

Isolation and Characterization of Pigment Producing Bacteria

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Abstract

Bacterial pigments are the best alternative to synthetic dyes used in the textile industries and synthetic colorant used in the food materials. Bacterial pigments also have antitumor, antimalarial, antibacterial, antimycotic, and immune-modulating activities. This study was directed to isolate and characterize strains of bacteria from the natural site of Jalandhar, Punjab, India. We optimized different pigment production on different concentrations of NaCl, nitrogen sources, carbon sources, and amino acids. Yeast extract, peptone fructose, and tryptophan substrates have shown maximum pigment production. The optimum temperature range was 25⁰C-37⁰C, and the optimum pH range was between 6 and 8. The potent pigment producing strain was codified as BBE was yellow-colored, Gram-negative, coccus, and non-motile. 16S rRNA gene sequence comparison revealed that isolated strain BBE was found to be *Acinetobacter oleivorans* strain JC3-1 based on nucleotide homology and phylogenetic analysis. We can use these organic pigments in industries like cosmetic, pharmaceutical or textile industry one screened for their safe effects.

Keywords: Antibacterial; BBE; Ladder DNA; Industries; Antitumor and immune-modulating.

Introduction

The freshness of food material is indicated by its safety and color that are also show good sensorial and aesthetic values. Bacterial pigments are used because of their harmless effects on humans as well as on environment (Ahmad, et al.,2012) There is a significant need to enhance the production of naturally occurring coloring compound as food-grade colors (Dufosse L,

2016 Microbial pigments have qualities of significance to numerous ventures. In food industries, purified microbial pigments used as food additives with properties such as antioxidants, color intensifiers, etc. (Rymbai, et al., 2011). Microbes are also a good source of organic acids, enzymes, vitamins, amino acids and organic acids. The extraction of pigment from microbial sources and then used as food colorant is an excellent alternative to synthetic dyes (Malik, et al. 2012). Bacteria produce pigment in response to some chemicals and radiations that induce bacteria to secrete secondary metabolites, which are pigments and may antibiotics or organic compounds. Some xanthophylls, carotenoids, flavonoids, tetrapyrroles are the most commonly used natural microbial pigments (Aberoumand, et al., 2011). The main benefits of pigment creation from bacterial species are fast and easy growth in the easily available and cheap culture medium, totally free from atmospheric conditions. The above mentioned literature evinces that microbial pigment production is one of the developing and advantageous areas of exploration. Bacterial pigments have outstanding potential for various industrial applications (Bhat et al. 2013).

Materials and research methodology

Sample Collection

Samples were collected aseptically from Baba Bhudha Enclave, Dhakoa, Jalandhar, India. Soil samples were taken from the depth of 5-10 cm, and sterile polythene bags and iron tools were used for sample collection. Isolated samples were transported on ice packs and were further kept under refrigeration (Pradeep et al., 2013). Samples details are provided in table 1.

Table-1 Sample list for isolation of pigment-producing bacteria.

S.No.	Sample type	Site of sample collection
01.	Soil	Pot soil, Lovely Professional University (LPU), Punjab
02	Plant (flower)	Flower garden, Lovely Professional University (LPU), Punjab
03	Plant Leaf	Flower garden, Lovely Professional University (LPU), Punjab
04	Soil	Agriculture land Sham Charasi, Hoshiapur, Punjab
05	Leaf	Plant nursery, Chaheru, Phagwara Punjab
06	Soil	Agriculture land, Baba Bhudha Enclave, Dhakoha, Jalandhar

Cultivation and Isolation of Bacteria

Nutrient agar and broth were used as a growth medium for bacterial growth. Autoclaving at 121⁰C, 15 min was used for sterilization of both agar and broth medium. Soil samples were serially diluted and inoculated through the spread plate method onto nutrient agar whereas plant samples like leaf and flower were inoculated by directly touching the leaf and flower surfaces on nutrient agar. Inoculation was carried out in laminar airflow and followed by incubation for 24 h at 30⁰C to make sure that plates were free from impurity (Ahmad *et al.* 2012). Samples were further serially diluted by using 1gm of soil with distilled water. 10⁻² -10⁻⁷ dilutions were made and further spread on the NAM plates. Incubation is carried at 37⁰C for 24 to 48 hours. Round yellow-colored colonies were observed on some plates. One potential isolate was isolated and used for analyses of pigment production. Serial sub-culturing was performed for maintenance of pure culture (Pradeep and Pradeep, 2013; Pradeep *et al.*, 2013).

Screening of Pigment Producing Bacteria

Physical screening

Isolated bacterial strains were cultured on nutrient agar. All these plates were incubated at 37⁰C for 24 to 48 hours. The colored bacterial colonies were then pure cultured on the same medium and were again incubated 37⁰C for 24 to 48 hours. Pure cultures were then preserved at 4⁰C for further study (Pradeep *et al.* 2013).

One potential isolate was further analyzed for screening purposes. The culture was maintained by using agar slants and stored at 4⁰C for further use. For screening 100mL conical flasks containing 50mL nutrient broth (g/L) were used and inoculated with 5% BBEMS strain (48 hours old inoculum), further flasks were incubated for 48 to 96 hours at 37⁰C in aseptic conditions in an orbital shaker at 210 rpm (Krithika K and Geetha Ramani D, 2013).

Preservation of BBE Culture

Agar slants were used to preserve the isolated BBE bacterial strains. This method is done for preserving culture for a few weeks to a few months. This is a short term preservation technique. The culture was preserved on agar slant, and slants were further stored at 4⁰C (Pradeep *et al.*, 2013).

Bacterial Stock cultures were maintained for long term storage in LB – glycerol solution. The bacterial colonies cultured were then transferred into LB-glycerol before storage at -20 °C (Ahmad *et al.*, 2012).

Characterization of pigment-producing bacteria

Morphological characterization

Isolated strain BBE was further selected for the characterization. Gram staining was performed to see the cell type and morphology. The smear of strain was prepared on a clean glass slide, and it was allowed to air dry and heat fix. The slide was flooded with Gram's iodine for 1 minute and washed with water. Gram decolorizer used to remove the excess strain. The slide was rewashed with water to remove the ethanol. Slide was then flooded with sefranin counterstrain for one minute, wash slide and blot dried it. Results were observed under light microscope and photos were taken. After observing the result we see that cells are Gram negative and coccus (Palanichamy *et al.*, 2011).

Colony morphology

Isolated strain BBE/MS was studied for the colony shape, size, edge, and surface (Bhat *et al.*, 2013).

Molecular characterization

Isolated strain BBE/MS was further studied for 16SrDNA sequencing by Samved Biotech, Ahmadabad.

Optimization of pigment-producing bacteria

Growth media were used for optimal production of pigments by isolated bacterial strain as per the method of Bhat *et al.* (Bhat *et al.*, 2013). Effect of temperature, pH, NaCl concentration, different amino acids, and different nitrogen and carbon sources were used for pigment optimization.

Effect of temperature

A total of four sets(I, II, III &IV) of petri plates were prepared for varied temperature effect on the pigment production. BBE strain was cultured at different temperatures of 22⁰C, 25⁰C, 30⁰C, and 37⁰C, respectively for 24 hours

Effect pH

Pigment production on varied pH effect from 5, 6, 7, and 8, respectively was studied. These plates from each set were inoculated with BBE strain and incubated at 37°C for 24 hours.

Effect of NaCl concentration

Effect of various concentrations of NaCl(2%, 4%, 6%, 8% and 10%)on pigment production, nutrient agar was supplemented with different. These plates with different salt concentrations were inoculated with the BBE strain in triplicates and incubated at 37°C for 48 hours.

Effect of different carbon sources

The isolated bacterial strain was inoculated in a nutrient broth supplemented with 1% of different carbon sources like glucose, fructose, maltose, sucrose, and lactose.

Effect of different nitrogen sources

The inoculated bacterial strain was cultured in a nutrient broth, which was supplemented with 1% different nitrogen sources, i.e., ammonium chloride, ammonium sulfate, ammonium nitrate, yeast extract, and peptone.

Effect of amino acids

The isolated bacterial strain was inoculated in a nutrient broth, which was supplemented with 1% different amino acids, i.e., cysteine, leucine, methionine, tryptophan, and proline.

Extraction of pigment

The first step was to identify the best solvent that would support a maximal yield of pigment from the isolated strain. Standardization of solvent done by using different solvents like acetone, ethanol, chloroform, petroleum, methanol, diethyl ether, hexane, ethanol, ethyl acetate, and distilled H₂O. Maximum solubility is observed in water between all solvents. Culture was

prepared in 5mL of broth by inoculating it with a loop full of the culture of isolated strain and incubating it at 37⁰C overnight. Further 50mL of freshly prepared broth was taken in 250mL Erlenmeyer flasks and inoculated it with the previously prepared culture (OD 1.0) as inoculum at 1% (v/v). Incubation of this culture was done at room temperature (28-30⁰C) overnight on a rotary shaker. Standardized extraction procedure for pigment extraction is; (i) 1mL of broth was taken in a microfuge tube and centrifuge it at 7000 rpm for 10 minutes at 4⁰C. (ii) The colored pellet was taken, and the supernatant was discarded. The cell pellet was resuspended in 1mL of dissolvable, i.e., refined water. (iii) The microfuge tube with suspended cell pellet at that point kept in a water shower at 60⁰C for 20 minutes and again rotator for 10 minutes at 40⁰C at 7000 rpm. The shaded supernatant was then investigated by UV obvious spectroscopy for identifying the focus(Slater *et al.*, 2003).

Results

Pigment-producing bacteria

After the screening of all the samples, it was found that BBE (Baba bhudhaencave) strain (Fig; 1) was found to be potent pigment-producing bacteria based upon the physical appearance and its ability to produce pigment under submerged culturing (Pradeep and Pradeep, 2013). After the characterization and strain identification by using molecular characterization methods, this bacterium was found to be novel, and we were the first group that has reported for the first time in India and was named as *Acinetobacter oleivorans*. This bacterium was further assessed for pigment production and characterization. It was successfully isolated from soil samples collected from the Agricultural field and Plant Nursery of Baba Bhudha Enclave Dakoha, Jalandhar, India. Yellow-colored colonies obtained from the manure of plant pot from Baba Bhudha Enclave DakhohaPhatak, Jalandhar. Yellow-colored colonies further subcultured on a plate to obtained pure culture.



Fig. 1-Yellow-colored pigment-producing bacteria (BBE).

Characterization of pigment-producing bacteria

Strategic manipulation of the medium was done for maximum pigment production by the bacteria *Acinetobacter oleivorans*. Different temperatures and pH conditions were given to observe the different effects of pigment production.

Morphological characterizations

With the help of Gram staining, shape and type of cells were studied, as shown in following in **Figure 2**. Cells were Gram-negative and *coccus* in shape. Somewhere they were present in clustered form and somewhere segregated. Isolated strain BBE was studied for the colony shape, size, edge and surface. However, the size of the cells was varied. Colonies show a minimum diameter of 0.1 cm. The morphological characters were showed in the table below Table 2.

Table: 2. Cell and colony morphology

Colony characteristics	Characters	Cell morphology	Character
Shape	Round	Cell shape	Cocci
Size	0.1 cm	Gram staining	Gram negative
Edge	Smooth	Motility	Non-motile
Surface	Smooth		
Color	Yellow		

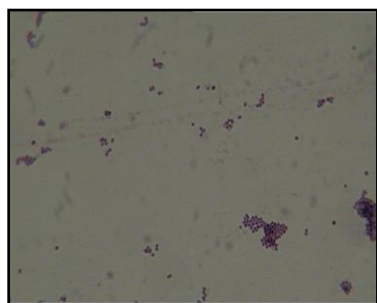


Fig. 2- Gram staining of isolated strain BBE

Molecular characterization

Isolated strain BBE/MS was further studied for 16SrDNA sequencing by Samved Biotech, Ahmadabad. The isolated BBE/MS culture was found to be *Acinetobacter oleivorans* strain JC3-1 16S ribosomal RNA gene with GenBank accession number: KM983423.1. It was based on nucleotide homology and phylogenetic analysis (Fig. 3). Electrophoresis gel was run with a DNA ladder and sample of BBE. The result shows that the genome size of the sample was 1.5 Kb. Lane 1 contains 16s rDNA of BBE strain, and Lane 2 contains ladder DNA, Fig 3.

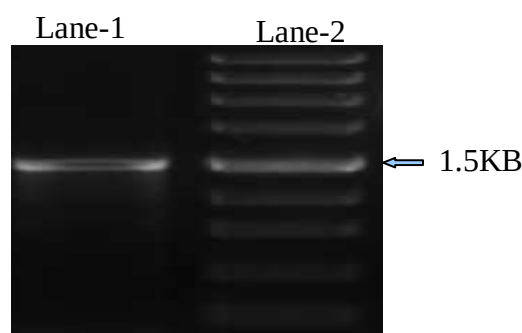


Fig. 3-Gel image of 16SrDNA of BBE sample

Relationship on the basis of evolutionary of 11 taxa

The evolutionary history was concluded using the Neighbor-Joining method (Saitou *et al.*, 1987). An optimal tree with the sum of branch length of 0.00181736 was built. Evolutionary distances were computed using the Kimura 2-parameter method (Tamura *et al.*, 2007) and these are in the units of the number of base substitutions per site. This analysis had involved 11 nucleotide sequences, and all positions comprising gaps and missing data were excluded. There were a total of 1102 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 shown in figure 3 (Felsenstein *et al.*, 1985)

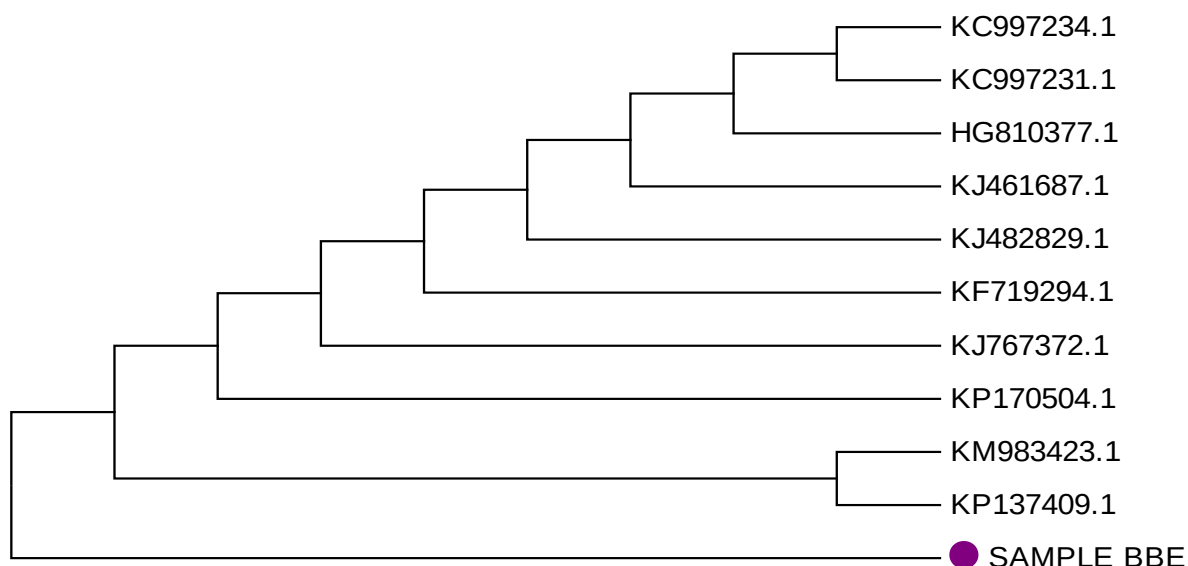


Fig. 4- Phylogenetic tree

Optimization of the isolated strain

Effect of different nitrogen source on pigment production

Acinetobacteroleivorans was inoculated in a nutrient broth, which was supplemented with 1% different nitrogen sources, i.e., ammonium chloride, ammonium nitrate, ammonium Sulphate, yeast extract, and peptone. The maximum yield of pigment was observed in the case of yeast extract as a nitrogen source. Peptone also gives good pigment production but less than yeast extract (Fig. 5).

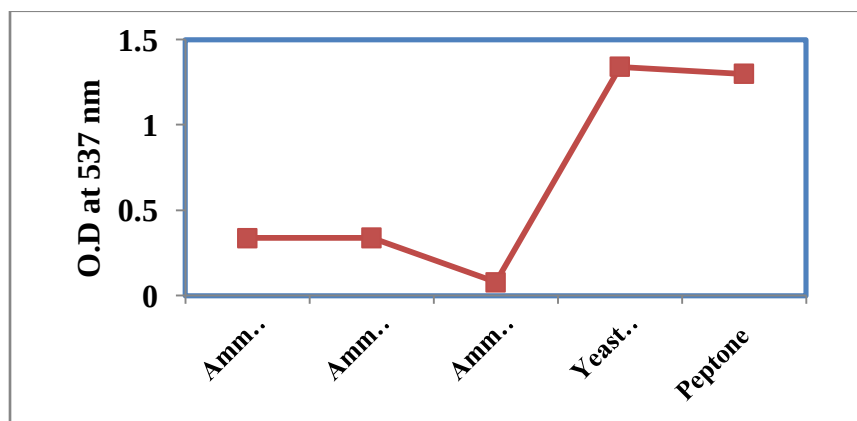


Fig. 5-Effect of different nitrogen sources supplemented in 1% concentration.

Effect of different carbon sources on pigment production

1% of glucose, fructose, maltose, sucrose, and lactose was taken for checking the effect of different carbon source. The maximum yield of pigment was observed in case fructose as carbon source. Glucose also gives good yield but less than fructose (Fig. 6).

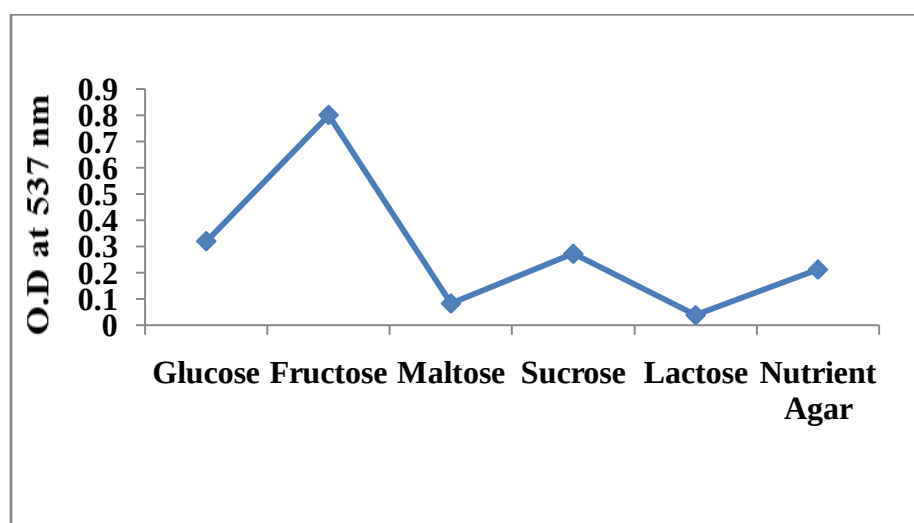


Fig.6- Effect of different carbon sources on pigment production.

Effect of amino acids on pigment production

1% different amino acids, i.e. cysteine, leucine, methionine, tryptophan, and proline were selected for checking the effect of different amino acid. Isolated bacteria *Acinetobacteroleivorans* were inoculated in a nutrient broth, which was supplemented with. OD was taken at 537nm, and the maximum yield of the pigment was observed in the case of tryptophan (Fig. 7). Out of all the amino acids, only tryptophan had shown maximum pigment production.

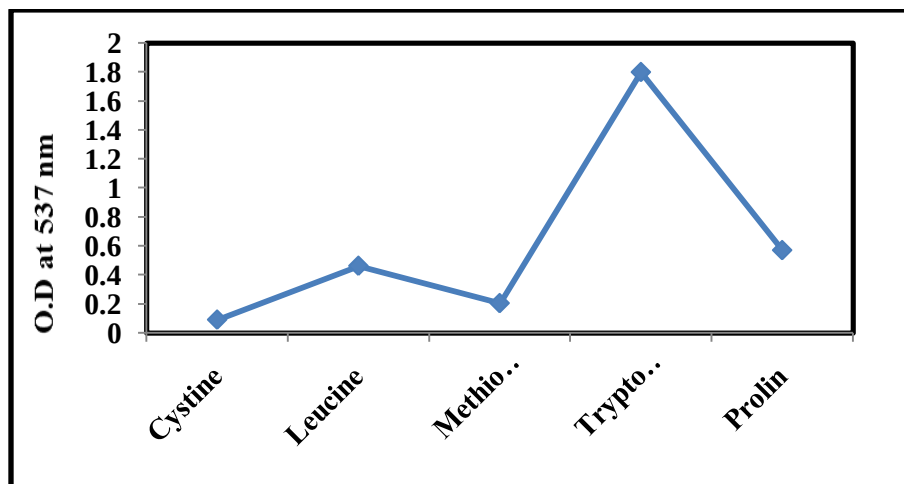


Fig. 7- Nutrient broth supplemented with 1% different amino acids after the inoculation of *Acinetobacteroleivorans*.

Effect pH

The pH range between 6 and 8 shows optimum pigment production.



Fig.8-*Acinetobacteroleivorans* grow that different pH.

Effect of NaCl concentration

Different salt concentrations that were used are, 2%, 4%, 6%, 8% and 10%. With an increase in salt concentration, first, there was an increase in pigment production, but it starts decreasing when we added 6% of NaCl. The pigment production was maximum at 2% and 4% of NaCl concentration. Beyond 6% pigment production decreases and almost no growth was observed at 10% of NaCl concentration.

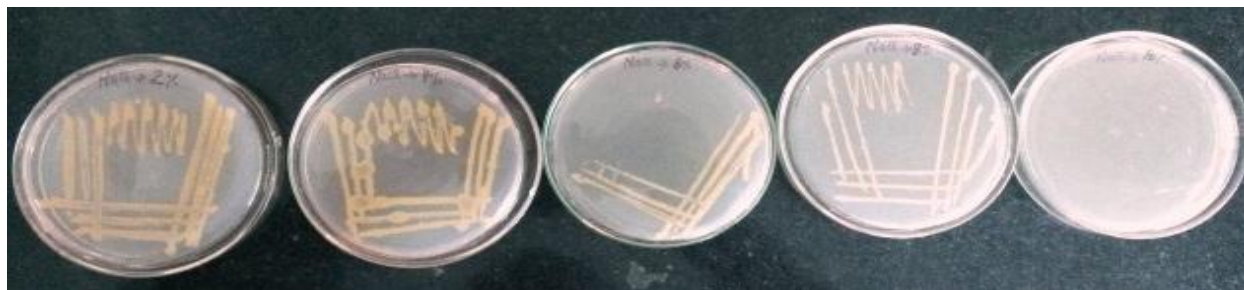


Fig. 9- Growth on different concentration of NaCl

Discussion

The isolated strain of bacteria showed maximum yield when supplemented with 2% of NaCl, 1% fructose as carbon source, 1% yeast extract as nitrogen source at pH of 6, and temperature 37⁰C. As pigment was water-soluble, it was a bit easier to extract pigment from bacterial cells. The utilization of engineered colors is unsafe to human wellbeing, and there are just some restricted sorts of such colors allowed to be utilized. In this way, there is an earnest requirement for regular nourishment colorants to elective the current one. When all is said in done, the normal hues are very normal and rich in color creating microorganisms, for example, microbes, yeast, growths, and so forth These shades can be utilized in various mechanical territories to assist humankind.

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