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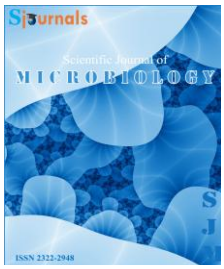

Original article

Molecular characterization of thermophilic *Streptomyces* using amplified 16s ribosomal DNA restriction analysis (ARDRA)

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ABSTRACT

The present study aims to characterize the 19 thermophilic *Streptomyces* isolates on the basis of their restriction profile obtained by Amplified Ribosomal DNA Restriction Analysis (ARDRA) method. Nineteen thermophilic *Streptomyces* isolates, characterized and identified on the basis of cultural, morphological, physiological and biochemical characteristics using PIB Win (Probabilistic identification of Bacteria) software, have been further characterized by Amplified Ribosomal DNA Restriction Analysis method. DNA of all the 19 selected isolates and two reference *Streptomyces* cultures of MTCC (*S. phaeochromogenes* (MTCC 2614) and *S. thermovulgaris* (MTCC 1822) were isolated and the 16S rRNA gene was amplified using primer F1 (5'-AGAGTTTGATCITGGCTCAG-3'; I= inosine) and primer R5 (5'-ACGGITACCTTGTTACGACTT-3'). Two restriction endonuclease enzymes *i.e. Eco R* I and *Hae* III were used for restriction digestion. The 16S rDNA were amplified by PCR using specific primers and the amplified product were cleaved separately by both endonucleases, yielding 4 to 5 bands by *Hae* III and 2 to 3 bands by *Eco R* I. Digestion with two endonucleases indicate that *Hae* III enzyme is better than *Eco R* I for differentiating *Streptomyces* isolates.

1. Introduction

The genus *Streptomyces* was proposed by Waksman and Henrici (1943). The genus currently accommodates aerobic gram-positive actinomycetes that are highly oxidative, form extensively branching substrate hyphae, aerial hyphae bearing long spore chains, contain L-DAP and glycine but no characteristic sugar in the cell wall (Lechevalier and Lechevalier, 1970) and have a DNA base composition within the range of 69 to 73 mol% G+C. They are the rich source of bioactive compounds, notably antibiotics, enzymes, enzymes inhibitors and pharmacologically active agents (Berdy, 1995). The traditional methods used for the identification of the aerobic filamentous actionmycetes are laborious, time consuming and often requires a series of specialized tests (Harvey et al., 2001; Steingrube et al., 1995b; Steingrube et al., 1997; Wilson et al., 1998). During taxonomic studies it has been noticed that even after combining chemotaxonomic and biochemical characteristics some isolates cannot be identified to a single genus and/or species level. In order to solve such problems molecular characterization becomes essential. Recently, identification of actinomycetes has been revolutionized by introduction of molecular methods involving comparative sequencing and re-association of macromolecules (Schleifer and Stackebrandt, 1983).

Among the molecular approaches PCR based methods have provided a rapid and accurate way to identify actinomycetes (Beyazova and Lechevalier, 1993; Mehling et al., 1995; Telenti et al., 1993). In particular, Amplified rDNA Restriction Analysis (AR-DRA) has proved to be very useful (Harvey et al., 2001). ARDRA has also been shown to be useful in identifying several medically important species of aerobic actinomycetes belonging to the genera *Actinomadura, Gordonia, Nocardia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Streptomyces* and *Tsuckamurella* (Steingrube et al., 1997; Wilson et al., 1998). In this study, amplified ribosomal DNA restriction analysis (ARDRA) was applied to identify 19 *Streptomyces* cultures, isolated from soil of Raipur district of Chhattisgarh. For this 16S rDNA gene was amplified and digested by two endonucleases enzyme *i.e. Eco R* I and *Hae* III. The restriction patterns of the isolated *Streptomyces* were compared with the restriction pattern of the reference *Streptomyces* for conformation. All isolates were identified initially to the species level by conventional biochemical tests.

2. Materials and methods

2.1. Isolation and biochemical characterization of isolates

Twenty four soil samples were collected from 16 blocks of Raipur district of Chhattisgarh, state (22^{o} 33' N to 21^{o} 14'N latitude and 82^{o} 6' to 81^{o} 38' E longitude) India. Total 42 thermophilic actinomycetes were isolated using serial dilution spread plate method with starch casein agar medium supplemented with cyclohexamide (25µg/ml). Twenty were selected on the basis of temperature optima for further characterization on the basis of morphological, physiological and different biochemical tests such as degradation activity of xanthine and elastin was performed by the method of William's et al. (1972). Lipolytic and lecithinase activities were performed according to the method of Nitsch and Kutzner (1969). Hydrolysis of pectin was detected by method of Hankin et al. (1971). Hydrolysis of hippurate was tested using method of Ziegler and Kutzner (1973). Nitrate reduction and hydrogen sulfide production were also performed. Effect of different concentration of NaCl (1-9%) and different pH (4-11) were recorded on the growth of different *Streptomyces* isolates. The growth of different isolates of *Streptomyces* was observed in presence of different chemical inhibitors *viz*. Potassium tellurite (0.01%, 0.001%], sodium azide (0.01%, 0.02%), phenol (0.1%) and crystal violet (0.0001%) according to the method describe in Bergey's manual of systematic bacteriology, volume IV (Locci, 1989). Growth of isolates was recorded in the presence of different carbon (Shirling and Gottlieb, 1966) and nitrogen sources. Anti-biotic sensitivity profiling (Bauer et al., 1966) and antimicrobial property of all selected isolates against 8 test organism were also recorded. All isolates were identified (morphological, physiological and biochemical) with the help of PIB Win software (Probabilistic identification of bacteria for windows) version 2.0 (Bryant, 2003).

2.2. Molecular characterization of isolates

2.2.1. DNA extraction of streptomyces isolates

For ARDRA the genomic DNA was extracted following the method of (Magarvey et al., 2004). *Streptomyces* strains were grown in 25 ml starch casein broth medium in 100 ml conical flask for 7 days at 45^{o}C and then centrifuged (Cooling centrifuge, Remi) at 10,000 rpm for 10 min. The mycelial pellet was resuspended in 500 µl of 5M NaCl and transferred to a 2-ml eppendorf tube. The cells were centrifuged (Remi) at 10,000 rpm for 5 min, and the pellet was resuspended in 1 ml of 10 mM Tris-Cl, 1mM EDTA (TE buffer) (pH 7.5) containing 20 mg of lysozyme/ml and 20 mg of RNase A/ ml and incubated at 37^{o}C for 1 h in water bath. Following incubation, 250 µl of 0.5 M EDTA, 250 µl of TE containing 5mg of proteinase K/ml, and 100 µl of 10% sodium dodecyl sulfate were added to each tube and incubated at 37^{o}C for 1 h in water bath. The tubes were mixed by inversion after the addition of 250 µl of 5 M NaCl. Immediately thereafter, 200 µl of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB plus 0.7 M NaCl) was added , and the tubes were heated at 65^{o}C in water bath for 10 min. Cellular debris was removed by centrifugation at 8,000 rpm for 5 min, and the supernatant solution was transferred to a new 2 ml micro centrifuge tube. Proteins and lipids were removed by the addition of 300 µl of phenol-chloroform, and the phases were mixed by inversion and centrifuged at 12,500 rpm for 5 min. The aqueous phase was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. After the genomic DNA was centrifuged (10,000 rpm for 2 min) the pellet was rinsed with 70% ethanol to remove traces of salt, dried and redisolved in 200 µl of TE for immediate use or storage at -20^{o}C.

2.2.2. PCR amplification

For polymerase chain reaction (PCR), extracted genomic DNA (2.5 µl) was added to 22.5 µl reaction mixture containing 0.5 µl of 2U *Taq* DNA polymerase enzyme, 1.3 µl of deoxriboynucleoside triphosphate at a concentration of 2.5 mM each, 0.5 µl of each primers (Eurofins genomics india Pvt. Ltd, Banglore, India), 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of 10X PCR buffer (pH 8.3) and 15.7 µl of Mili Q water. The primer sequence were F1 (5'-AGAGTTTGATCITGGCTCAG-3'; I= ino-sine) and primer R5 (5'-ACGGITACCTT-GTTACGACTT-3'). The primers were used to amplify16S rDNA sequences. The PCR programme used was an initial denaturation (96^{o}C for 2 min), followed by 30 cycles of denaturaion (96^{o}C for 45 s), annealing (56^{o}C for 30 s), and extension (72^{o}C for 2 min), and a final extension (72^{o}C for 5 min). The PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide (0.5ug**/**ml), to ensure that a fragment of the correct size had been amplified (Cook and Meyers, 2003).

2.2.3. Restriction endonuclease digestion

PCR amplified product was separately digested with two restriction endonuclease enzymes *i.e. Eco R* I and *Hae* III. Each 10 µl reaction mixture contained 5 µl PCR amplified product, 3.5 µl Mili Q water, 0.5 µl restriction endo-nuclease enzyme, 1 µl restriction buffer (1X) and incubated at 37^{o}C for 2 h in water bath. The digested product was electrophoresed on 2% agarose gel containing ethidium bromide (0.5µg/ml) and photographed using digital camera (Nikon, Coolpix 995, Japan). The restriction fragments patterns were compared manually.

2.2.4. Statistical analysis

ARDRA bands were scored as either present (1) or absent (0). All binary data were entered and genetic distances were calculated through Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), version 2.02 using Euclidean distance and then assembled a dendogram using “Unweighted Paired Group Method using Arithmetic average criterion” (UPGMA).

3. Results and discussion

Twenty cultures identified through PIB Win software are TS1 *S. violaceus,* TS3 *S. exfoliates,* TS5 *S. diastaticus,* TS9 *S. chromofuscus,* TS10 *S. microflavus,* TS15 *S. microflavus,* TS18 *S. phaeochromogenes,* TS19 *S. microflavus,* TS21 *S. violaceus,* TS22 *S. cyaneus,* TS29 *S. cyaneus,* TS30 *S. cyaneus,* TS32 *S. chromofuscus,* TS34 *S. halstedii,* TS35 *S. diastaticus,* TS36 *S. microflavus,* TS37 *S. cyaneus,* TS39 *S. chromofuscus,* TS40 *S. cyaneus,* TS42 *S. olivaceoviridis.* (Thakur and Rai, 2011).

In order to characterize and differentiate isolates by molecular method, all 19 isolates with 2 reference *Streptomyces* cultures *i.e S. phaeochromogenes* (MTCC 2614) and *S. thermovulgaris* (MTCC 1822) were examined

by Amplified Ribosomal DNA restriction Analysis. (Deng et al., 2008) also reported that Amplified ribosomal DNA restriction analysis (ARDRA) is a commonly used tool to study microbial diversity that relies on DNA polymorphism. The 16S rDNA were amplified by PCR using specific primers and the amplified product were cleaved separately by both endonucleases, yielding 4 to 5 bands by *Hae* III (Fig.1) and 2 to 3 bands by *Eco R* I (Fig.3).

ARDRA profile generated by *Eco R* I restriction enzyme revealed that out of 21 isolates 17 isolates had 100% similarity to each other and form one clade. Four isolates *i.e. S. violaceus* TS1 (Lane 13), *S. chromofuscus* TS39 (Lane 15), *S. chromofuscus* TS32 (Lane 23) and *S. violaceus* TS21 (Lane 17) had 100 % similar to each other and form second clade. Likewise clade 1 shows 49% similarity to clade 2. Thus dendogram generated on the basis of above restriction pattern showed 3 different clades (Fig.3 and 4).

ARDRA profile generated by *Hae* III restriction enzyme revealed different banding patterns. *S. violaceus* TS1 (Lane 13) and *S. violaceus* TS21 (Lane17) had 100% similarity and forms I clade. *S. diastaticus* TS5 (Lanes 3) and *S. diastaticus* TS35 (Lane 4) had 100% similarity and forms II clade. *S. phaeochromo-genes* TS18 (Lane 14) and Lane 16 TS100 *S. phaeochromogenes* (MTCC 2614) had 100% similarity and forms III clade. *S. cyaneus* TS22 (Lane 5); *S. cyaneus* TS30 (Lane 6); and *S. cyaneus* TS29 (Lane 7) had 100% similar banding were found to constitute the IV clade. *S. microflavus* TS15 (Lane 20), *S. microflavus* TS10 (Lane 21), *S. microflavus* TS19 (Lane 22), and *S. microflavus* TS36 (Lane 23) had 100% similar band pattern comprises V clade. *S. chromofuscus* TS9 (Lane 12) had approx 60% similarity to clade I. *S. exfoliatus* TS3 (Lane 2) had approx 50% similarity to II clade. *S. chromofuscus* TS39 (Lane 15) had approx 40% similarity to clade III and IV. *S. olivaceoviridis* TS42 (Lane 24), *S. halstedii* TS34 (Lane 19), *S. thermovulgaris* TS93 (MTCC 1822) present in Lane 25, *S. chromofuscus* TS32 (Lane 8) and *S. cyaneus* TS37 (Lane 37) had 30 to 60 % similarity to V clade (Fig 1 and 2).

Table 1

ARDRA pattern with restriction enzymes *Eco R* I and *Hae* III. Sizes were expressed in base pairs (bp).

S.N.	Accession no.	*Streptomyces sps.*	*Eco R*I digested 16s rDNA fragments (bp)	*Hae* III digested 16s rDNA fragments (bp)
1.	TS1	*S. violaceus*	600,800,1500	210,410,600,1000,1100
2.	TS3	*S. exfoliates*	600,800	210,290,900
3.	TS5	*S. diastaticus*	600,800	190,210,290
4.	TS9	*S. chromofuscus*	600,800	210,410,600
5.	TS10	*S. microflavus*	600,800	120,190,220,300
6.	TS15	*S. microflavus*	600,800	120,190,220,300
7.	TS18	*S. phaeochromogenes*	600,800	210,300
8.	TS19	*S. microflavus*	600,800	120,190,220,300
9.	TS21	*S. violaceus*	600,800,1500	210,410,600,1000,1100
10.	TS22	*S. cyaneus*	600,800	210,290,300
11.	TS29	*S. cyaneus*	600,800	210,290,300
12.	TS30	*S. cyaneus*	600,800	210,290,300
13.	TS32	*S. chromofuscus*	600,800,1500	200,300,400,500
14.	TS34	*S. halstedii*	600,800	120,220,250,300,500,600
15.	TS35	*S. diastaticus*	600,800	190,210,290
16.	TS36	*S. microflavus*	600,800	120,190,220,300
17.	TS37	*S. cyaneus*	600,800	200,250,300
18.	TS39	*S. chromofuscus*	600,800,1500	210,300,500,600
19.	TS42	*S. olivaceoviridis*	600,800	120,190,250,300
20.	TS93 (MTCC 1822)	*S. thermovulgaris*	600,800	120,240,250,300
21.	TS100 (MTCC 2614)	*S. phaeochromogenes*	600,800	210,300

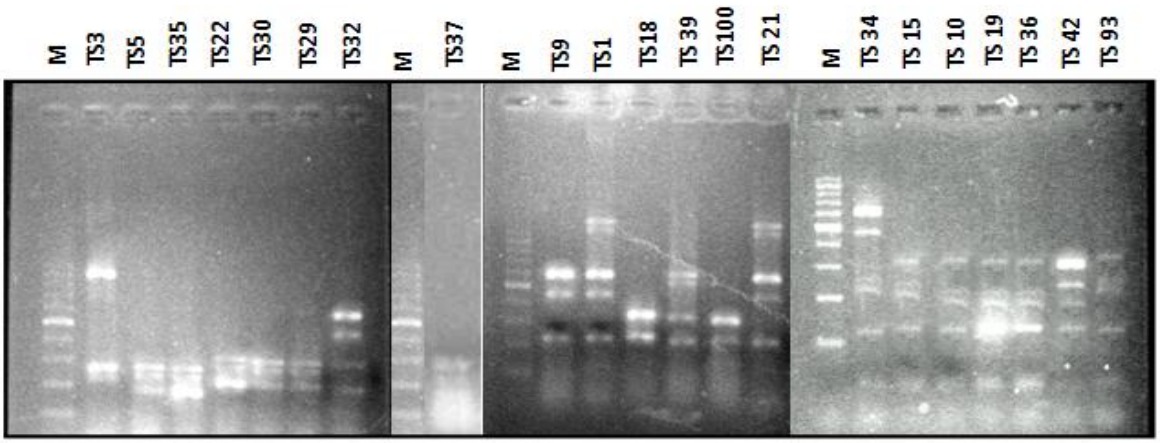

Fig. 1. 16S rDNA amplification (ARDRA) of *Streptomyces sps.* using *Hae* III restriction enzyme.

Whereas, M: DNA Marker :: Lane 1: M; 2: *S. exfoliatus* TS3; 3: *S. diastaticus* TS5; 4: *S. diastaticus* TS35: 5: *S. cyaneus* TS22; 6: *S. cyaneus* TS30; 7: *S. cyaneus* TS29; 8: *S. chromofuscus* TS32; 9: M; 10: *S. cyaneus* TS37; 11: M; 12: *S. chromofuscus* TS9; 13: *S. violaceus* TS1; 14: *S. phaeochromogenes* TS18; 15: *S. chromofuscus* TS39; 16: *S. phaeochromogenes* TS100; 17: *S. violaceus* TS21; 18: M; 19: *S. halstedii* TS34; 20: *S. microflavus* TS15; 21: *S. microflavus* TS10; 22: *S. microflavus* TS19; 23: *S. microflavus* TS36; 24: *S. olivaceoviridis* TS42; 25: *S. thermovulgaris* TS93.

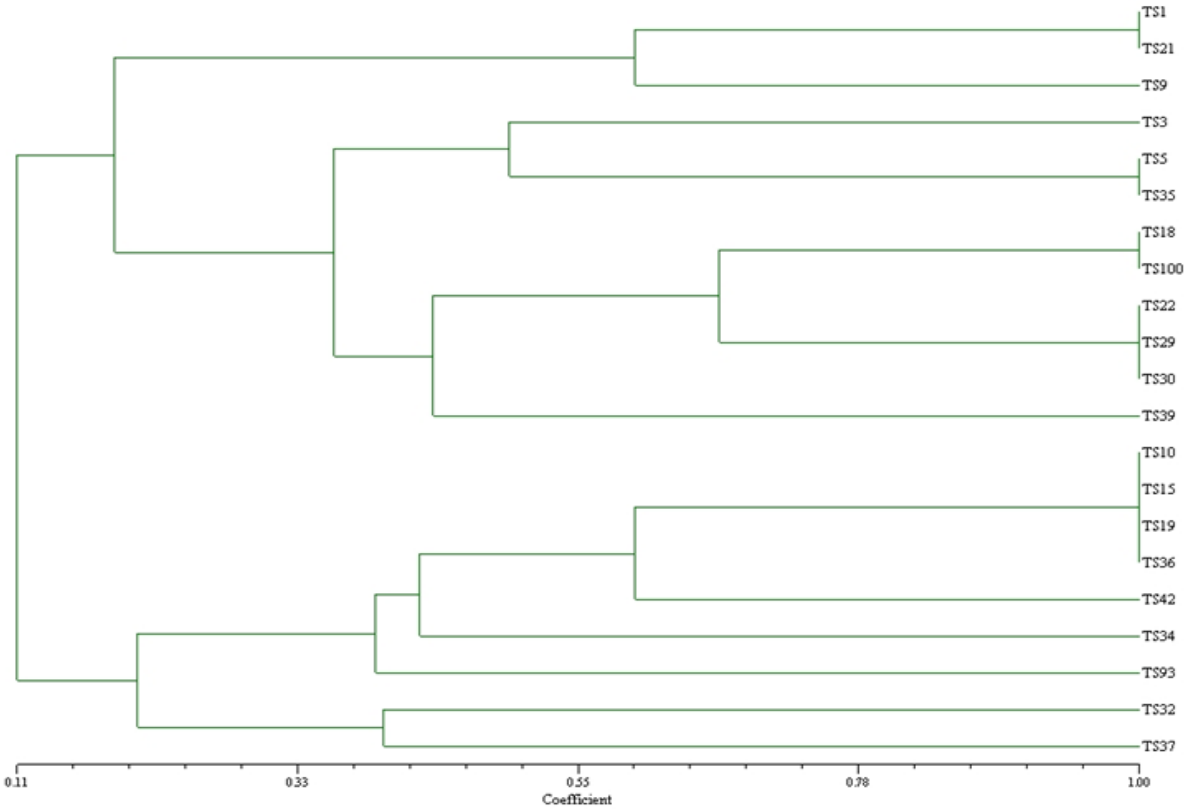

Fig. 2. Dendogram showing relatedness among *Streptomyces* based on restriction digestion of DNA using *Hae* III. Whereas, TS1: *S. violaceus*; *S. violaceus* TS21; *S. chromofuscus* TS9; *S. exfoliatus* TS3; *S. diastaticus* TS5; *S. diastaticus* TS35; *S. phaeochromogenes* TS18; *S. phaeochromogenes* TS100; *S. cyaneus* TS22; *S. cyaneus* TS29; *S. cyaneus* TS30; *S. chromofuscus* TS39; *S. microflavus* TS10; *S. microflavus* TS15; *S. microflavus* TS19; *S. microflavus* TS36; *S. olivaceoviridis* TS42; *S. halstedii* TS34; *S. thermovulgaris* TS93; *S. chromofuscus* TS32; *S. cyaneus* TS37.

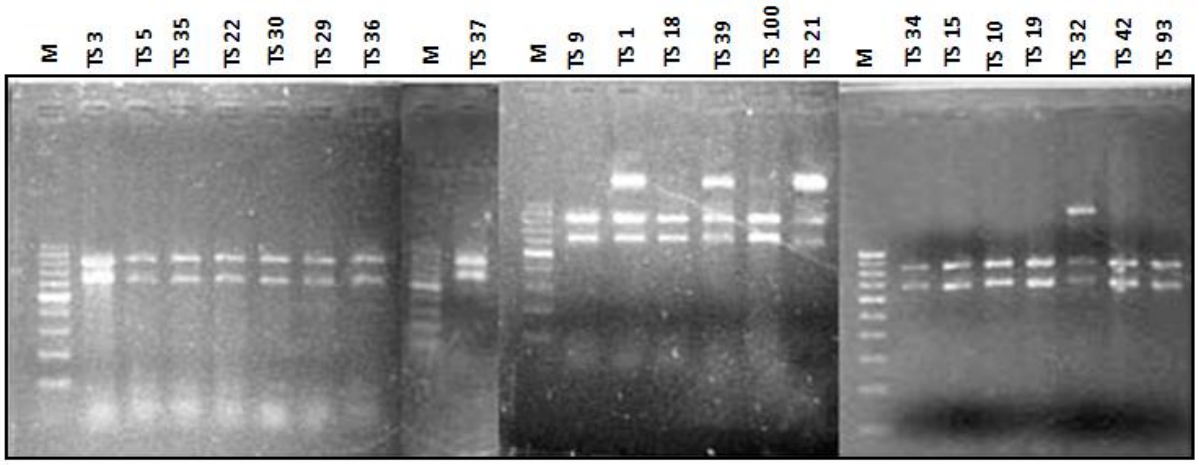

Fig. 3. 16S rDNA amplification (ARDRA) of *Streptomyces sps.* using *Eco R* I restriction endonuclease enzyme.

Whereas, M: DNA Marker: Lane 1: M; 2: *S. exfoliatus* TS3; 3: *S. diastaticus* TS5; 4: *S. diastaticus* TS35: 5: *S. cyaneus* TS22; 6: *S. cyaneus* TS30; 7: *S. cyaneus* TS29; 8: *S. microflavus* TS36; 9: M; 10: *S. cyaneus* TS37; 11: M; 12: *S. chromofuscus* TS9; 13: *S. violaceus* TS1; 14: *S. phaeochromogenes* TS18; 15: *S. chromofuscus* TS39; 16: *S. phaeochromogenes* TS100; 17: *S. violaceus* TS21; 18: M; 19: *S. halstedii* TS34; 20: *S. microflavus* TS15; 21: *S. microflavus* TS10; 22: *S. microflavus* TS19; 23: *S. chromofuscus* TS32; 24: *S. olivaceoviridis* TS42; 25: *S. thermovulgaris* TS93.

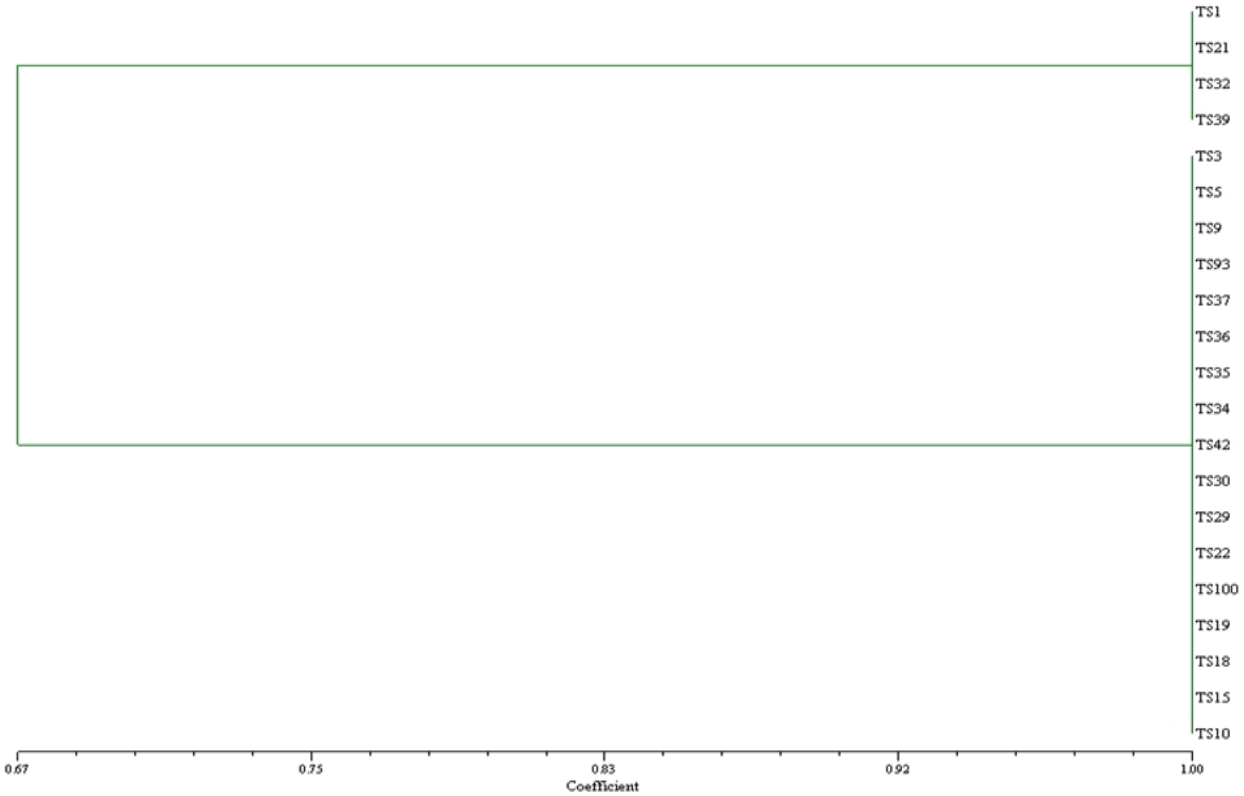

Fig. 4. Dendogram showing relatedness among *Streptomyces* based on restriction digestion of DNA using *Eco R* I.

Whereas, *S. violaceus* TS1; *S. violaceus* TS21; *S. chromofuscus* TS32; *S. chromofuscus* TS39; *S. exfoliatus* TS3; *S. diastaticus* TS5; *S. chromofuscus* TS9; *S. thermovulgaris* TS93; *S. cyaneus* TS37; *S. microflavus* TS36; *S. diastaticus* TS35; *S. halstedii* TS34; *S. olivaceoviridis* TS42; *S. cyaneus* TS30; *S. cyaneus* TS29; *S. cyaneus* TS22; *S. phaeochromogenes* TS100; *S. microflavus* TS19; *S. phaeochromogenes* TS18; *S. microflavus* TS15; *S. microflavus* TS10.

4. Conclusion

The results obtained with two endonucleases revealed that *Hae* III enzyme is better than *E.co R* I for differentiating isolates and closely justifies the result obtained by biochemical identification using PIB Win software. It appears that *Hae* III enzyme is useful for differentiating isolate in the present study.

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