food colors have been manufactured but many of them comprise various hazardous effects (Fabre et al., 1993). Due to the toxicity of several artificial colorants uses of natural additives are of increasing interest. Increasing consumer awareness put string emphasis on the production of biocolors or natural colors extracted from fruits, vegetables, roots and microorganisms (Pattnaik et al., 1997). The industrial production of natural food colorants is already well-established and expanding. However, the range of natural color-shades is still limited compared to synthetic dyes. Natural colors used in food industry are largely plant extracts having several disadvantages such as instability against light, heat or adverse pH, low water solubility and non-availability throughout the year. Microbial pigments are a promising alternative source for natural food grade pigments and have a great potential for food application due to their natural

color and safety to use, medicinal properties, nutrient like vitamins, production being independent of season and graphical conditions with controllable and predictable yield (Francis et al., 2000; Johnson and Schroeder, 1996).

Production of microbial food grade pigments are likely to cut down the high production cost of natural colors, thus leading to a cheaper source of natural food colorants among the modern consumers. Hence, microbial pigment production is now one of the emerging fields of research and in nature a wide range color rich and pigment producing microorganisms (bacteria, fungi, yeast, protozoa) (Duffose, 2009) offer considerable scope for commercial production of biopigments like carotenoids, anthraquinone, chlorophyll, melanin, flavins, quinones, prodigiosins, monascins, violacein etc. (Keneni and Gupta, 2011; Sasidharan et al., 2013; Tarangini and Mishra, 2013; Moss, 2002).

Of the various microbial pigments carotenoids, prodigiosin, astaxanthin and violacein have found application in medical areas due to their activities as immunosuppressive, anticancer, anti-aging and antioxidative agents (Williamson et al., 2007; Guerin et al., 2003; Raj et al., 2009). Red biopigments produced as a typical secondary metabolite by some *Serratia* species (*Serratia marcescens*), actinomycetes and fungus *Monascus* sp show antimicrobial activity (Mekhael and Yousif, 2009) and have a strong potential to develop antitumor drugs (Perez-Thomas et al., 2003).

Materials and Methods

Chemicals

Analytical grade chemicals and solvents were obtained from SRL, E. Merck India. Gram staining kit and reagents required for biochemical tests were obtained from E. Merck, India. Bacteriological media were obtained from Himedia Pvt. Ltd.

Screening of pigment producing microorganisms

Soil sample was collected from the local area of Kolkata using sterile spatula and aseptically transferred into sterile polythene bag and stored under refrigeration until the analysis were carried out.

The soil sample was serially diluted and plated on Nutrient Agar (NA) plates and incubated at 30^{o}C for 72 hrs. Of the different pigmented colonies, single colony exhibited red coloration was subcultured only in nutrient agar slants and incubated at 30^{o}C for 7 days. The slants were stored at 4^{o}C for further analysis. The purity of the strains was verified by microscopic examination.

Identification of the red pigment producing microorganism

Red pigment producing bacterium isolate was plated on Nutrient Agar and allowed to grow for 7 days and studied for morphological, physiological and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The bacterial strain was studied for different cell morphological parameters such as colony evaluation, colony configuration, colony margin, colony surface, colony texture, colony opacity and colony pigmentation.

Gram's reaction, spore formation and motility tests of the bacterial cells were performed using standard methods.

In order to determine the physiology of the isolated red pigment producing bacteria, a

series of biochemical tests were performed. To determine the different enzymes such as oxidase, catalase, arginine hydrolase, lysine decarboxylase and urease that the bacteria may have, different tests were done. Urea broth was used to see if the isolated bacteria can degrade urea. The presence of tryptophanase in the bacteria to degrade tryptophan into indole was determined by using SIM medium. The nitrate broth was used to see if the bacterium has the ability to reduce the nitrate to nitrite. Starch Agar Media and Nutrient Gelatin Agar were used to know if the isolate produced the enzymes amylase and exoenzyme gelatinase respectively. The utilization of citrate as only carbon source was measured using Simmons Citrate Agar slants. To detect the conversion of pyruvic acid in acetoin, the Methyl Red (MR) test was used and to see the fermentation of glucose and its transformation to pyruvic acid, Voges-Proskauer (VP) test was performed. To determine the carbohydrate utilization, assays were performed with lactose, maltose, mannitol, mannose, cellobiose and arabinose broths with phenol red as indicator.

Preparation of inoculums

Pure culture of bacterium from the NA slant was transferred in pre-sterilized 100ml nutrient broth and incubated with shaking for 24 hr at 30^{o}C. One percent of the above cell suspension was used as inoculums.

Pigment production and extraction

The inoculums of the isolated strain were grown in 250 ml Erlenmeyer flasks containing 100 ml of nutrient broth. Fermentation was carried out at 30^{o}C for 7 days under stationary condition. Extraction of the pigment from the fermentation broth was done by solvent extraction method. The organism was harvested by centrifuging at 6,000 rpm for 10 mints. The supernatant was discarded and the pellet was resuspended in acidified ethanol (4% of 1M HCl in 96 ml ethanol). The mixture was vortexed and the suspension was centrifuged at 6000 rpm for 10 mins. The concentration of clear supernatant was determined by measuring the absorption under UV-VIS spectrophotometer (JASCO V-630). All the results shown in tables are average of triplicate sets of experiments.

Optimization of growth conditions for red pigment production

To determine the optimum temperature for pigment production, the isolate was streaked on Nutrient agar plates. Then the plates were incubated for 7 days at different temperatures ranging from 4^{o}C to 55^{o}C.

To determine the optimal pH for the bacteria, tubes with Nutrient Broth at different pH ranging from 5-12. After inoculation tubes were incubated for 7 days at 30^{o}C.

To determine the range of NaCl that the bacteria can grow, 1% fresh culture of the isolate was incubated into Nutrient Broth adjusted with NaCl concentration with different percentage from 1-8%.

Antimicrobial activity of red pigment

Well diffusion assay method was used for the detection of antimicrobial activity of red pigment against different species of Gram positive (*Staphylococcus aureus, Bacillus subtilis*) and Gram negative bacteria (*Escherichia coli, Pseudomonas spp).* 24 hr broth culture of target strains were inoculated on solid Muller-Hinton agar medium by spread plate method. 4 wells were made in each of the plates. These wells

were filled with different concentrations of extracted red pigment. Target strain inoculated plate with un-inoculated red pigment served as control. The plates were incubated at 37^{o}C for 24 hr and the inhibitions zones were visualized.

Antibiotic susceptibility test

Disk diffusion method described by Andrews was followed to determine the sensitivity of the red pigment producing organismto different antibiotics. Five different antibiotics such as Penicillin, Rifampin, Metrogyl, Amoxicillin and Tetracycline were collected and varying concentrations (0.25-1 mg/ml) of all the selected antibiotics were prepared in Nutrient Broth. The test inoculum was prepared by incubating the isolated culture into Nutrient Broth at 37^{o}C for 12 hr and 100 µl of it was inoculated to Muller-Hinton agar plates by spread plate method. 4 wells were made in each of the plates. These wells were filled with 100 µl of selected antibiotics each of different concentrations. Agar plates were then incubated for 24 hr at 37^{o}C. The zone of inhibition was visualized.

Results and Discussion

Isolation and identification of the red pigment producing microorganism

Out of different colonies isolated in Nutrient agar medium, only one strain showed luxurious growth and intense red color pigment production. Colony characteristics of the isolate was studied by picking up a single well isolated colony aseptically and transferred to selective medium to observe the growth pattern of isolates on Nutrient Agar medium. Colonies appeared off-white colored, irregular (large) in configuration with flat elevation and wavy margin (Table 1).

For detail characteristics of the red pigment producing organism, isolated strain was sent for morphological, physiological and biochemical characterization to Institute of Microbial Technology Chandigarh (IMTECH), Chandigarh, India. Their distinguishing features are Shown in Table1. The isolate was found rod shaped, spore producing, non-motile and Gram positive bacteria. Results on various biochemical tests revealed that the red pigment producing bacteria was able to utilize/hydrolyse starch, esculin, gelatin, casein. Though the bacterium expressed a positive catalase reaction, it showed a negative reaction for oxidase, urease, lysine decarboxylase and arginine decarboxylase.

Based on the results in the Table 1 the microorganism was identified as *Bacillus* species and is closely relate to *Bacillus cereus.*

Optimization of growth conditions for red pigment production

Appearance of red pigment from *Bacillus* spp. in nutrient broth was started after 72 hrs of incubation in the culture medium. Pigment production increased to a maximum after 6 days of incubation followed by decrease on day 7. In subsequent period both growth and pigmentation gradually decreased. Broth culture collected after 6 days were centrifuged to obtain the red colored cell pellet. The color was extracted by acidified ethanol and the extract was analyzed by scanning the absorbance with a UV-VIS spectrophotometer and peak was obtained at 740 nm.

Table.1 Morphological, Physiological and Biochemical Characterization of the Red Pigment Producing Microorganism

Colony Morphology	
Configuration	Irregular (Large)
Margin	Wavy
Elevation	Flat
Surface	Mucoid
Texture	Dry
Pigment	Off-White
Opacity	Opaque
Gram's reaction	+
Cell shape	Rod
Spore(s)	+
Motility	_
Biochemical Tests	
Oxidase	_
Catalase	+
MR	_
VP	+
Casein hydrolysis	+
Citrate	+
Nitrate reduction	+
Indole	_
Arginine Dihydrolase	_
Lysine decaroboxylase	_
Urease	_
Gelatin hydrolysis	+
Starch hydrolysis	+
Esculin hydrolysis	+
Acid Production from	
Lactose	_
Maltose	+
Mannitol	_
Mannose	_
Cellobiose	+
Arabinose	+

'+' sign stands for positive and '-' stands for negative result.

Table.2 Effect of medium pH, incubation temperature and NaCl on growth and pigmentation by the isolated *Bacillus* sp.

Parameter	**Medium pH**							
	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0
Growth	+	++	+++	++	+	+	+	+
Pigmentation	+	++	+++	++	+	+	+	+
	Incubation Temperature (^{o}C)							
	4	15	25	30	37	42	55	
Growth	-	+	+	++	++	+	-	
Pigmentation	-	+	+	+++	+++	+	-	
	NaCl (%)							
	1	2	3	4	5	6	7	8
Growth	+	++	+	+	+	-	-	-
Pigmentation	+	++	+	+	+	-	-	-

'_' or '+' sign stands for no or Positive visual growth and pigmentation. Number of '+' signs stands for relative extent of visual growth and pigmentation.

Table.3 Antibacterial activity of the isolated bacterial strain

Test Organisms	**Gram Character**	**Growth of test organisms**
Escherichia coli	Negative	Inhibited
Bacillus subtilis	Negative	Inhibited
Staphylococcus aureus	Positive	Inhibited
Pseudomonas spp.	Negative	Inhibited

From the experimental results (Table 2) it was observed that growth and pigment production of the isolated *Bacillus* sp occurred within the pH range from 5-12 and the optimum pH value for intense pigment production was at pH 7. Like pH, temperature is an important factor in bacterial growth. In the present study the isolated *Bacillus* sp was able to grow within 15^{o}C-42^{o}C and the optimum temperature for maximum growth and pigmentation efficiency was observed at 30^{o}C-37^{o}C. No growth and pigment production were observed at 4^{o}C and at 55^{o}C. Table 2 revealed that isolated *Bacillus* sp showed its ability to grow and

produce red pigment in the range of 1-8.0% NaCl concentration but optimum growth and pigmentation was observed at only lower salt concentration (1-5%). Higher concentration than this exhibited inhibitory influence on both the growth and pigment production by *Bacillus* sp.

Antimicrobial activity of red pigment

Table 3 represents the antimicrobial activities exhibited by *Bacillus* sp. which indicates that the red pigment extracted from the isolated *Bacillus* sp. were able to inhibit the growth of all the test microorganisms. This experiment clearly indicates that the inhibitory metabolites produced by isolated *Bacillus* species were extracellular and diffusible.

Results concerning the sensitivity of the isolated *Bacillus* species to different antibiotics are shown in Table 4 which reveals that isolate was resistant to all the five selected antibiotics including amoxicillin indicating that antibiotics will not affect the growth of the isolated *Bacillus* sp.

Based upon morphological, physiological and biochemical studies, the red pigmented bacterial strain was identified as a novel strain of *Bacillus* species. The red pigment produced as a typical secondary metabolite by bacteria such as *Serratia marcescens* (Keneni and Gupta, 2009; Raj D. N. et al., 2009; Mekhael and Yousif, 2009), by filamentous fungus *Monascus* sp (Hamano et al., 2005) was reported but very few literature review was found on the production and extraction of red pigment from *Bacillus* sp isolated from soil.

The present study revealed that the red pigment production is influenced by different parameters and optimum growth and pigment production was observed at 30-37^{0}C and pH 7. The red pigment exhibited antimicrobial activity against some indicator pathogens and the isolated strain was resistant to different antibiotics.

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