The Bioscan AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES 19(2): 150-155, 2024 www.thebioscan.com

ISOLATION AND IDENTIFICATION OF PIGMENT PRODUCING BACTERIA FROM SOIL

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DOI: https://doi.org/10.63001/tbs.2024.v19.i02.S2.pp150-155

Received on:
20-07-2024
Accepted on:
04-11-2024

ABSTRACT

In present study the pigment producing microorganisms were isolated from different regions of Aurangabad district. Upon preliminary investigation, viz. colony characterization and grams staining, the suspected colony was observed to have a entire margin with a slightly raised elevation and gram negative, asporogenous. Biochemical analyses of the organism was done . The identity of the isolate was established via 16S rRNA, which strongly suggested it was *Pseudomonas aeroginosa.* The pigment produced by the isolate was applied for dying bioplastic. The use of synthetic dyes in these industry cause problems in environment such as toxicities. The synthetic dyes are not environment friendly. The natural colours are more advantage than synthetic colours, they have antibacterial, antifungal, anti-oxidant, anticancer activities etc

INTRODUCTION

Pigments are the chemical substances that absorb the light of visible region. The produced colour is because of the chromophore, a molecule specific structure which captures the sunenergy and causes an excitation of electron from external orbital to higher orbital, where the non-absorbed energy is refracted or reflected to be captured by eye [6].

The modern meaning related to the word pigment has its origin in the twentieth century, meaning a substanceconstituted of small particles which is practically insoluble in the applied medium and is used due to its colorant, protective or other properties. Pigments are compounds with uniquenessof importance to many industries. In the food industry they are used as additives, antioxidants, color intensifiers, etc. Pigments come in a wide selection of colors, some of which are water-soluble [10, 16].

Pigments have become an essential part of our daily lives and have extensive applications in many areas. They provide attracting appearance to marketable products such as food product textiles and pharmaceutical. There are many synthetic colorants but they possess various hazardous and harmful effects to that substrate to avoid these hazardous effects one of great alternative is the natural sources. Pigments produced from natural sources are of worldwide interest and is gaining significance. Natural pigments are obtained from ores, insects, plants and microbes. Natural pigments possess anticancer activity, contain pro-vitamin A and have some desirable properties like stability to light, heat and pH2. Natural colorants or dyes derived from flora and fauna are believed to be safe because of non-toxic, non-carcinogenic and biodegradable in nature. The advantages of pigment production from microorganisms comprise easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades [9].

Prokaryotes are known to synthesize pigments, just like animals and plants do. Some of these pigments aid in the synthesis of complex carbohydrates by photosynthetic bacteria, while others protect them from ultraviolet (UV) damage. The biosynthesis of these accessory pigments is crucial for the taxonomic characterization and identification of novel bacteria and helps establish genetic relatedness amongst new species and the pre-existing ones. Natural pigments sourced from the micro-organism are preferred over those from plants because of their stability and availability for cultivation through the year. Microbial pigments have many advantages over artificial and inorganic colours. Many natural pigments besides fulfilling their function of giving colour are known as interesting bioactive compounds with potential health benefits. Such naturally synthesized pigments are now used in various industries, such as textile, cosmetics, pharmaceuticals, and food, because of their exotic properties and advantageous characteristics, which are useful for both human health and the environment. Microbes can grow easily and fast in the cheap culture medium and independent from whether conditions. Pigmented micro-organisms, also known as chromogenic microorganism, belonging to, e.g., bacteria, microalgae, archaea have been isolated from diverse environmental and geographical conditions, including terrestrial, aerial, and marine locations. Microbial pigments are available in different shades and are biodegradable and environment friendly they also have numerous clinical characters like antioxidants, anticancer, antiproliferative, immunosuppressive, treatment of diabetes mellitus, etc. Extensive studies proved that microbes are known to produce a large amount of stable pigments [2]. Large amounts of agro-industrial and domestic residues are generated from diverse economic activities; utilization of these residues as inexpensive substrates to support the growth of microorganisms to generate value-added products like pigments are of biotechnological interest in recent years [5, 19,17].Hence, microbial pigments production is now one of the emerging fields of research to demonstrate it's potential for various industrial applications.

METHODOLOGY

Collection of sample:

Many different soil samples were collected from various sites from forest areas, forts, city areas, and near river areas, caves

of Aurangabad city, Maharashtra. The soil samples were collected and transferred to the laboratory in aseptic conditions.

Isolation and Screening of Actinomycetes

In order to isolate bacteria from different soil samples, 1g of the respective soil sample was suspended in 9ml of distilled water taken in a pre–autoclaved sterile test tube. The suspension was then serially diluted up to 10^{-5}. The diluted suspension (100µl) of10^{-3},10^{-4}, and $10^{-5}$was spread plated on nutrient agar medium and incubated at 37^0C for 3 ~ 7 days. Post incubation, all the plates were screened for pigment producing colonies based on morphology and pigmentation. The pigment producing colony that showed significant coloration was sub–cultured onto fresh cetrimide agar medium by streaking until a pure culture was obtained.

Characterization of the Isolate

The isolate was characterized by studying its morphological (colony characters and Grams nature) and biochemical characteristics using standardized protocol. Colony characters such as form, margin, elevation, pigmentation, and texture were visually studied, and the observations were noted as described in Bergey's Manual of Systemic Bacteriology. Biochemical tests such as Carbohydrate fermentation, Simmons' citrate (citrate utilization), IMViC test , enzyme hydrolysis test were performed using standard protocol.

Genomic analysis and identification of potent isolate.

The identity of the isolate was determined using 16S rRNA molecular sequencing analysis. The isolate was outsourced to Progenome bio laboratory, chhatrapati Sambhajinagar for this purpose. The molecular identification technique was carried out as per the standard genome sequencing protocol.

Genomic analysis of potent pigment producing bacteria: The isolated strain was identified by using 16SrRNA sequencing method and noted.

The given sample was provided by the researcher. The DNA was extracted by Nucleospin

Microbial DNA Kit and quality checked on 1% agarose gel electrophoresis. Gel was visualized using UV Transilluminator (Himedia). Fragment of gene 16S rRNA was amplified by 27_F and 1492_R primers. A single discrete PCR amplicon band was observed when resolved on 1.2 % Agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out withforward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using Bioedit software.The 16S rRNA gene sequence was used to carry out BLAST with the database of NCBI genbank database.Based on maximum identity score and alignments using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 11.Species confirmation using 16S rRNA marker

Amplification of 16S rRNA

The 16S rRNA region was amplified by using primers. Amplified PCR products were visualized on 1.2% agarose gel

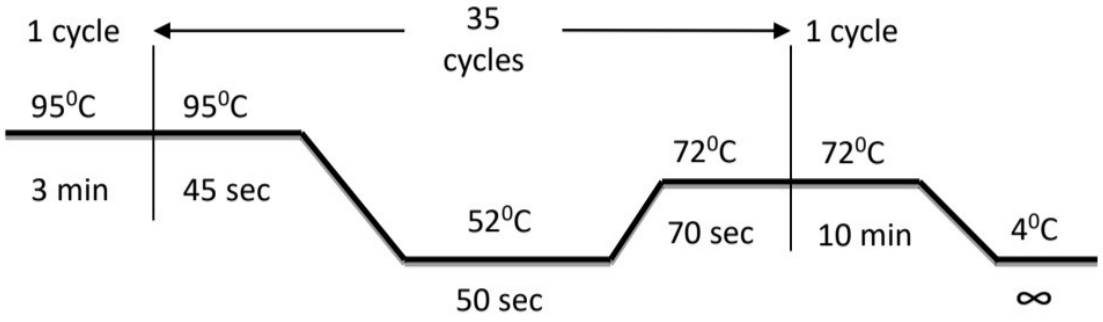

Figure 1. 16SrRNA thermal cycling conditions used for amplification Sequencing

PCR products were processed for cleanup to remove unincorporated nucleotide and residual primers using Exonuclease-I and Shrimp Alkaline phosphatase enzyme followed by cycle sequencing reaction using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). The Cycle sequencing is followed by sequencing cleanup by ethanol precipitation followed by dissolving template in HiDi formamide and bidirectionally sequenced in ABI 3730 Genetic analyzer.

Sequence alignment and assembly PCR products were then processed for direct bi-directionally sequencing using ABI PRISM 3730 × 1 Genetic Analyzer (Applied Biosystems, USA). The resulting DNA sequences were aligned using CLUSTALW in MEGA 11, manually trimmed and edited to obtain complete sequences. The confirmation of species depends on the sequence similarity score.Homology searches were carried out using the BLASTn program against the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast. cgi). ML tree was constructed using MEGA 11 with all positions containing gaps and missing data were included for analysis. Clade supports were calculated based on 1,000 bootstrap resamplings. Table 3. Sample IDs showing Similarity Searches in Sequence Alignment

RESULT

Among all the soil samples collected, only one colony exhibited a sticky consistency with greenish pigmentation and distinctively fruity oudour . The suspected colony was obtained from a 10^{-6} serial dilution of a soil sample which was procured from the Aurangabad caves area. A pure culture was obtained by sub – culturing a loop full of the colony onto fresh cetrimide agar medium.

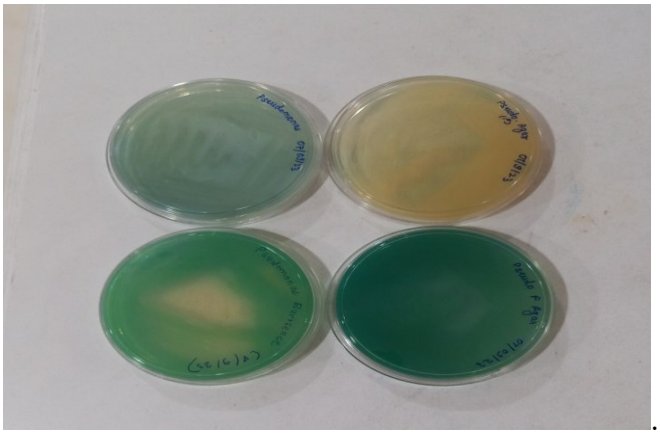

Fig: 1 Pigment producing bacteria isolated from various soil samples

Characterization of the potent isolate

The morphological (colony characters and Grams nature) and biochemical characteristics of the isolate as observed have been summarized in Table.1 The isolate was found to be gram negative in nature and was found to rod shaped.

Table no. 1 : Colony characteristics of pigment producer.

COLONY CHARACTERS	CHARACTERS
Colour	Green
Size	0.5 mm
Shape	Circular
Elevation	Raised
Consistency	Thick
Margin	Complete
Opacity	Opaque
Gram nature	Gram –ve, rod shaped

Fig 2: **Bacterial isolate producing pigment**.

- Biochemical Analysis of potent isolate:

Different enzyme hydrolysis test and IMViC test were performed for identification of microorganism summarized in Table no. 2. According to biochemical analysis the organism was found to be of *Pseudomonas* species by referring to Bergeys manual Vol 4.

Table no. 2: Biochemical Analysis of the pigment producing isolate.

Tests	Result
Gelatin Hydrolysis	+ve
Amylase Test	-ve
Casein Hydrolysis	-ve
Urease Test	-ve
Catalase test	-ve
Citrate Test	+ve
Methyl Red Test	+ve
Indole Test	-ve

Table 2. Biochemical analysis

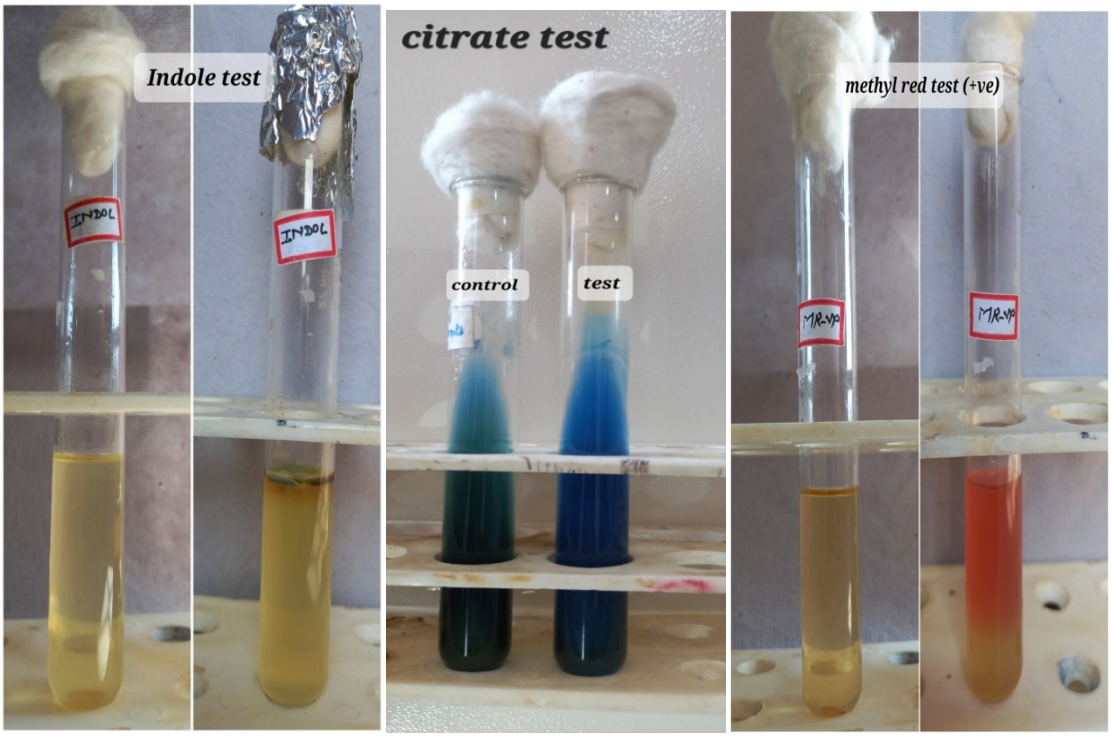

Fig 3: Imvic tests results

- Molecular identification of pigment producing isolate.

  Genomic analysis of potent pigment producing bacteria: The isolated strain was identified by using 16SrRNA sequencing method The length of the sequence was found to be 539bp. The 539bp sequence of the isolate was aligned using the BLAST n tool with pre-existing 16S rRNA gene sequences of microorganisms in the NCBIGen Bank database. The nucleotide alignment showed high similarity (95%) with *Pseudomonas aeruginosa*. The sequence data was submitted to the NCBI GenBank database under accession number PP493975 as *Pseudomonas aeruginosa* VVAA1.

  >AZ-R

  TCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCCGA
  GGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGG
  ACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG
  CAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCT
  GGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGC
  GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGG
  CCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTC
  CCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGAT
  CTCAAGGATCCCAACGGCTAGTCAACATCGTTTACGGCGTG
  GACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCG
  CACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCA
  CTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAG
  GAGATTCCACCACCCTCTACCGTACTCTAGCTCAATACTTTT
  GGATGCAGTTCCCAGGGTGAGCCCGGGGATTTCTCATCCGA
  CTTGGCTGAACCACCTACGCGGGGCTTTATGCGCCATAATT
  CCCAATAAACGCTTGCACCCTTCGTTNTACTTGGNTGCTGG
  AGAAAAATAAAAAGGGGGGTTGTCTGCTTGGGAAAACAAAA
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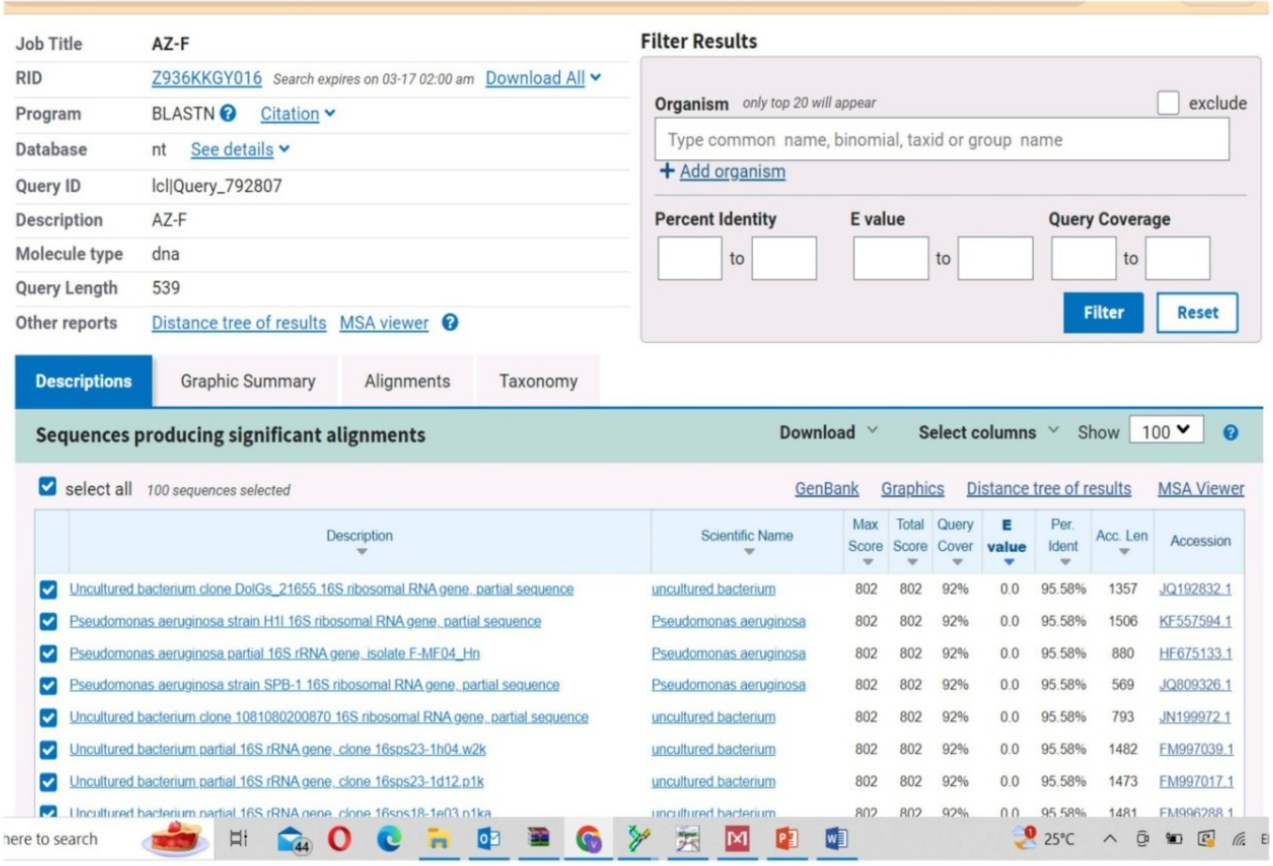

DISCUSSION

Biological pigments, especially from microbes are of great importance due to the some of the following reasons: microbes have rapid multiplication rates, they can be grown in low-cost media with ease, pigments can be processed easily with simple techniques when compared to plant pigments, etc. At present, there is high demand for microbial pigments in the global market because they are preferred over plant pigments and synthetic dyes due to their low production cost and eco – friendly nature respectively[13]. Due to the aforementioned reasons, the present study was aimed at isolating and identifying a pigment producing bacteria from soil. The main focus was to isolate a pigment-producing bacteria because pigment production is more widely present in this group.

In attempts to isolate a pigment-producing a, a greenish pigment producing colony was isolated from one of soil samples by serial dilution metho. The isolate had a entire margin, and an elevation that appeared to be slightly raised with prominent greenish pigmentation on the underside of the colony. The colony exhibited sticky consistency and on gram staining the organism appeared to have gram-negative rod shaped organism. Biochemical analyses showed the organism belong to Pseudomonas species. The identity of the isolate was confirmed using 16S rRNA which revealed it to be *Pseudomonas aeruginosa*.

CONCLUSION

The present study was conducted so as to report a potential source for the extraction of microbial pigment. The main objective was to isolate and identify a pigment-producing bacteria. This objective was driven by the existence of reports that are suggestive of pigment production occurring predominately in bacteria and the supporting evidence conclusive of their biological activities has proven these bacteria to be the quint essential targets for microbial pigments.

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