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MOLECULAR CHARACTERIZATION OF CHITINOLYTIC STRAINS ISOLATED FROM TERRESTRIAL HABITATS

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ABSTRACT

Three actinomycete strains producing extracellular chitinase, designated as colony 130, 194 and 184 were isolated from soil samples collected from NTPC chemical plant, (Faridabad) Haryana, landfill site (Sarai Kale Khan) Delhi and sugar plant Mandi Dhanaura, U.P respectively. During primary screening diameter of zones of hydrolysis were in the range of 8 to 16 mm as reported in our previous paper Das et al., 2017. Isolates 130, 194, 184 and Streptomyces albidoflavus (NRRLB 16746) as positive control were subsequently subjected to secondary level of screening and purification. Activity of enzyme in crude extracts was in the range of 7.16 -14.12 IU/ml and in partially purified samples in the range 12.1 to 23.10 IU/ml as reported in our earlier studies [14]. Comparison and analysis of the 16S rRNA gene sequence revealed that colonies 130, 194 and 184 belonged to the genus Streptomyces. Colony 130 (Accession no: KY357309) had 97.23% sequence similarity with Streptomyces calvus ISP 5010T (AY999780), colony 194 (Accession no: KJ934595) had 96.20% similarity with Streptomyces albidoflavus DSM 40455T (Z76676) and colony 184 (KY357314) showed 98.8% similarity with Streptomyces enissocaesilis NRRL B-16365T (DQ026641). Random amplified polymorphic DNA (RAPD) was assayed to determine the genetic diversity of colonies 130 and 194 with reference strain, Streptomyces albidoflavus. Colony 130 showed around 80-90% interspecific variation, the colony has different genetic origin in comparison to the reference strain. On the other hand, colonies 194 and 184 showed around 50-60% and 20% variation respectively and hence have almost same genetic origin as S. albidoflavus.

KEY WORDS

Extracellular chitinase from actinomycetes, Primary and secondary screening, Polyphasic taxonomy, Analyses of genetic diversity by RAPD.

INTRODUCTION

Phylum Actinobacteria is widely distributed in nature and its members have potential to degrade organic matter as they are source of extracellular enzymes [1-5]. *Streptomyces* is the most dominant genus in actinomycetes and has been extensively studied over the past decades for their applications in agricultural, pharmaceutical and food industry [1,6]. Discovery of new *Streptomyces* species may help to find new and commercially beneficial products like highly thermotolerant and pH tolerant extracellular enzymes and novel antibiotics [7-9]. One way to explore new species is by mining of various underexplored habitats. Extracellular chitinases of microbial origin have widespread applications in diverse industries ranging from textile, biorefineries, food, pulp and paper, agriculture, detergent and pharmaceuticals [10-13].In the course of our study, actinomycete isolate 130 collected from NTPC chemical plant, (Faridabad) Haryana, 194 from landfill site (Sarai Kale Khan) Delhi

and 184 from sugar plant Mandi Dhanaura, U.P were screened for production of chitinase enzyme. During primary screening diameter of zones of hydrolysis were in the range of 8 to 16 mm as reported in our previous paper Das *et al.* 2017 [14]. Based on the results, colonies showing appreciable zones of clearance were selected for quantitative screening and partial purification along with the positive control strain. Enzyme activity in crude extracts was in the range of 7.16 -14.12 IU/ml and in partially purified samples in the range 12.1 to 23.10 IU/ml as reported in our earlier studies [14]. This was followed by taxonomic characterization of selected colonies using polyphasic taxonomy based on a judicious combination of morphological, biochemical and genotypic features. Genetic diversity of isolates was ascertained by Randomly Amplified Polymorphic DNA (RAPD) analyses.

Materials and Methods

1. Collection of soil samples

Soil samples were taken from different habitats. Actinomycetes were isolated by plate dilution method. Purification of colonies was done on actinomycete specific medium (Yeast Extract-Malt extract) and stocks were stored in 20% glycerol at -80ºC [15,16]. Strain NRRL B-16746 (*Streptomyces albidoflavus)* from ARS culture collection USA, was used as positive control.

2. Qualitative (primary) and quantitative (secondary) screening of strains for production of chitinase

Primary screening was done using spot inoculation on chitin agar media having 1% colloidal chitin as substrate [17,18]. Secondary screening was performed using p-dimethyl amino benzaldehyde (p-DMAB) method [19,14].

3. Polyphasic taxonomy

In present course of our studies, colonies were subjected to polyphasic characterization based on a judicious combination of morphological, biochemical and genotypic features (16s rRNA gene studies) [20,21,11].

3.1 Morphological studies

Growth studies were done by streaking the cultures on various ISP media. The plates were incubated for 10-14 days at 28ºC and were examined for growth, sporulation, release of diffusible pigments, color and texture of vegetative and aerial mycelium. Microscopic observation of spores was made by phase contrast microscope (Nikon Eclipse, TS 100) at 40X [20,21, 11].

3.2 Biochemical studies

Colonies were checked for their ability to utilize various carbon sources (Raffinose, Meso-inositol, L-Arabinose, Mannitol, L-Rhamnose, Sucrose, D-Xylose, D-Fructose), skimmed milk and hydrolysis of tween, starch and hypoxanthine [20,21,11].

3.3 16S rRNA gene studies

Standardized methods were used for isolation of genomic DNA from actinomycetes [22]. PCR-mediated amplification of 16S rRNA gene from the strain was performed. Sequencing of PCR products was done by using Micro SeqR sequencing kit (Applied Biosystems, USA) and Genetic Analyzer Sequencer (Applied Biosystems 3100 Avant™). Results were aligned manually with corresponding sequences of available *Streptomyces* drawn from the Eztaxon. An evolutionary tree was made by neighbor-joining method using Clustal_X 1.81 and Phylip 3.69. The tree topology was also confirmed by using Maximum likelihood and Maximum Parsimony method. Bootstrap resampling method (1,000 replicates) was used for evaluation of topology of the phylogenetic tree. The evolutionary tree was rooted with *Actinomadura hibisca* JCM 9627T (AF163115) as the out-group [23, 24, 11].

4. Analysis of genetic diversity by Random amplified polymorphic DNA (RAPD)

Actinomycete colonies were subjected to RAPD fingerprinting analyses using 4 different random primers (P1- ACCCCAGCCG, P2- GCAGCAGCCG, P3-CACCTGCCGC and (P4-CGCCAGGATC). Each primer was 10-mer long (Sigma Aldrich, USA). Reproducibility of bands was checked by repeating the experiment three consecutive time with same set of primers. The output of each experiment was compared to the previous one. PCR products were screened on 1.5% (w/v) agarose gel. DNA ladders of 100bp (Bangalore Genei Pvt. Ltd.) and λ DNA, double digested with *Eco*RI/*Hind*III (Bangalore Genei) were used for estimating molecular size of the bands.

4.1 Scoring, Data analysis and construction of dendrogram

For scoring the reproducibility of amplified bands obtained from each primer, the bands were manually scored as 1 or 0 on the basis of presence or absence of particular bands against each primer. For every strain, each band that was amplified was treated as a unit character. Bands were divided into three types: 1) Presence of normal bands, 2) appearance of extra bands

and 2) disappearance of normal bands. In the gel pictures, one lane with the control profile of NRRL B-16746 (*S. albidoflavus)* was taken as reference; the banding pattern of the reference lane was then compared with other lanes [25-27]. Evaluation of fragment patterns were done using Cliqs 1D pro and Alpha Imager software's.

Estimation of pair wise genetic similarities among all pair of samples was done with Jaccard's co-efficient. The matrix of binary characters prepared manually was validated by the matrix made by the software [25-27]. The matrix of binary characters prepared manually was validated by the matrix made by the software [25-27]. Dendrogram was constructed by using binary character matrix for cluster analysis based on UPGMA method using Cliqs 1D pro and Alpha Imager software's [25-27].

RESULTS

1. Collection of soil samples

Total 105 randomly selected colonies were screened for chitinase production. Colonies 130, 194 and 184 were the highest producers.

2. Qualitative (primary) and quantitative (secondary) screening of strains for production of chitinase

In our previous studies colonies 130, 194, 184 and *S. albidoflavus* (Positive control) showed high zones of hydrolysis (8 to 16mm) and subjected to quantitative screening for estimation of enzyme activity. In crude extracts enzyme activity was in the range of 7.16 -14.12 IU/ml and in partially purified samples in the range 12.1 to 23.10 IU/ml as reported in our earlier studies [14].

3. Polyphasic taxonomy

3.1 Morphological studies

Results of phenotypic characterization of isolates indicated that all the colonies were aerobic, Gram-positive actinomycetes showing formation of aerial and vegetative mycelium. Colonies showed difference in their morphological characteristics perhaps due to adaptation to their respective habitats (Table 1).

Table 1: Morphological and biochemical characteristics comparison of chitinase producing isolates among their closest clade members.

Characteristics	Isolate no 130	***Streptomyces calvus*** **(ISP 5010) *T*** (Backus et al., 1957)	Isolate no 194	***Streptomyces albidoflavus*** **(DSM 40455T)** (Rong et al., 2009)	Isolate no 184	**Streptomyces rochei (NBRC 12908)** (Berger et al., 1953)
Color of: Substrate mycelium	Light brown/white/dark grey on ISP 1,2,3,4,5,6, 7	Black grey on ISP 2 and 4 Beige on ISP3, 5, 6 Brown beige on ISP 7	Cream (ISP 3) Yellow (ISP 1, 2,6) Beige (ISP 5) Yellow-Black (ISP 4,7)	N.P.	Beige/brown on ISP 1,2,3,4,5,6, 7	N.P.
Aerial mycelium	White/dark grey on ISP 1,2,3,4,5,6, 7	N.P.	Grey (ISP 4) White (ISP 1,2, 3, 5,6) Yellow-White (ISP 7)	White/Grey (ISP 2, 5), No sporulation seen on other ISP media	White/Light brown/grey on ISP 1,2,3,4,5,6, 7	N.P.

Characteristics	Isolate no 130	*Streptomyces calvus* (ISP 5010) *T* (Backus et al., 1957)	Isolate no 194	*Streptomyces albidoflavus* (DSM 40455T) (Rong et al., 2009)	Isolate no 184	Streptomyces rochei (NBRC 12908) (Berger et al., 1953)
Growth	Good on ISP 1,2,3,4,5,6, 7	N.P.	Moderate (ISP 4,5,7) Poor (ISP 1,6) Good (ISP 2,3)	N.P.	Moderate on ISP 1,2,3,4,5,6, 7	N.P.
Sporulation	Good on ISP 1,2,3,4,5,6, 7	N.P.	Poor (ISP 1,3,6) Moderate (ISP 4,5,7)	N.P.	Moderate on ISP 1,2,3,4,5,6, 7	N.P.
Production of pigment	Brownish, weak (ISP 3) No diffusible pigment (ISP 1,2,4,5,6,7)	N.P.	No diffusible pigment on any of the ISP media	No diffisible pigment on any of ISP media	No diffusible pigment on (ISP 1,2,3,4,5,6,7)	N.P.
Spore chain	Spirales	N.P.	Rectiflexibles	Rectiflexibles	Rectiflexibles	N.P.
Utilization of:						
D-fructose	+	+	+	+	++	+.
D-Mannitol	+++	+	Weak	+	++	.+
D-Xylose	-	-	+	+	-	-
Sucrose	+	+	+	-	+	-
Meso-inositol	++	N.P.	-	+	-	N.P.
L-arabinose	-	+	+	+	-	-
L-rhamnose	-	-	+	-	++	+
Raffinose	-	+	+	-	-	-
Tween	-	NP	+	NP	+++	NP
Hypoxanthine	+++	NP	+	NP	++	NP
Urea	++	NP	+	NP	++	NP
Caesin	-	NP	+	NP	++	NP
Starch	-	NP	+	NP	+++	NP

NP = Not present in literature.

Table 2: Percentage polymorphism and monomorphism calculated for chitinase strains.

%Polymorphism/monomorphism using 4 sets of primers	Colony 130	Colony 194	Colony 184
% Polymorpism	80-90%	56-60%	20%
% Monomorphism	10-20%	40-50%	80%

Colony 130 has light brown/white/dark grey vegetative mycelium, white/dark grey aerial mycelium, moderate to good growth and good sporulation on ISP media. Spore chain morphology is Spirales (Figure 1(a)).

Colony 194 has yellow-black/yellow/beige/ creamy vegetative mycelium and gray/white/yellowish-white aerial mycelium, abundant sporulation and growth on ISP media. Spore chain morphology is Rectiflexibles (Figure 1(b)).

Colony 184 showed brown/beige substrate mycelium, light brown/ white /grey aerial mycelium, moderate

sporulation and growth. Spore chain morphologyis Rectiflexibiles (Figure 1(c)).

3.2 Biochemical studies

Colony 130 Utilizes D-mannitol, Meso-inositol, D-fructose, Sucrose. Degrades Urea, Hypoxanthine but not Starch, Caesin and Tween (Table 1).

Colony 194 Utilizes D-fructose, D-xylose, Sucrose Arabinose, Raffinose but weak utilization of D-mannitol. Good Caseinase activity. Degrades Tween 80. Good metabolism of Urea and Hypoxanthine (Table 1).

Colony 184 Utilizes D-fructose, Sucrose, D-mannitol and L-rhamnose. Degrades Urea, Casein, Tween, Starch and Hypoxanthine efficiently (Table 1).

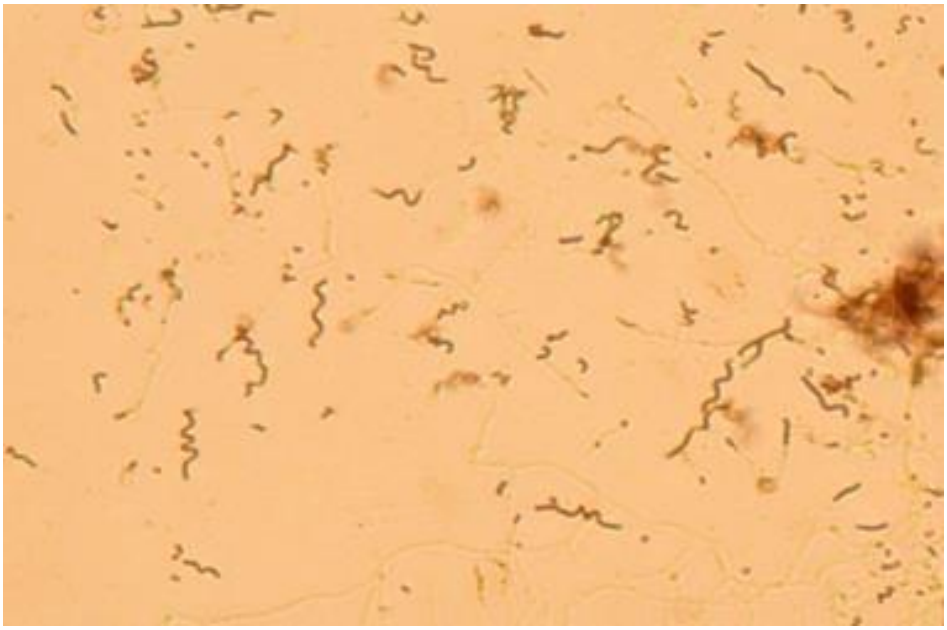

Fig. 1(a): Spore chain morphology of Colony 130: Spirals (Spirales)

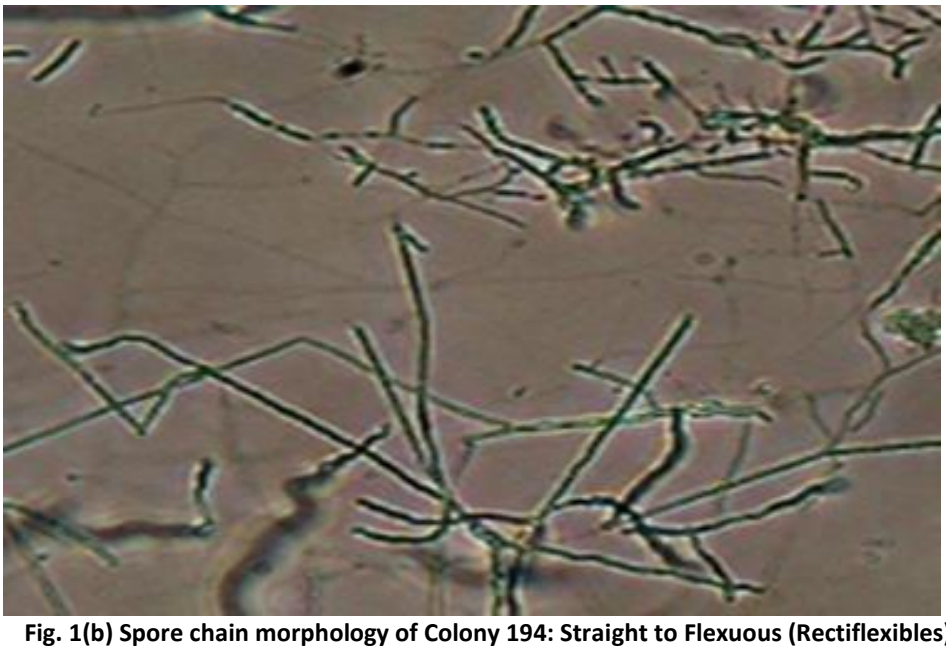

Fig. 1(b) Spore chain morphology of Colony 194: Straight to Flexuous (Rectiflexibles)

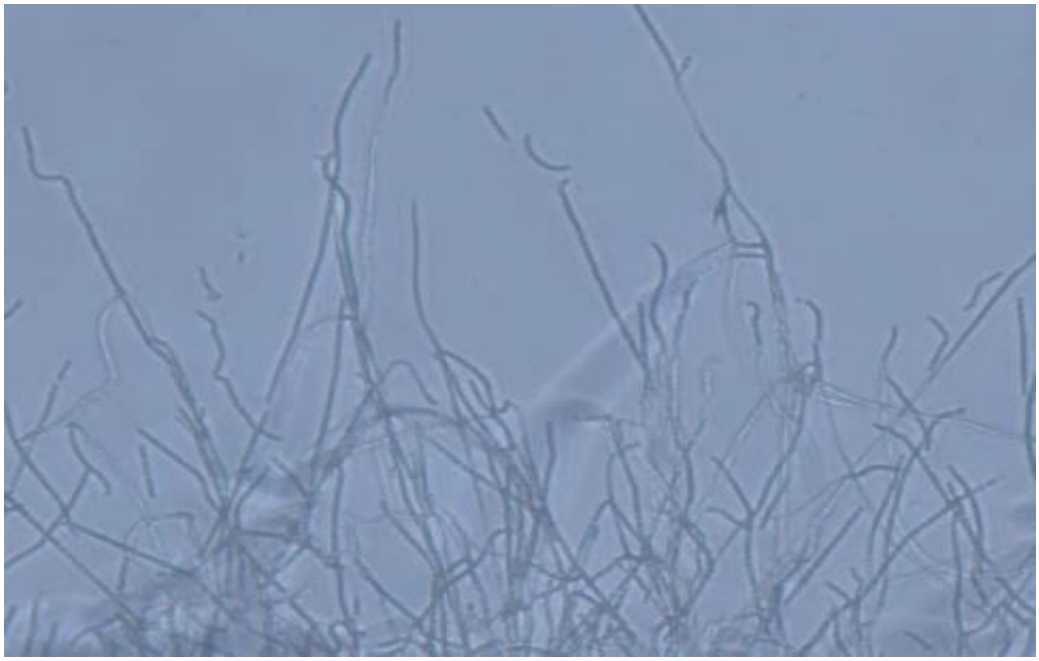

Fig. 1(c) Spore chain morphology of Colony 184: Straight to Flexuous (Rectiflexibles)

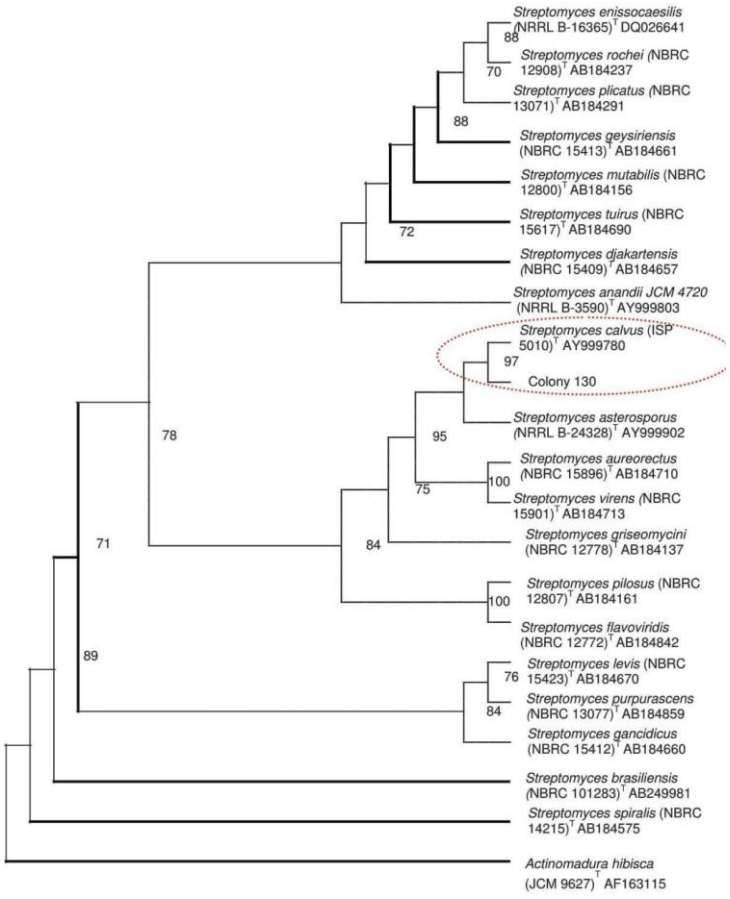

Fig. 2: Rooted evolutionary tree designed based on 16S rRNA gene sequences, depicting the phylogenetic relationship among isolate 130 (KY357309) and the related representative species of *Streptomyces*. *Actinomadura hibisca* was taken as the outgroup. This tree was designed using neighbor- joining method, Clustal_X 1.81, PHYLIP 3.69 as well as bootstrap values based on the analysis of 1,000 resampled datasets Note: Bootstrap values below 70% were omitted).

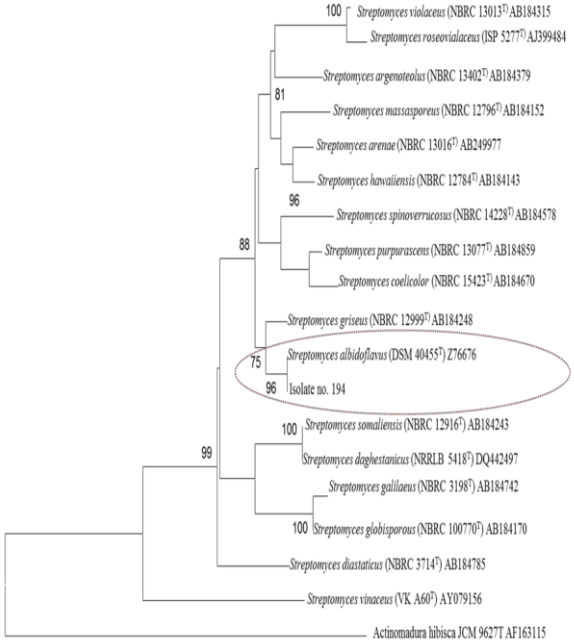

Fig. 3: Rooted evolutionary tree designed ased on 16S rRNA gene sequences, depicting the phylogenetic relationship among isolate 194 (KJ934595) and related representative species of *Streptomyces*. *Actinomadura hibisca* was taken as the outgroup. This tree was designed using neighbor- joining method, Clustal_X 1.81, PHYLIP 3.69 as well as bootstrap values based on the analysis of 1,000 resampled datasets (Das et al., 2014). (Note: Bootstrap values below 70% were omitted).

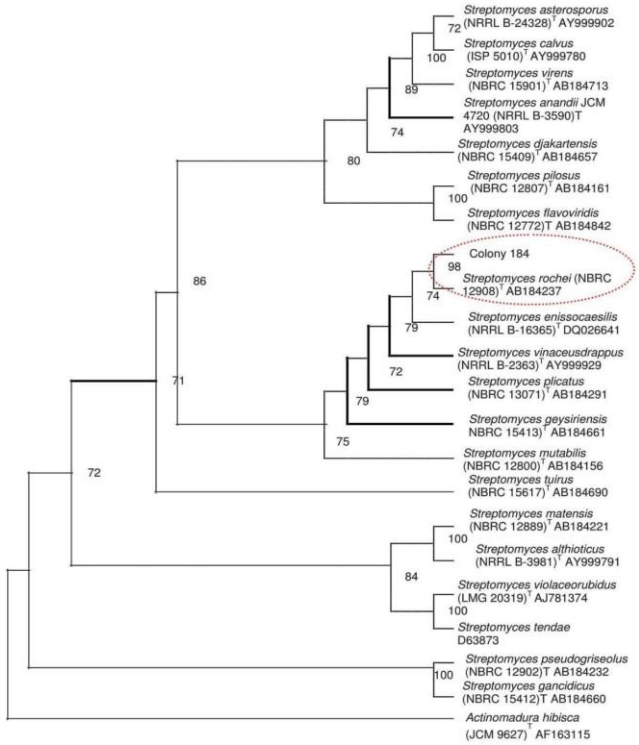

Fig. 4: Rooted evolutionary tree designed based on 16S rRNA gene sequences, depicting the phylogenetic relationship among isolate 184 (KY357314) and related representative species of *Streptomyces*. *Actinomadura hibisca* was taken as the outgroup. This tree was designed using neighbor- joining method, Clustal_X 1.81, PHYLIP 3.69 as well as bootstrap values based on the analysis of 1,000 resampled datasets. (Note: Bootstrap values below 70% were omitted).

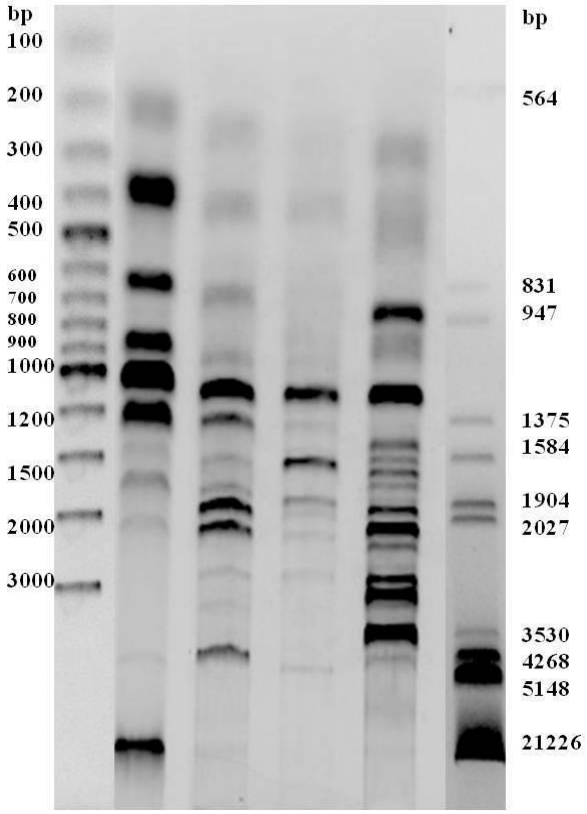

Fig. 5 (a): RAPD fingerprints of chitinase producing *Streptomyces* strains amplified using Primer 1.

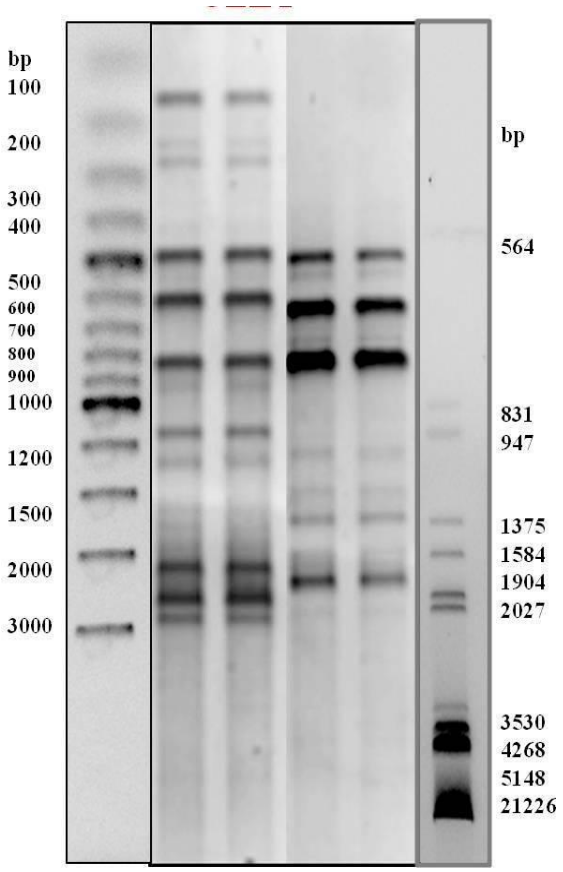

Fig. 5 (b): RAPD fingerprints of chitinase producing *Streptomyces* strains amplified using Primer 2.

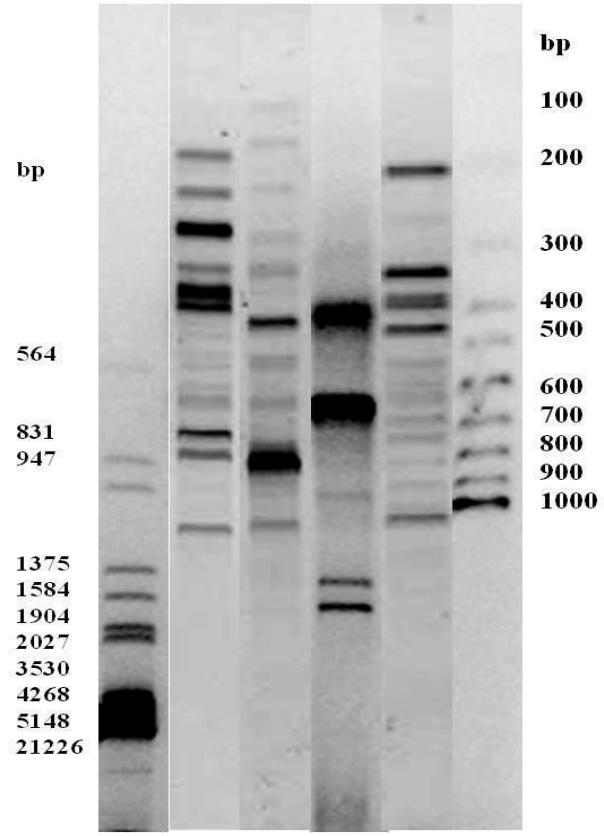

Fig. 5 (c): RAPD fingerprints of chitinase producing *Streptomyces* strains amplified using Primer 3.

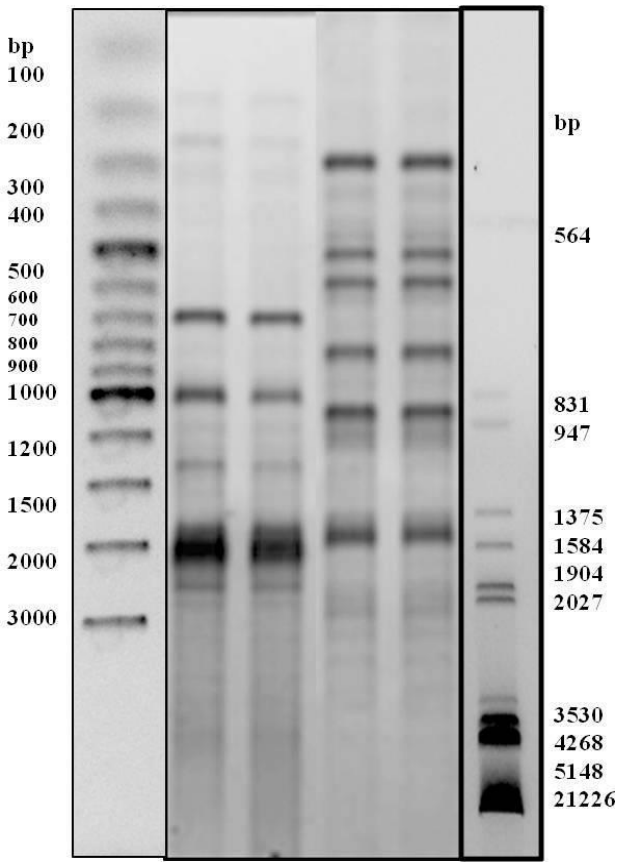

Fig. 5 (d): RAPD fingerprints of chitinase producing *Streptomyces* strains amplified using Primer 4.

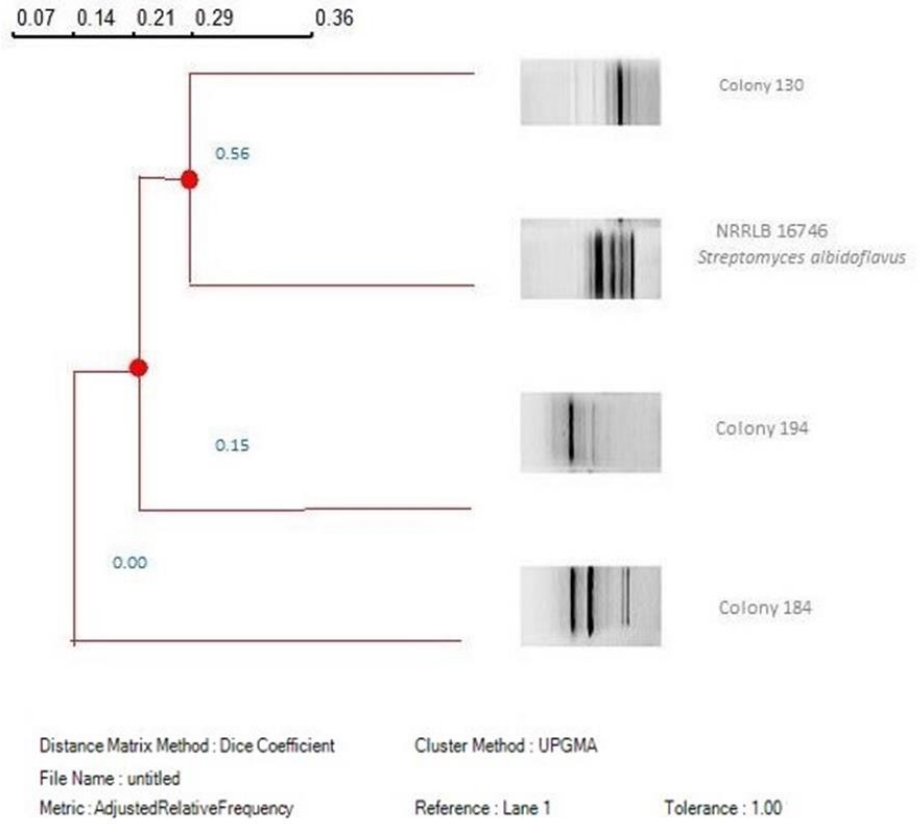

Fig.6: Dendrogram for chitinase strains using Primer 1.

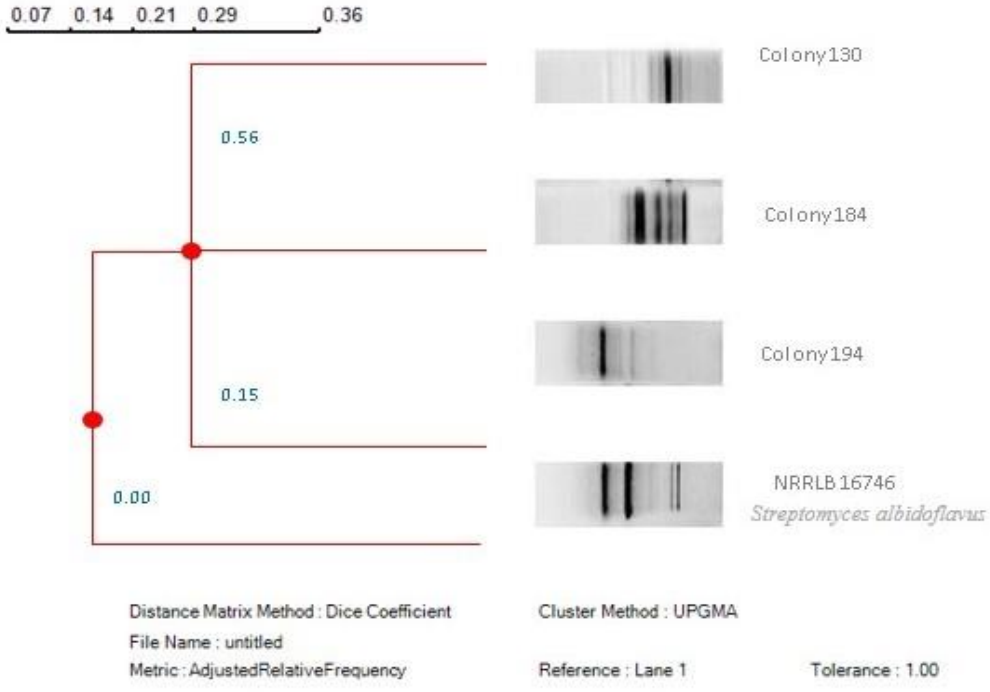

Fig.6: Dendrogram for chitinase strains using Primer 2.

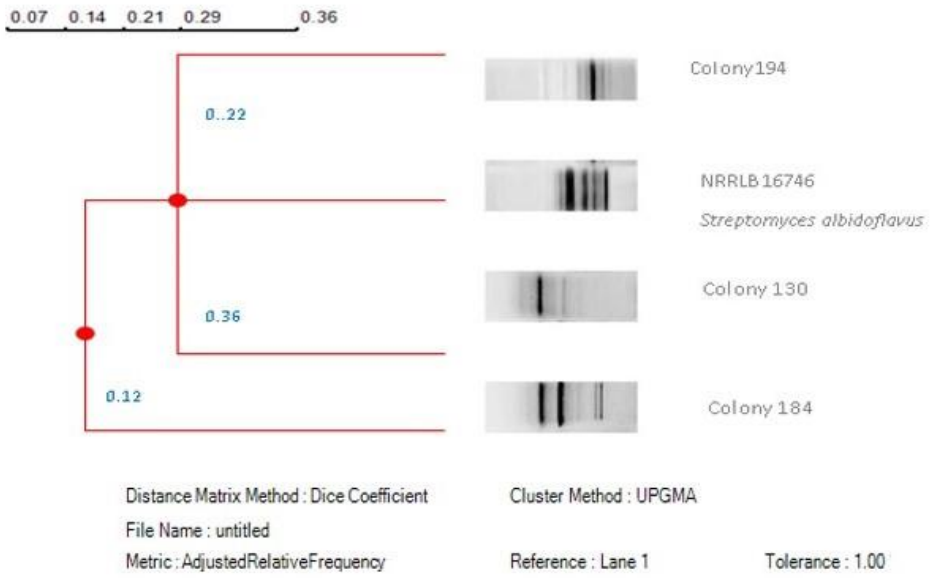

Fig.6: Dendrogram for chitinase strains using Primer 3.

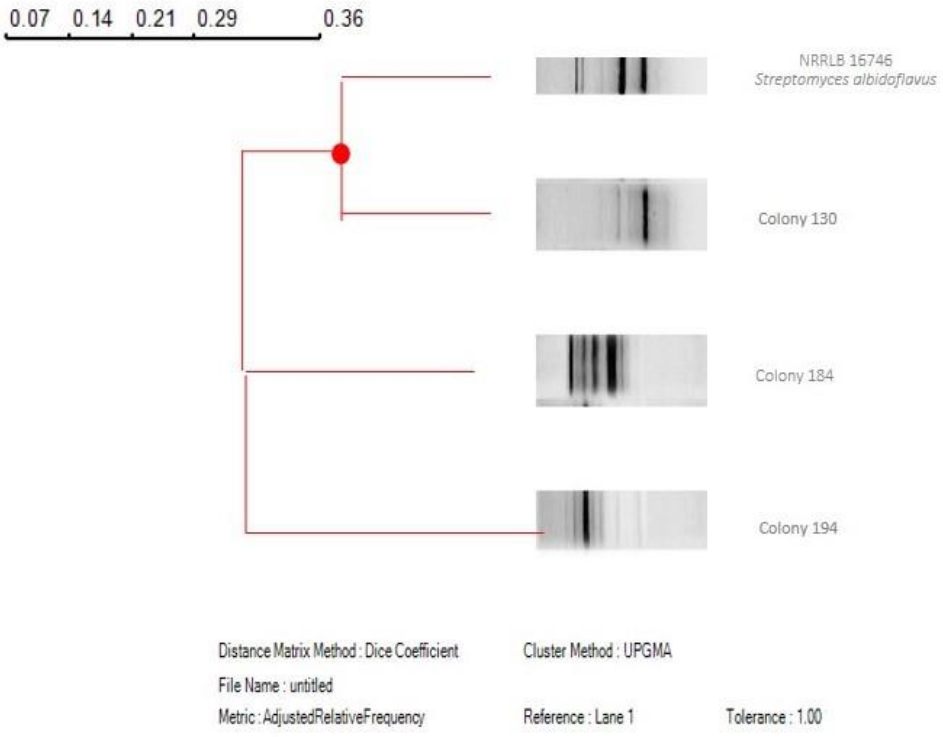

Fig.6: Dendrogram for chitinase strains using Primer 4.

3.3 16S rRNA gene studies

Comparison of 16S rRNA gene sequences of chitinase producing colonies 130 (1412bp), 194 (1432bp) and 184 (1415bp) with sequences of close *Streptomyces* species deposited in databases, indicated that these isolates belong to genus *Streptomyces.* These actinomycete isolates were represented as distinct clades in their respective rooted evolutionary trees designed by neighbor-joining method. Colony 130 showed 97.23% similarity with *Streptomyces calvus* ISP 5010T

(AY999780) (Figure 2). Similarity of 96.20% with *Streptomyces albidoflavus* DSM 40455T (Z76676) was observed in case of colony 194(Figure 3). Colony 184 showed 98.8% similarity with *Streptomyces enissocaesilis* NRRL B-16365T (DQ026641) (Figure 4). The 16S rRNA gene sequences of isolates were submitted to GenBank nucleotide database of NCBI and were assigned accession numbers KY357309 (colony 130), KJ934595 (colony 194) and KY357314 (colony 184) respectively. Comparison of biochemical and morphological features of these isolates with their closest clade members has been shown in Table 1.

4. Analysis of genetic diversity by Random amplified polymorphic DNA (RAPD)

RAPD profiles were generated using a set of 4 primers for amplification of genomic DNA from chitinase producing strains. Colonies 130, 194 and 184 showed approximately 80- 90%, 50-60% and 20% polymorphism in comparison to the reference strain (NRRL B- 16746 (*S. albidoflavus)*) as per the band matrix calculated using Cliqs 1D Pro and Alpha- Innotech softwares. Based on the observations, colony 130 was found to be genetically diverse as compared to the reference strain whereas colonies 194 and 184 were genetically related to reference (Table 2 and Figure 5).

4.1 Scoring, Data analysis and construction of dendrogram

Dendrogram assembled all the chitinase producing colonies in one super cluster. In case of primers 1 and 4, super cluster got further divided into three clusters, one containing the outgroup, one for colony 194 and the last branch bifurcates into two and contains colony 130 and NRRL B-16746 (Figure 6). In case of primers 2 and 3, the super cluster got divided into two clusters, one for outgroup and one for colonies 130, 194 and NRRL B-16746 (Figure 6). A hypothetical correlation of RAPD results with enzyme activity showed primers 1 and 4 cluster highest enzyme producers (colony 130 and NRRL B-16746) together in one clade and lower enzyme producers (colony 194 and 184) in another clade.

DISCUSSION

Actinobacteria are well known for extra cellular enzyme production. This group of bacteria have been isolated and studied by researchers for their potential to produce chitinase [10,11,21,24,28,29]. In the present study, phenotypic and taxonomic characterization of colonies 194,130 and 184 (chitinase producers) showing extracellular enzymatic activity was done as per the protocol reported by Shirling and Gottlieb, 1966 [30]. Genus of the colonies was tentatively determined by morphological studies which included color, sporulation of bacterial colonies, size, shape, production of diffusible pigment and absence or presence of aerial mycelium on different ISP media as reported in literature [23, 31,32, 33, 34, 35, 36, 37, 38,]. Observation of spores and mycelia was made by phase contrast microscope [23,33,36,39,40,]. Biochemical studies were based on utilization of carbon sources, urea, skimmed milk, degradation of organic compounds such as hypoxanthine, xanthine, tyrosine, casein [30,32,34,38]. The results revealed that all the colonies were Gram-positive, aerobic actinomycetes capable of forming both vegetative and aerial mycelium.

However, differences in biochemical or morphological features observed among the colonies could be because of their adaptation to diverse habitats [23,31,32,33,34,35,36,37,38]. Comparison of strains with their nearest relatives in terms of phenotypic and biochemical features indicated similarities and dissimilarities on the basis of literature review [23,32,34,37,38,41,42]. Thirumurugan *et al.,* 2015 [21] characterized actinobacteria strains showing maximum chitinase activity on the basis of their morphological features (aerial spore mass color, melanoid pigments, reverse side and spore chain morphology). From the 101 colonies tested, 27 were morphologically distinct isolates that produced powdery colonies with different aerial mass color and reverse side pigments (red, black, white, ash, brown and yellow/orange). Isolates were positive for the biochemical characteristics such as production of citrate, amylase, cellulase, catalase and oxidase and negative for H2S production, haemolysis and urease. Wang *et al.,* 2015 [43] studied the culture characteristics of chitinase producing strain Bn035 such as growth and color of mycelium, as well as the amount of soluble pigment by streaking the cultures on different ISP media. Morphological features like aerial hyphae, base mycelium, spore body and spore wire was also studied. It was concluded from the results that Bn035 was Gram-positive strain, with spirales spore chain morphology, showed yellow or white mycelium on plates without producing any diffusible pigment, hydrolyzed starch and cellulose, reduced nitrate, utilized D-galactose, D-glucose, D-xylose, sucrose, L-arabinose, D-mannitol, L-inositol, maltose, L-rhamnose,

cellobiose, D-raffinose, lactose, glycerol and mannitol. Comparison of 16S rRNA gene sequences of chitinase producing colonies 130 (1412bp), 194 (1432bp) and 184 (1415bp) with sequences of close *Streptomyces* species deposited in databases, indicated that these isolates belong to genus *Streptomyces.* Chitinolytic *Streptomyces* strain FS2 was identified using 16S rRNA sequence analysis by Santhi [24]. For this purpose, genomic DNA was isolated using the small-scale method, 16S rDNA was amplified using PCR with universal primers. PCR amplified product was sequenced for validation of the results. The sequence was later subjected to sequence similarity search using BLAST tool for the identification of the strain *Streptomyces* sp. FS2. Based on the observations made during phylogenetic analyses, it can be concluded that colonies 130 and 194 which showed 97.2% and 96.20% similarity with their respective clade members were more diverse as compared to colony 184 which showed 98.8% similarity with its close relatives. Hence, colony 130 and 194 could be novel chitinase producers that have been isolated from two diverse habitats: NTPC Chemical Plant (Faridabad), Haryana and landfill site (Sarai Kale Khan), Delhi respectively.

Isolates were subjected to RAPD genetic diversity analyses using 4 different random primers. The data obtained during this study was comparable to the results reported in literature. Boroujeni et al., 2012 [44] reported molecular characterization of 13 multiple enzyme producing (chitinase, cellulose and xylanase) actinomycete strains by RAPD fingerprinting technique using 20 random primers. PCR amplifications were done, and the amplified products were run on agarose gel and photographed. Data was analyzed by band-based Dice method [45] and clustered in dendrogram by UPGMA method using software (AlphaImager HP 3.4.0.0). In case of enzyme activity, it was found that most of the isolates of cluster 1 were able to produce all the three enzymes.

Isolate I-7 which showed maximum chitinase and xylanase activities was added in cluster I whereas I-27 which showed maxiumum cellulase production was placed into cluster 3.

CONCLUSION

Isolates 130 and 194 are efficient chitinase producers, results of present study revealed that these isolates could be novel producers of chitinase that have been isolated from two diverse habitats. Taxonomic studies of these strains may be helpful for the future series of works such as in growing field of enzyme engineering and waste biodegradation.

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