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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 fulvissimus* DSM 40593^T, *S. microflavus* NBRC13062^T, *S. setonii* NRRL ISP-5322^T, *S. anulatus* RRL B-2000^T 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 18 major families now standard within the Domain Bacteria **(Sharma *et al.*, 2018)**. Most of the antibiotics of biological origin are of actinobacteria origin with *Streptomyces* being especially prolific. Based on several studies, antibiotics produced by the actinobacteria make 80% of the known antibiotics (**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 marsences* and *Streptomyces* and this pigment belong to the family called prodigosin **(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-moleculae have been reported to play an active role as the driving force for the design of greener, healthy and environmentally friendly nanoparticles' syrthesis 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 (**Bais *et al.*, 2006).** This condition attracts a microbial community unique to plants **(Essarioui *et al* ., 2017**) hence, both plants and microorganisms are altered. Therefore, rhzospheric soil was used for isolation of actinobacteria. Ten grams of rhizosperic soil samples were collected from JollyGrant, Uttarakhand, India during February 2019. Three samples were collected from three different sites of Jollygrant. 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 (**Saitou and Nei, 1987)**. 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 agr (g/L, Sodium caseinate-2.0, L-Asparagine-0.1, Sodium proponate-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, HM 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 ; yeast 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,Banglore, 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

$AgNO_3$ 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 (HNO_3:HCl , 3:1 (v/v) and washed thoroughly using distilled water **(Karthika *et al.*, 2015)**. 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 the 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 discarded and the black-colored precipitate was re-dissolved in double-distilled water and scanned for λ max from 200-1100 nm using UV-Vis spectrophotometer (UV-1800 Shimadzu, Japan). The potential biomolecules present in the pigment of actinobacteria responsible for reducing and capping the bio-reduced silver nanoparticles was studied by using Fourier Transform Infrared (FTIR) Spectroscopy Measurements **(Raut *et al.*, 2009)**. To analyze the shape and size of nanoparticles, SEM analysis was performed. The sample was sonicated for 14 min. then loaded on the carbon tape. The solvent was allowed to evaporate and then the sample was coated 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 $AgNO_3$ was used as control. The tested bacteria were *Staphylococcus aureus* MTCC 2940, *Bacillus subtilis* MTCC 441 *Salmonella typhi*, *Proteus vulgaris* MTCC 6380, and *Escherichia coli* MTCC 739.

RESULTS AND DISCUSSION

Isolation and characterization of isolates

The occurrence and distribution of actinobacteria in different soil samples are given in table 1. Based on colony morphology, 25 isolates were selected. These isolates were tentatively isolates up to genus level as described by **Kumar *et al.* (2012)**. During the study, it was recorded that *Streptomyces* sp. was dominant (58%) followed by *Streptosporangium* sp (19%), *Nocardia* sp. (11%), *Actinomadura* sp. (8%) and *Micromonospora* sp. (4%) as shown from Fig.1

Table 1 Occurrence and distribution of actinobacteria in rhizospheric soil

Sample	C.F.U	Morphotypes
S-1	15.0×10^5	5
S-2	30.0×10^5	10
S-3	25.0×10^5	10
	Total	**25**

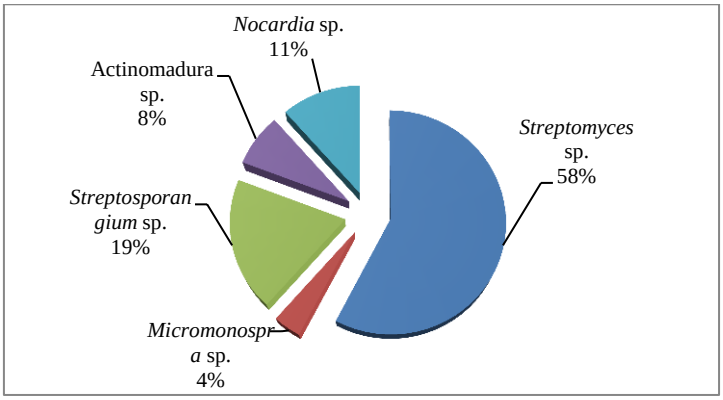

Figure 1 Distribution of actinobacteria in rhizospheric soil

These isolate 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 entensively branched substrate and aerial hyphae. It bears light pink colored flexous spore chain on the aerial mycelium (Fig. 2). . The cultural characteristics of the actinobacterial isolate NS-05 are given in table 2. Good growth was recored in all the media tested. However, pigment was only produed 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 waas 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 fulvissimus* DSM 40593T, *S. microflavus* NBRC13062T, *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 chacteristics. The isolate NS-05 produced flexous spore chains while most closly related species produced spirals and rectiflexibiles. The spore mass color was light pink in case of NS-05 whereas it was red, gray and yellow towhile for *Streptomyces fulvissimus* DSM 40593T, *S. microflavus* NBRC13062T, and *S. setonii* NRRL ISP-5322T respectively. NS-05 showed negative test for lipase actvity whereas type strains showed postive results.Comparatively no growth was recorded at 0.001(w/v) Potassium terullite and in cysteine. Therefore, NS-05 may represent a novel species of *Streptomyces*.

Table 2 The cultural characteristics of the actinobacterial isolate NS-05

Media	Aerial Mycelium	Substrate mycelium	Spores	Pigment
AIA	Dull white	Dark Brown	++	Dark Pink
NAM	Light cream	Dark brown	++	Light Pink
YEMEA	White	Yellow	++	-
PDA	White	Brown	++	-

AIA- Actinomycetes isolation agar, NAM-Nutrient agar media, YEMEA-Yeast extract malt extract agar,PDA-Potato dextrose agar

Table 3 Differentiation of *Streptomyces* sp. NS-5 with most closely related type strains (Locci, 1989)

Characteristics	Isolate NS-05	*Streptomyces fulvissimus* DSM 40593T	*S. microflavus* NBRC13062T,	*S. setonii* NRRL ISP-5322T
Spore chain	Flexous	Rectiflexibiles	Spirals	Rectiflexibiles
Spore mass	Light pink	Red	Gray	Yellow to white
Mycelial pigment	Brown	Red orange	Yellow-brown	Yellow brown
Diffusible pigment	Pink	-	-	Yellow brown
Lipase activity	-	+++	+	+++
Growth at				
7% (w/v) NaCl	+++	+	-	++
0.01% (w/v) Sodium azide	+++	-	++	+
0.1% (w/v)Phenol	+	++	+++	+++
0.001(w/v) Potassium terullite	-	+++	++	++
Nitrogen source				
L-cysteine	-	++	++	++

L- valine	+	+++	+	+
L-phenylalanine	++	+++	+++	++
L-histidine	++	+++	+	++
Carbon source				
Sucrose	+++	+	+++	+
Mannitol	++	+++	+++	+++
Raffinose	+	++	+++	+
Melibiose	+	++	+	+
Dextran	-	+	-	+++
Inositol	+++	+++	+	+

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

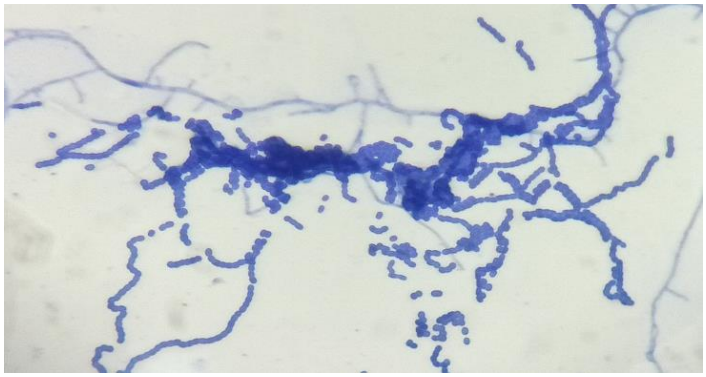

Figure 2 Spore chain morphology (Flexous)

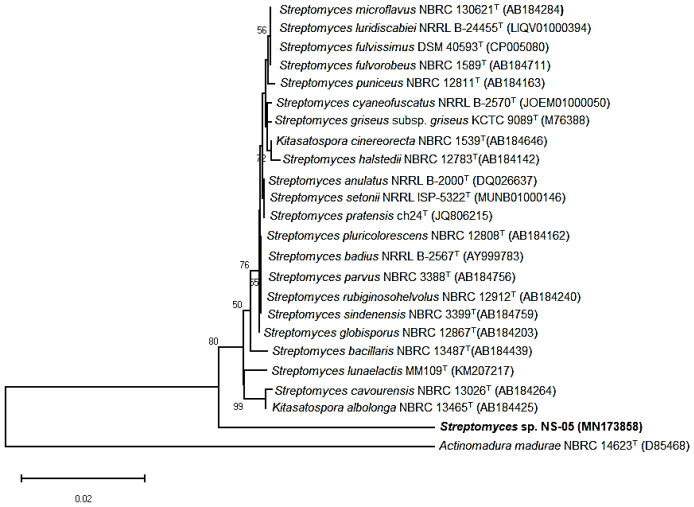

Figure 3 Neighbour-joining phylogenetic tree based on 16S rDNA gene sequences, showing the relationships between tested strain *Streptomyces* sp. NS-06 and the most closely related with type strains of *Streptomyces*. Only values above 50% are given.

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)		T1 (Pigment +$AgNO_3$)	
1	λ max (nm)	Abs	λ) (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.5 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 3454.83cm^{-1} corresponding to OH stretching in alcohol and phenolic compound and peak at 1636 corresponds to amide group due to carbonyl stretch in proteins, and the 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)**.

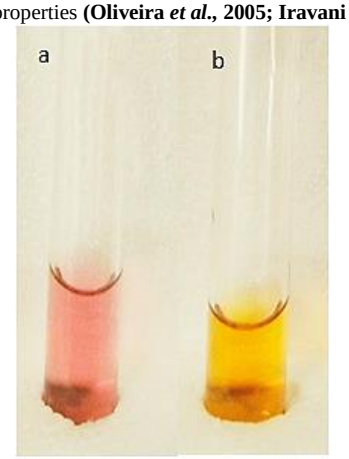

Figure 4 The colour of the extracted pigment (a) before addition of silver nitrate (b) after addition of silver nitrate

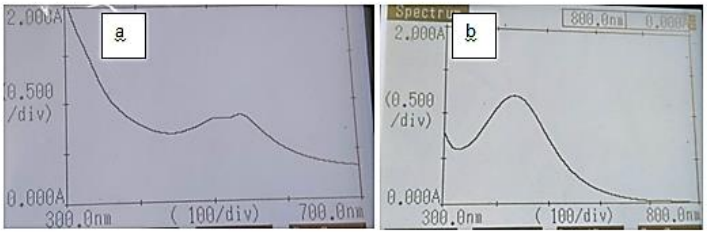

Figure 5 UV-Vis spectroscopy, a, Control-Pigment (λ max, 504 and 580nm**)**; b-Synthesized nanoparticle: peak at 433 nm

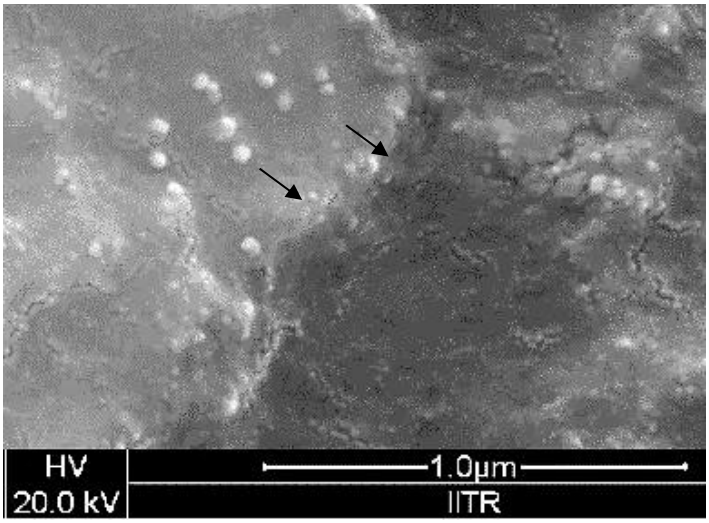

Figure 6 SEM analysis of silver nanoparticles synthesized from pigment produced by *Streptomyces* sp. The black arrows indicates the nanoparticles.

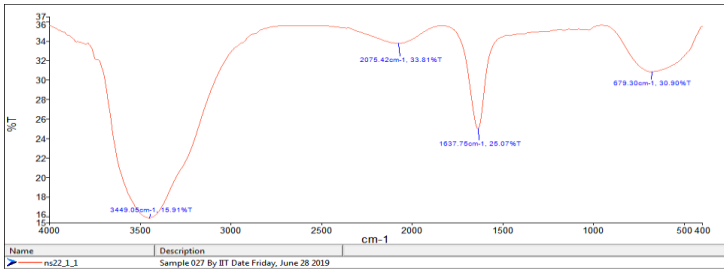

Figure 7 FTIR analysis of silver nanoparticles synthesized from pigment produced by *Streptomyces* sp.

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 interaction 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-Baz *et al.*, 2016)**.

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

Test organism	**Inhibition zone diameter in mm**		
	Synthesized nanoparticles	**Pigment (Control)**	**$AgNo_3$ (Control)**
S. aureus MTCC 2940	18.00± 1.00	-	9.00± 1.00
E. coli MTCC 739	14.00± 1.00	5.00± 1.00	8.00± 1.00
P. vulgaris MTCC 6380	16.00± 1.00	8.00± 1.00	5.00± 1.00
S. typhi	10.00± 0.47	_	9.00± 1.00
*Bacillus subtilis*MTCC 441	19.00± 1.00	_	10.00± 0.3
Streptococcus pyogens	17.00± 0.47	_	10.00± 0.40

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

CONCLUSION

The rhizosperic 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 fulvissimus* DSM 40593T, *S._microflavus_*NBRC13062T, *S. setonii* NRRL ISP-5322T, *S. anulatus* 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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