ACTINOBACTERIAL PIGMENT ASSISTED SYNTHESIS OF NANOPARTICLES AND ITS BIOLOGICAL ACTIVITY

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ABSTRACT

Recently, the green synthesis of nanoparticles has gained considerable attention due to its benefits such as cost efficiency, simplicity, eco-friendly nature, biocompatibility and broad applications over conventional chemical and physical techniques. In this context twenty actinobacteria were isolated from the rhizospheric soil of wild carrot and screened for their pigment producing ability. These isolates belong to the genus Streptomyces (58%), followed by Streptosporangium sp. (19%), Nocardia sp. (11%), Actinomadura sp. (8%), and Micromonospora sp. (4%). The most promising isolate (NS-05) producing the pink pigment has been taken for the synthesis of silver nanoparticles. The isolate NS-05 was identified as Streptomyces sp. based on cultural characteristics and 16S rDNA sequence analysis. It was most closely related with type strain Streptomyces fulvisinus DSM 40593T, S. microflavus NBRC13062T, S. setonii NRRL ISP-5322T, S. anulatus RRL B-2007T with a sequence similarity of 95.6% which shows that it may belong to novel species of Streptomyces. The bio-pigment assisted synthesized nanoparticles were characterized using UV-Vis, FTIR and Scanning electron microscopy studies. The average size of synthesized silver nanoparticles was 42.5nm and has λ max at 433 nm. The synthesized nanoparticles showed promising activity against major pathogens like Staphylococcus aureus MTCC 2940, Bacillus subtilis MTCC 441 Salmonella typhi, Proteus vulgaris MTCC 6380, Escherichia coli MTCC 739. The findings of present research are promising, and this pigment can also be used for the green synthesis of other nanoparticles.

Keywords: Streptomyces, pigment, nanoparticles, antimicrobial activity

INTRODUCTION

Actinobacteria are Gram-positive, filamentous bacteria widely distributed in water, soil, and other natural ecosystems. The phylum Actinobacteria carries out a life cycle, which is complex and represents the main taxonomic parts between different niches (Waksman, 1961). Likewise pigments such as red, violet, and orange produced by most of the genera of the actinobacteria. The red pigment synthesized by certain bacteria including actinobacteria, Serratia marcescens and Streptomyces and this pigment belong to the family called prodigiosin (Khanafari et al., 2006). In recent years, the demand for the natural product is increasing day by day and these natural products has become a site of interest and have been successful in attracting the population, while leaving the synthetic one. The synthetic colors are being replaced by natural colors (pigments). These pigments both natural and synthetic colors are widely used in various fields like food industries, paper industries, agricultural process, cosmetics, water science, researches, clothes and other technologies (Tuli et al., 2015).

In the development of nanoparticles with distinct shapes and sizes, the natural bio-molecule have been reported to play an active role as the driving force for the design of greener, healthy and environmentally friendly nanoparticles' synthesis protocols (Sharma et al., 2019). The synthesis of nanoparticles has got attention because it can be applied in various field. Similarly, the certain pigment-producing microorganisms can be a good source of natural pigment which can be used effectively. The pigments contains some functional group liable for the reduction of silver ions (Carvalho et al., 2011). The reduction is usually carried out by using pigments in aqueous/ethanol or with microbial cells with a silver solution, usually silver nitrate. The presence of active chemicals in the extracted pigment and cells are responsible for the complex bio-reduction reaction process (Sadeghi et al., 2015; Baraka et al., 2017; John et al., 2020).

Many researchers have synthesized nanoparticles based on chemical, physical and biological method (San Diego et al., 2020). In this context, we undertook the present study to isolate the pigment-producing actinobacteria and synthesis of pigment assisted nanoparticles and study its biological activity.

MATERIAL AND METHODS

Isolation and characterization of actinobacteria

The rhizosphere is in immediate contact with the plant roots and is actively enriched by the complex mixture of sources such as amino acids, sugar and other plant-based nutrients (Buis et al., 2006). This condition attracts a microbial community unique to plants (Essariouli et al., 2017) hence, both plants and microorganisms are altered. Therefore, rhizospheric soil was used for isolation of actinobacteria. Ten grams of rhizospheric soil samples were collected from Jolly Grant, Uttarakhand, India during February 2019. Three samples were collected from three different sites of Jolly Grant. The soil samples were taken to the laboratory for its further analysis in sterilized polyethylene bags. The isolation and characterization of the actinobacteria was done by the according to a method described by Krishnamoorthy and Ekambaram, 2018; Kumar et al., 2012 (a &b). The neighbor-joining method was used to study the evolutionary history (Salitou and Niel, 1997). 1000 replicates selected for bootstrap analysis (Felsenstein, 1985). Kimura 2-parameter method was used for the study of evolutionary distances (Kimura, 1980). MEGA X software was used to perform phylogenetic analysis (Kumar et al., 2018).

Culture media

The culture media used during study were Actinomycetes isolation agar (g/L, Sodium caseinate-2.0, L-Asparagine-0.1, Sodium propanone-4.0, Dipotassium phosphate-0.5, Magnesium sulphate-0.1, ferrous sulphate-0.001, Agar-15.0) Nutrient agar media (g/L, Peptone-5.0, Yeast extract-5.0, H2O peptone-1.0, Yeast extract-1.5, NaCl-5.0, Agar-15.0, pH-7.2), Yest extract malt extract Agar (g/L, Peptone-5.0; yeat extract-3.0, malt extract-3.0, dextrose-10.0, agar- 20.0), Potato dextrose agar (g/L, Potatoes, infusion-200.0, dextrose -20.0, agar- 15.0). All the media were purchased from Himedia, Bangalore, India.
Production of pigment from actinobacteria

The isolates were grown on Actinomycetes isolation agar media (Himedia, India) and incubated at 27°C for 3 days until an extracellular water soluble pigment was produced. The produced pigment was extracted by crushing the agar with methanol and filtered through Whatman filter paper. The filtered solution concentrated by using Rota vacuum and converted into powder.

Nanoparticle synthesis

AgNO₃ was the silver precursor and the solution was prepared using sterilized distilled water and kept in dark to avoid photo-reduction. All the glasswares were cleaned using aqua-regia (HNO₃:HCl, 3:1 (v/v)) and washed thoroughly using distilled water. The solid pigment was dissolved in sterile double distilled water, out of which 600µl transferred to a test tube containing 400µl of 1mM solution of silver nitrate. The reaction mixture was incubated at room temperature for 16 to 24 hrs in dark. After incubation, the change in color was observed from red to brown.

Characterization of synthesized nanoparticles

After 16-24 hrs of incubation, the reaction mixture centrifuged at 15000 rpm for 30 min. (REMI, India). The supernatant was allowed to evaporate and then the sample was coagulated with gold and analyzed using SEM (Duran et al., 2005).

Antibacterial assay

Antibacterial assay was carried out using agar well diffusion method according to the method described by Kumar et al., 2012 (a &b). Antimicrobial activity was detected by measuring the zone of inhibition in mm (including the wells diameter) appeared after 24 hrs at 37°C. Pigment and AgNO₃ was used as control. The tested bacteria were Staphylococcus aureus MTCC 2940, Bacillus subtilis MTCC 441, Salmonella typhi, Proteus vulgaris, K. pneumoniae, S. typhi, P. vulgaris, K. pneumoniae, S. typhi and E. coli were screened for their pigment producing ability and only one isolate (NS-05) was able to produce pink colored pigment and was further identified by using polyphasic approach. Isolate NS-05 produced extensively branched substrate and aerial hyphae. It bears light pink colored flexuous spore chain on the aerial mycelium. The cultural characteristics of the actinobacterial isolate NS-05 are given in Table 1. Good growth was recored in all the media tested. However, pigment was only produced in AIA and NAM. The physiochemical characteristic of NS-05 with most closely related type stains has been given in table 3. Based on the 16S rDNA sequence (1016 Nucleotides) analysis the tested isolate was identified as Streptomyces species and the sequence was submitted to GenBank under the accession number MN173858. Based on pairwise sequence analysis the isolated was most closely related to Streptomyces fulvisissimus DSM 40593T, S. microflavus NRBC13062T, S. setonii NRRL ISP-5322T and S. anulatus NRRL B-2000T with a sequence similarity of 95.6%. According to Stackebrandt and Goebel (1994) the organisms having a sequence similarity of 97% or less may belong to novel species. Hence, this isolate may be the novel species of Streptomyces. Moreover, isolate NS-05 is out-group with all the most closely related species which further confirms its novelty (Fig. 3). The isolate NS-05 can also be distinguished from type strains in many other characteristics. The isolate NS-05 produced flexuous spore chains while most closely related species produced spirals and rectiflexibles. The spore mass color was light pink in case of NS-05 whereas it was red, gray and yellow tallow for Streptomyces fulvisissimus DSM 40593T, S. microflavus NRBC13062T, and S. setonii NRRL ISP-5322T respectively. NS-05 showed negative test for lipase activity whereas type strains showed positive results. Comparatively no growth was recorded at 0.001%(w/v) Potassium tellurite and in cystine. Therefore, NS-05 may represent a novel species of Streptomyces.
L-valine  +  +++  +
L-phenylalanine  ++  +++  +++  +
L-histidine  ++  +++  +
Carbon source
Sucrose  +++  +  +++  +
Mannitol  ++  +++  +++  +
Raffinose  +  +++  +
Melibiose  +  +
Dextran  -  -
Inositol  +++  +

*Symbols used: +++ good; ++ Fair; + poor; - negative

Table 4 λ max (peak detection) for the synthesized nanoparticles

| S. No | Control (Pigment) | Ti (Pigment +AgNO₃) |
|-------|-------------------|---------------------|
| 1     | λ max (nm) | Abs λ max (nm) | Abs |
| 2     | 540.00 | 0.137 | 735.00 | 0.018 |
| 3     | 508.00 | 0.064 | 433.00 | 1.213 |

After 16 hrs of incubation reaction mixture was scanned (between 200-1100nm) using UV-Vis spectroscopy as a result of which the bio-reduction of silver ion was monitored. The λ max was recorded at 430 nm (Fig.3 and Table 4), which indicates that the nanoparticles were synthesized. The dark brown color was exhibited by the silver nanoparticles (Mulvaney, 1996; Gao et al., 2014). SEM analysis of the silver nanoparticles reveals that they are predominantly spherical (Fig. 6). The average size of the synthesized nanoparticles was 42.5 nm. The action of nanoparticles on disease causing organism is related to the shape size and concentration with the synthesized nanoparticles. The smaller the nanoparticle the more is its activity against pathogen (Chauhan et al., 2013). Possible bio-molecules responsible for Ag⁺ ions reduction and capping were identified using FTIR analysis. The major spectra (Fig.7) of nanoparticles obtained spectrum resulted in peak value at 3454.83 cm⁻¹ corresponding to CO stretching in alcohol and phenolic compound and peak at 1636 corresponds to amide group due to carbonyl stretch in proteins, and peak identified at 655.84 cm⁻¹ as halogen compounds. Researchers have proved that presence of thiols, amino acids and alcohols protect particles from sedimentation, agglomeration, or losing their surface properties (Oliveira et al., 2005; Iravani et al., 2014).

Figure 3 Neighbour-joining phylogenetic tree based on 16S rDNA gene sequences, showing the relationships between tested strain Streptomyces sp. NS-06 and the most closely related with type strains of Streptomyces. Only values above 50% are given.

Characterization of the synthesized nanoparticles

In modern technology, synthesis of nanoparticles is one of the lime-lighted topic, especially biosynthesis of nanoparticles from the pigment produced naturally by microorganisms under exploitation. Hence, in the present study was focused on the synthesis of silver nanoparticles using the pigment produced from Streptomyces sp. NS-05. The silver nanoparticles' formation was indicated by observing the change in color from pink to brown after addition of silver nitrate (Shah et al., 2015) as shown in Fig.4.

Table 4 λ max (peak detection) for the synthesized nanoparticles

| S. No | Control (Pigment) | Ti (Pigment +AgNO₃) |
|-------|-------------------|---------------------|
| 1     | λ max (nm) | Abs λ max (nm) | Abs |
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Figure 4 The colour of the extracted pigment (a) before addition of silver nitrate (b) after addition of silver nitrate

Figure 5 UV-Vis spectroscopy, a, Control-Pigment (λ max, 504 and 580nm); b-Synthesized nanoparticle: peak at 433 nm

Figure 6 SEM analysis of silver nanoparticles synthesized from pigment produced by Streptomyces sp. The black arrows indicates the nanoparticles.
Antimicrobial activity of silver nanoparticles synthesized

The antimicrobial activities of the synthesized nanoparticle are given in table 5. It showed maximum zone of inhibition against B. subtilis MTCC 441 (19.00 ± 1.00 mm) followed by S. aureus MTCC 2940 (18.00 ± 1.00 mm). S. pyogenes (17.00 ± 0.47 mm), P. vulgaris MTCC 6380 (16.00 ± 1.00 mm) and E. coli MTCC 739 (14.00 ± 1.00 mm). The pigment alone showed activity against E. coli and P. vulgaris only, while the activity was enhanced when silver nanoparticles was synthesized. It is usually recognized that the antimicrobial activity of synthesized nanoparticles are due to electrostatic interactions between cell wall of bacteria (negative charged) and nanoparticles (Positive charge). This electrostatic interaction finally leads to the death of the microbial cells (Hajipour et al., 2012; Wnag et al., 2017). The enhancement of antimicrobial activity may be due to the free conjugation form of silver nanoparticles (AgNPs) as revealed by FTIR data (El-Bat et al., 2016).

Table 5 Antimicrobial activity of silver nanoparticles synthesized by well diffusion method

| Test organism       | Inhibition zone diameter in mm | AgNPs (Control) | Synthesized nanoparticles |
|---------------------|--------------------------------|-----------------|--------------------------|
| S. aureus MTCC 2940 | 18.00± 1.00                   | -               | 9.00: 1.00               |
| E. coli MTCC 739    | 14.00± 1.00                   | -               | 8.00: 1.00               |
| P. vulgaris MTCC 6380 | 16.00± 1.00              | -               | 5.00: 1.00               |
| S. pyogenes         | 17.00± 0.47                   | -               | 5.00: 1.00               |
| Bacillus subtilis MTCC 441 | 19.00± 1.00 | -               | 10.00: 0.3               |
| Streptococcus pyogenes | 17.00± 0.47     | -               | 10.00: 0.40              |

Average of triplicates ± Standard deviation; -, No zone of inhibition

CONCLUSION
The rhizospheric soil is rich source of Actinobacteria mainly the genera Streptomyces, Streptosporangium sp, Nocardia sp, Actinomadura sp and Micromonospora sp. The most promising isolate (NS-05) producing the pink pigment was most closely related with the type strain Streptomyces falvissimus DSM 40593T, S. microflavus NBRIC13062T, S. setonii NRRL ISP-5322T, S. amnolus NRRL B-2000T with a sequence similarity of 95. 6% which indicates, that it may belong to novel species of Streptomyces. Average size of synthesized AgNPs were found to 42.5 nm and have λ max at 433 nm. Synthesized nanoparticles showed promising activity against both Gram-positive and Gram-negative bacterial pathogens. The findings of present research are promising, and this pigment can also be used for the green synthesis of other nanoparticles.

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Conflict of interest: None

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