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Isolation and Screening of Cellulolytic Actinomycetes from Diverse Habitats

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ABSTRACT

Cellulases have become the focal biocatalysts due to their wide spread industrial applications. Wide variety of bacteria in the environment permits screening for more efficient cellulase producing strains. The study focuses on isolation and screening of cellulase producing actinomycetes. The isolates from varied ecological habitats were subjected to primary screening. Between 80-90 % isolates were found to produce cellulase enzyme. Among the isolates tested, colonies 51, 157, 194 and NRRL B-16746 (*S. albidoflavus*) showing appreciable zones of clearance were selected for secondary screening. Enzyme activity was determined in crude and in partially purified extracts. NRRL B-16746 *S. albidoflavus* (1.165 U/ml/min) showed maximum activity followed by colony 194 (0.995 U/ml/min), colony 51 (0. 536 U/ml/min), colony 157 (0.515 U/ml/min) and NRRL B-1305 (*S. albogriseolus*; positive control) (0.484 U/ml/min). The taxonomic status of isolates 51,157 and 194 was studied by polyphasic approach including morphological and biochemical characterization. The 16S rRNA gene sequence similarity between isolates 51 and its phylogenetic relative *S. griseochromogenes* NBRC 13413^T (AB184387), isolate 157 and its relatives *S. rochei* NBRC 12908^T (AB184237), *S. enossicasilus* NRRL B-16365^T (DQ026641), *S. plicatus* NBRC 13071^T (AB184291) and isolate 194 with *S. albidoflavus* DSM 40445 (Z76676) was 100, 100 and 96.20% respectively. The 16S rRNA gene sequences of isolates 51, 157 and 194 were submitted to GenBank nucleotide database of the National Centre for Biotechnology Information (NCBI) and assigned accession numbers KJ995861, KJ934594, KJ934595 respectively.

Keywords: Actinomycetes, Cellulolytic activity, Primary and secondary screening, 16S rRNA gene, Polyphasic taxonomy.

[I] INTRODUCTION

Enzymes are complex proteins, produced by all living beings [15]. Soil micro-organisms including bacteria, fungi and nematodes secrete extra cellular enzymes for the degradation of organic matter [42]. Actinomycetes are an important group of filamentous gram positive micro organisms, which are widely distributed in different habitats and are degraders of organic matter in the natural environment, producers of antibiotics [19, 44, 45, 46] and extracellular

enzymes such as cellulases [7, 28], chitinases [48], xylanases [16], peptidases, proteases, amylases [47], pectinases, hemicellulases and keratinases [40].

Due to increasing demand for energy and rising cost of fossil fuels, the need of utilization of renewable resources for the production of alternative energy has become important [51]. Lignocellulose is one such important bio-residue generated from different industries [29] and includes renewable biopolymers such as cellulose, hemicellulose and lignin [30]. Cellulose is commonly degraded by an extracellular enzyme called cellulase [28, 52]. Cellulase complex consists of a group of free enzymes- endoglucanase, exoglucanase and cellobiase which together convert cellulosic substrates into fermentable products [22]. The high cellulase producing bacterial genera include *Streptomyces*, *Bacillus*, *Pseudomonas, Thermonospora*, *Actinosynnema and Nocardiopsis* [2, 5, 35, 52].

[II] MATERIALS AND METHODS

2.1 Isolation of actinomycetes

Soil samples were collected from diverse ecological habitats for isolation of actinomycetes (Table 1). Samples were dried and appropriate aliquots of the dilutions prepared in saline were spread on actinomycete specific media including Yeast Extract-Malt Extract (YM agar) [43], Starch Casein (SC agar) [21], Arginine Glycerol (AG agar) [8], Organic Agar Gause 2 (OMG 2) [11] and Glycerol Asparagine (GA agar) [43]. Single actinomycete colonies were purified by restreaking on YM agar plates and stored as 20% glycerol stocks at -20 ºC/-80 ^{0}C.

2.2 Primary Screening of isolates for production of Cellulase enzyme

Basal agar medium containing (gL^{-1}) $(NH_4)_2SO_4$ 2.64, KH_2PO_4 2.38, $K_2HPO_4.3H_2O$ 5.65, $MgSO_4.7H_2O$ 1, 1 ml of trace salt solution (composition gL^{-1}, $CuSO_4.5H_2O$ 6.43, $FeSO_4.7H_2O$ 1.1, $MnCl_2.4\ H_2O$ 7.9, $ZnSO_4.7\ H_2O$ 1.5), agar 15 (pH 6.8-7) [12] supplemented with 2% cellulose was prepared. Cultures were spot inoculated and incubated at 28^{0}C for 7 days to allow for secretion of cellulase. Cellulose degradation was indicated by the formation of cleared zone of hydrolysis around the colonies [41] which was measured to select cellulase producing isolates [9].

2.3 PCR and Sequencing

Genomic DNA was isolated by methods standardized for actinomycetes [20, 23]. PCR (Master cycler gradient, Eppendorf) mediated amplification of 16S rRNA gene was done by universal primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- TACGGTTACCTTGTTACGACTT-3'); 27F (5'-AGAGTTTGATCCTGGCTCA-3') and 1542R (5'-AAGGAGGTGATCCAGCCGCA-3') (SIGMA). Eluted amplicons were purified with Bangalore Genei kit and were sequenced by the Micro Seq^R 16S rRNA gene sequencing kit (Applied biosystems, USA) and Applied Biosystems 3100 Avant^{TM} Genetic Analyzer Sequencer. Resultant sequences 1437nt for isolate no 51, 1450nt for isolate no 157 and 1432nt for isolate no 194 were aligned manually with sequences of available *Streptomyces* drawn from the Eztaxon [6]. Phylogenetic trees were constructed by neighbor-joining method [39] using Clustal_X 1.81 and MEGA 4.1 [4, 33, 46, 49]. Topology of the phylogenetic trees was evaluated by bootstrap resampling method with 1000 replicates [17, 26]. The evolutionary trees were rooted with *Actinomadura hibisca* JCM 9627^T (AF163115) as the out-group [46, 50]. The 16S rRNA gene sequences of isolates 51, 157 and 194 were submitted to GenBank nucleotide database of the National Centre for Biotechnology Information (NCBI) for accession numbers.

2.4 Bacterial characterization

Morphological and biochemical characterization of strains was carried out as per protocols of International *Streptomyces* project [20, 33, 43]. Observation of spores and mycelia of isolates 51, 157 and 194 was made by phase contrast microscope (Nikon E600) [33].

2.5 Secondary screening of enzyme activity by submerged fermentation process

Isolates showing appreciable zones of clearance were inoculated in 25ml of 148G medium (g/L^{-1})

Glucose 22, Beef extract 4, Bacto peptone 5, Yeast extract 0.5, Tryptone 3, NaCl 1.5, (pH 7.5). CFU's/ml was calculated [9] and the inoculum having an average viable count of 10^5 to 10^7 CFU's/ml was transferred to basal medium, incubated at 28^0C for 5-6 days on rotary shaker (New Brunswick Scientific, Excella E24R) at 200 rpm [41,42].

2.6 Protein content in crude culture broth

Protein concentration in crude enzyme was determined by Lowry's method [24] with Bovine serum albumin (BSA) as a standard.

2.7 Enzyme assay in crude culture broth

Cellulase activity was estimated in crude culture broth. Well grown, week old cultures were centrifuged to obtain cell free supernatant, used as a source of crude enzyme [52]. Cellulase activity was measured, using Dinitrosalicyclic acid (DNS) method [13, 38]. 0.5 ml of crude culture filtrate was added to 1.5 ml of 2% cellulose prepared in 0.05M sodium citrate buffer (pH 4.8) were incubated at 40°C for 30 minutes. The reaction was terminated by adding 3 ml DNS reagent and by subsequent incubation at 100°C for 5 minutes. Absorbance was recorded at 575 nm using spectrophotometer (UV Vis Elico SI-159) [27, 32, 36]. By using a calibration curve for glucose, one unit of enzyme activity was defined as the amount of enzyme that releases 1µM of glucose/ml/min [41]. Enzyme activity (IU/ml/min) was calculated by the formula- Concentration of glucose (µg/ml)* x reaction volume / Molecular weight of glucose x reaction time x volume of enzyme x dilution factor, (*where, Concentration of glucose = Actual OD/ Slope from the graph) [3, 36].

2.8 Purification of enzyme by ammonium sulphate saturation and dialysis method

50 ml of crude culture extract was saturated upto 80% by addition of 25.8g ammonium sulphate and the mixture was kept overnight at 4^0C for protein precipitation [41, 42, 52]. Precipitate was recovered by centrifugation at 8000 rpm, for 20 min, 4^0C and brought in 0.5 M Tris HCl buffer (pH 8.0) by dialysis through dialysis membrane-150 (HiMedia, 5KDa), overnight at 4^0C with continuous stirring. After dialysis, the enzyme volume was made up to 15 ml using 0.5 M Tris HCl buffer (pH 8.5) and was concentrated upto 1 ml using the Amicon ultra centrifugation tubes (membrane cut-off of 3 kDa) at a speed of 3000 rpm for 2 hours at 4 ^{0}C. The partially purified sample was assayed for enzyme activity and protein estimation.

2.9 Protein content in partially purified products

Protein content in samples was determined as mentioned earlier.

3.0 Enzyme assay in partially purified products

Protein concentration was equalized in all samples by using 0.5M Tris-Cl (pH 8.0). Enzyme activity was measured in the equalized protein samples by DNS method using glucose standard curve.

[III] RESULTS AND DISCUSSION

3.1 Isolation of actinomycetes

Soil samples were collected from diverse ecological habitats (Table 1, Figure 1). 646 actinomycete bacterial colonies were isolated using actinomycete specific media; AG agar, GA agar, OAG 2 were found to be more effective (Table 2).

Table 1: Number of isolates from different ecological habitats

S.No.	Habitat	Total no. of colonies
1.	AGRICULTURAL SOILS	
	Agricultural soil, Dhanaura, U.P	86
	Agricultural soil, Yamuna Bank, Delhi	37
	Agricultural soil, Nainital	13
	Agricultural soil, Kashipur	17
2.	INDUSTRIAL SOILS	
	Sugar Plant, Dhanaura, U.P	7
	Chemical Plant, Faridabad	10
3.	LANDFILL SOILS	
	Dumping site, Sarai Kale Khan, Delhi	28
4.	RIVER/LAKE SOILS	
	Yamuna Bank, Delhi	6
	Lake soil, Purana Quila, Delhi	14
5.	DIVERSITY PARK SOILS	
	Diversity Park, Sarai Kale Khan, Delhi	8
	Great Himalayan National Park, Teerthan Valley, H.P	42

	Great Himalayan National Park near a narrow spring, Teerthan Valley, H.P	38
6.	SEA/BEACH SOILS Catamaran Beach Hotel, Colombo, Sri Lanka Havelock Islands, Andaman & Nicobar Islands Carbon Island, Andaman & Nicobar Islands	 20 76 98
7.	FOREST SOILS Killingpong 4000ft, Kolkata Pine Forest, Teerthan Valley, H.P	 44 102

Table 2: Comparison of efficacy of different media for the selective isolation of actinomycetes

+++ large number of colonies, ++ moderate number of colonies, + less number of colonies

Figure 1: Some collection sites for soil samples

Agricultural Soil, Yamuna Bank, Delhi, India — Agricultural soil Dhanaura, UP, India

Dumping site, Sarai Kale Khan, Delhi, India — Sugar plant, Dhanaura, UP, India

3.2 Primary screening of isolates

Preliminary screening revealed that extent of degradation of cellulose varied from isolate to isolate (Table 3). Between 80-90% isolates showed cellulase activity. Colonies 4, 51, 157, 196, 222, 186, 194, *Streptomyces albogriseolus* (NRRLB 1305), *S. subrutilus* (NRRLB 12377), *S. mexicanus* (NRRLB 24196), *S. albidoflavus* (NRRLB 16746), *S. venezuelae* (ISP 5230), *S. stramineus* (NRRLB 12292) and *S. coelicolor* (NRRLB 16638) showed substantial cellulase production.

It was found that the cleared zone diameters produced by *S. albidoflavus* (NRRL B-16746), isolate no 194 , isolate no 51, isolate no 157 and *S. albogriseolus* due to production of cellulase were 33mm, 31mm, 26mm, 18mm and 17mm respectively (Figure 2, Table 4, Graph 1). Based on the results of primary screening, colonies 51, 157, 194, *S. albidoflavus* and S. *albogriseolus* (control) which showed substantial cellulase activity and representing diverse ecological habitats were selected for further analyses.

Medium	Appearance of actinomycete colonies
Arginine Glycerol (AG) agar	+++
Glycerol asparagine agar	+++
Organic Gause agar	+++
Starch caesin agar	++
Yeast extract Malt extract agar	+

Table 3: Primary Screening results for cellulase enzyme

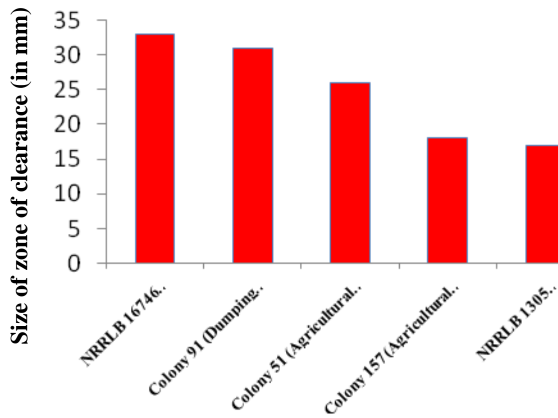
	Activity
AGRICULTURAL SOIL, DHANAURA	
Colony no 4	+++
Colony no 23	-
Colony no 43	++
Colony no 51	+++
Colony no 157	+++
AGRICULTURAL SOIL, YAMUNA RIVER	
Colony no 85	++
Colony no 101	+
Colony no 138	++
Colony no 196	+++
Colony no 222	+++
DUMPING SITE, SARAI KALE KHAN	
Colony no 102	-
Colony no 112	++
Colony no 136	++
Colony no 186	+++
Colony no 194	+++
Streptomyces albogriseolus (NRRL B-1305) (Cellulase Control)	+++
Streptomyces mexicanus (NRRL B-24196) (Xylanase control)	+++
Streptomyces thermocoprophilus (NRRL B-24314) (Xylanase control)	-
Streptomyces albidoflavus (NRRL B-16746) (Chitinase control)	+++
Streptomyces venezuelae (ISP 5230) (Chitinase control)	+++
Streptomyces stramineus (NRRL B-12292) (Phosphatase control)	+++
Streptomyces coelicolor (NRRL B-16638) (Phosphatase control)	+++

+++ = High activity, ++ = Moderate activity, + = Low activity, - = No activity

Table 4: Clear zone produced by isolates due to production of cellulase

S.NO	Strains	Clear zone diameter (Diameter of clear zone-inoculum size) (mm)
1.	NRRL B- 16746 (*Streptomyces albidoflavus)*	33
2.	Colony 194 (Dumping site, Sarai Kale Khan, Delhi)	31
3.	Colony 51 (Agricultural soil, Dhanaura, UP)	26
4.	Colony 157 (Agricultural soil, Dhanaura,UP)	18
5.	NRRL B-1305 *(Streptomyces albogriseolus)*(Control)	17

Graph 1: Comparative bar graph of zone of clearance of various isolates



Strains/isolates

Figure 2: Plates showing zone of clearance of isolates due to production of cellulase

Figure 2.1: *Streptomyces albidoflavus* (NRRL B-16746) culture showing zone of clearance (size- 33 mm) on basal medium with cellulose

Figure 2.2: Colony no 194 culture showing zone of clearance (size- 31 mm) on basal medium with cellulose

Figure 2.3: Colony no 51culture showing zone of clearance (size- 26 mm) on basal medium with cellulose

Figure 2.4: Colony no 157culture showing zone of clearance (size- 18 mm) on basal medium with cellulose

Figure 2.5: *Streptomyces albogriseolus* (NRRL B-1305) culture showing zone of clearance (size- 17 mm) on basal medium with cellulose.

3.3 Identification of isolates

Comparison of 16S rRNA gene sequences of isolate 51 (1437nt), isolate 157 (1450nt), isolate 194 (1432nt) with sequences of close *Streptomyces* species deposited in databases indicated that these isolates belong to genus *Streptomyces.*

Rooted phylogenetic trees based on neighbor joining method, prepared separately for isolates 51, 194 and 157 indicated that these were included in distinct clades in their respective trees (Fig 3, 4, 5). Isolate 51 showed 100% similarity with *Streptomyces griseochromogenes* NBRC 13413^T (AB184387) [39], isolate 157 shows 100% similarity with *Streptomyces rochei*

NBRC 12908^T (AB184237) [40], *Streptomyces enissocaesilis* NRRL B-16365^T (DQ026641) [41] and *Streptomyces plicatus* NBRC 13071^T (AB184291) [42]. Isolate 194 showed 96.20%

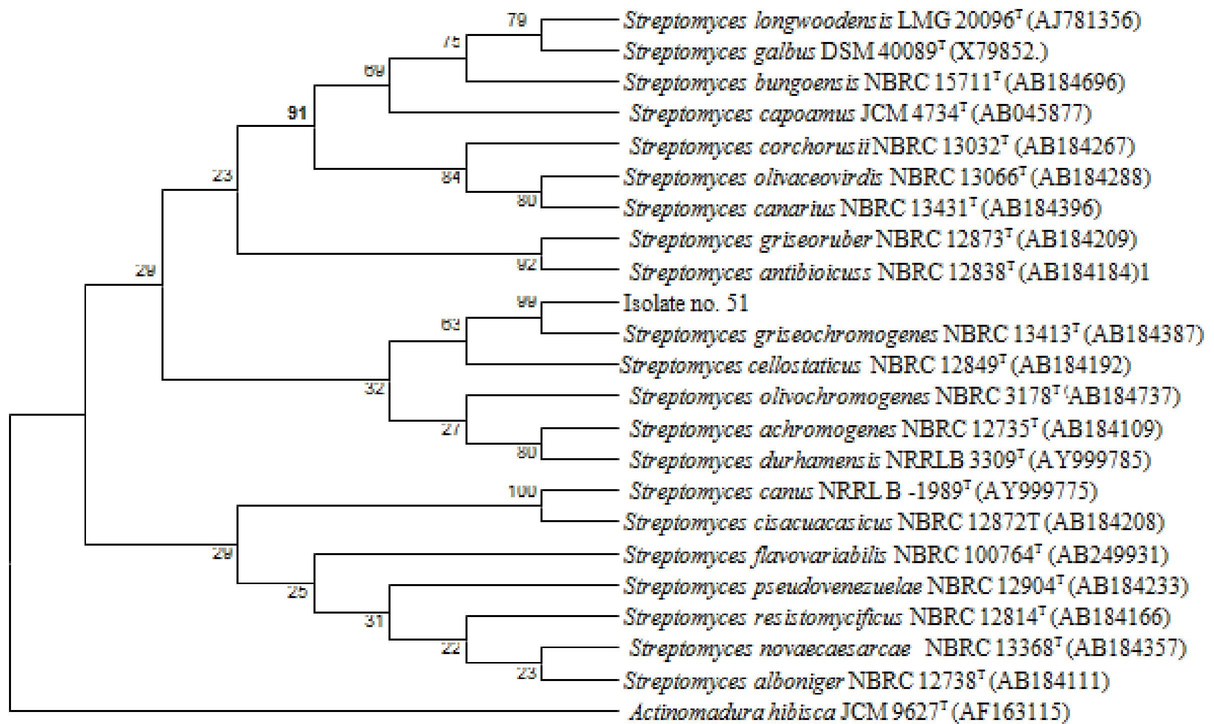


Fig. 3 Rooted phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between isolate no. 51 (KJ995861) and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^T (AF163115) was used as an outgroup. The tree was generated using the neighbor-joining method (Clustal_X version 1.81 and MEGA version 4.1) and includes bootstrap percentages based on the analysis of 1,000 resampled datasets.

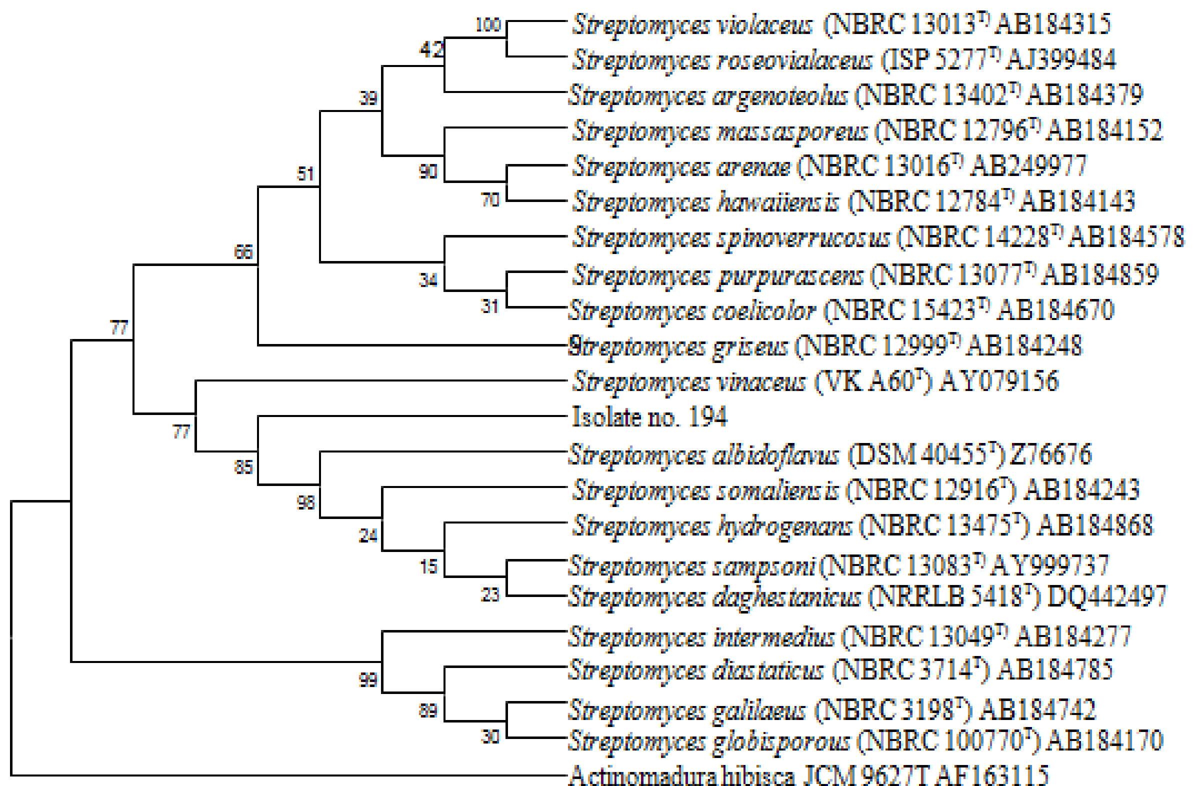


Fig. 4 Rooted phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between isolate no. 194 (KJ934594) and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^T (AF163115) was used as an outgroup. The tree was generated using the neighbor-joining method (Clustal_X version 1.81 and MEGA version 4.1) and includes bootstrap percentages based on the analysis of 1,000 resampled datasets.

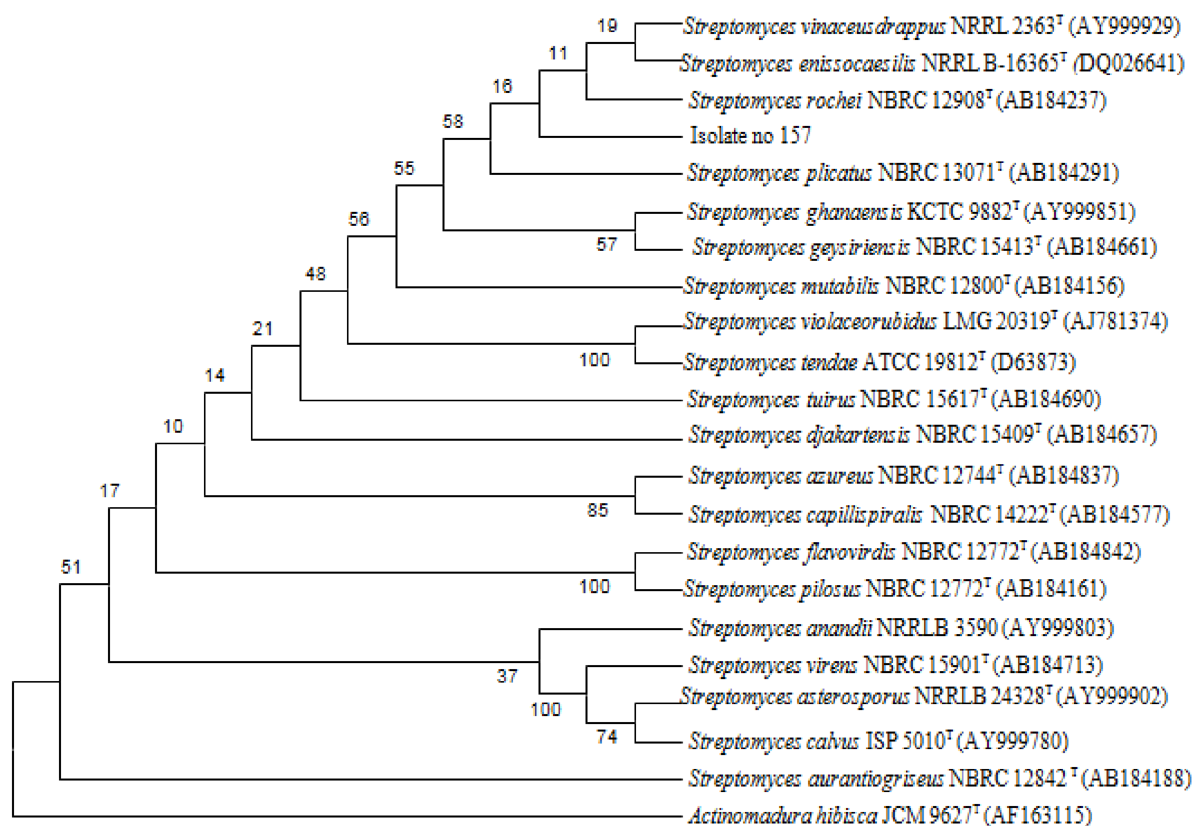


Fig. 5 Rooted phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between isolate no. 157 (KJ934594) and related representative species of the genus *Streptomyces*. The sequence of the 16S rRNA gene of *Actinomadura hibisca* JCM 9627^T (AF163115) was used as an outgroup. The tree was generated using the neighbor-joining method (Clustal_X version 1.81 and MEGA version 4.1) and includes bootstrap percentages based on the analysis of 1,000 resampled datasets.

similarity with *Streptomyces albidoflavus* DSM 40455^T (Z76676) [43].

The 16S rRNA gene sequences of isolates 51, 157 and 194 were submitted to GenBank nucleotide database of NCBI and assigned accession numbers KJ995861, KJ934594 and KJ934595 respectively.

3.4 Description of isolates

The results of phenotypic characterization of isolates indicated that all the isolates were aerobic, Gram-positive actinomycetes showing formation of vegetative and aerial mycelium. They however differed in morphological and biochemical characters (Table 5) which could be due to adaptation of isolates to their respective habitats.

Isolate no. 51 (KJ995861) has cream/yellow/beige/brown substrate mycelium, white/gray aerial mycelium, abundant sporulation on most ISP media. Spore chain morphology Retinaculiaperti (Figure 6). Utilizes Fructose, Mannitol, Sucrose, Meso-inositol, Arabinose, Raffinose.

High Caseinase activity. Degrades Tween 80 (Table 5).

Isolate no. 157 (KJ934594) shows yellow/beige substrate mycelium, white/gray aerial mycelium, abundant sporulation on most ISP media. Spore chain morphology Retinaculiaperti (Figure 6). Utilizes Xylose, Sucrose, Meso-inositol, Arabinose, Moderate Caseinase activity.

Degrades Tween 80. Good metabolism of Urea (Table 5).

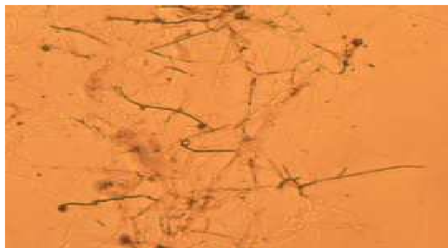
Isolate no. 194 (KJ934595) has yellow/yellow-black/cream/beige substrate mycelium, white/gray/yellow-white aerial mycelium, abundant sporulation on many ISP media. Spore chain morphology is Rectiflexibles (Figure 6). Utilizes Fructose, Xylose, Sucrose

Table 5: Comparison of morphological and biochemical characteristics of isolates with their respective phylogenetic relatives

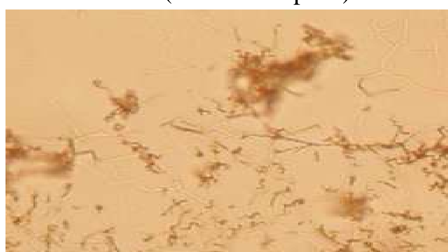
Characteristics	Isolate no 51	*Streptomyces griseochromogenes* (*NBRC 13413^{T}*) (Fukunaga 1955)	Isolate no 157	*Streptomyces rochei* (NBRC 12908^{T}) (Kavitha and Vijayalakshmi, 2007)	*Streptomyces enissocaesilis* (*NRRL B-16365^{T}*) (Gause 1986)	*Streptomyces plicatus* (NBRC 13071^{T}) (Pridham et al., 1958)	Isolate no 194	*Streptomyces albidoflavus* (*DSM 40455^{T}*) (Rossi Doria 1891) Waksman and Henrici 1948)
Color of: Substrate mycelium	Cream (ISP 1, 3) Yellow (ISP 2, 5,7) Beige (ISP 4) Brown (ISP 6)	Greyish-yellow-orange brown (ISP 2) Greyish-yellow-light olive brown/ gray greenish yellow (ISP 3,4,5)	Yellow (ISP 1,2,7) Beige (3,4,6,)	Light to dark yellow (ISP 2)	Brown (ISP 3) Black greyish brown (ISP 5)	N.R.	Yellow (ISP 1, 2,6) Yellow-Black (ISP 4,7) Cream (ISP 3) Beige (ISP 5)	N.R.
Aerial mycelium	White (ISP 1,2, 3,4, 5) Grey (ISP 7) No aerial mycelium on ISP 6	Grey (ISP 2,3,4,5)	White (ISP 1, 3,4) Grey (ISP 2,5,6,7)	Grey-white (ISP 2)	Poorly developed (ISP 3) Absent or poorly developed, whitish (ISP 5)	Grey (ISP 2)	White (ISP 1,2, 3, 5,6) Grey (ISP 4) Yellow-White (ISP 7)	White/Grey (ISP 2, 5) No sporulation observed in any other ISP media
Growth	Good (ISP 2, 4, 5, 7) Moderate (ISP 3) Poor (ISP 1,6)	N.R.	Good (ISP 2,3,4,7) Moderate (ISP 3,6,7)	Good (ISP 3,4,5,7)	N.R.	N.R.	Good (ISP 2,3) Moderate (ISP 4,5,7) Poor (ISP 1,6)	N.R.
Sporulation	Good (ISP 2, 4, 5,7) Moderate (ISP 3) Poor (ISP 1,6)	N.R.	Good (ISP 2,5,6,7) Moderate (ISP 1,3,4)	N.R.	N.R.	N.R.	Good (ISP 2) Moderate (ISP 4,5,7) Poor (ISP 1,3,6)	N.R.
Production of diffusible pigment	No diffusible pigment on ISP 1,2,3,4,5,7 Brown (ISP 6)	No pigment on ISP 2,3,4,5 Melanoid pigment on ISP 1,6,7	No diffusible pigment on any ISP media	No diffusible pigment	Brownish, weak (ISP 3) No diffusible pigment (ISP 5)	N.R.	No diffusible pigment on any ISP media	No diffisible pigment on any ISP media
Spore chain	Retinaculiaperti	Spirales to retinaculiaperti	Retinaculiaperti	Spirales	Retinaculiaperti	Spirales	Rectiflexibles	Rectiflexibles
Utilization of:								
D-fructose	+	+	-	+	+	+	+	+
Mannitol	+	+	Weak	+	+	+	Weak	+
Xylose	Weak	N.R.	+	+	+	+	+	+
Sucrose	+	+	+	+	-	-	+	-
Meso-inositol	+	+	+	N.R.	+	+	-	+
L-arabinose	+	+	+	-	+	-	+	+
L-rhamnose	Weak	-	+	-	-	+	+	-
Raffinose	+	+	+	+	-	-	+	-
Tween	+	N.R.	+	N.R.	N.R.	N.R.	+	N.R.
Hypoxanthine	-	N.R.	+	N.R.	N.R.	N.R.	+	N.R.
Urea	+	N.R.	+	N.R.	N.R.	N.R.	+	N.R.
Caesin	+	N.R.	+	-	N.R.	N.R.	+	N.R.
Starch	+	N.R.	+	+	N.R.	N.R.	+	N.R.
Production of extracellular enzymes	Cellulase, Xylanase, Chitinase		Cellulase, Xylanase, Chitinase	Amylases, Asparaginases, Chitinase, Cellulase, Nitrate reductase, Xylanase	Cellulase, Xylanase, Lipase, β-amylase	Chitinase, Endoglycosidase H	Cellulase, Xylanase, Chitinase	Cellulase, pectinase, isomerase

Arabinose, Raffinose but weak utilization of Mannitol. Good Caseinase activity. Degrades Tween 80. Good metabolism of Urea and Hypoxanthine (Table 5). Comparison of taxonomic features of isolates with their clade members is shown in Table 5.

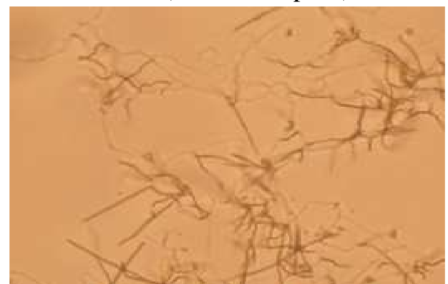
Figure 6: Spore chain morphology of strains



Colony 51: Hooks, loops or spirals with one to two turns (Retinaculiaperti)



Colony 157: Hooks, loops or spirals with one to two turns (Retinaculiaperti)



Colony 194: Straight to flexuous (Rectiflexibles)

3.5 Secondary screening for enzyme activity by submerged fermentation process

Due to appreciable cellulase activity observed in *S. albidoflavus* (NRRL B-16746), isolates 194, 51 and 157 these cultures along with the control were selected for subsequent secondary screening. Standard inoculum having an average viable count of 10^5 to 10^7 CFU's/ml was transferred to production medium.

3.6 Protein content in crude culture broth (in 50 ml volume)

Protein content in crude culture extract was calculated using BSA standard curve (Table 6).

3.7 Cellulase activity in crude culture broth (in 50 ml volume)

Cell free culture extract was used as a source of crude cellulase enzyme. The amount of glucose (mg) secreted by 50 ml culture broth was determined using the glucose standard curve. Cellulase enzyme activity units were calculated.

Highest enzyme activity (0.756 U/ml/min) was shown by *S. albidoflavus* (NRRL B- 16746) followed by enzyme activity of colony 194, colony 51 and colony 157 and *S. albogriseolus* (NRRL B-1305) (Table 6).

Table 6: Cellulase enzyme activity and protein content in crude culture extract (in 50 ml volume)

S.No.	Cultures	Absorbance at 575 nm (for enzyme activity)	Concentration of glucose (mg)	Enzyme activity (U/ml/min)	Absorbance at 675 nm (for protein content)	Protein content (mg)
1.	NRRLB 16746	1.001	0.8	0.756	1.241	0.88
2.	Colony 194	0.831	0.65	0.627	1.070	0.71
3.	Colony 51	0.549	0.43	0.414	0.715	0.51
4.	Colony 157	0.431	0.34	0.325	0.684	0.49
5.	NRRLB 1305 (control)	0.429	0.34	0.324	0.632	0.45

3.8. Purification of Cellulase enzyme by ammonium sulphate saturation and dialysis method

Crude culture extracts were partially purified by dialysis followed by concentration [45].

3.9 Protein content in partially purified samples of Cellulase enzyme

Protein content was determined in the partially purified protein samples by the earlier mentioned procedure (Table 7). For enzyme activity comparison in partially purified extract, protein contents in all samples were equalized.

4.0 Enzyme assay in partially purified samples (25 ml after dialysis, 1 ml after concentration)

Cellulase enzyme activity was found maximum in NRRL B-16746 (1.165 U/ml/min) followed by colony 194 (KJ934595) (0.995 U/ml/min), colony 51 (KJ995861) (0.536 U/ml/min),

colony 157 (KJ934594) (0.515 U/ml/min) and NRRL B-1305 (0.414 U/ml/min) in 1 ml of concentrated samples (Table 8, Graph 2).

During primary screening NRRL B-16746 (*S. albidoflavus*) showed maximum zone of clearance followed by colonies 194, 51 and 157. The results of primary screening were confirmed during secondary screening. The enzyme activity was found maximum in NRRL B-16746 followed by colony 194, colony 51, colony 157 and NRRL B-1305 both in case of crude extract as well in partially purified extract of samples (Table 9).

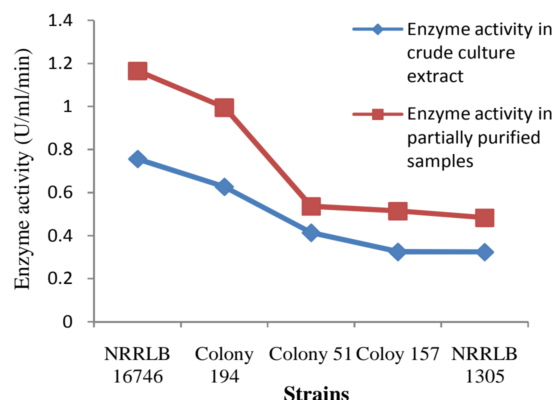
Table 7: Protein content and cellulase enzyme activity in partially purified protein samples (in 1 ml of concentrated volume)

S.No	Cultures	Absorbance at 675 nm	Total protein content after purification (mg)	Protein content after equalization (mg)	Absorbance at 675 nm	Conc of glucose (mg)	Enzyme activity (U/ml/min)
1.	NRRLB 16746	0.792	1.08	0.59	1.543	1.2	1.165
2.	Colony 194	0.756	0.9	0.56	1.318	1.02	0.995
3.	NRRLB 12377 (control)	0.721	0.67	0.55	1.10	0.86	0.83
4.	Colony 51	0.710	0.60	0.52	0.710	0.55	0.536
5.	Colony 157	0.704	0.52	0.51	0.682	0.54	0.515
6.	NRRLB 1305 (control)	0.700	0.51	0.51	0.641	0.52	0.484

Table 8: Comparison of cellulase enzyme activity of crude culture extracts and partially purified samples

S.No	**Cultures**	**Clear zone diameter (mm)**	Enzyme activity in crude culture extract (U/ml/min) (in 50 ml volume)	Enzyme activity in partially purified samples (U/ml/min) (in 1 ml concentrated volume)
1.	NRRLB 16746	33	0.756	1.165
2.	Colony 194	31	0.627	0.995
3.	Colony 51	26	0.414	0.536
4.	Colony 157	18	0.325	0.515
5.	NRRLB 1305 (control)	17	0.324	0.484

Graph 2: Comparison of cellulase enzyme activity of crude culture extracts and partially purified samples



S.No	Cultures	Enzyme activity in crude culture extracts (U/ml/min) (in 50 ml volume)	Enzyme activity in partially purified samples (U/ml/min) (in 1 ml of concentrated volume)
1.	NRRLB 16746	0.756	1.165
2.	Colony 194	0.627	0.995
3.	Colony 51	0.414	0.536
4.	Colony 157	0.325	0.515
5.	NRRLB 1305 (control)	0.324	0.484

Table 9: Comparison of zone size and enzyme activity of cultures

[IV] DISCUSSION

Actinomycetes are well known for production of extra cellular enzymes [16, 31, 40, 47, 48]. In the present study, actinomycetes were screened for production of extra cellular Cellulase (Table 1). Actinomycetes have been isolated by researchers from various ecological environments for identifying cellulase producing actinomycetes [7, 28].

During primary screening, a total of 80-90% isolates showed cellulase activity (Table 3). Mohanta, 2014 [28] found 18% isolates as cellulase producing organism.

Based on the results of primary screening, isolate nos. 51, 157, 194, *S. albidoflavus* (NRRL B-16746) along with *Streptomyces albogriseolus* (NRRL B-1305, control) (Table

4, Graph 1, Figure 2) were selected for secondary or quantitative analyses. Nayaka & Vidyasagar, 2012 [31], tested 57 thermotolerant actinomycetes isolates from different habitats for production of different enzymes, and found isolate no. VSAC 1-57 produced zone of clearance in the range of 19-52mm.

Protein content and enzyme activity (U/ml/min) in 50 ml crude extracts was determined. Enzyme assay was done by dinitrosalicyclic acid (DNS) method (Table 6). Golinska and Dahm, 2011 [14] estimated cellulase activity in different *Streptomyces* sp. and found enzyme activity in the range of 0.011–0.1539 units/ml. FPase and CMCase activity was determined by Mohanta 2014 [28] in nine actinomycete isolates from Bhitarkanika National Park, Odisha. Cellulase activity using whatman filter paper as the substrate ranges from 0.266- 0.734 U/ml and in case of CMCase it ranges from 0.501-1.381 U/ml.

The crude supernatant was subjected to partial purification by ammonium sulphate saturation and dialysis followed by concentration. Amore et al., 2012 [1] precipitated secreted proteins from *Streptomyces* spp using ammonium sulphate. Partially purified protein samples were used for estimation of protein content and for assay of enzyme activity (Table 7, 8, Graph 2). Yassein et al., 2014 [52], purified enzyme samples produced by *Streptomyces* spp. by ammonium sulphate precipitation followed by dialysis and then using DEAE cellulose and Sephadex columns. The enzyme obtained after purification was 38.85 fold purified. Enzyme activity recorded in crude enzyme extract was 3.8 U/50 ml, 2.5 U/ 25 ml after ammonium sulphate and dialysis and 0.876 U/ml in 3 ml and 0.517 U/ml in 2 ml after purification by DEAE-cellulose column and Sephadex column respectively.

[V] CONCLUSION

The present work was carried out for the isolation of cellulolytic bacteria from varied ecological habitats. Isolates were subjected to primary screening and based on the results isolates 51, 157 194 representing diverse habitats NRRLB 16746 *(S. albidoflavus)* along with the positive control NRRLB 1305 *(S. albogriseolus*) were selected for secondary screening Isolates were morphologically and biochemically characterized. The 16S rRNA gene sequencing and phylogenetic analyses showed that isolates 51 (KJ995861), 157 (KJ934594) and 194 (KJ934595) belong to the genus *Streptomyces.* Enzyme activity and protein content in crude culture extract was determined. The cell free extract was also subjected to partial purification, followed by determination of enzyme activity and protein content Maximum enzyme activity was found in NRRL B-16746 followed by colony 194, colony 51, colony 157 and NRRL B- 1305. Further work needs to be done for purification of cellulase enzyme by column chromatography, determining the enzyme activity and protein content in purified culture extracts followed by protein profiling on SDS-PAGE.

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REFERENCES:

1. Amore, A., Pepe, O., Ventorino, V., Birolo, L., Giangrande, C. and Faraco, V. (2012). Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces* sp. G12 isolated from compost. *Microbial Cell Factories,* **11:**1-12.
2. Anderson, I., Abt, B., Lykidis, A., Klenk, H.P., Kyrpides, N. and Ivanova, N. (2012). Genomics of aerobic cellulose utilization systems in actinobacteria. *PLoS One*, **7:**1-10.
3. Azzeddine, B., Abdelaziz, M., Estelle, C., Mouloud, K., Nawel, B., Nabila, B., Francis, D. and Said, B. (2013). Optimization and partial characterization of endoglucanase produced by *Streptomyces sp.* B-PNG23. *Arch. Biol. Sci.*, **65:** 549-558.

4. Charbonneau, D.M., Mouelhi, F.M., Boissinot, M., Sirois, M. and Beauregard, M. (2012). Identification of thermophilic bacterial strains producing thermotolerant hydrolytic enzymes from manure compost. *Indian. J. Microbiol.,* **52:**41-47.
5. Cheng, C.L. and Chang, J.S. (2011). Hydrolysis of lignocellulosic feedstock by novel cellulases originating from *Pseudomonas* sp. CL3 for fermentative hydrogen production. *Bioresour. Technol.,* **102:**8628-34.
6. Chung, J., Lee, J.H., Jhung, Y., Kim, M., Kim, B.K. and Lim. Y.W. (2007). Ez/Taxon: a web based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **57:**2259-2261.
7. Deepthi, M.K., Sudhakar, M.S. and Devamma, M.N. (2012) Isolation and screening of *Streptomyces* sp. from Coringa mangrove soils for enzyme production and antimicrobial activity. *International Journal of Pharmaceutical, Chemical and Biological Sciences* **2:**110-116.
8. El-Nakeeb and Lechevalier, H.A. (1963). Selective isolation of aerobic actinomycetes. *Appl. Microbiol.,* **11:**75-77.
9. El-Sersy, N.A., Abd-Elnaby, H., Abou-Elela, G.M., Ibrahim, H.A.H. and El-Toukhy, N.M.K. (2010). Optimization, economization and characterization of cellulase produced by marine *Streptomyces ruber*. *Afr. J. Biotechnol.,* **9:**6355-6364.
10. Fukunaga, K., Misato, T., Ishii, I. and Asakawa, M. (1955). Blasticidin, a new antiphytopathogenic fungal substance I. Bulletin of the Agricultural Chemical Society of Japan, **19:**181-188.
11. Gause, G.F., Preobrazhenskaya, T.P., Sveshnikova, G.V., Terekhova, L.P. and Maksimova, T.S. (1983). A guide for determination of actinomycetes, Nauka, Moscow, Russia.
12. Gautam, S.P., Bundela, P.S., Pandey, A. K., Jamaluddin, Awasthi, M. K. and Sarsaiya, S. (2012). Diversity of cellulolytic microbes and the biodegradation of municipal solid waste by two novel cellulolytic fungi. *Biotechnology Research Internationa,*, 1-8.
13. Ghosh TK (1987) Measurement of cellulose activities. Pure Appl Chem 59**:**257-68.
14. Golinska, P. and Dahm, H. (2011). Enzymatic activity of actinomycetes from the genus *Streptomyces* isolated from the bulk soil and rhizosphere of the *Pinus sylvestris. Dendrobiology,* **65:**37–46.
15. Haggag, K., Ragheb, A.A., EL-Thalouth, I.A., Nassar, S.H. and Sayed, H.E.L. (2013). A review Article on Enzymes and Their Role in Resist and Discharge Printing Styles. *Life Science Journal,* **10:**1646-1654.
16. Kamble, R. D. and Jadhav, A.D. (2012). Isolation, Purification, and Characterization of Xylanase Produced by a New Species of Bacillus in Solid State Fermentation. International Journal of Microbiology, 1- 8.
17. Kaur, J., Verma, M. and Lal, R. (2011). *Rhizobium rosettiformans sp. nov.,* isolated from hexachlorocyclohexane (HCH) dump site in India, and reclassification of *[Blastobacter]aggregatus* Hirsch et al. [1985] as *Rhizobium aggregatum* comb. *nov. Int. J. Syst. Evol. Microbiol.,* **61:**1218-1225.
18. Kavitha and Vijayalakshmi, M. (2007). Studies on cultural, physiological and antimicrobial activities of *Streptomyces rochei. Journal of Applied Sciences Research,* **3:** 2026-2029.
19. Khanna, M., Solanki, R. and Lal, R. (2011). Selective isolation of rare actinomycetes producing novel antimicrobial compounds. *Int. J. Adv. Biotechnol. Res.* **2:**357-375.
20. Khanna, M. and Solanki, R. (2012). *Streptomyces antibioticalis*: a novel species from sanitary landfill soil. *Indian. J. Microbiol.,* **52:**605-611.
21. Kuester, E. and Williams, S.T. (1964). Selection of media for isolation of *Streptomycetes*. *Nature*, **202:**928-929.
22. Kuhad, R.C., Gupta, R. and Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzyme Research*, 1-10.
23. Lal, R., Lal, S., Grund, E. and Eichenlaub, R. (1991). Construction of a hybrid plasmid capable of replication in *Amycolatopsis mediterranei. Appl. Environ. Microbiol.,* **57:**665-671.
24. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193:**265–275.
25. Maki, M., Broere, M., Leung, K.T. and Qin, W. (2011). Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers. *Int. J. Biochem. Mol. Biol.,* **2:**146-154.

26. Malhotra, J., Anand, S., Jindal, S., Raman, R. and Lal, R. (2012). *Acinetobacter indicus* sp. nov. isolated from hexachlorocyclohexane (HCH) dumpsite. *Int. J. Syst. Evol. Microbiol.,* doi:10.1099/ijs.0.037721-0.
27. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, **31:**426–428.
28. Mohanta, Y.K. (2014). Isolation of cellulose degrading actinomycetes and evaluation of their cellulolytic potential. *Bioengineering and Bioscience* **2:**1-5.
29. Moreno, A.D., Ibarra, D., Alvira, P., Tomas-Pejo, E. and Ballesteros, M. (2014). A review of biological delignification and detoxification methods for lignocellulosic bioethanol production. *Crit Rev Biotechnol.*[Epub ahead of print].
30. Naggar, E.L.N., Sherief, A.A. and Hamza, S.S. (2011). Bioconversion process of rice straw by thermotolerant cellulolytic *Streptomyces viridiochromogenes* under solid-state fermentation conditions for bioethanol production. *African Journal of Biotechnology,* **10:**11998-12011.
31. Nayaka, S. and Vidyasagar, G.M. (2012). Occurrence and extra cellular enzyme potential of actinomycetes of a thermoltolerant, northern region of Karanataka, India. *International Multidisciplinary Research Journal,* **2:**40-44.
32. Ponnambalam, A.S., Deepthi, R.S., Ghosh, A.R. (2011). Qualitative display and measurement of enzyme activity of isolated cellulolytic bacteria. *Biotechnol. Bioinf. Bioeng.,* **1:**33-37.
33. Prasad, P., Tanuja and Bedi, S. (2014). Characterization of a novel thermophilic cellulase producing strain *Streptomyces matensis* strain St-5. *Int. J. Curr. Microbiol. App. Sci.,* **3:** 74-88.
34. Pridham, T.G., Hesseltine, C.W. and Benedict, R.G. (1958). A guide for the classification of *Streptomycetes* according to selected groups; placement of strains in morphological sections. *Appl. Microbiol.,* **6:**52-79.
35. Rastogi, G., Bhalla, A., Adhikari, A., Bischoff, K.M., Hughes, S.R., Christopher, L.P. and Sani, R.K. (2010). Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. *Bioresour. Technol.,* **101:**8798-806.
36. Rathnan, R. K. and Ambili, M. (2011). Cellulase Enzyme Production by *Streptomyces sp.* Using Fruit Waste as Substrate. *Australian Journal of Basic and Applied Sciences*, **5:** 1114-1118.
37. Rong, X., Guo, Y. and Huang, Y. (2009). Proposal to reclassify the *Streptomyces albidoflavus* clade on the basis of multilocus sequence analysis and DNA-DNA hybridization, and taxonomic elucidation of *Streptomyces griseus* subsp. *solvifaciens*. *Syst. Appl. Microbiol*., **32:** 314-322.
38. Sadhu, S. and Maiti, T.K. (2013). Cellulase Production by Bacteria: A Review. *British Microbiology Research Journal*, **3:** 235-258.
39. Saitou, N. and Nel, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., **4:**406-425.
40. Selvam, K., Vishnupriya, B. and Yamuna, M. (2013). Isolation and description of keratinase producing marine actinobacteria from South Indian Coastal Region. *African Journal of Biotechnology*, **12:**19-26.
41. Shaikh, N.M., Patel, A.A., Mehta, S.A. and Patel, N.D. (2013). Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulase production. *Universal Journal of Environmental Research and Technology*, **3:** 39-49.
42. Shanmugapriya, S., Saravana, P.S., Krishnapriya, Manoharan, M., Mythili, A. and Joseph, S. (2012). Isolation, screening and partial purification of cellulase from cellulase producing bacteria. *Int. J. Adv. Biotechnol. Res.,* **3:**509-514.
43. Shirling, E.B. and Gottlieb, D. (1966). Methods for characterization of *Streptomyces species*. *Int. J. Syst. Bacteriol.,* **16:**313:340.
44. Solanki, R., Khanna, M. and Lal, R. (2008). Review article entitled "Bioactive compounds from marine actinomycetes". *India. J. Microb*., **48:**410-431.
45. Solanki, R., Lal, R. and Khanna, M. (2011). Antimicrobial activities of actinomycetes from diverse ecological habitats in Delhi and its adjoining states. *India. J. Microb. World* **13:**233-240.
46. Solanki, R., Das, P. and Khanna, M. (2013). Metabolic profiling of actinomycetes having

antimicrobial properties. *Int. J. Adv. Biotechnol. Res.* **4:**444-459.

47. Sonia, M.T., Hafesh, B., Abdennaceur, H. and Ali, G. (2011). Studies on the ecology of actinomycetes in an agricultural soil amended with organic residues II: Assessment of enzymatic activities of Actinomycetales isolates. *World Journal of Microbiology and Biotechnology*, **27:**2251-2259
48. Sowmya, B., Gomathi, D., Kalaiselvi, M., Ravikumar, G., Arulraj, C., and Uma, C. (2012). Production and purification of chitinase by *Streptomyces sp.* from soil. *J. Adv. Sci. Res.,* **3:** 25-29.
49. Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA 4: molecular evolutionary genetic analysis (MEGA) software version 4.0. *Mol. Biol. Evol.,* **24:**1596-1599.
50. Tomita, K., Nishio, M., Saitoh, K., Yamamoto, H., Hoshino, Y., Ohkuma, H., Konishi, M., Miyaki, T. and Oki, T. (1990). "Pradimicins A, B and C: new antifungal antibiotics. I. Taxonomy, production, isolation and physico-chemical properties*". J. Antibiot.,* **43:**755-762.
51. Wushke, S., Levin, D.B., Cicek, N. and Sparling, R. (2013). Characterization of enriched aerotolerant cellulose-degrading communities for biofuels production using differing selection pressures and inoculum sources. *Can J Microbiol.*, **59:**679-83.
52. Yassien, M.A.M., Jiman-Fatani, A.A.M. and Asfour, H.Z. (2014). Production, purification and characterization of cellulase from *Streptomyces sp. African Journal of Microbiology*, **4:**348-354.