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Detection, Screening and Molecular Characterization of Potential Actinobacterium from Lime-dwelling Powder for Extra Cellular Cellulase

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ABSTRACT

Actinobacteria, conventionally known as actinomycetes are the most unique microorganisms revealing a link between bacteria and fungi. They are highly adaptable to extreme environmental condition and also exhibit a high diversity in metabolic activities. Biochemical, physiological and genetic features are mainly responsible for their higher adoptability to harsh conditions and extra cellular synthesis of wider secondary metabolites in general and enzymes and antibiotics in particular. The limestone quarry and lime powder dwellings are the harsh habitats prevailing in the northern region of Karnataka. These are the typical habitats left behind after the exploration of limestone and lime powder for highly commercial industrial activities such as production of cement and petroleum refining process respectively.

In the present investigation, efforts were made to detect cellulolytic actinobacteria from lime powder dwellings. Actinobacteria confirmed by the basic colony characters, microscopic features, biochemical and physiological properties were screened for the potential cellulolytic activity. In all 54 isolates of actinobacteria were detected and screened to obtain three best cellulolytic actinobacteria, namely DSA22, DSA38 and DSA39. The maximum zone of hydrolysis on *carboxymethylcellulose* medium was an important criterion to screen the best cellulolytic isolates of actinobacteria. Further, the three best isolates of cellulolytic actinobacteria were screened for maximum production of extra cellular cellulase. The isolate DSA22 with higher enzyme activity (12 IU) was subjected to molecular characterization. Based on 16s rRNA analysis (BioEra Laboratory, Pune, Maharashtra) an isolate DSA 22 was identified as *Streptomyces enissocaesiles*.

Keywords- 16S rRNA, Cellulase, Lime dwelling, *Streptomyces*.

I. INTRODUCTION

The biosphere constitutes a huge number of microorganisms with the ability and capacity to degrade diverse biopolymers, which includes polysaccharides. Cellulose rich crop residues account for 50% of the dry weight in agriculture waste, and biodegradation of cellulose is a vital part of the carbon cycle in the biosphere (Haruta *et al.*, 2002). For these processes thermophilic and or alkalophillic or acidophilic microorganisms as sources of thermostable and wide range of pH stable enzymes are needed, because of their higher stability and activity over a wider range of temperatures and pH (Bakare *et al.*, 2005; Viikari *et al.*, 2007; Sadhu *et al.*,2013). Selected microbes are then employed for the scale up and production processes for the industries. The source needs to be unique which, cannot be utilized by majority of microbes other than the specific one. For the specific selection, primary and secondary screening is carried out which employ different techniques. However, numerous microorganisms that are able to degrade cellulose have been isolated and identified.

The possibility of obtaining high yields of commercially useful cellulase from actinomycetes has attracted the attention of researchers, and several mesophilic and thermophilic strains have been isolated for this purpose (Higerdal *et al.*, 1978; Ishaque & Kluepfel, 1980; Su & Paulavicius, 1975 and Thayer *et al.*, 1984). In recent years, reports have shown cellulase production by different actinomycetes belonging to genera viz., *Streptomyces* (Nurkanto, 2009), *Micromonospora* (Eida *et al.*, 2012), *Actinopolyspora*, *Actinoplanes*, *Microbiospor*a, *Thermomonospora*, *Rhodococcus*, *Nocardia* and *Thermoactinomyces* (Saini, 2015).

Cellulolytic microbes play an important role in recycling the cellulosic waste materials in biosphere. Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity. These cellulolytic bacteria include aerobes (Pseudomonas and Cytophaga) actinomycetes (*Streptomyces* and *Thermomonospora*), facultative anaerobes (*Bacillus* and *Cellulomonas*) and strict anaerobes (*Clostridium* and *Fibrobacter*). Among the microbes, actinobacteria (actinomycetes) are one of the largest taxonomic units within the Bacterial

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domain (Niva *et al.*, 2006) and they are the most efficient prokaryotes to be used economically and biotechnologically for their production of about half of the discovered bioactive secondary metabolites (Berdy, 2012). Actinobacteria can metabolize many different compounds including sugars, alcohols and amino acids. Additionally, many of the species of *Streptomyces* and *Rhodococcus* produce extracellular hydrolytic enzymes to obtain nutrients from cellulose, hemicellulose, proteins and fats. Furthermore, some strains are degrading compounds of macromolecules (lignin, cellulose, chitin, in part starch and aromatic hydrocarbons). Therefore, actinobacteria often occur in materials where organic matter is degraded (Schafer *et al.*, 2010), such as soils, organic compost heaps and building materials. Their metabolic diversity is due to their extremely large genome which codes for a variety of transcription factors that in turn control gene expression, allowing them to respond to specific needs.

Genus *Streptomyces* is known as the largest producer of cellulases (Jang and Chang 2005). Extensive research revealed that cellulases produced from *Streptomyces* sp. are of optimum alkaline pH and highly thermostable (Jones *et al.*, 2004). Besides *Streptomyces*, several other genera like *Micromonospor*a and *Thermobifida* are known to be produced recombinant cellulases (Zhang *et al.*, 2011). Chellapandi and Jani (2008) reported a recombinant cellulase with thermal and pH stability from *Streptomyces thermoviolaceus*. *Streptomyces lividans, Streptomyces albaduncus, Streptomyces reticuli* and *Streptomyce*s sp. Akurathi and Thoti (2018) worked on biocatalysis of agro-processing waste using marine *Streptomyces fungicidicus* strain RPBS-A4 for cellulase production.

The morphological, physiological, and biochemical characters are mainly used for classification of actinomycetes. The classical approach of identification of actinomycetes was described by two main sources namely Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974). These methods are very much useful in the identification of *Streptomycetes.*

Since, from the last few decades, sequence analysis of cloned 16S ribosomal RNA genes is considered as a powerful tool for investigating the microbial diversity of both cultivated and uncultivated microorganisms from environmental samples (Izquierdo *et al.*, 2010). The application of molecular techniques to the study of microbial diversity has changed the face of microbial ecology and opened a new era of microbial molecular ecology (Pace *et al.*, 1986, Amana *et al.*, 1990 and Liesack *et al.*, 1991). Currently, microbes can be recognized at the DNA or RNA level without the cultivation of pure cultures on selective media. An updated taxonomy of the phylum Actinobacteria that is based on 16S rRNA trees was recently reported (Ludwig *et al.*, 2012).

II. MATERIALS AND METHODS

Soil samples

Soil samples were collected from selected field stations as per the procedure prescribed by Skinner (1951). The top layer (about 4-5 cm) soil was removed and with a sterile spatula, about 250g of soil was collected from 5-15 cm deep in sterile polythene bag. Bags containing samples were labelled indicating the date of sample collection and the sample number (Kuster, 1963).

The collected soil samples were brought to the laboratory and cleaned to remove gravels, stones, debris and air dried for 3-4 days. Cleaned and air dried soil samples were refilled in fresh sterile polyethylene bags, labelled and stored at 4 ^{o}C for further studies.

The chemical properties of the soil were analyzed using standard methods, pH (Jackson, 1973); organic matter (Walkley and Black, 1934); alkalinity (Walkley and Black, 1934).

Detection of cellulolytic microorganisms

Preserved soil samples were kept at the normal laboratory conditions for 24 hrs and used for the preparation of serial dilution to isolate cellulolytic bacteria and fungi. Further the soil samples were pretreated with calcium carbonate, phenol and heat, as follows, for the better isolation of actinobacteria (Pridham *et al.*, 1956; 1957).

Nutrient Agar (Sagardoy, 1984), Sabouraud Dextrose Agar (Sabouraud, 1892) and Starch Casein Agar (Kuster and William, 1964) were prepared for the isolation of bacteria, fungi and actinobacteria respectively, as per the standard methods.

Further, in all these basal media, the sources of carbon were replaced with carboxy methyl cellulose, aiming at the selective isolation of cellulolytic bacteria, fungi and actinobacteria.

Serial dilutions of the respective soil samples were prepared as per the standard method (Cochrane *et al.*, 1961), up to 10^{-6} dilutions.

0.5ml of 10^{-5} and 10^{-6} dilutions, 10^{-4} and 10^{-3} dilutions and 10^{-3} and 10^{-2} dilutions were inoculated on Nutrient Agar, Sabouraud Dextrose Agar and Starch Casein Agar respectively for the isolation of cellulolytic bacteria, fungi and actinobacteria. All the inoculated plates were kept for incubation in the incubator at 35^{o}C for six days.

The typical colonies of bacteria, fungi and actinobacteria grown on the respective media were observed and recorded at every 24 hrs for six days. The characters of prominent colonies of bacteria, fungi and actinobacteria were described.

Screening of Cellulolytic Microorganisms

The prominent isolates of cellulolytic bacteria, fungi and actinobacteria were subjected for qualitative screening to assess the synthesis of extracellular cellulase on carboxy methyl cellulose (Hankin and Anagnostakis, 1976), medium at pH 7. Plate culture method (Mandel,

1957) was followed by a point inoculation of two days old test isolates and plates were incubated at 35 °C for 5 days. The extracellular synthesis of cellulase was determined using congo red solution (John, 1986) as an indicator using 1M sodium chloride solution (4%) as de-staining agent. The clear zone (mm) of hydrolysis around the colony was recorded with the help of zone measuring scale (Hi-Media made) for every 24 hrs up to 120 hrs of incubation to measure the degree of synthesis of cellulase.

Quantitative screening of selected cellulolytic bacteria, fungi and actinobacteria was carried out by assessing the extra cellulase activity. Extra cellulase activity in terms of endoglcanase (CMCase), exoglucanase (avicelase), cellobiase (β-glucosidase) and total cellulase (Filter paper-Fpase) as per the standard protocol (Ghose, 1987).

Assay of cellulase

The cellulase activity was determined by incubating 1ml assay mixture containing 0.5 ml of crude enzyme extract and 0.5% specific substrate in citrate buffer (0.5 ml with pH- 4.8) for 30 min at 50 °C. The reducing sugar formed after the incubation was estimated by Di nitro salicylic acid (DNS) method (Miller, 1959) with UV-vis spectrophotometric analysis at 540 nm and the enzyme activity was calculated. Carboxy methyl cellulose, Avicel, p-nitro phenyl β-glucoside and filter paper were employed as suitable substrates for the assay of endoglucanase, exoglucanase, cellobiase and F-pase respectively.

Characterization of the Cellulolytic Microorganisms

Morphological study including substrate mycelium, aerial mycelium and sporulation status of the actinobacteria (Williams and Wellington, 1980), after their growth on Starch Casein Agar was carried out as per the standard protocol prescribed in Bergey's Manual of Systematic Bacteriology (Goodfellow,1989). The colony characters including aerial mycelium, substrate mycelium, pigmentation of all potential cellulollytic actinobacteria was studied on ISP (International Streptomyces Project) media.

The typical colonies of actinobacteria were reinoculated on Starch Casein Agar in which a cover slip was placed inclined position and incubated for 3 days. After the growth of actinobacteria, the coverslip was removed and observed for the substrate mycelium and aerial mycelium (Shinobu, 1958; Kawato and Shinobu, 1959). The colour and arrangement of spore were also recorded (Flaig and Kutzner, 1960).

All the three selected cultures of actinobacteria were subjected for hydrolysis of casein, cellulose, gelatin and starch, reduction of hydrogen peroxide, nitrate and production of hydrogen sulphide, as per the standard methods prescribed in Bergey's Manual of Systematic Bacteriology (Goodfellow and O'Donnell, 1989) and described by Gottlieb (1961).

The utilization of important sugars and amino acids by all test isolates of actinobacteria and also the influence of pH, temperature and sodium chloride on their growth was observed by following the standard methods prescribed in Bergey's Manual of Systematic Bacteriology (Goodfellow and O'Donnell, 1989) as described by Gottlieb (1961).

Basic morphological features of aerial mycelium, substrate mycelium and Gram staining nature of colonies were observed under microscope as per the methods described in the Bergey's Manual of determinative Bacteriology (Goodfellow, 1989).The scanning electron microscopic analysis of the potential cellulolytic *Streptomyces* DSA 22 was carried out as per the protocol prescribed by Tresner *et al.* (1961) and Dietz and Mathews (1969). The study of sporulation pattern under electron microscope was facilitated at SAIF, IIT Madras, Tamil Nadu.

Molecular characterization

Molecular analysis of an efficient isolate of actinobacteria was carried out as per the standard protocols, described in brief as follows.

The purified fragments of DNA molecule containing 16S rRNA genes after PCR amplification were processed at BioEra Pvt. Ltd., Pune for 16S rRNA gene sequencing. The genomic DNA was isolated using Qiagen DN easy Extraction Protocol and the desired gene was amplified using specific primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACCTTGTTACGACTT) described by Lane (1991). After amplification, accuracy of the PCR product was visualized on agarose gel and purified using a gene O-spin PCR product purification kit (gene Ombio technologies, Pune; India). The purified 16S rRNA gene was directly sequenced using an ABI PRISM Big Dye Terminator V3.1 kit (Applied Biosystems, USA). The Sequencing Analysis 5.2 software was used to analyze the sequences (Chun *et al*, 2007). BLAST analysis was performed at BlastN site at NCBI to restore closest relatives of the celluloytic actinobacterium and phylogenetic tree was constructed (BioEra, Pune).

III. RESULTS AND DISCUSSION

Detection of Cellulolytic microorganisms

In the present study, different field sites selected from various habitats for the purpose of detection of cellulolytic microorganisms are presented in Figure 1. All these unique habitats were surveyed and selected at regional place called Kalldevanahalli of Surpur (Tq) and Yadgir (Dist). A heap of natural unearthed white soil (A), a deep quarry after removal of pinkish white natural soil (B), a surface wet quarry along with accumulated runoff water(C) and a heap of natural unearthed pinkish soil (D) are the characteristic geographical spots indentified for the collection of soil samples. In addition to that, regionally predominant agricultural field with black and red soils were also selected for the collection of soil samples.

Important physiochemical properties such as texture, pH, alkalinity and organic matter content of the collected soil samples were analyzed and recorded in Table 1. All collected soil samples from six different sites are dry and powdery in nature, except sediment soil sample, which was wet. pH and alkalinity of all six soil samples were highly varied. pH of the soil samples C, B and A shows the high range of alkaline pH with 10.1, 10.0 and 9.8 respectively. Samples D and E both shows a low range of alkaline pH with 8 however agricultural field red soil (F) sample shows an acidic pH of 5.5. Alkalinity condition of all soil samples are in coordination with high range of alkaline and acidic range of pH showing variation from 1026 mg/L (B) to 220 mg/L (E). Surprisingly, organic matter content of all the soil samples did not reveal much variation and are in range of 6.0% (E) to 6.8% (A).

Table 1: Phyiscochemical properties of soil samples collected for the isolation of microorganisms

Sample code	Sample site	Soil Texture	Soil pH	Soil Alkalinity (mg/L)	Soil Organic matter (%)
A	Unearthed heaps	Dry, powdery & white	9.8	980	6.8
B	Quarry	Dry, powdery & pinkish to light brown	10	1026	6.3
C	Sediment	Moist & greyish white	10.1	920	6.5
D	Unearthed heap	Dry, powdery & Pinkish	8.0	450	6.0
E	Agricultural black soil	Cottony black	8.0	460	6
F	Agricultural red soil	Reddish to brown	5.5	220	6.7

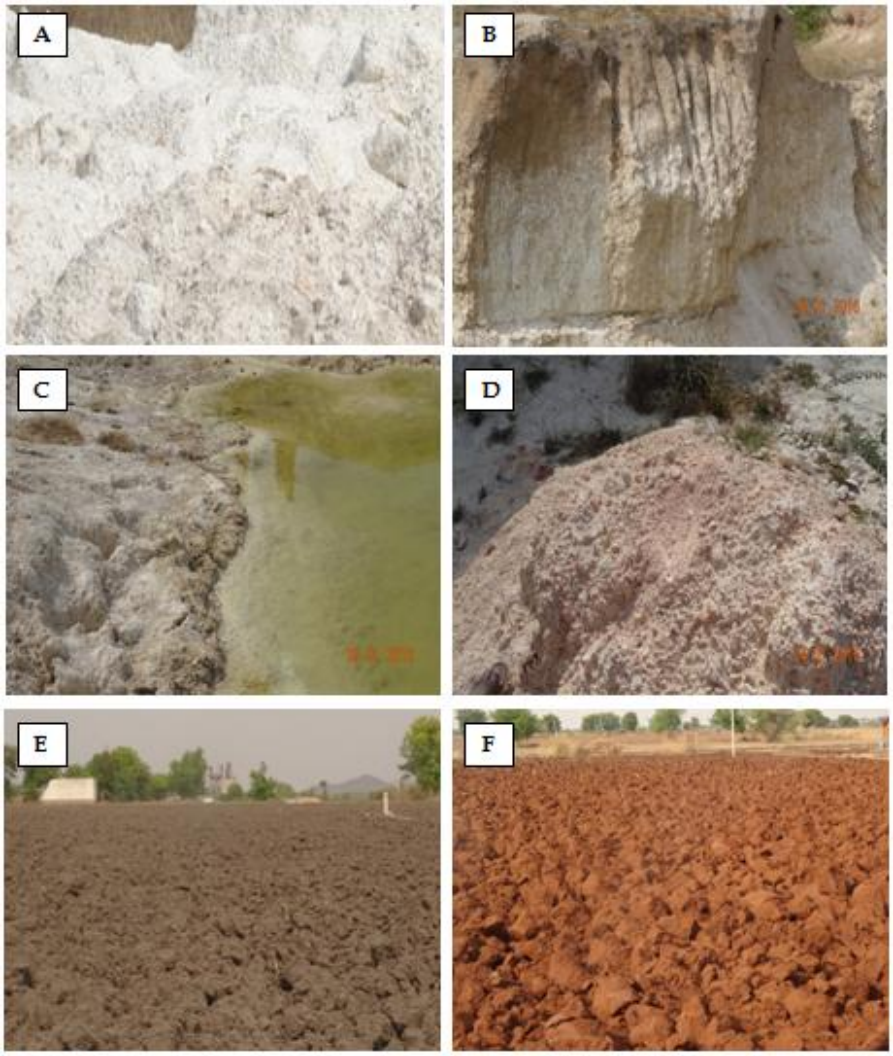

Plate 1: Selected field sites for the collection of soil samples

Screening of Cellulolytic actinobacteria

Primary screening of cellulolytic actinobacterium carried out using CMC Na as a sole carbon source in selective media for 49 isolates. Table 5 gives the details of prominent cellulolytic microorganisms isolated from different sites/soil samples. Based on the results of primary screening, among the 49 isolates, bacterial isolated did not show much zone of hydrolysis, fungal isolated showed lesser zone of hydrolysis (Table 6) compared to actinomycetes DSA 12, DSA 13, DSA 22, DSA 27, DSA 28, DSA 29, DSA 38 and DSA 39 that showed higher activity in terms of maximum zone of hydrolysis 25mm, 26-27mm, 27mm, 36-37mm, 27mm, 28mm, 30mm, 32mm and 33mm respectively (Table 2 and Figure 2) at 96h of incubation period.

Table 2: Zone of hydrolysis by selective isolates on CMC plate using congo red

Sl. No	Isolates	Clear zone on CMC
1	DSA12	25mm
2	DSA13	26-27mm
3	DSA19	27mm
4	DSA22	36-37mm
5	DSA27	27mm
6	DSA28	28mm
7	DSA29	30mm
8	DSA38	32 mm
9	DSA39	33 mm

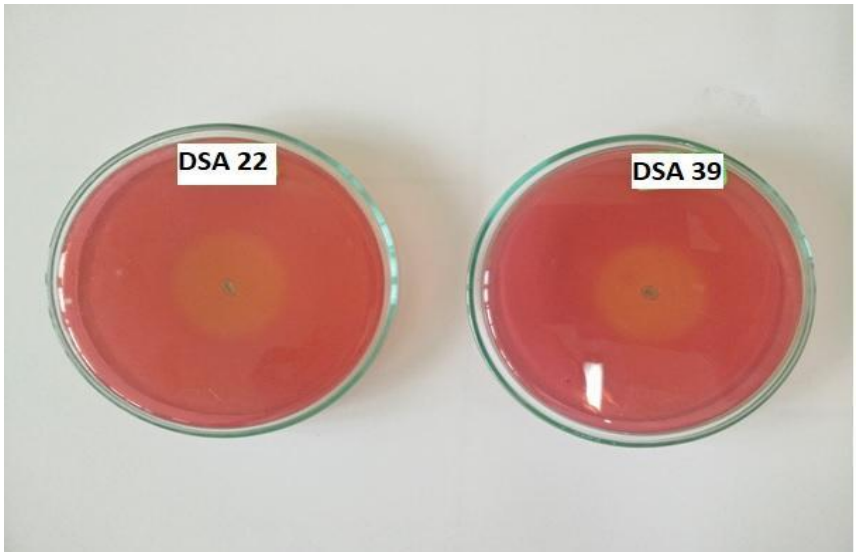

Figure 2: Colonies of DSA 22 and DSA 39 culture showing zone hydrolysis on CMC

Functional potential of seventeen bacteria, twenty fungi and nineteen actinomycetes isolates was confirmed by endoglucanase assay (Game *et al.,* 2018**).** Prakash *et al.,* (2013) isolated total 14 actinomycetes from the southeast coast of India. Recently, Sirisha *et al.,* (2013) reported bioactive compounds obtained from marine actinobacteria isolated from the sediments of the Bay of Bengal, among which 24% of the strains exhibited cellulase activity. Meena *et al.,* (2013) isolated 26 strains of actinobacteria from the marine sediments of the Andaman and Nicobar Islands and among them, two species (NIOT-VKKMA02 and NIOT-VKKMA26) showed excellent cellulase activity. Reyad (2013) also reported the actinobacterial production of cellulase and ascertained that the halo tolerant actinobacteria associated with mangroves are a good source of cellulase. Mohanta (2014) isolated nine isolates of cellulose-degrading actinomycetes from different sediment samples from the Bhitarkanika Mangrove Forest. Gobalakrishnan and Sivakumar (2017) isolated and screened cellulase enzyme producing 19 morphologically distinct marine actinobacterial strains from the sediments of the Havelock Island, The Andamans.

Morphology, Biochemical and Physiological properties of selected isolates

All the nine selected isolates are gram positive actinobacteria showing mycelium and spores under microscopic observation (Table 3). However, they differ in biochemical and physiological properties.

Isolate DSA 12 have small, circular and smooth dry colonies with grey aerial mycelium and brownish substrate mycelium with moderate mycelial branching and spiral sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and producse H2S and Utilizes, Dextrose, Lactose, Maltose, Mannitol, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 12 showed maximum zone of hydrolysis at pH: 7.5, 8.0 and 8.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 13 have small, irregular and rough dry colonies with grey aerial mycelium and colourless to yellow substrate mycelium with moderate mycelial branching and coiled sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and Utilizes Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Mannitol, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 13 showed maximum zone of hydrolysis at pH: 6.0, 6.5, 7.0 and 7.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 19 have medium, circular and smooth dry colonies with whitish to greyish aerial mycelium and greyish to light red substrate mycelium with moderate mycelial branching and spiral sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and Utilizes Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Mannitol, Raffinose, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 19 showed maximum zone of hydrolysis at pH: 6.0, 6.5, 7.0 and 7.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 22 have small, circular and smooth dry colonies with grey aerial mycelium and whitish to grey substrate mycelium with moderate mycelial branching and spiral sporulating pattern (Figure 3 and Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and Utilizes Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Mannitol, Raffinose, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 22 showed maximum zone of hydrolysis at pH: 8.0 and 8.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 27 have small, irregular and rough dry colonies with greyish to white aerial mycelium and colourless substrate mycelium with moderate mycelial branching and linear sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and produce H_2S and Utilizes Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Mannitol, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 27 showed maximum zone of hydrolysis at pH: 6.0, 6.5, 7.0 and 7.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 28 have small, irregular and smooth dry colonies with whitish to grey aerial mycelium and colourless substrate mycelium with moderate mycelial branching and spiral sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and produce H_2S and Utilizes Dextrose, Lactose, Maltose, Mannitol, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 28 showed maximum zone of hydrolysis at pH: 7.5, 8.0 and 8.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 29 have medium, irregular and smooth dry colonies with grey aerial mycelium and whitish substrate mycelium with moderate mycelial branching and spiral sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and produce H_2S and Utilizes Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Mannitol, Raffinose, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 29 showed maximum zone of hydrolysis at pH: 6.0, 6.5, 7.0 and 7.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 38 have medium, circular and smooth dry colonies with greyish to brown aerial mycelium and colourless substrate mycelium with moderate mycelial branching and linear sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and produce H_2S and Utilizes Arabinose, Dextrose, Lactose, Maltose, Mannitol, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 38 showed maximum zone of hydrolysis at pH: 7.5, 8.0 and 8.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

Isolate DSA 39 have small, circular and smooth dry colonies with grey aerial mycelium and whitish to grey substrate mycelium with moderate mycelial branching and linear sporulating pattern (Table 3). It hydrolysis Casein, Cellulose, Gelatin, Starch; Reduces H_2O_2, Nitrate and Utilizes Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Mannitol, Sucrose, Xylose, Cysteine, Glutamine, Histidine, Phenyl Alanine, Tyrosine, Tryptophan and Valine (Table 4). DSA 29 showed maximum zone of hydrolysis at pH: 6.0, 6.5, 7.0 and 7.5; Temperature (^oC): 35; NaCl concentration (%): 2.0 (Table 5).

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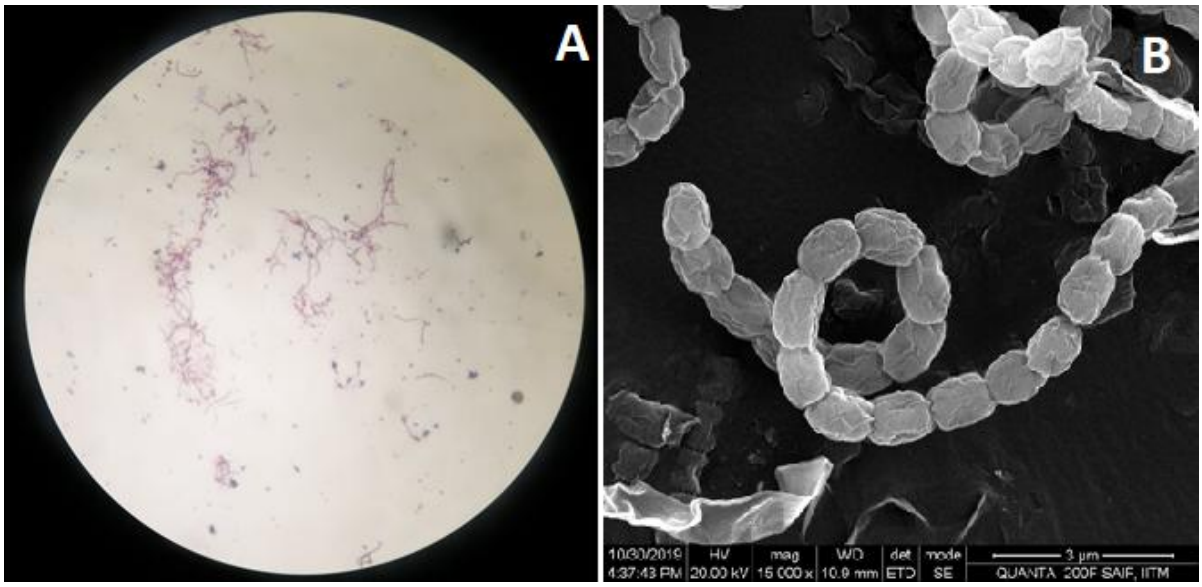

Figure 3: Microscopic image of DSA 22: A- Compound microscope image showing mycelium, B- Scanning Electron Microscopic image showing spore chain morphology

Table 3: Morphological characters of the prominent cellulolytic actinobacteria

Isolate Code	Nature of colony	Pigmentation			Microscopic characters
		Aerial mycelium	Substrate mycelium	Diffused	
DSA12	Small [A] Circular [B] Smooth dry [C]	Grey	Browish	Nil	Positive[a] Moderate[b] Spiral[c]
DSA13	Small Irregular Rough dry	Grey	Colourless to yellow	Nil	Positive[a] Moderate[b] coiledl[c]
DSA19	Medium Circular Smooth dry	Whitish to grayish	Grayish to light red	Nil	Positive[a] Moderate[b] Spiral[c]
DSA22	Small Circular Smooth dry	Grey	Whitish to gray	Nil	Positive[a] Moderate[b] Spiral[c]
DSA27	Small Irregular Rough dry	Grayish to white	Colourless	Nil	Positive[a] Moderate[b] linear[c]
DSA28	Small Irregular smooth dry	Whitish to grey	Colourless	Nil	Positive[a] Moderate[b] Spiral[c]
DSA29	Medium Irregular Smooth dry	Grey	Whitish	Nil	Positive[a] Moderate[b] Spiral[c]
DSA38	Medium Circular Smooth dry	Grayish to brown	Colourless	Nil	Positive[a] Moderate[b] linear[c]
DSA39	Small Circular Smooth dry	Grey	Whitish to gray	Nil	Positive[a] Moderate[b] Spiral[c]

A – Size of the colony; B – shape of the colony; C – surface of the colony
a – Gram staining; b - mycelial branching; c – sporulation pattern

Table 4: Biochemical properties of prominent cellulolytic actinobacteria

Properties	Isolates								
	DSA12	DSA13	DSA19	DSA22	DSA27	DSA28	DSA29	DSA38	DSA39
Hydrolysis of									
Casein	+	+	+	+	+	+	+	+	+
Cellulose	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+
Reduction of									
H_2O_2	+	+	+	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	+
Production of H_2S	+	-	-	-	+	+	+	+	-
Utilization of sugars									
Arabinose	-	+	+	+	+	-	+	-	+
Dextrose	+	+	+	+	+	+	+	+	+
Fructose	-	+	+	+	+	-	+	-	+
Galactose	-	+	+	+	+	-	+	-	+
Lactose	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+
Raffinose	-	-	+	+	-	-	+	-	-
Sucrose	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+
Cysteine	+	+	+	+	+	+	+	+	+
Glutamine	+	+	+	+	+	+	+	+	+
Histidine	+	+	+	+	+	+	+	+	+
Phenyl alanine	+	+	+	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+	+

Table 5: Physiological properties of prominent cellulolytic actinobacteria

Variables	Isolates								
	DSA12	DSA13	DSA19	DSA22	DSA27	DSA28	DSA29	DSA38	DSA39
pH									
6.0	+	++	++	+	++	+	++	+	++

6.5	+	++	++	+	++	+	++	+	+++
7.0	+	++	++	+	++	+	++	+	++
7.5	++	++	++	++	++	++	++	++	++
8.0	++	+	+	+++	+	++	+	++	+
8.5	++	+	+	+++	+	++	+	++	+
9.0	+	+	+	+	+	+	+	+	+
9.5	+	+	+	+	+	+	+	+	+
Temperature (^{0}C)									
30	+	++	+	++	+	++	+	++	+
35	+++	+++	+++	+++	+++	+++	+++	+++	++
40	++	++	++	++	++	++	++	++	++
45	+	+	+	+	+	+	+	+	++
50	-	-	-	-	-	-	-	-	-
NaCl Concentration (%)									
0.0	+	+	+	+	+	+	+	+	+
1.0	+	+	++	+	+	+	+	+	+
2.0	+++	++	++	+++	+++	++	+++	++	+++
3.0	+	+	++	+	+	+	+	+	+
4.0	-	-	-	-	-	-	-	-	-

Note: 25-30mm: +; 31-35mm: ++; 36-40mm: +++; 0-24mm: -

Identification of DSA 22

16S rRNA gene sequence (1170 nt) was determined for strain 7627. Primary sequence analysis with those of representatives of the family *Streptomycetaceae*. The highest 16S rRNA gene sequence similarity values were found with the type strains of *Streptomyces enissocaesilis* (99.83%), *Streptomyces plicatus* (99.80%) and *Streptomyces rochei* (99.79%) (Figure 3).

16S rRNA gene sequence of DSA22

CTAATACCGGATACTGATCCTCGCAGGCATCTGCGAGGTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCG
GCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
AAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGA
AGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCG
GGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCCTGGTG
TAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGC
TGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACT
AGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAACTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCC
GCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCA
ACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGT
GTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
CTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAAATCGGAGG
AAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACA
TGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGT
CTGCAACTCCGACCCCATGAAGTCGGAGTCGCTA

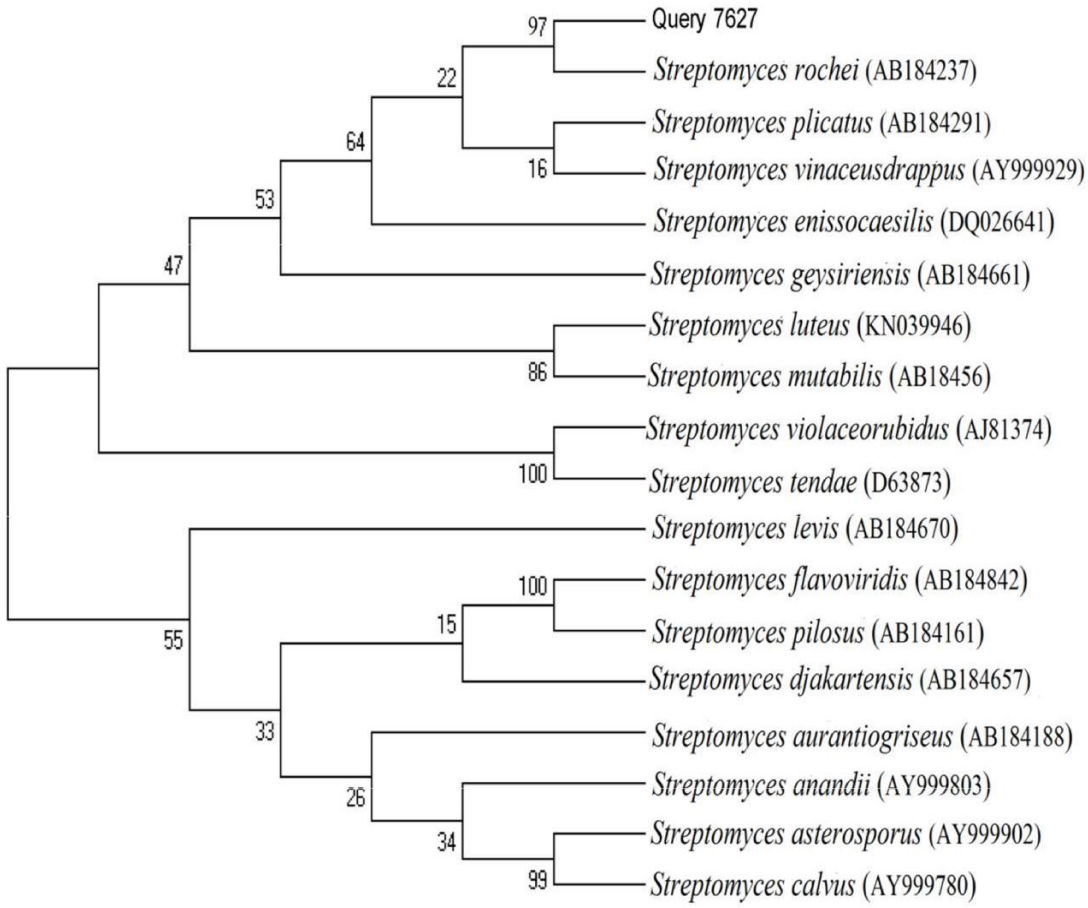

Figure 3: Phylogenetic tree of the 16S rRNA sequence of the isolated strain

Results demonstrates that strain 7627, together with the above-mentioned species, and do not form a distinct phyletic line supported by a 53% bootstrap value with the neighbour-joining method. The summary of the accession number of the first ten closest strains to SA22 is listed in table 6.

Table 6: First ten closest neighbours for DSA22

Sl. No	Name	Strain	Accession	Similarity (%)	Mismatch/Total NT
1	*Streptomyces enissocaesilis*	NRRL B-16365	DQ026641	99.82	2/1167
2	*Streptomyces plicatus*	NBRC 13071	AB184291	99.80	2/1167
3	*Streptomyces rochei*	NBRC 12908	AB184237	99.79	2/1167
4	*Streptomyces geysiriensis*	NBRC 15413	AB184661	99.74	3/1167
5	*Streptomyces luteus*	TRM 45540	KN039946	99.65	4/1167
6	*Streptomyces mutabilis*	NBRC 12800	AB184156	99.65	4/1167
7	*Streptomyces vinaceusdrappus*	NRRL 2363	AY999929	99.65	4/1162
8	*Streptomyces tuirus*	NBRC 15617	AB184690	99.22	9/1167
9	*Streptomyces djakartensis*	NBRC 15409	AB184657	99.22	9/1167
10	*Streptomyces aurantiogriseus*	NBRC 12842	AB184188	98.97	12/1166

The successful use of scanning electron microscope in studies of actinomycetes was made by Williams and Davies (1967). Since the initial work on actinomycetes by Williams and Davies (1967), a large number of strains have been examined and further information on the range of forms of the members of this group has been obtained. The fine structure of reproductive and vegetative organs of the genus *Streptomyces* has been investigated by many workers (Painter and Bradley, 1965; Ki-hyeong, 2003; Castillo *et al.*, 2006; Kaltenpoth *et al.*, 2006; Malviya *et al.*, 2010). *Streptomyces* species were compared on the basis of their cultural characteristics and spore chain morphology and spore surface. Spore surface are observed under electron microscope and are characterized as smooth, spiny, hairy and warty (Sivakumar *et al.*, 2011). Spores of *Streptomyces* were examined by transmission and scanning electron microscopy (Skujins *et al.*, 2002; Slim *et al.*, 2017). Priyanka *et al.*, (2019) observed for spore chain morphology under scanning electron microscopy (SEM).

Most potential actinomycete identified as *Streptomyces rochei*, which was confirmed based on morphological, biochemical and phylogenetic characterization. Mujoko *et al.*, (2014) isolated actinomycetes from rhizospheric soil of tomato and chili orchards in Malang and were confirmed as *Streptomycetes* by molecular characterization. Amsaveni *et al.*, (2015) collected soil samples from forest soil, river and well. Total 45 samples were collected and isolated actinomycetes. Out of 45 soil samples, 26 actinomycetes were isolated and identified by genotypic characterization like 16s rRNA gene sequencing. Singh *et al.*, (2016) isolated 15 active actinomycetes. Out of 15 isolates, 13 showing strong antimicrobial activities were selected for detailed taxonomic, physiological, and biochemical studies. Ganesan *et al.*, (2017) isolated 400 actinomycetes from different soil samples and cross streaked them against different microbial pathogens. Rajivgandhi *et al.*, (2018) carried out molecular characterization and antibacterial investigation of marine endophytic actinomycetes *Nocardiopsis* sp. Feina *et al.*, (2019) obtained 843 isolates from mangrove soil in Futian and Maoweihai of China. Among these, 539 isolates were identified as actinobacterial strains by partial 16S rRNA gene sequence comparison analysis and further assigned to 39 genera in 18 families of 8 orders.

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