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16S rRNA gene sequencing and PCR amplification of gelatinase-producing *Streptomyces* strains of fruit orchard

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Abstract: *Gelatinase as a tool in biotechnological applications has attracted the attention because of the prevalent use in detergents, pharmaceutical and leather industries. The present investigation to search gelatinase activities rested with eight strains of the group SK703, which were isolated in our laboratory from fruit orchard of Muzaffarpur and neighbouring district. The gelatinase activities of 59.6 U/ml on 4th day of incubation at optimum pH of 6.5 and optimum temperature at 35^{0} C was successfully recorded in the group of strains SK703. Thus, microscopical, cultural, biochemical and physiological measures were considered as per Bergey's manual to recognise the strains to be Streptomyces species. Further similarity in 16S rRNA gene sequencing data established its relationship with ten stains which helped to create the phylogenetic tree. The closest member was found to be that of Streptomyces flavoviridis strain ZG084 with 99 % similarity in the 1389 base sequence of 16S rRNA gene, however, the difference of 1% in the sequence established the strain SK703 as Streptomyces flavoviridis strain SK61^{B}after getting NCBI accession no. as KF815976.*

Keywords*: Streptomyces; gelatinase; 16S rRNA gene sequencing; NCBI accession no. KF815976.*

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I. Introduction

Actinomycetes that include *Streptomyces* as one of the prominent members, are the organisms that share characters common to both bacteria on one hand and fungi on the other. Besides, they possess distinctive features which delimit the group into a distinct category. Thus, taxonomically actinomycetes are placed with bacteria under class Schizomycetes and order Actinomycetales. They are unicellular with a mycelium which is coenocytic and more slender. Their cell wall is without chitin and cellulose as against that of fungi [1]. . On culture media, actinomycetes colonies grow slowly, show powdery consistency and stick firmly to agar surface as against slimy distinct colonies of true bacteria which grow quickly,. They produce hyphae and conidia sporangia like fungi. Certain actinomycetes whose hyphae undergo segmentation resemble bacteria, both morphologically and physiologically.[2]

Actinomycetes are numerous and widely distributed in soil and are next to bacteria in abundance. The present study remained confined on the actinomycetes strains of the soil of fruit orchard, viz., litchi, banana and guava. They are widely distributed in the soil, compost etc. Plate count estimates give values ranging from 10^4 to 10^8 per gram of soil. They are sensitive to acidity / low pH (optimum pH range 6.5 to 8.0). The population of actinomycetes increases with depth of soil even up to horizon 'C' of a soil profiler They are heterotrophic, aerobic and mesophilic, growing at 25-30^{0} C organisms (*Sreptomyces)* but some species are commonly present in compost and manures are thermophilic growing at 55-65° c temperature (*Thermoatinomycetes*).

Streptomyces species are Gram positive filamentous bacteria having a high G + C content (55-75%). They occupy a prominent place among secondary metabolite producing microbes such as bacteria, fungi and actinomycetes [3]. They are ubiquitous in habitat. However the diversity and distribution of the group actinomycetes to which the investigating strains belong, that produce secondary bioactive metabolites can be determined by different physical, chemical and geographical factors [4, 5]. Among many species, *Streptomyces* is the largest known genus as the producer of many secondary metabolites having different biological activities such as actinobacterial, antifungal, antiparasitic, anticancer and immunosuppressive actions.[6,7]. More recently, they have been reported to be a promising source in the soil of fruit orchard of Muzaffarpur for producing wide range of important enzymes, viz., phytase [8], cellulase [9] and caseinase [10] from our laboratory. Most of the studies on actinobacteria have focused on antibiotic production while limited no. of reports is on their enzymatic activities [11, 12]. The present study was designed to isolate gelatinase producing actinomycetes from the fruit orchard and their morphological, biochemical and molecular characterization along with the optimization of culture conditions required for enzyme production.

II. Materials and methods

Collection and processing of Soil Samples

The soil samples were collected from rhizosphere of different locations of fruit orchard of Muzaffarpur (26^0 7'N &85^0 25'E) and neighbouring district in Bihar, India. The soil samples were taken from 5-15 cm below the surface using sterilized spatula and zip lock bags. To minimize contamination the samples were heated at 70^0 C for 15 minutes followed by air dried and then mixed thoroughly. Thus obtained soil was used as sample.

Isolation of Actinomycetes from soil samples

The collected soil sample was used for the isolation of actinomycetes. 1 gram of the soil was transferred to 10 ml distilled water in a flask. It was then filtered through a two layered muslin cloth. There after the sample was diluted to 10^{-3}, 10^{-4} and 10^{-5}. 0.2 ml of each dilution was placed on a starch agar medium (Starch 9.0 g, L-Asparagine 9.0 g , Ammonium sulphate 2.0 g, Tris 2.0 g, Sodium chloride 1.0 g, Dipotassium sulphate 0.5 g, Magnesium sulphate 0.2 g, Calcium chloride 0.1g, Trace solution 1ml, Potassium Dihydrogen Phosphate 0.5 g, agar 15 g, dissolved in one litre distilled water; pH 7.0) plates, supplemented with antifungal nystatin (50µg ml^{-1}) and incubated at 35 ± 2^0C for seven days. Plates with around 200 colonies were selected. Streaking of single colony was carried out to purify the selected colonies on the same medium.

Screening of gelatinase activity

Primary screening:

The selected actinomycetes SK701 and SK703 were initially screened for their proteolytic activity on Difco nutrient agar with gelatin as protein substrate, containing (g/l) : Peptone- 5, NaCl-5, Beef extract-3 and Agar agar-20 at pH 6.0. Sterilized aqueous solution of gelatin (8%w/v) was added to obtain 0.4% gelatin concentration in the medium. The medium was streaked in the middle of the plate with the isolated actinomycetes and incubated at 35 ± 2^0 C for 7 days. The plates were then flooded with 1.5 % $HgCl_2$ in 20% Conc. HCl. The plate showing halo transparent zone against an opaque whitish non hydrolysed medium indicated the protease producing strains. Relative enzyme activity of protease positive strain was calculated using the formula:

REA=Diameter of zone of enzyme activity in mm/ Diameter of the colony in mm

On the basis of REA test, actinomycetes have been categorised as excellent (REA≥8.5), good (REA≥5 to ≤8.4), fair (REA≥2 to≤5) and poor (≤2) protease activity [13].

Secondary screening

The test strains SK701 and SK703 were grown in nutrient broth (peptone-5; NaCl -5; Beef extract-3 g/l) in presence of 0.4% gelatin for 7 days at 35 ± 2^0C in incubator shaker at 100 rpm. The cell free culture filtrate was obtained by centrifugation at 5000 rpm for 10 minutes at 4^0C and the clear supernatant was taken as crude enzyme. An amount of 500 µL of it was mixed with 500 µL of gelatin solution (0.2%) in phosphate buffer at pH 7.The reaction mixture was incubated at 35 ± 2^0C for 1 hour. After that 150 µL of TCA solution (0.44M TCA, 0.88M CH_3COONa, 1.32M CH_3COOH) was added to terminate the reaction. Acetonitrile (3ml) was added in order to precipitate the unhydrolyzed gelatin in the solution and the absorbance was measured at 700nm. One unit of protease activity was defined as the amount of enzyme that caused 1% reduction in the optical density of the gelatin solution (0.2%) per hour.

Characterization of the isolates

The selected isolates SK701 & SK703 were examined for morphological and cultural studies as per the directions of the International *Streptomyces* Project [14] on different agar medium. Gram staining smears were prepared and examined under the microscope to ascertain the nature of isolates. Various biochemical and physiological tests were performed for the identification of the isolates [15].

Optimization of temperature and pH

The fermentation was carried out at various temperature such as 25^0C, 30^0C, 35^0C, 40^0C , 45^0C ,50^0C and 55^0C to study their effect on enzyme activity. Different pH concentrations viz. 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7 of the gelatine broth were prepared using buffer solution. All the experiments were carried out in triplicate and average values were calculated

Molecular characterization

Genomic DNA Extraction

Genomic DNA of SK703 was isolated using standard protocol [16] and purity of DNA was checked A280/A260. Further bands were visualized by running on (0.8% w/v) agarose gel electrophoresis.

PCR amplification & sequencing of16S rRNA gene

16S rRNA gene of isolated genomic DNA of SK703 was amplified in a PCR thermal cycler (Genetix) by universal primers, 9F (5'GAGTTTGATCCTGGCTCAG3') & 1541R (5'AAGGAGGTGATCCAACC3'). The reaction mixture contained 2µg of dNTPs mixture (1.25 mM each), 1µg of each of the primers, 2.5µg of DNA, 1µl of Taq polymerase and sterile deionised water to make final volume 100 µl . PCR consists of an initial denaturation at 94^0C for 1 min, annealing at 63^0C for 1 min and 72^0 for 1 min & final 5 min extension at 72^0C [17]. The PCR amplified product were stained with ethydium bromide and run on agarose (1.2%) gel electrophoresis and examined under gel documentation system. The complete 16S rRNA gene of SK703 was sequenced by using PCR products at Samved Biotech, Ahmedabad.

Construction of phylogenetic tree

The 16S rRNA gene sequence of SK703 were compared with other bacterial sequences by using NCBI BLAST programme. Multiple sequence alignment & phylogenetic tree was constructed using software programme MEGA 4.0 [18]. The 16S rRNA gene sequence of strain SK703 was submitted to GenBank (Nucleotide database of NCBI).

III. Results

Isolation & Identification of protease producing microbes

Bacterial strains, isolated from the fruit orchard were grown on starch agar medium, supplemented with nystatin and were screened for colony colour, spore morphology, mycelia colour, etc. (Table 1). Two bacterial strains, SK701 and SK703 of the orchard had shown gelatinase activities as they developed halo regions on Difco nutrient agar supplemented with gelatin. But the isolate SK703 was more prominent having approximately 22 mm clear halo zones around colonies or streaked mycelia as compared to 9 mm halo zone of SK701 (Table 1). Purified strains were examined under microscope for gram staining and both the isolate were found to be gram positive. Further both isolates reacted positively in different biochemical and physiological tests (Table 2).

Table 1: Source, colony colour spore, morphology and clear zone formation on gelatine supplemented medium .

Bacterial isolate	Source of isolation	Colony colour	Spore morphology	Presence of halo zone
SK 701	Litchi, Muz.	White –grey	Smooth oval	+ (9 mm)
SK 703	Banana, Hajipur	White	Oval round	++ (22 mm)

Gelatinase estimation

The quantitative screening of gelatinase production was found to be maximum after 96 hrs of incubation. Both strains produced maximum amount of gelatinase in between 96-120 hrs. After 144 hrs a sharp drop by 50% in the protease activity was noticed. The gelatin-degradation activities by releasing gelatinase was found maximum (59.6 U/ml) on 4^{th} day (Table 3). The effective temperature for maximum protease activities was noticed in between $35\text{-}40^0$ C. At high temperature ($>45^0$ C), the gelatinase activities dropped to the level of 20% (Table 4). The optimum pH for gelatinase production was found at 6.5 in SK703 whereas it is 5.5 in case of SK701 (Table 4).

Table 2: Physiological and Biochemical characteristics of Isolated actinomycetes strains SK701 and SK703

Character	Result		Character	Result	
	SK701	SK703		SK701	SK703
Gram staining	**+ve**	**+ve**	Tolerance to NaCl %	2-5	2-4
Enzyme activities			pH range	5-7	4-7
Indole production	**-ve**	**-ve**	Temp(±2°C)	35	35
Methyl red	-ve	-ve	Carbon & nitrogen source		
Voges Proskauer	**-ve**	**-ve**	Glucose(1%)	**+ve**	**+ve**
Citrate utilization	**+ve**	**+ve**	Sucrose(1%)	**+ve**	**+ve**
Casein hydrolysis	**+ve**	**+ve**	Fructose(1%)	**+ve**	**+ve**
Urea hydrolysis	**+ve**	**+ve**	Lactose(1%)	**-ve**	**-ve**
Starch	**-ve**	**-ve**	Glucose	**+ve**	**+ve**

hydrolysis			+Sucrose(0.5%)		
Gelatin hydrolysis	**+ve**	**+ve**	Glycine	**+ve**	**+ve**
H_2S production	-ve	-ve	Malt extract	**+ve**	**+ve**

Table 3: Quantitative estimation of gelatinase enzyme in shake culture of actinomycetes strains SK701 and SK 703 on different hours of incubation

No. of hours	Gelatinase activities	
	SK701	SK703
24	0	10±0.5
48	6±0.2	14±o.5
72	13±0.5	29±1.0
96	20±1.5	59.5±2.5
120	18±1.5	52±2.0
144	9±0.5	31±1.0
168	4±0.5	15±0.5

Table 4: Effect of temperature on proteolytic activity in strains SK701 and SK 703

S.No.	Temperature (^{0} C)	Protease activity	
		SK701	SK703
1	25	11±1.5	20±1.5
2	30	15±.5	**42**±1.5
3	35	19±1.5	55±1.5
4	40	20±0.5	59±o.5
5	45	14±2.5	38±1.0
6	50	8±0.2	22±0.5
7	55	0	15±0

Table 5: Effect of pH on gelatinase activity in strains SK701 and SK 703

S N	pH	Protease activity	
		SK701	SK703
1	3	0	0
2	3.5	0	0
3	4	3±0.5	5±0.5
4	4.5	11±0.5	8±0.5
5	5	18±1.5	12±1.0
6	5.5	20±1.0	30±1.15
7	6	16±	50±2.0
8	6.5	9±1.0	59.5±2.0
9	7	0	55±

DNA Amplification

The genomic DNA of the proteolytic isolate SK703 was isolated by standard protocol [16] and the samples were run on 0.8% agarose gel electrophoresis. Thereafter PCR amplification using universal primers resulted into a single discrete amplicon band of 1.5 kb of 16S rRNA gene (Fig 1).

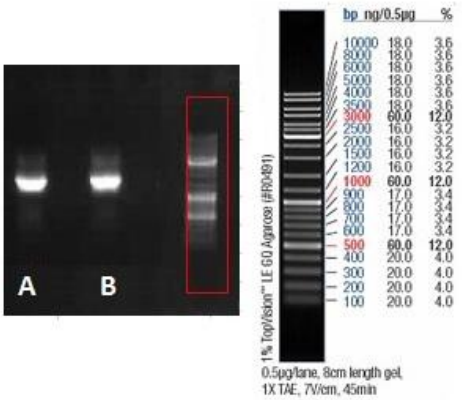

Figure 1. PCR amplified product of 16S rRNA gene of 1.5 kb of strain SK703 *Streptomyces flavoviridis* SK 61^B on agarose gel electrophoresis (A) with Control (B).

Molecular characterization of the selected isolate

The NCBI BLAST homology analysis [19] of 16S rRNA gene of SK703 showed maximum similarity (≥99%) with *Streptomyces flavoviridis* strain ZG084. Based on the result, phylogenetic tree [20] was constructed using neighour joining method (Fig.2). The sequence (Table 6) was published after trimming and assembling to NCBI (http//:www.ncbi.nlm.nih.gov). It was revealed that the strain SK703 showed1375 base similarity out of 1389 (99%) with *Streptomyces flavoviridis* strain ZG084 (NCBI Accession no. GQ985452.1). Thus, the difference of 14 nucleotides in the 16S rRNA gene qualified the strain SK 703 to earn a separate organism *Streptomyces flavoviridis* strain SK61^{B} with NCBI Accession no. KF815976.

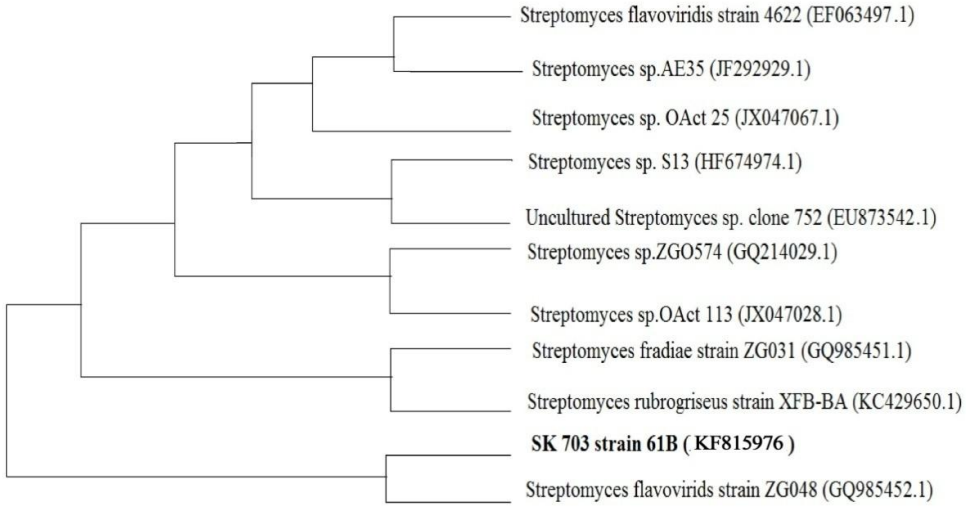

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence showing relationship between Isolate SK703 (*Streptomyces* sp. Strain SK61^{B}) and ten related members.

Table 6. The 16S rRNA gene sequencing of bacterial isolate SK703 Strain SK61^{B} (1398 bp).

Agagtttgatcctggctcaggacgaacgctggcagcgtgcttaacacatgcaagtcggac gatgaaccacttcggtggggattagtggcgaacgggtgagtaacacgtgggcaatctgcc ctgcactctgggacaagccctggaaacggggtctaataccggatactgacccccgcaggc atctgcggggttcgaaagctccggcggtgtaggatgagcccgcggcctatcagcttgttg gtgaggtaacggctcaccaaggcgacgacgggtagccggcctgagagggcgaccggccac actgggactgagacacggcccagactcctacgggaggcagcagtggggaatattgcacaa tgggcgaaagcctgatgcagcgacgccgcgtgagggatgacggccttcgggttgtaaacc tctttcagcagggaagaagcgaaagtgacggtacctgcagaagaagcgccggctaactac gtgccagcagccgcggtaatacgtagggcgcgagcgttgtccggaattattgggcgtaaa gagctcgtaggcggcttgtcacgtcggttgtgaaagcccggggcttaaccccgggtctgc agtcgatacgggcaggctagagttcggtaggggagatcggaattcctggtgtagcggtga aatgcgcagatatcaggaggaacaccggtggcgaaggcggatctctgggccgatactgac gctgaggagcgaaagcgtggggagcgaacaggattagataccctggtagtccacgccgta aacggtgggcactaggtgtgggcgacattccacgtcgtccgtgccgcagctaacgcatta agtgccccgcctggggagtacggccgcaaggttaaaactcaaatgaattgacgggggccc gcacaagcggtggagcatgtggtttaattcgaagcaacgcgaagaaccttaccaggcctt gacatccaatgaactttctagagatagattggtgccttcgggaacattgagacaggtgct gcatggctgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgtaacgagcgcaac ccttgtccttagttaccagcacgtaatggtgggcactctaaggagactgccggtgacaaa ccggaggaaggtggggatgacgtcaagtcatcatggcccttacggcctgggctacacacg tgctacaatggtcggtacagagggttgccaagccgcgaggtggagctaatcccacaaaac cgatcgtagtccggatcgcagtctgcaactcgactgcgtgaagtcggaatcgctagtaat cgcgaatcagaatgtcgcggtgaatacgttcccgggccttgtacacaccgcccgtcacac catgggagt.

IV. Discussion:

Gelatinase, a proteinase enzyme, produced by microorganisms commands importance not only in protein chemistry but also in detergents, pharmaceutical and leather industries. Besides microbes, plants and animals are involved in enzyme production but microbial gelatinase is preferred. [21]. The possibility of using

actinomycetes for gelatinase production in particular *Streptomyces* has been investigated because of their capacity to secrete the enzyme into extracellular media. *Streptomyces* species are heterotrophic feeders which can utilize both complex and simple molecules as nutrients [22]. They produce variety of extracellular proteinase that has been related to aerial mycelium and sporulation [23]. The present study was experienced for gelatinase production from the isolate and its identification on the basis of morphological, biochemical and taxonomic characterization according to Burgey's manual of Determinative Bacteriology [24,25] as well as similarity in PCR amplified product of 16S rRNA gene sequence.

The strains SK701 lagged behind in case of gelatinase activities as compared to SK703, though initially it gave some positive results. The maximum gelatinase activity of strain SK703 in gelatine supplemented broth was observed after 96h of incubation during the stationary phase in the range of pH was also noticed within 6.5 to 7.0 and pH 7.8, confirming its slightly nature and at 35 to 40° C establishing its mesophilic nature. Final recognition of strain SK703 as the member of actinomycetes was on the basis of similarity in 16S rRNA gene sequencing data with that of *Streptomyces flavoviridis* strain ZG084 but the difference of 14 nucleotides established the strain SK703 as *Streptomyces flavoviridis* strain $SK61^B$

V. Conclusion

The isolate SK703 with gelatinase activity on gelatine supplemented agar medium was identified as *Streptomyces* species on the basis of morphological, cultural, biochemical and physiological characteristics as well as 16S rRNA gene sequencing. On the basis of 99% blast homology data base similarity with *Streptomyces flavoviridis* strain ZG084 (NCBI Accession no. GQ985452.1), the isolate SK703 was christened as *Streptomyces flavoviridis* strain $SK61^B$ and because of the difference of 14 nucleotides which amounts to nearly 1%, the strain earned a fresh accession no. KF815976.

Conflict of interest statement

The authors have no conflict of interest associated with this study.

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