JJBS

Volume 6, Number 3, September .2013
ISSN 1995-6673
Pages 239 - 242

Jordan Journal of Biological Sciences

Short Communication

Molecular Characterization and Phylogenetic Analysis of Cellulase Producing *Streptomyces griseorubens* (Strain St-1) Isolated from Indian Soil

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Received: January 15, 2013, Accepted March 9, 2013

Abstract

The present study was aimed at characterization of the cellulose degrading actinomycete strain St-1, isolated from soil samples of Patna region in India, on the basis of morphological, cultural, physiological and molecular characterization. Based on morphological and analysis of 16S rRNA sequence, the strain St-1 was identified as *Streptomyces griseorubens* (Accession no. AB 184139). The strain was able to grow in a wide range of pH (5-11) and temperature (4-45 ^{0}C) with 7 and 28^{o}C being the optimum pH and temperature, respectively. The strain was able to survive at up to 6 % NaCl concentration through the optimum growth was observed at 1% NaCl concentration.

Keywords: Cellulase Producing Actinomycete, *Streptomyces griseorubens*, 16S rRNA Analysis

1. Introduction

Actinomycetes, phylogenetically defined as a number of taxa within the high G+C subdivision of the gram-positive phylum (Embley and Stackebrandt, 1994), are involved in important processes in a wide range of habitats (Mincer *et al*., 2002). Among actinomycetes, *Streptomyces* are quite significant as they bring about the decomposition of various plant based polysaccharides by the production of wood hydrolytic enzymes, such as cellulases and hemicellulases (Crawford and Sutherland, 1979). Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al*., 2005) for the possible use in large scale biorefining. Glucose, from appropriate hydrolysis of this cellulosic biomass, under the treatment of advanced biotechnology can be used in different applications such as production of fuel ethanol, single cell protein, feed stock, industrially important chemicals and so on (Lynd *et al*., 2002). The objective of this study was to describe the morphological, biochemical, physiological, cultural and the molecular characteristics of the cellulase producing *Streptomyces griseorubens* Strain St-1 isolated from soil samples collected from Patna region in India

2. Materials and Methods

2.1. Isolation of Microorganism

Cellulase producing strain St-1, displaying the greatest cellulase activity on CMC agar (carboxymethylcellulose 0.5 g/l, $NaNO_3$ 0.1 g/l, K_2HPO_4 0.1g/l, $MgSO_4$ 0.05g/l, yeast extract 0.05g/l, agar 15 g/l) (Kasana *et al*., 2008) plates using Congo red test, was selected among the cellulolytic strains isolated from soil samples collected from Patna region on Starch Casein Agar medium (Soluble starch 10 g/l, Casein 0.3 g/l, K_2HPO_4 2 g/l, $CaCO_3$ 0.02 g/l, $FeSO_47H_2O$ 0.01 g/l, KNO_3 2 g/l, $MgSO_47H_2O$ 0.05 g/l, NaCl 2 g/l, Agar 18 g/l) by standard serial dilution technique. The strain was then purified by single streak plate technique (Thakur *et al*., 2007) and preserved on slants of Nutrient Agar at 4^{o}C with periodic sub culturing for further investigation. Colony morphology on Starch Casein Agar and Nutrient Agar regarding the color of the mycelium, pigmentation and texture of the culture were observed and recorded after an incubation period of 7days at 28 ^{0}C. Micro-morphological studies and photographic observations were done with a light microscope (Carl Zeiss Microscope with camera) using cover-slip method in ISP2

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agar (yeast extract 4 g/l, malt extract 10 g/l, glucose 4 g/l, agar, 20 g/l) (Shirling and Gottlieb, 1966) after Gram staining.

2.2. Cultural characteristics

The cultural characteristics were studied by growing the strain on different ISP and non ISP media according to the guidelines of the International Streptomyces Project (Shirling and Gottlieb, 1966). Nine different media, including ISP_1, ISP_2, ISP_3, ISP_4, ISP_5, ISP_6, ISP_7, Starch Casein Agar and Nutrient Agar were selected for describing the culture characteristics.

2.3. Physiological characteristics

Physiological criteria included the ability of the isolate to utilize different carbons as described by Pridham and Gottlieb (1948) using plates containing basal medium ($(NH_4)_2SO_4$, 2.64g; KH_2PO_4, 2.38g; K_2HPO_4, 5.65g; MgSO4, $7H_2O$, 1g; standard saline solution, 1ml; agar, 15g; H_20, 1000 ml, pH 7.2) supplemented separately with 1% dextrose, sucrose, lactose, fructose, mannitol and inositol. The plates were incubated at 28°C and the growth was read after 7 days of incubation. The pH and temperature for growth were determined by growing the strain on NA plates with variable pH values (3, 5, 7, 9 and 11) and temperatures (4°C, 15°C, 26°C, 37°C, 45°C, 50°C, 55°C and 60°C) for 7 days. The growth of strain St-1 on 10 different concentrations of NaCl (1% to 10%) in the medium was studied according to the method of Tresner *et al*., 1968.

2.4. Molecular characterization

2.4.1. Isolation and amplification of chromosomal DNA

The chromosomal DNA of the strain St-1 was isolated as described by Houda *et al*. (2009). Amplification of the 16S rRNA was performed using a PCR kit (Sino-American Biotechnology Co., Beijing). PCR mixture consisted of 1 µl (80 ng) chromosomal DNA, 0.5 µl (200 µM) of each deoxynucleoside triphosphate (New England Biolabs), 0.8 µl (80 ng) Universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 0.8 µl (80 ng) Universal primer 1492R (5'-TACGGYTACCTTGTTA CGACTT-3'): *Escherichia coli* 16S rDNA numbering system (Brosius *et al.*, 1978), 0.5 µl (2.5 Units) Taq Polymerase, 2.5 µl of 1X Standard Taq buffer and 18.9 µl autoclaved water to make the final volume of 25 µl. PCR was carried out in a thermocycler (Eppendorf Mastercycler gradient) using PCR program that included initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and primer extension at 75°C for 2 min.

At the end of the cycles, the mixture was kept at 75°C for 10 min for a final extension.

Approximately 1.5 kb amplified 16S rRNA gene fragment was separated by agarose gel electrophoresis, extracted using Qiaquick gel extraction kit (Qiagen) and purified.

2.4.2. Sequencing of 16S rRNA gene

The amplified and purified PCR product was sequenced by the dideoxy chain terminator method using a Big Dye Terminator kit followed by capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystem). The primers used for sequencing were 27F, 341F (5'-CTGGGAGGCAGCAGTGGG-3'), 786 F (5'-GATTAGATACCCTGGTAG- 3'), 536 R (5'-GTATTACCGCGGCTGCTG – 3') and 1492R. The 16S rRNA sequence of the strain was compared with sequences deposited in the NCBI GenBank database and EZTaxon Version 2.1 using BLAST program.

2.5. Phylogenetic analysis

Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA program version 4.1 (Tamure *et al.*, 2004). The 16S rDNA gene sequences of the type strains of the various genera used in this study were retrieved from the GenBank database and used for cladistic analysis. These sequences have been chosen as reference sequences.

Nucleotide sequence accession number:

The 16S rRNA gene sequence of the strain was deposited in GenBank and an accession number (AB184139) was obtained.

3. Results

3.1. Morphological characteristics

The strain was identified as an actinomycete by colony morphology. Morphological observation of the cultures of the strain St-1 on Starch Casein Agar showed luxuriant grayish white aerial mycelium and yellow substrate mycelium while no diffusible pigmentation was observed. Micro-morphological observation revealed Gram positive filamentous structure.

3.2. Cultural characteristics

The cultural characteristics of the strain listed in Table 1 showed different degrees of growth on most media. The strain was able to grow luxuriantly on all media except ISP_2 and ISP_3. Typically, the colony showed white to grey aerial mycelium. The substrate mycelium was colorless on all the media except ISP_1, ISP_6 and Starch Casein Agar on which it was brownish yellow, brown and yellow, respectively. The strain produced a vivid yellow diffusible pigment on ISP_1 media.

Table 1. Cultural characteristics of *S.griseorubens* (strain St-1)

S. No. Name of the medium	*S.griseorubens* (strain St-1)
1. **Tryptone Yeast Extract Agar (ISP_1)**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Brownish yellow
Pigmentation	Yellow
2. **Yeast Extract Malt Extract Dextrose Agar (ISP_2)**	
Growth	Poor
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	Nil
3. **Oatmeal Agar (ISP_3)**	
Growth	Poor
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	Nil
4. **Inorganic Salt Starch Agar (ISP_4)**	
Growth	Luxuriant
Aerial mycelium	Grayish white
Substrate mycelium	Colourless
Pigmentation	Nil
5. **Glycerol Asparagine Agar (ISP_5)**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	Nil
6. **Peptone Yeast Extract Iron Agar (ISP_6)**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Brown
Pigmentation	Nil
7. **Tyrosine Agar (ISP_7)**	
Growth	Luxuriant
Aerial mycelium	Light grey
Substrate mycelium	Colourless
Pigmentation	Nil
8. **Starch Casein Agar**	
Growth	Luxuriant
Aerial mycelium	Grayish white
Substrate mycelium	Yellow
Pigmentation	Nil
9. **Nutrient Agar**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	

3.3. Physiological characteristics

The physiological factors influence the rate of growth of actinomycetes and an essential tool for their classification and identification as reported by Kampfer *et al.* (1991). Six carbon sources were used in our study and the strain St-1 was able to utilize all the tested carbon sources efficiently except dextrose. Growth of the strain St-1 was observed in the pH range of 5-11 with the optimum being 7. The temperature range for growth was 4-45^{0}C and optimum growth was observed between 26 and37^{o}C. The strain showed salt tolerance up to 6% with optimum growth at 1% NaCl. Hence, it can be inferred that the strain is not a halophile. The physiological characteristics are presented in Table 2.

Table 2. Physiological characteristics of *S.griseorubens* (strain St-1)

Physiological characteristics	Growth of *S. griseorubens* (strain St-1)
Carbon utilization	
Dextrose	++
Sucrose	+++
Lactose	+++
Fructose	+++
Mannitol	+++
Inositol	+++
Range of pH	
3	Nil
5	+++
7	+++
9	+++
11	+++
Range of temperature	
4^{0}C	+
15^{0}C	++
26^{0}C	+++
37^{0}C	+++
45^{0}C	++
50^{0}C	-
55^{0}C	-
60^{0}C	-
NaCl tolerance	
1%	+++
2%	++
3%	++
4%	++
5%	++
6%	++
7%	-
8%	-
9%	-
10%	-

- No growth; + poor growth

3.4. Molecular characterization and Phylogenetic analysis

The phenotypic characteristics showed that the isolate *Streptomyces griseorubens* strain St-1 belongs to the genus *Streptomyces*. The taxonomic position of the isolated strain was confirmed by 16S rRNA gene sequencing (Figure 1). BLAST analysis of 1411 bps of the 16S rRNA gene sequence of strain St-1 in EZTaxon Version 2.1 software revealed 100% similarity with *Streptomyces griseorubens*. The sequences of the first ten hits were retrieved from the database and used to construct a phylogenetic tree by Neighbour-Joining method (Saitou and Nei, 1987) in MEGA Version 5.1 software (Tamura *et al*., 2011**)**. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) in the units of the number of base substitutions per site.

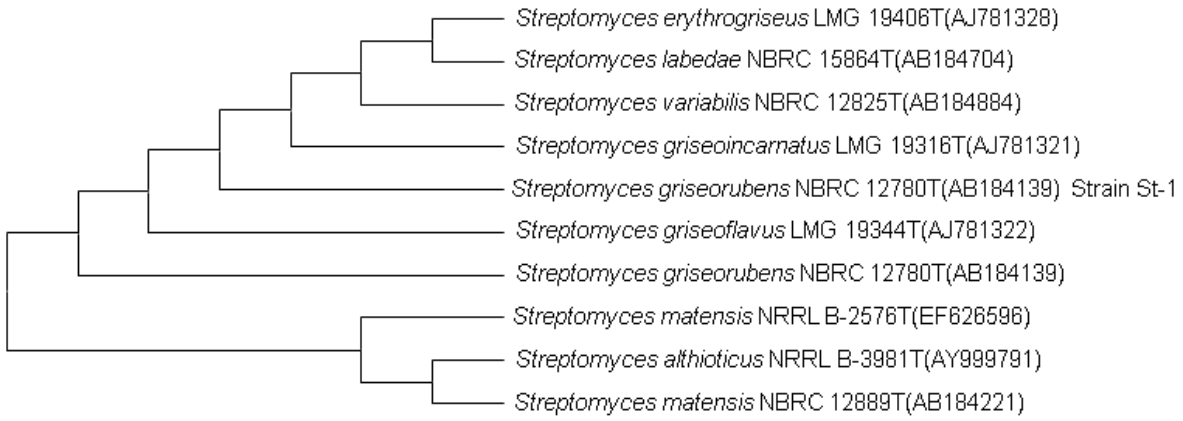

Figure 1. Evolutionary relationships of taxa: The optimal tree with the sum of branch length = 0.60185941 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

4. Conclusion

The present study was aimed at isolation and characterization of the isolated cellulose degrading actinomycete *Streptomyces griseorubens* strain St-1 from soil on the basis of morphological, cultural, physiological and molecular characterization. Growth of the strain at different pH and temperature reveals that the strain possesses high tolerance to the variability of pH and temperature that can be helpful in the survival of this cellulose degrader in the fluctuating growth conditions predominant in nature.

Acknowledgements

We gratefully acknowledge DST, Ministry of Science and Technology, Govt. of India for financial support under WOS-A scheme and Dr. (Sister) Doris D'Souza, A.C., Principal, Patna Women's College for providing necessary infrastructural facilities.

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