Isolation and Identification of Pigment Producing Actinomycete Saccharomonospora azurea SJCJABS01

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Given the rising demand for biological pigments, especially of microbial origin – 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 actinomycete because pigment production is prevalent in this group. A powdery, greenish–blue colony with a chalky azure aerial mass was isolated from one of the many rhizosphere soil samples. Upon preliminary investigation, viz. colony characterization and grams staining, the suspected colony was observed to have a filamentous margin with a slightly raised elevation and gram–positive filamentous hyphae. Biochemical analyses of the organism revealed positive results for carbohydrate fermentation and Triple Sugar Iron (TSI) test with no signs of gas production during the former but gas & H2S production during the latter. The identity of the isolate was established via 16S rDNA and phylogeny analysis, which strongly suggested it was Saccharomonospora azurea. Limited research pertaining to morphology, physiology, genomics and secondary metabolite production with no reports on the physicochemical properties of the pigment produced by S. azurea adequately suggests that it is relatively novel. Hence, further studies related to the same could be beneficial to the scientific community.

Keywords: Actinobacteria; actinomycetes; Greenish-Blue Pigment; Microbial Pigment; Rhizosphere Soil; Saccharomonospora azurea.

Actinobacteria are a ubiquitous, heterogenous group of Gram–positive bacteria characterized by their fungal morphology and high GC content (>55 mol %). These bacteria exhibit physiological diversity as apparent from their ability to produce, synthesize, and excrete numerous primary and secondary metabolites. As a result of their extensive physiological diversity, actinomycetes have great biotechnological potential. One such note worthy metabolite originating from actinomycetes are pigments. Actinomycetes are capable of synthesizing a wide range of dark pigments referred to as melanin/melanoid pigments, some well-established pigments from actinomycetes are-blue (Streptomyces coelicolor), brown (Streptomyces sp.), green (Actinomyces viscosus and Saccharomonospora viridis), orange (A.naeslundii), red (Streptomyces echinoruber), violet (Streptomyces mauvecolor), yellow (Streptomyces hygroscopicus), etc. Pigment production is influenced by chemical, physical and physiological factors such as anaerobic
conditions and low temperatures (28 - 30°C), that boost pigment production in *Streptomyces coelicolor*. Pigments are considered to be a helpful criterion for taxonomical investigations. Testing melanin production by using L-tyrosine/L-DOPA as a substrate is considered to be a potential criterion for the identification and classification of *Streptomyces*. Besides their significance in taxonomic studies, these microbial pigments have a broad spectrum of biological activities such as antibiotic, antimicrobial, antioxidant, and antitumor. With rising demands for biological pigments over synthetic colorants, there is a lot of work tending towards the identification and utilization of pigments and the microorganism producing them. Since pigment production seems to be more widely present in actinomycetes than in any other microbial groups, they are considered as the quintessential targets.

The genus *Saccharomonospora* comprises of a group of bacteria that are of keen interest as their genome is so far poorly characterized. The bacteria of this genus occur in diverse habitats (compost, leaf litter, manure, peat, etc.,) and are assumed to play a role in the primary degradation of plant material by digesting hemicellulose. Nonomura & Ohara (1971) described the genus *Saccharomonospora* for bacteria that predominantly produced single spores, and occasionally spores in pairs and short chains, on aerial hyphae. Members of this genus contain a type IV cell wall (i.e., meso-diaminopimelic acid in the peptidoglycan together with arabinose and galactose in cell hydrolysates) distinguishing them from other groups of the same family. *Saccharomonospora azurea* is one of the 9 species of the genus *Saccharomonospora*. Scientific reports on *S. azurea* are meagre with few reports on morphological characterization, physiology, genomics & secondary metabolite production and no significant studies on the nature of the pigment produced.

This study aims to isolate and identify a pigment-producing actinomycete from rhizosphere soil followed by morphological and biochemical characterization of the isolate and its 16S rDNA molecular sequence & phylogeny analysis, so as to report a potential source for extraction of microbial pigment.

### MATERIALS AND METHODS

**Sample Collection**

Rhizosphere soil samples were collected from the gardens and pots at St. Joseph’s College (Autonomous), Bengaluru, Karnataka, India with a geographical location N:12° 57’ 45.72", E:77° 35’ 49.56". The samples were procured from a depth of 8 – 10cm using a sterile spatula and transferred to sterile glass bottles. The samples were brought to the laboratory and processed further immediately.

**Isolation & Screening of Actinomycetes**

In order to isolate actinomycetes from different rhizosphere soil samples, 1g of the respective 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) of 10⁻³, 10⁻⁴, and 10⁻⁵ was spread plated on Actinomycetes Isolation Agar (AIA) medium and incubated at 37°C for 3 ~ 7 days. AIA medium was prepared by dissolving 2.17g AIA (Hi-Media) and 2g Agar–Agarin 100ml distilled water. 100 µl of Erythromycin and Fluconazole was added to the medium post autoclaving. Appropriate amount (30ml) of hot media was then poured into sterile petri plates and allowed to solidify at room temperature. Post incubation, all the plates were screened for actinomycetes colonies based on morphology and pigmentation. The actinomycete colony that showed significant coloration was sub-cultured onto fresh AIA 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. Grams nature of the isolate was determined using the standard protocol that uses Crystal Violet as the primary stain, an iodine solution as a mordant, 95 % ethanol as a decolourizer and Safranin as the counterstain. Grams nature of the isolate was determined using the standard protocol that uses Crystal Violet as the primary stain, an iodine solution as a mordant, 95 % ethanol as a decolourizer and Safranin as the counterstain. Biochemical tests such as Carbohydrate fermentation, Simmons’ citrate (citrate utilization), Triple Sugar Iron (TSI) and Rapid Urease Test (RUT) were performed.
using standard protocols. Glucose was used as the sole carbon source for the carbohydrate fermentation test. A lead acetate strip was used to detect H$_2$S production for TSI test.

**Molecular Sequencing and Phylogeny analysis**

The identity of the isolate was determined using 16S rDNA molecular sequencing analysis. The isolate was outsourced to Yaazh Xenomics, Coimbatore, Tamil Nadu, India for this purpose. The molecular identification technique was carried out as per the standard genome sequencing protocol. Genomic DNA was isolated using the EXpure Microbial DNA Isolation Kit developed by Bogar Bio Bee Stores Pvt. Ltd. The procedure for Genomic DNA isolation involved lysis, homogenization, centrifugation, binding (using binding buffer), washing and elution. The isolated Genomic DNA was amplified using PCR by following the regular thermal cycling conditions of denaturation, annealing and extension. Purification

![Fig. 1. Isolation and Screening of Actinomycetes; A: Master plate with bacterial isolate as obtained on AIA; B: Pure culture of the suspected colony](image1)

![Fig. 2. Morphological & biochemical analyses of the pigment-producing actinomycete strain; A: Microscopic field showing gram-positive filamentous hyphae; B: Carbohydrate fermentation test results exhibiting glucose fermentation with no gas production; C: Negative Citrate utilization test; D: Triple sugar iron test indicating only Dextrose fermentation with gas & H$_2$S production; E: Negative Urease test](image2)
of the PCR products was done using the Montage PCR Clean Up Kit (Millipore) and PCR products were sequenced using primers with the help of the Terminator Cycling Sequencing Kit. Single pass sequencing was performed on each template using 16S rRNA universal primers. The fluorescent labelled fragments were purified using ethanol precipitation and the samples were resuspended in distilled water and subjected to electrophoresis in a sequencer. The sequence obtained was subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/) to identify its phylogenetic origin through homology. The generic and specific epithet of the strain was confirmed based on percentage similarity with the homologous sequences. The sequence data was submitted to the NCBI GenBank Database for public access. The most significant matches obtained from the NCBI GenBank Database (https://www.ncbi.nlm.nih.gov/genbank/) through BLASTn were utilized for constructing a phylogenetic tree under the Maximum Likelihood (ML) criterion using MEGA-X software.

## RESULTS

### Isolation & Screening of Actinomycetes

Among all the rhizosphere soil samples collected, only one colony exhibited a powdery consistency with pigmentation. The suspected colony was obtained from a 10⁻³ serial dilution

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### Table 1. Morphological & biochemical properties of the pigment-producing actinomycete strain

| S. No. | Characteristics          | Inference                                                        |
|--------|--------------------------|------------------------------------------------------------------|
| 1      | Form                     | Filamentous                                                      |
| 2      | Margin                   | Filamentous                                                      |
| 3      | Elevation                | Raised                                                           |
| 4      | Pigmentation             | Greenish – blue pigmentation on the underside of the colony     |
| 5      | Texture                  | Powdery with chalky azure aerial mass                            |
| 6      | Grams nature             | Gram – positive                                                  |
| 7      | Carbohydrate fermentation| Glucose fermentation with no gas production                     |
| 8      | Citrate utilization      | Negative                                                         |
| 9      | Triple sugar iron        | Dextrose fermentation only, with gas (CO₂ and H₂) and H₂S production |
| 10     | Urease                   | Negative                                                         |

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![Fig. 3. Phylogenetic tree highlighting the position of S. azurea SJCJABS01](#)
Table 2. Sequencing analysis of 16S rDNA of isolated actinomycete

| GenBank Accession No. | MT509549 |
|-----------------------|----------|
| Target gene           | 16S rDNA |
| No. of Base pairs     | 1307bp   |
| Organism              | *Saccharomonospora azurea* |
| Strain                | SJCJABS01 |

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FASTA Gene Sequence >Pigment producing *Saccharomonospora azurea* SJCJABS01TAC
CGGATA GGACA CACTGTGCAGATTGTTGTTGGAAGTCTCCGGCGGTACAGG
TTGAGC CCGCGCCTGAGAGGAGGTACCGGCTATGCACTGGTGGGCTTGCAGG
GACGGGTA GCCGCGCCTGAGAGGAGGTACCGGCTATGCACTGGTGGGCTTGCAGG
CCAGCTCTACGGGCAGCAGTGGGATATTTGCAAAATCCGGCGGTTCGGGCTTGCAGG
GCTAAGAAGCCTCTGGATGCAAGTGCCTCAGCTCCGGCTTGAACATTCGGGCTTGCAGG

Characterization of the Isolate

The morphological (colony characters and Grams nature) and biochemical characteristics of the isolate as observed have been summarized in Table 1. The colony had a filamentous margin and an elevation that appeared to be slightly raised. Prominent greenish–blue pigmentation was observed on the underside of the colony. The colony had a powdery consistency with a chalky azure aerial mass. On Gram staining the organism appeared to have gram–positive filamentous hyphae (Figure 2A). Biochemical analyses showed the organism to be positive for carbohydrate fermentation with no gas production (Figure 2B) and exhibited glucose fermentation only, with gas and H₂S production for TSI test (Figure 2D). The organism tested negative for citrate utilization (Figure 2C) and RUT (Figure 2E).

Molecular sequencing and Phylogeny analysis

The details of the 16S rDNA molecular sequencing analysis have been summarized in Table 2. The length of the sequence was found to be 1307bp. The 1307bp sequence of the isolate was aligned using the BLASTn tool with existing 16S rDNA gene sequences of microorganisms in the NCBI GenBank database. The nucleotide alignment showed high similarity (100%) with *S. azurea*. The sequence data was submitted to the NCBI GenBank database under accession...
number MT509549 as ‘Saccharomonospora azurea strain SJCJABS01’. The phylogeny analysis of the sequence has been summarised in Figure 3. The phylogenetic tree constructed under the ML criterion using MEGA.X software highlights the position of S. azurea strain SJCJABS01 among its phylogenetic neighbours.

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 respectively13. Due to the aforementioned reasons, the present study was aimed at isolating and identifying a pigment–producing actinomycete because pigment production is more widely present in this group.

In attempts to isolate a pigment–producing actinomycete, a greenish–blue pigment–producing colony was isolated from one of soil samples by serial dilution method using AIA as the medium. The isolate had a filamentous margin, and an elevation that appeared to be slightly raised with prominent greenish–blue pigmentation on the underside of the colony. The colony exhibited powdery consistency with a chalky azure aerial mass. On Gram staining the organism appeared to have gram–positive filamentous hyphae. Biochemical analyses showed the organism to be positive for carbohydrate fermentation with no gas production and exhibited dextrose fermentation only, with gas and H₂S production for TSI test. The identity of the isolate was confirmed using 16S rDNA and phylogeny analysis which revealed it to be Saccharomonospora azurea.

CONCLUSION

By definition, a pigment refers to any substance capable of absorbing light. Basically, it is the property of electromagnetic radiation with wavelengths ranging from 300–400 nm and 700–800 nm10. During the 19th century, development of synthetic colors gained popularity due to their chemical stability, economical production cost and expansive spectrum of shades. However, subsequent discoveries enumerating the possible antagonistic effects of synthetic colorants like – allergenicity, carcinogenicity, hyperactivity, toxicity, etc., in humans and environmental pollution to name a few, demeaned their popularity leading to the increase in demand for natural alternatives. Microbial pigments, also referred to as biological pigments/biochromes are simply pigments produced by microorganisms. A multitude of such pigments have been isolated and characterized from various microbes because of their wide-ranging bioactivity, for example: Phycoerythrin, Phycocyanin and Scytonemin from Cyanobacteria exhibit antioxidant, antitumor and immunoregulatory effects; Canthaxanthin from Haloferax alexandrinus, Bradyrhizobium spp. and Lactobacillus pluvalis showcases anti-inflammatory, antioxidant, antitumor and photo-protectant activity; Anthraquinones from Penicillium oxalicum are antifungal and virucidal; while Melanin from Neoformans is an antibiofilm, antimicrobial and antioxidant. Even though many such microbial pigments may be rendered useless because of their instability against chemical and physical condition, various techniques have been developed to produce more stable pigments, for instance: microencapsulation, nano-emulsion formation and nano-formulations have been utilized to improve stability and deliverance of biological pigments to food matrices8. Moreover, pigments obtained from plants are limited and require complex production processes making them expensive in contrast to microbial pigments that are available in varying shades and can be easily produced in sufficient amounts with simpler processing techniques making them inexpensive2. Hence, due to these reasons pigment production in microorganisms is currently one of the most promising fields of research.

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 actinomycete. This objective was driven by
the existence of reports that are suggestive of pigment production occurring predominately in actinomycetes and the supporting evidence conclusive of their biological activities has proven these bacteria to be the quintessential targets for microbial pigments. A study conducted on *Streptomyces sp.* showed that a melanin pigment produced through solid state fermentation is a potential source of antioxidants like tocopherol and trolox. Another study on actinomycete pigment Prodigiosin from *Streptomyces sp.* revealed its antiproliferative property against Human Cervical Adenocarcinoma (HeLa) cell line, therefore, deducing actinomycetes to be extremely useful for pharmaceuticals. An investigation undertaken by Parmar, R. S. & Singh, C., (2018) aimed at identification, characterization, and application of actinomycetes pigment showed that the isolate - actinomycete ARITM02, selected on the basis of its ability to produce a diffusible pigment was tested safe to be used as a natural food and pharmaceutical colorant. The conclusion of the study suggested that the novel versatile pigment was safe to be utilized in cosmetic, food, pharmaceutical and textile industries. Miscellaneous research pertaining to aspects such as lip balm production and textile dyeing from actinomycete pigments exhibited prominent results for dye retention hence, making actinomycetes useful in cosmetic and textile industries.

A powdery, greenish–blue colony with a chalky azure aerial mass was isolated on AIA from one of the many rhizosphere soil samples collected from our college campus. Upon preliminary investigation, viz. colony characterization and grams staining, the suspected colony was observed to have a filamentous margin with a slightly raised elevation and gram–positive filamentous hyphae. Biochemical analyses of the organism revealed positive results for carbohydrate fermentation and TSI test with no signs of gas production during the former but gas & H₂S production during the latter. The isolate tested negative for citrate utilization and urease production. Identification of the isolate was achieved by 16S rDNA molecular sequence and phylogeny analysis. Results of the analysis strongly suggested that the pigment–producing soil isolate was *Saccharomonospora azurea*. Correlations of morphology with previously reported *S. azurea* strains i.e., NA–128 and AP–11/18 further substantiate our observations. Sparse research restricted tomorphology, physiology, genomics & secondary metabolite production with no reports on the physicochemical properties of the pigment produced by the identified organism clearly suggests that it is a relatively novel theme. Hence, further research pertaining to the nature of the pigment could be a benefactor to the scientific community.

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**Conflict of Interest**

No conflict of interest for this work.

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