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Characterization and Phylogenetic Analysis of Cellulase Producing *Streptomyces noboritoensis* SPKC1

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Abstract: A cellulase producing strain of Actinomycetes was isolated from soil samples which were collected from Southwest ghats, Kerala, India at a depth of 6 – 12 inches and Actinomycetes was characterized by morphological, cultural, physiological, chemotaxonomical and phylogenetic analysis. The 16S rRNA region of this strain was amplified and sequenced. The Neighbor-joining and Maximum Parsimony algorithm with topology tree of 16S rRNA was constructed. Based on results of observation and phylogenetic analysis, the strain SPKC1 was proved to belong to the species *Streptomyces noboritoensis* with cellulase activity. The Carboxy Methyl Cellulose (CMCase) activities of the strain SPKC1 on eighth day an amount of 910 μg/ml of glucose, 210 μg/ml of protein and 850 mg/100 ml of growth (biomass) on ninth day were recorded. In exocellulase activity strain SPKC1 on first day an amount of 500 μg/ml glucose was produced.
Key words: *Streptomyces noboritoensis*, cellulose, CMCase, 16S rRNA, phylogeny.

1 Introduction

Streptomycetes are Gram-positive soil microorganisms that produce a wide variety of enzymes and secondary metabolites, many of which have potent biological activities. They produce more than half of the known biologically active microbial products, including many commercially important antibiotics, immunosuppressive compounds, animal health products, and agrochemicals. They also produce various enzymes that are commercially and academically valuable. This vast reservoir of diverse products makes *Streptomyces* one of the most important industrial microbial genera (Herai *et al.*, 2004).

The chemical diversity of microbial products anticipates their functional diversity. Enzymes have been produced commercially from plant, animal and microbial sources. The processing of plant and animal litter by the soil population is vital for the maintenance of soil fertility. It involves the co-operative action of many different groups of organisms including bacteria, fungi, actinomycetes, protozoa and nematodes (Beare *et al.*, 1992). There are a number of cellulose degrading microorganisms, which includes bacteria, fungi and actinomycetes. Actinomycete's, one of the known cellulase-producers, has attracted considerable research interest due to its potential applications in recovery of fermentable sugars from cellulose that can be of benefit for human consumption and to the ease of their growth (Jang and Cheng, 2003) compared to anerobic cellulase producers such as *Paenibacillus curdlanolyticus* (Pason *et al.*, 2006). The biotechnology applications of cellulases began in the early 1980s in animal feed followed by food applications (Harchand and Singh, 1997). Today, these enzymes account for approximately 20% of the world's enzyme market (Jaradat *et al.*, 2008).

The *Streptomyces* are able to utilize a wide range of organic compounds as a carbon source, including complex biological materials such as cellulose and lignin and can also utilize an inorganic nitrogen source (Kutzner, 1986). Taxonomy of actinomycetes is very difficult because of their morphological and physiological similarities. Bacterial analysis by 16S rRNA has become popular because these sections of genes are easy to sequence. Determination of difference in the sequence of the 16S rRNA gene is well established as a standard method

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for the identification and phylogenetic classification of prokaryotic species, genera and families (Michael and Sharon, 2007), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. In that way, in this investigation cellulase producing strain SPKC1 was isolated from soil samples and characterized to belong to *Streptomyces noboritoensis* with cellulase activity by morphology, biochemical and phylogenetic analysis.

2 Material and methods

The soil samples containing rich of actinomycetes were collected from Southwest ghats, Kerala, India at a depth of 6 – 12 inches. The soil samples were air dried in a hot air oven at 45℃ for 1 hr to reduce the proportion of bacteria other than Actinomycetes (Williams *et al.*, 1972). Standard dilution plate technique was followed for the isolation of Actinomycetes (Kuster and Williams, 1964). 10 gram each of the soil samples were added to 90 ml distilled water in a 250 ml Erlenmeyer flask under sterile condition and kept in a rotary shaker (120 rpm) at room temperature for 30 min. Vacuum filtration was used for collecting supernatant, and then the supernatant was serially diluted to obtain 10^{-4}, 10^{-5} and 10^{-6} dilutions. Each dilution was plated on sabouraud's agar. After inoculation of 7 – 9 days at 27±1℃ the actinomycetes colonies were selected, counted and made into pure culture following single spore culture technique. The culture was maintained on sabouraud's agar by periodical sub-culturing.

Fifteen colonies were used for screening process; the inorganic salts cellulose agar was prepared. 20ml of the medium was poured in to the petriplates. The isolated Actinomycetes were inoculated into different petriplates and incubated at room temperature (27±1℃) for 7 days then the plates were taken and 0.1% congored was spreader over the plates. After half hour incubation 0.5 M NaCl was spread. Then the 0.5 M NaCl was discarded, the presence of a clear zone indicates the cellulolytic activity of the strain. The diameter of the zone was measured and based on this activity of the organisms were categories as weak and promising ones.

2.1 Assay of cellulase

The inorganic salts cellulose broth supplemented with 1% Carboxy Methyl Cellulose (CMCase) and 1% yeast extract was prepared. 100 ml of the broth was taken in 250 ml Erlenmeyer flasks and inoculated heavily using the individual stock cultures maintained on sabouraud's slants. The flasks in duplicate were incubated at room temperature at 120 rpm. During incubation, every day 1 ml of the broth was collected up to 12^{th} day for CMCase assay. In endocellulase determination the ß (1 – 4) glucanase was determined by measuring the reducing sugar as glucose by dinitrosalicyclic acid (DNS) method (Miller, 1959). The reaction mixture was contained 1 ml of w/v CMC in citrate buffer pH 5.0 and 1ml of the broth, which was collected on first day to seven days. The assay was started by incubating the reaction mixture at 50℃ for 20 min. The reaction was stopped by the addition of 3 ml DNS reagent and boiling for 5 min. After cooling the absorbance was read at 540 nm in a colorimeter. Gluscose was used as the standard and the reducing sugar formed was calculated as the glucose equivalent. One unit of CMCase was expressed as 1 μmol of reducing sugar released per min per ml. In exocellulase activity, the Whatman No.1 filter papers were cut into 4 mm dia pieces. A reaction mixture was contains 1 ml of 1% citrate buffer pH 5.0 and 1 ml of the broth was prepared. 30 mg filter papers were added to it. Further assay was similar to the above. For determining the amount of cellulase (protein estimation) produced by strain SPKC1 was performed by the method of Lowry *et al.* (1951).

2.2 Protein extraction

Extraction was carried out with buffers used for the enzyme assay. 500 mg of the sample was weighed, ground well and grinded well with a mortar and pestle in 5 – 10 ml of the buffer. Protein estimation was carried out based on method described by Lowry *et al.* (1951). Standard graph was sketched and calculated the amount of protein in the sample. Expressed the amount of protein in IU/ml of culture broth was used for calculation. Estimating a biomass, 100 ml culture was filtered through a pre-weighed and dried Whatman filter paper. The filter paper was dried at 40℃ for 24 hrs and the weight was taken in a weighing balance. The biomass was expressed in mg/ml. Based on the performance of cellulose activity out of 15 one colony was taken for further study.

2.3 Morphology

The determination of Aerial mass colour, reverse colony colour and the 7 to 14 days old cultures growth characters in different culture media which were reported by International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). Medium ISP-1 to ISP-7 was used according to description of Shirling and Gottlieb (1966). The colour of soluble pigments were noted and compared with Methuen hand book of colour which was noted from Kornerup and Wanscher (1967). The microscopic studies were prepared according to the method described by Chakrabarthi (1998). The pure cultures of the Actinomycetes strains were inoculated to the contract line of immersed cover slips at an angle of 45° on Sabouraud's agar plates prepared aseptically. The plates with cover slips were incubated at 28℃ for 4 – 8 days. After attaining suitable growth with spore chains the cover slips were removed and placed on micro slides with the growth on the upper surface. The slides were viewed under a phase contrast microscope (Nikon Optiphot-II). The details of

the conidial chain/sporangium, spores, Arial and substrate mycelium and fragmentation of the mycelia were noted and the slides were photographed at suitable magnifications. In physiological and biochemical studies, melanin production was determined on ISP-6 media; after 4 days of growth the colour change of media were noted by the methodology described by Shirling and Gottlieb (1966).

2.4 Carbon source utilization

Different organic carbon sources were sterilized using di-ethy1 ether and incorporated to carbon utilization agar (Shirling and Gottlieb, 1966). These include D-Fructose, meso-Inositol D-mannitol, Raffinose, L-Rhamnose, Sucrose and D-Xylose at 1% w/v. Results were determined after 7, 14, and 21 days by comparing with a negative control (without carbon source) and a positive control containing D-Glucose. In Chemotaxonomical studies, the Actinomyces strains were inoculated in 100 ml sabaraud's broth in Erlenmeyer flask and incubated for 5 days at room temperature in a rotary shaker (120 rmp). The cells were killed with formalin (con.1%) for 24 hr at room temperature and harvested by centrifugation. The cells were washed once in double distilled water and once in 95% ethanol and then dried by overnight heating in a hot air oven at 45℃. The dried cells were analyzed for Diaminopimilic acid (DAP) and carbohydrates (Staneck and Roberts, 1974).

2.5 Amplification and phylogenetic analysis of 16S rRNA

Samples used for DNA extraction were collected from a highly sproulated growth on sabouraud's agar were inoculated to 25 ml yeast extract malt extract (YEME) broth supplemented with 0.5% glucose and 5 mM $MgCl_2$ in a 100 ml Erlenmeyer flask, incubated for 2 – 3 days at room temperature at 10 rpm. Genomic DNA was isolated by the method described by Murray and Thompson (1980). The integrity of the obtained genomic DNA was detected by electrophoresis in 1% agarose gel (Sigma Aldrich, India) stained with ethidium bromide. The 16S rRNA region was amplified with forward 5' GATCCTGGCTCAGGATGAAC 3' and reverse 5' GGACTACCAGGGTATCTAATC 3' bacterial universal primer pairs. The reaction mixture for PCR amplification was prepared in a total volume of 50 μl with 10 x PCR buffer, 10 mM of dNTPs, 1.0 μl of the taq polymerase and 200 pmol of forward and reverse primers. The amplifications were performed in a DNA thermal cycler 480 (Perkin Elorer, USA). The PCR reaction details were as follows: 5 min at 95℃ for initial denaturation, 1 min at 95℃ for denaturation, 2 min at 56℃ for annealing, 1 min at 72℃ for extension with total 34 cycles of amplification and 10 min at 72℃ for the final extension. The 16S rRNA (FJ769838) was purified using GenElute^{TM} Gel Extraction Kit (Sigma Aldrich, USA) and sequencing of 16S rRNA gene was done in an automated ABI-3100 Genetic Analyser (GeNei, India).

BLASTN (optimized for megablast) searches were manipulated with the sequences of *Streptomyces noboritoensis* SPKC1. The corresponding sequences of representative species were used for phylogenetic analyses. MEGA 4.1 software programme was started with a set of aligned sequences using Clustal W, and searches for phylogenetic trees that are optimal according to Neighbor-Joining (NJ) and Maximum Parsimony (MP) algorithms (Tamura *et al.*, 2007). The GenBank accession number of sequence reported in this paper is FJ769838.

3 Results and discussion

3.1 Morphology

In this study, 15 strains were obtained and one cellulase producing colony was found, it could be selected for further studies and termed as SPKC1. Cellulase activity of strain SPKC1 on inorganic salt cellulose agar was found to be 26 mm diameter zone after 7 days of incubation at 28±1℃. The isolate SPKC1 gives morphology similar to that of a *Streptomyces* isolate with a distinct substrate and aerial mycelium with conidial chains observed on microscopic examination. The conidial chains with more than 50 spores were observed and these were oblong in shape. The spore chains of *Streptomyces* may be of different type, straight to flexuous (Rectus-flexibilis), open loops (Relinaculam – apertum), pen of closed spirals (spira) and verticillate (Pridham *et al.*, 1958). The microsopic examinations of the present isolate SPKC1 shows that the conidial chains are Rectus – flexibilis. The cell wall analysis shows the presence of LL-Diaminopimilic acid (LL-DAP) as major cell wall peptidoglycan content. There were no diagnostic sugars detected in cell wall. So the isolate SPKC1 was identified as a representative of genus *Streptomyces* (Waksman and Henrici, 1943).

Table 1 shows the cultural characteristic of the isolate after an inculbation period of one week at 28±1℃. The spore mass colur of the *Streptomyces* members may vary from white, gray, red, yellow, green and blue to violet. Colour of the aerial mycelium is one of the prominent identification characters of *Streptomyces* isolates at species level (Pridham and Tresner, 1974). On further examination of the morphological characters the isolate is having a spore mass colour of predominantly white and occasionally grey. The International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) has recommended recording of aerial mycelial colour in different media for use as a taxonomic character, which was also followed in the present study. ISP-6 was showed (Figures are not shown) the reverse colony colour is yellow brown and is not producing any diffusible pigments. The isolate was showing good growth on ISP media and other suggested media (Table 1).

The above-mentioned characters suggest that the isolate SPKC1 can be identified as *Streptomyces* groups of white series (Nonomura, 1974).

The isolate was utilizing D-fructose, Meso-ionsitol, Mannitol, Raffinose, Rhamnose and D-xylose as the carbon source. In addition, isolate SPKC1 was not utilizing sucrose as the carbon source. The utilization of carbon compounds is an important aid for species determination among actinomycetes (Pridham and Gottlieb, 1948). The isolate SPKC1 utilized almost all carbohydrates, but not sucrose. Likewise, the pigment production stands out as a characteristic tool in identifying the various microorganisms. However, SPKC1 does not produce any of these pigments, these minor physiological characters are not delaminating the identification of this strain as *Streptomyces noboritoensis.* The keys of Nonomura (1974), Szabo *et al.* (1975) and Shirling and Gottlieb (1966) with based on ISP description giving emphasis to aerial mass colour, melanin pigment production, spore chain morphology and carbon source utilization pattern, the isolate SPKC1 was identified as *Streptomyces noboritoensis.*

3.2 Cellulase activity of the isolate SPKC1

Streptomyces species have been always a source of thousands of bioactive compounds. Enzyme is one of the important products of this unusual group of bacteria. Endocellulase activity, the CMCase activites of the isolate SPKC1 on different days of incubation is shown in Fig. 1a. The isolate was showing good CMCase activity on the eighth day. On the eighth day an amount of 910 μg/ml of glucose and 210 IU/ml of protein were recorded at pH 5 and temperature is 50℃. The growth (biomass) was 850 mg/100 ml on the ninth day. Exocellulase activities of the isolate SPKC1 on different days of incubation are shown in Fig. 1b. The isolate was showing maximum exocellulase activity on first day of incubation. On the first day an amount of 500 μg/ml glucose was produced. Jaradat *et al.* (2008) have observed that *Streptomyces* strain J2 is showed the highest crude enzyme activity (432 U/l) after 3 days of incubation at pH 7 and 60℃ of temperature. This result is considerably similar to what was reported by Theberge *et al.* (1992) who showed that the optimum pH for endoglucanase from a strain of *Streptomyces* lividans was 5.5. However, the results appeared to contradict previous results reported by Solingen *et al.* (2001) of an alkaline novel *Streptomyces* species isolated from east African soda lakes that have an optimal pH of 8, highlighting the effect of alkaline environment on the adaptation of these *Streptomyces.* Furthermore, the maximum CMCase activity of isolate SPKC1 was recorded at 50℃ with no significant difference between 50 and 60℃. These results are in agreement with results reported by McCarthy (1987), who reported an optimal temperature for cellulase activity in the range of 40 – 55℃ for several *Streptomyces* species including *S. lividans, S. flavogrisus, and S. nitrosporus.* Jang and Chen (2003) described a CMCase produced by a *Streptomyces* T3-1 with optimum temperature 50℃, whereas Schrempf and Walter (1995) described a CMCase production by a *S. reticuli* at an optimum temperature 55℃.

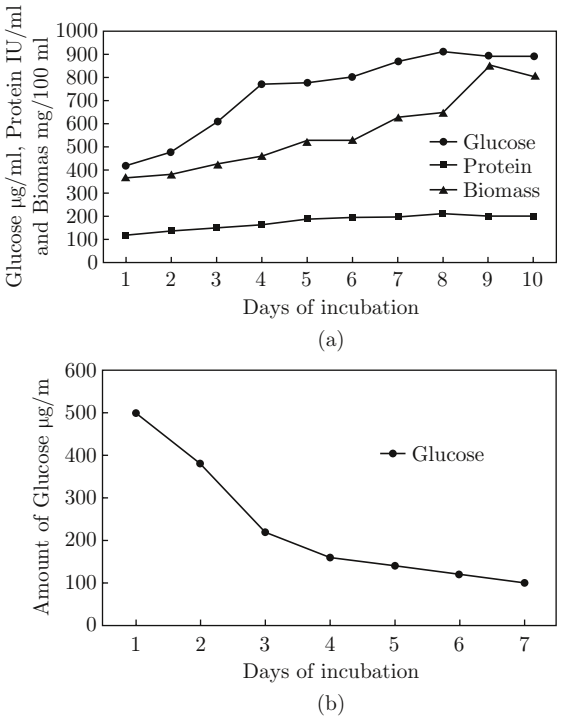

Fig. 1 Cellulase activities of strain SPKC1. (a) CMCase activities of the *Streptomyces* isolate SPKC1. (b) Exocellulase activities of *Streptomyces* isolate SPKC1

Isolate SPKC1 was showed high cellulolytic activity in the preliminary screening. Schlochetermeier *et al.* (1992) and Schrempf (1989) reported that Actinomycetes are playing an important role in degradation of cellulose and *Streptomyces reticuli* is a soil bacterium, which hydrolyzes crystalline cellulose due to the action of exocglucanase. Kamini *et al.* (1999) also reported *Streptomyces* species with cellulose activity. In this study the present strain SPKC1 was showing good endocellulolytic activity on the eighth day and exocellulolytic activity on the first day. According to the above evidences, the present strain SPKC1 was identified as a high cellulose producing *Streptomyces* species.

3.3 Phylogenetic analysis

A 567 bp sequence was amplified from the genome DNA with the bacterial universal primers, its sequence was submitted to GenBank (FJ769838). As is shown in Table 2, strain SPKC1 16S rRNA gene had 100% identity (E value 0.0) with 16S rRNA gene of *S. noboritoensis* (EU857664). The 16S rRNA gene showed high similarity with 16S rRNA genes deposited in the GenBank (Table 2). In this study, 16S rRNA gene

Table 1 Cultural characteristics of isolate SPKC1

Medium	Growth	Reverse Colony Color	Aerial mycelium (Growth and Color)	Pigment production
ISP1	Poor	Yellow Brown	Absent	None
ISP2	Good	Dark Brown	Good and light gray	Melanin
ISP3	Good	Yellow Brown	Good and light gray/white	None
ISP4	Poor	Yellow Brown	Absent	None
ISP5	Good	Dark Brown	Absent	Melanin
ISP6	Good	Yellow Brown	Good and light gray	Melanin
ISP7	Good	Yellow Brown	Good and light gray/white	Melanin
Sabouraud's	Good	Yellow Brown	Good and light gray/white	None

of different *Streptomyces* species (different strains of a species) was obtained by BLASTN search, however 20 strains of *Streptomyces* species were selected on the basis of high identity (%) with good E value for phylogenetic analysis. As the number of sequences available for analysis continues to grow, the structure of phylogenetic trees derived from 16S rRNA sequences becomes both more intricate and more accurate. Pernodet *et al.* (1989) reported that 16S rRNA and 23S rRNA of various *Streptomyces* species were partially sequenced and used for defining all members of the genus, groups of species or individual species. As shown in Fig. 2(a) (NJ Algorithm) four strains belonging to Streptomycetaceae were relatively closely related to *Streptomyces*; strain SPKC1 was on the branch with *S. noboritoensis* (EU857664). As shown in Fig. 2(b) (NJ Algorithm with topology) two strains belonging to Streptomycetaceae were relatively closely related to *Streptomyces*. Strain SPKC1 had the own branch with *S. noboritoensis* (EU857664). Figs. 2(c) and 2(d) (MP Algorithm and MP Algorithm with topology) shown strain SPKC1 had own branch and cluster with *S. phaechromogenes* (FJ486378), *S. noboritoensis* (EU857664) and *S. melanogenes* (AB184222). These results supported that the phylogenic position of strain SPKC1 in the genus *Streptomyces* might belong to the family Streptomycetaceae (Kataoka *et al.*, 1997).

Table 2 Results of similarity searches between 16S rRNA genes isolated in the present investigation and GenBank accessions using BLASTN Algorithm (optimized for megablast)

GenBank Accession No. (SPKC1)	Highly Identical Species	Matched Sequence Accession No.	Sequence Identity (%)	E-value
FJ769838	*Streptomyces noboritoensis*	EU857664	100	0.0
	Streptomyces phaeochromogenes	FJ486378	99	0.0
	Streptomyces melanogenes	AB184222	99	0.0
	Streptomyces olivochromogenes	EU841608	99	0.0
	Streptomyces crystallinus	AB184652	99	0.0
	Streptomyces flavotricini	FJ532405	98	0.0
	Streptomyces polychromogenes	FJ547116	98	0.0
	Streptomyces erythrochromogenes	AB184746	98	0.0
	Streptomyces globosus	EU196532	98	0.0
	Streptomyces lavendulae	EF371426	98	0.0
	Streptomyces bikiniensis	EF620359	98	0.0
	Streptomyces gobitricini	AB184666	98	0.0
	Streptomyces lavendofoliae	AB184217	98	0.0
	Streptomyces herbaricolor	DQ442505	97	0.0
	Streptomyces lilaceus	AB184457	97	0.0
	Streptomyces katrae	EF654092	97	0.0
	Streptomyces racemochromogenes	AB184235	97	0.0
	Streptomyces lavendulocolor	AB184216	97	0.0
	Streptomyces luridus	AB184150	97	0.0
	Streptomyces toxytricini	EU841711	97	0.0

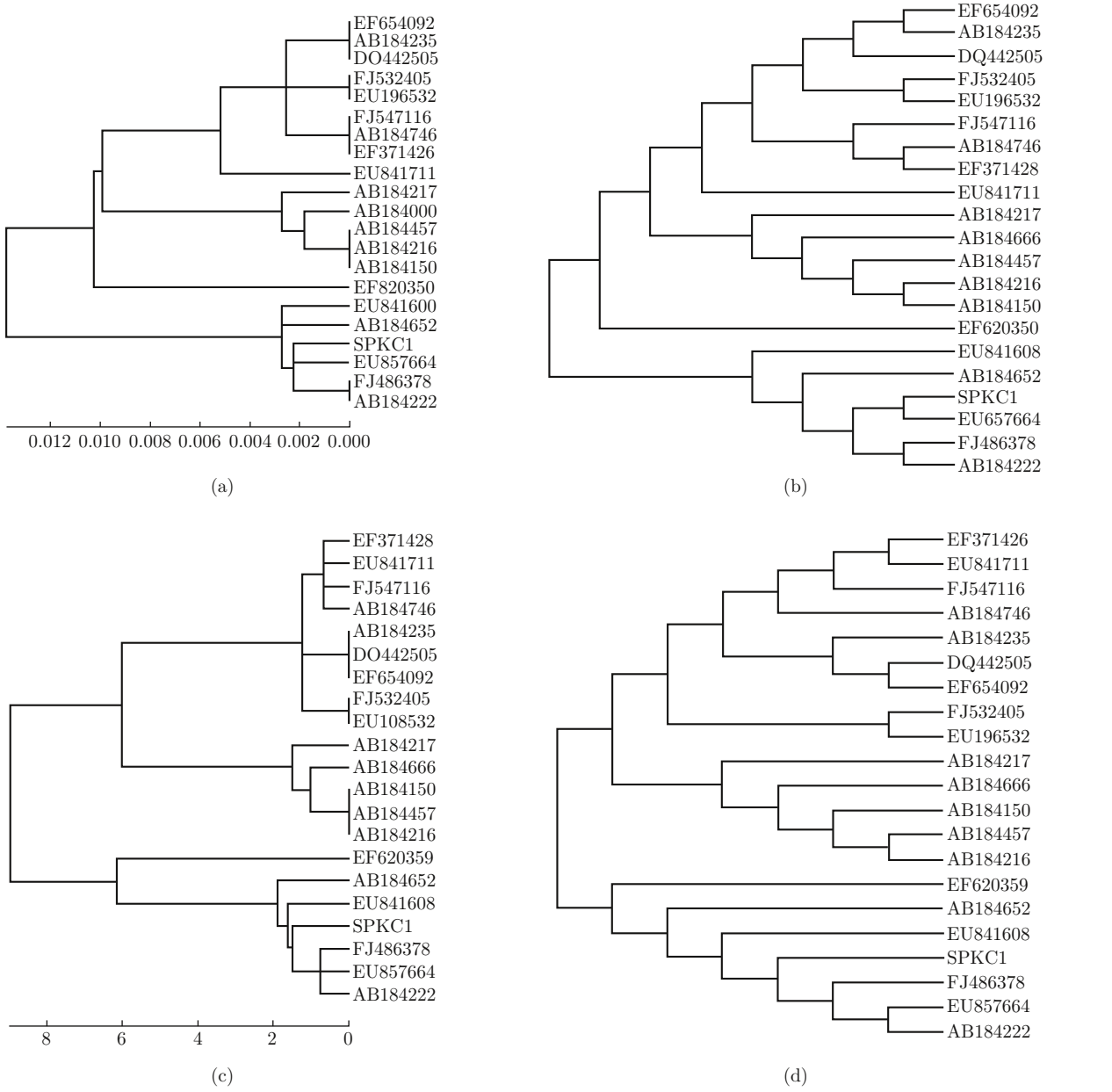

Fig. 2 Phylogenetic trees are based on the nucleotide sequence of 16S rRNA genes. The trees were constructed by using MEGA 4.1 software. (a) Neighbor-Joining algorithm was used for tree construction. (b) Neighbor-Joining algorithm with topology was used for tree construction. (c) Maximum Parsimony algorithm was used for tree construction. (d) Maximum Parsimony with topology was used for tree construction

The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 – 83%), with regard to species from 1 – 14% of the isolates remaining unidentified after testing. Michael and Sharon (2007) reported that, the minimum 500 – 525 bp essential for phylogenetic analysis, and also for species identification minimum >99% similarity and ideal >99.5% similarity should be desirable. <0.5% similarity and other properties such as phenotype should be considered to final species identification. E value is related to the probability that the observed degree of similarity could have arisen by change: E is the number of sequences that would be expected to match as well or better than the one being considered, if the same database were probed with random sequences. Values of E below about 0.05 would be considered significant; at least they might be worth considering (Arthur, 2005). According to Michael and Sharon (2007) and Arthur (2005) reports, in this study, isolate SPKC1 (567 bp) had significant (100%) ideal >99.5% similarity and E value (0.0)

<0.05 with *S. noboritoensis*. On the basis of BLASTN search with phenotypic results SPKC1 was identified as a strain of *S. noboritoensis*. The above the results of a phylogenetic analysis based on the 16S rRNA sequences were suggested strain SPKC1 positioned in the genus *Streptomyces*. In addition, the special characteristics mentioned in the study suggest that novel strain SPKC1 belong to the species *Streptomyces noboritoensis* with cellulose activity. On the basis of these results, the strain *Streptomyces noboritoensis* SPKC1 has potentiality for industrially important in this world for the production of the yield of cellulase. It has been reported that the biosynthesis of cellulase is induced during growth on cellulose or other cellulose derivatives (Fernandez-Abalose *et al.*, 1997; Godden *et al.*, 1989). In all cases, it has been found that it is essential to keep the required nutrients at low level to insure maximum accumulation of fermentation products (Priest, 1984). Overall, the study indicated that cellulase production from SPKC1 isolate was constitutive in natural world, as apparent from the very high number of *Streptomyces* isolates producing CMCase in India soils.

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