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Original Research Article

Characterization of a novel thermophilic cellulase producing strain *Streptomyces matensis* strain St-5

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A B S T R A C T

In the present study, a novel thermophilic cellulolytic bacterium labeled as St-5 was isolated from the soil of agricultural field of Patna area with an aim to screen microbes that can degrade various recalcitrant lignocellulosic biomaterials. Macroscopic and microscopic observations revealed actinomycete nature of the isolate, while 16S rRNA sequencing analysis identification showed St-5 as *Streptomyces matensis* and has been deposited in the GenBank database with the Accession No. KF553639. The isolate was found to utilize different non ISP and ISP growth media; and carbohydrates except Stanier's basal medium, ISP 6 and sucrose, respectively. Growth optimization parameters were found to be pH 7 and temperature 37°C while optimum production of reducing sugar on 4th day of incubation with variations of pH as well as temperature was found at pH 7 and temperature 50°C (62 µg/ mL). The enzyme assay was carried out at different pH (5 – 11) and temperature (20 – 50 °C). The optimum endoglucanase activity was observed at pH 7 (0.02072 IU/ mL) and at temperature 50°C (0.01998 IU/ mL).

Keywords

Cellulolytic enzyme; endoglucanase activity; exoglucanase activity; *Streptomyces matensis*; thermophilic.

Introduction

Cellulose is one of the most abundant biological compounds on terrestrial earth and also probably the most dominant waste material in different forms of plant matter. They represent sustainable resources of veritable biotechnological value. But cellulose itself is among the least degradable of the natural polymers due to its high molecular weight, high degree of structural order, insolubility and low surface area. Utilization of cellulosic biomass is even more complex than that of pure cellulose because in cellulosic biomass, the cellulose occurs in association with lignin and hemicelluloses. Therefore, much of the cellulosic waste is often disposed of by biomass burning. With the help of cellulolytic enzyme system, cellulose can be converted to glucose which is a multi utility product, in

a much cheaper and biologically favorable process. There are several microorganisms that play important role in conversion of lignocelluloses wastes into valuable products like biofuels produced by fermentation (Lynd et al., 2002).

Microbial cellulases are the most economic and available sources, as they can be grown on large scale on inexpensive media such as agriculture and food industries by-products. Microbial degradation of plant structural polysaccharides requires an array of microbial enzymes which work synergistically to achieve effective hydrolysis (Baker et al., 1995; Beguin, and Aubert, 1994) that include endo-β-1,4-glucanases, exo- β -1,4-glucanases (also called cellobiohydrolases), and β-glucosidases (Lynd et al., 2002; Warren, 1996). The endo-1, 4-glucanases cleave at random at internal amorphous sites in the cellulose glucan chains, and the exo-1, 4-β-glucanases act progressively to release cellobiose primarily from the chain ends (Teeri, 1997). The β-glucosidase is considered an integral part of this complex because its presence shows significant improvement in the saccharification process as it relieves inhibition of cellulase action caused by accumulation of the cellobiose in the hydrolysate (Reese and Levinson, 1952).

Research on fungal degradation of lignocellulose started with the investigation of Reese et al. (1950) and since then, has traditionally been concentrated on the fungi by other researchers (Knowles et al., 1987; Ogawa et al., 1991; Bhat and Bhat, 1997; Lusta et al., 1999). But lignocellulose-degrading prokaryotes are beginning to receive more attention due to their easy large scale production and investigations by researchers (Hagerdal et al., 1978; Garcia-Martinez et al., 1980; Saddler and Khan, 1981; Coughlan and Mayer, 1992; Gilbert and Hazlewood, 1993 and others) have revealed that several bacterial genera produce cellulolytic enzymes.

The objectives of this study were to isolate and identify a novel bacterial strain with cellulolytic activity and to characterize it by optimizing the growth conditions and cellulase production.

Materials and Methods

Isolation of cellulolytic bacteria

Cellulolytic bacterial strains were isolated from soil samples collected locally from different sites in Patna region of Bihar in India by using standard serial dilutions and pour plate technique (Dubey and Maheshwari, 2002) on CMC Agar medium (carboxymethylcellulose, 0.5 g; $NaNO_3$, 0.1 g; K_2HPO_4, 0.1 g; $MgSO_4$, 0.05 g; yeast extract 0.05 g; Agar, 15.0 g; distilled H_2O, 1000 mL, pH 7) (Kasana et al., 2008) at 37˚C. Bacterial colonies thus obtained were purified by single streak method and the purified colonies were preserved at 4˚C on Nutrient Agar (HiMedia) slants for further investigations.

Screening of cellulolytic bacteria

Pure cultures of bacterial isolates were individually transferred on CMC Agar plates. After an incubation period of 7 days, CMC Agar plates were stained with 0.1 % Congo red and counterstained with 1N HCl for 15 min at room temperature. Clear area in the otherwise red CMC Congo red stained background appeared around growing bacterial colonies indicating cellulose hydrolysis (Andro et al., 1984). The bacterial strain showing the

largest clear zone was selected for identification and characterization.

Identification of cellulolytic bacteria

Microscopic observation at 400× magnification was recorded using compound microscope provided with camera (Carl Zeiss). Identification of cellulolytic bacteria was carried out using 16S rRNA based molecular technique. DNA was extracted from 48 h old culture broth using DNA Isolation Kit (Qiagen) according to the manufacturer instructions and its quality was evaluated on 0.8% (w/v) Agarose Gel (HiMedia) in an electrophoresis apparatus (BioRad). Fragment of 16S rRNA gene was amplified using PCR kit (Sino-American Biotechnology Co., Beijing) from the above isolated DNA according to the manufacturer instructions. PCR cycling parameters included an initial denaturation at 95 ºC for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55 °C for 1 min and extension at 75 °C for 2 min and a final extension for 10 min at 75 °C. After completion of PCR, the sample was loaded in a 0.8% Agarose Gel with a 1 kb DNA marker supplied by NEB. The amplified product was visualized on a UV transilluminator after running for about 1 h at 100 Volts in the electrophoresis apparatus. The gel slice was excised using a clean scalpel and the PCR amplicon band of 1500 bp was extracted using a Qiagen Gel extraction kit. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-TACGGYTACC TTGTTA CGACTT-3' primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rRNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W and the phylogenetic tree was constructed using MEGA 4 (Tamura et al., 2007).

Polyphasic characterization of the isolate

The isolate was characterized biochemically (Cappuccino and Sherman, 1999). and tested for its substrate utilizing capability by growing it on different media that included Nutrient Agar, CMC Agar, Starch Casein Agar (Soluble starch, 10 g; Casein, 0.3 g; K_2HPO_4, 2 g; $CaCO_3$, 0.02 g; $FeSO_4$ $7H_2O$, 0.01 g; KNO_3, 2 g; $MgSO_4$ $7H_2O$, 0.05 g; NaCl, 2 g; Agar, 18 g; Distilled H_20, 1000 mL, pH 7.2), Cellulose Congo Red Agar with slight modification (K_2HPO_4, 0.50 g; $MgSO_4$, 0.25 g; cellulose powder, 1.88 g; Congo red, 0.20 g; Agar, 15 g;, Gelatine, 2 g; Distilled H_20, 1000 mL, pH 7), Stanier's basal medium ($(NH_4)_2SO_4$,1g; K_2HPO_4, 1 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.1 g; $FeCl_3$, 0.02 g; Distilled H_20, 1000 mL, pH 7.5), Mcbeth medium (K_2HPO_4, 1 g; $CaCO_3$, 2 g; Na_2SO_4, 2 g; $MgSO_4.7H_2O$, 1 g; $(NH_4)_2SO_4$, 2 g; CMC, 1.0 %; Agar, 15 g; Distilled H_20, 1000 mL, pH 7.2); CSPY-ME medium (K_2HPO_4, 0.5 g; Casein, 3 g; Maize starch, 10 g; Peptone, 1 g; Yeast extracts, 1 g; Malt extracts, 10 g; Agar, 15 g; Distilled H_20, 1000 mL, pH 7.5). Morphological features were observed on ISP1, ISP2, ISP3, ISP4, ISP5, ISP6 and ISP7 media according to the guidelines of the International *Streptomyces* Project (Shirling and Gottlieb, 1966) and on different carbohydrates including dextrose, fructose, lactose, sucrose, inositol and mannitol. The effect of different pH (3, 5,

7, 9 and 11) and temperature (4, 15, 26, 37, 45, 50, 55 and 60 ˚C) on growth of the isolate were studied to determine the optimum physicochemical conditions for the cultivation of the isolate. To investigate the effect of pH, five plates of Nutrient Agar medium (HiMedia) each with pH adjusted to 3, 5, 7, 9 and 11, respectively, using 1N HCl and 1N NaOH as per the requirement, were inoculated with the isolate and incubated at 37˚C for 4 days and the observations were recorded. To study the effect of temperatures on growth of the isolate, five plates of Nutrient Agar medium (HiMedia) were inoculated with the isolate and incubated separately at the previously specified temperatures for 4 days and the observations were recorded. All the experiments were done in triplicates. The susceptibility of the isolate to different antibiotics that included Amoxyllin (A), Chloremphenicol (C), Gentamicin (G) and Nalidixic acid (N) were also investigated. For this the isolate was plated on Nutrient Agar medium (HiMedia) plates and discs of antibiotics were placed on it. The diameter of zone of inhibition of the culture growth, if any, was measured in mm after 4 days of incubation period at 37˚C.

Measurement of enzyme activity

Crude cellulase enzyme preparations

A loopful of pure culture of the isolate St-5 was inoculated in 50 mL of CMC broth medium at pH 7 in a 150 mL conical flask and incubated at 37˚C in shaker incubator at 100 rpm for the production of enzyme. After 4 days of incubation period the culture broth was subjected to centrifugation at 5000 × g for 15 min at 4˚C. The clear supernatant was used as crude cellulase enzyme for enzyme assay.

Enzyme assay

The enzyme activity was determined according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission on biotechnology (Ghose, 1987) with slight modifications. Endoglucanase activity was determined by incubating 0.5 mL of supernatant with 0.5 mL of 1% amorphous cellulose in 0.05 M sodium citrate buffer (pH 6.0) in a test tube at 50 ˚C for 30 min. Exoglucanase activity was determined by incubating 0.5 mL of supernatant with 1.0 mL of 0.05 M sodium citrate buffer (pH 6.0) containing Whatman no.1 filter paper strip—1.0 × 6.0 cm (=50 mg) in a test tube at 50 ˚C for 60 min. The reactions were terminated by adding 1 mL of 3, 5-dinitrosalicylic acid (DNS) reagent. The tubes were transferred to a test tube stand and boiled for 5 min in a water bath. The liberated reducing sugars were quantified spectrophotometrically by taking the absorbance at 540 nm (Miller, 1959) and estimated using glucose as standards. The enzymatic activity of endoglucanase and exoglucanase were defined in International Units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 μmol reducing sugar (measured as glucose) per mL per minute.

Production of reducing sugar

Reducing sugar analysis was conducted by adding 1 mL of DNS reagent to 1mL of crude enzyme and boiling for 5 min in the water bath. The absorbance was recorded at 540 nm using a spectrophotometer against the blank of distilled water and was compared with standard glucose curve to estimate the amount of reducing sugar (mg or µg) released.

Effect of pH and temperature on production of reducing sugar

The effect of pH and temperature on enzyme production was studied by inoculating the isolate in CMC broth medium with a range of pH 5-11 (pH 5, 7, 9 and 11) and incubating the production media over a temperature range of 20 -50 ˚C (20, 30, 40 and 50 ˚C) for 4 days. The total amount of reducing sugar released at the specified pH and temperatures were estimated as stated above.

Characterization of enzyme

The endoglucanase enzyme was characterized for its activity at pH 5, 7, 9 and 11 and temperatures 20, 30, 40, 50 and 60 ˚C. The pH of the aliquots of the enzyme solution was adjusted to the specified pH using 1N HCl and 1N NaOH and were incubated at 50 ˚C. The reactions were stopped after 30 min by adding DNS reagent and the activities were assayed as described above. The temperature profile for cellulase activity was determined by incubating the aliquots of the enzyme solution with a pH value 7 at the specified temperatures for 30 min and assaying the enzyme by the method stated above.

Results and Discussion

Isolation and screening of cellulolytic bacteria

The amount and the ease of its availability make cellulose a rich and renewable source of energy. Cellulolytic microorganisms produce a wide variety of different catalytic and non catalytic enzyme modules which form the cellulases and act synergistically on cellulose to yield soluble glucose. Cellulases are inducible extracellular enzymes (McCarthy, 1987) and are secreted in presence of cellulosic substrates. Habitats where these substrates are present are the best sources for isolation of cellulolytic microorganisms (Das, 2010). Fresh water and soil samples are good sources of yielding cellulose degrading bacteria after enrichment (Ivanen, 2009).

In the present study, soil samples were used as source of cellulolytic bacterial strains. Among the several bacterial isolates obtained from different soil samples by serial dilution and pour plate technique, a bacterial strain labeled as strain St-5 was selected for the present study on the basis of observation of largest clear zone of cellulose hydrolysis around the growth of the colonies on CMC Agar plates (Fig. 1).

Identification of cellulolytic bacteria

Microscopic examination revealed that the isolate was Gram positive and filamentous in nature (Fig. 2). The fragments of the genomic DNA extracted from the culture broth using DNA Extraction Kit, after PCR amplification and resolving on Agarose Gel with a 1 kb DNA marker was visualized on a UV transilluminator. A single discrete PCR amplicon band of 1500 bp was observed (Fig. 3). The consensus sequence of 1462 bp 16S rRNA gene generated from forward and reverse sequence data using aligner software showed 100% sequence homology with the strain *Streptomyces matensis* and a phylogenetic tree (Fig. 4) was constructed using the Neighbor-Joining method in MEGA program version 4.

Nucleotide sequence accession number

The almost complete 16S rRNA sequence of strain *Streptomyces matensis* strain St-5 determined in the present study (1462

nucleotides) has been deposited in the GenBank database with the Accession No. KF553639.

Polyphasic characterization of the isolate

Biochemically the isolate gave positive results for the production of amylase, catalase and sucrose fermentation. The biochemical activities of the isolate are tabulated in Table 1. Microbial enzymes have the enormous advantage of being able to be produced in large quantities by mass cultivation of the cellulolytic culture. Production of microbial enzymes is directly associated with the growth of the culture (Tholudur et al., 1999) that depends on a complex relationship involving a variety of factors like substrate utilization, pH value, temperature, aeration and growth time etc. as reported by Immanuel (2006). Thus attention has been focused to optimize the growth of the culture in various environments of substrates, pH and temperatures and the strain St-5 expressed good growth on almost all the specified non ISP and ISP media except the Stanier's basal medium and ISP 6, respectively; and on all the carbohydrates used in the study except sucrose. The growth, texture and the color of aerial and substrate mycelium of the isolate on different non ISP, ISP and carbohydrates are recorded in Table 2. The isolate showed considerable growth at pH 5-11 indicating its wide spectrum tolerance to various pH of the environment. However, the isolate was unable to tolerate highly acidic condition and the growth was nil at pH 3. The isolate showed moderate growth at low temperatures but from 26 ˚C onwards the isolate exhibited luxuriant growth indicating the strain to be thermophilic. This property of the isolate would be helpful in the survival of the isolate in the Indian climate. Besides the use of higher temperatures decrease the possibility of microbial contamination.

The isolate was also tested for antibiotic susceptibility to determine its antibiotic resistance potential. The isolate was resistant to the antibiotics Amoxyllin and Chloramphenicol; and was more susceptible towards Gentamicin showing 29 mm of diameter of zone of inhibition as compared to Nalidixic acid in which the zone of inhibition was 8 mm of diameter. The data regarding the antibiotic susceptibility of the isolate are presented in Table 4.

Enzyme Assay

The amount of the endoglucanase and the exoglucanase secreted by the strain St-5 on the 4^{th} day of incubation at pH 7 and at temperature 37˚C were estimated in terms of cellulase units. Enzyme assay for cellulose activity on filter paper (exoglucanase activity) was found to be much more (0.011 IU/mL) in comparison to the endoglucanase activity (0.008 IU/mL). The reducing sugar released on 4^{th} day of incubation at pH 7 and temperature 37˚C was found to be 118 µg/ mL. The data are shown in Table 5.

Native cellulose is recalcitrant substrate in nature due to its insolubility and heterogenous structure comprising of crystalline and amorphous topologies (Schwarz. 2001). The crystallinity degree of cellulose is one of the most important structural parameters which affect the rate of enzymatic degradation by hydrolysis (Petre *et. al.*, 1999). The complete degradation of cellulose occurs with the action of endo-1,4-β-glucanases attacking the cellulose chain internally and exo-1,4-β-glucanases attacking the reducing or

Fig.1 The isolate showing clear zone of cellulose hydrolysis around the growth of the colonies on CMC Agar plates after Congo red test (Reverse view)

Fig. 2 Microscopic view of the isolate at 400× magnification

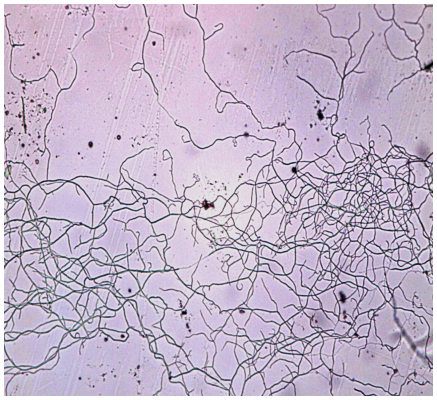


Fig. 3 Gel image of 16S rRNA Amplicon band of the isolate

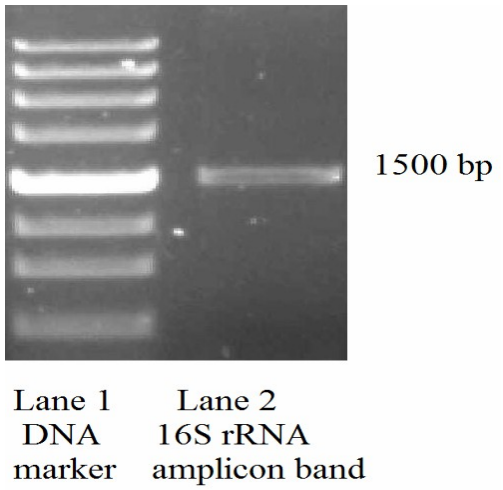


Fig. 4 Phylogenetic tree: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

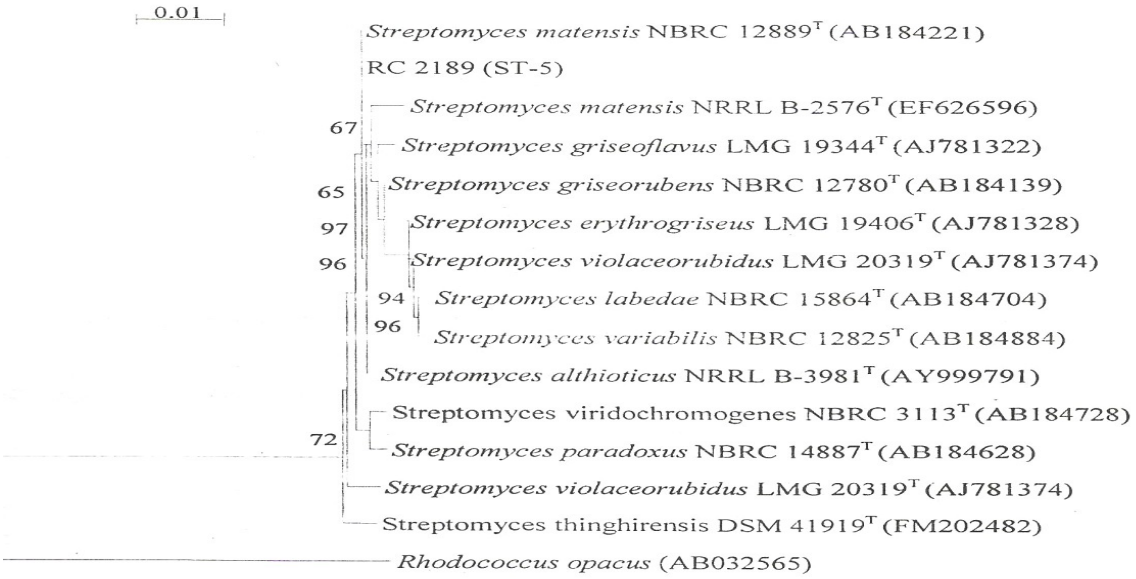
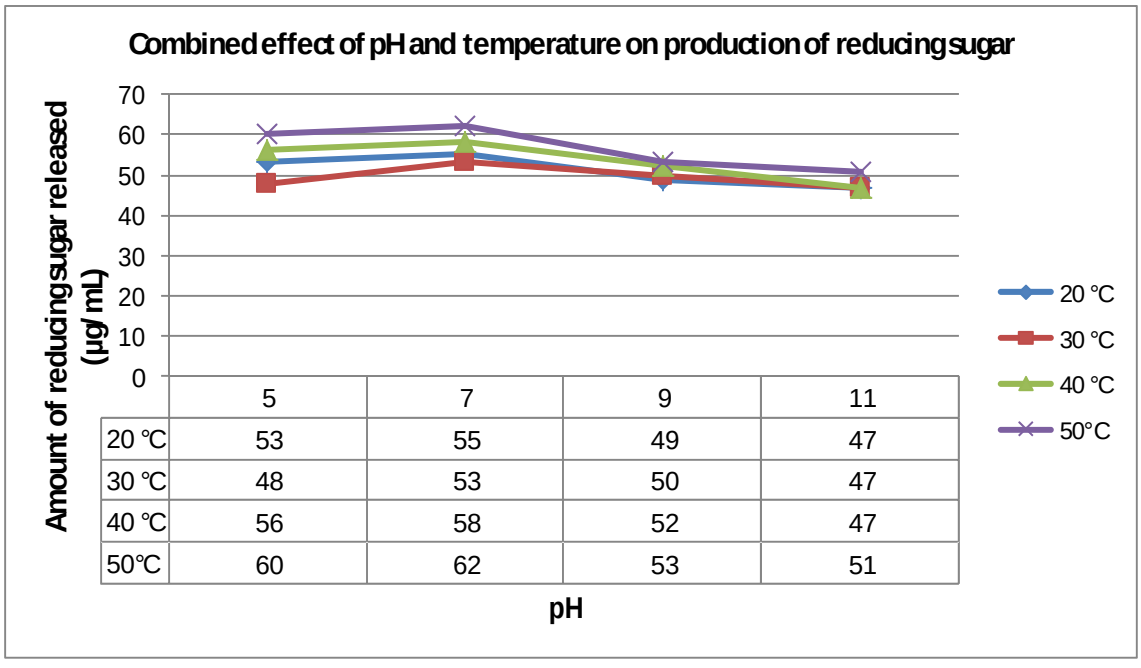


Fig.5 Effect of pH and temperature on production of reducing sugar by *Streptomyces matensis* strain St-5



	5	7	9	11
20 °C	53	55	49	47
30 °C	48	53	50	47
40 °C	56	58	52	47
50°C	60	62	53	51

Fig.6 Effect of pH on endoglucanase activity of *Streptomyces matensis* strain St-5

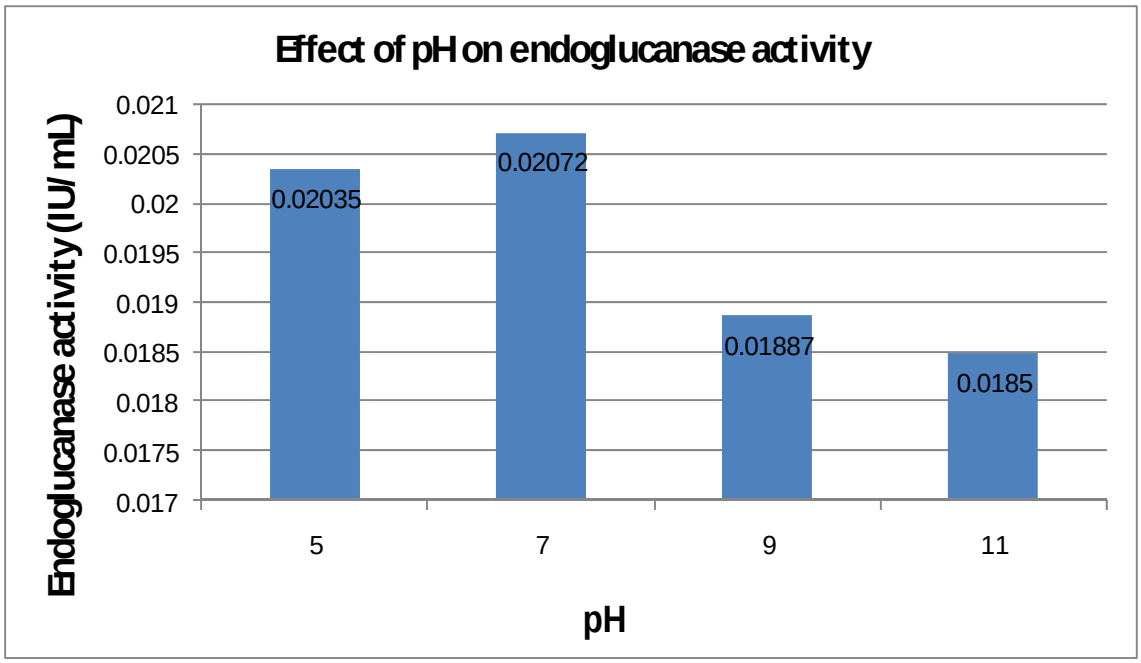


Fig.7 Effect of temperature on endoglucanase activity of *Streptomyces matensis* strain St-5

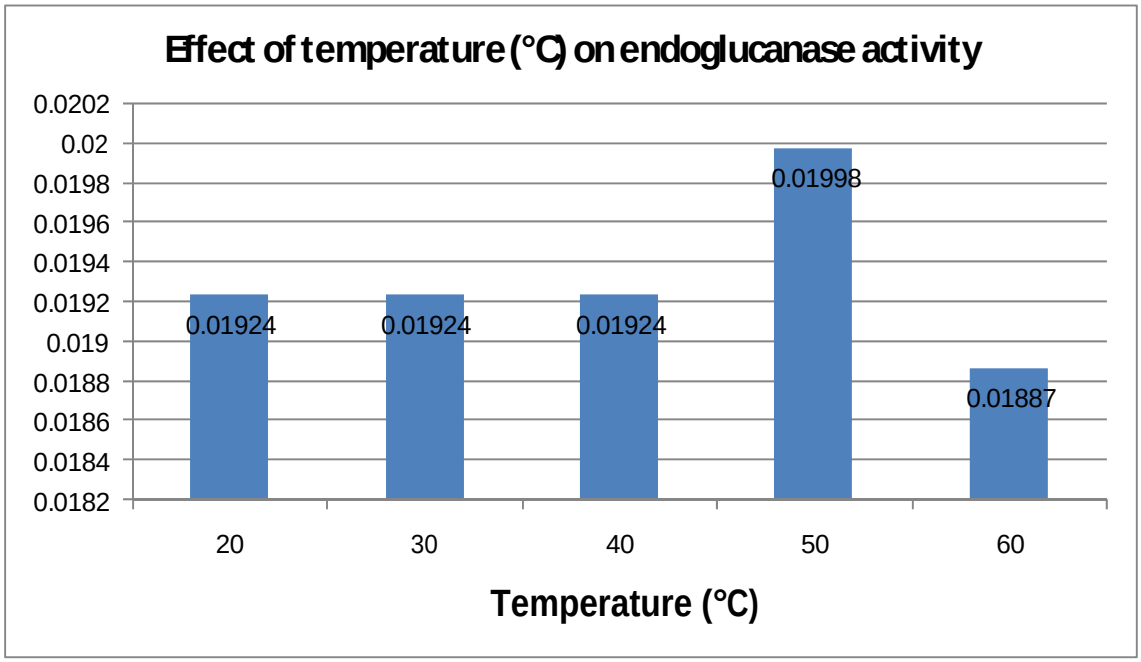


Table.1 Biochemical characteristics of *Streptomyces matensis* strain St-5

Biochemical characteristics	*Streptomyces matensis* strain St-5
Indole production	-
Citrate utilization	-
Voges proskauer	-
Catalase	+
Nitrate reduction	-
Amylase	+
Dextrose fermentation	-
Lactose fermentation	-
Sucrose fermentation	+
Gelatin hydrolysis	-
Hydrogen sulphide production	-
Casein hydrolysis	-

Negative; + Positive

Table.2 Morphological features of *Streptomyces matensis* strain St-5 on different media and carbohydrates

Medium	Growth	Texture	Color of aerial mycelium	Color of substrate mycelium
Non ISP medium				
Nutrient Agar	+++	Powdery	White	Nil
Starch Casein Agar	+++	Powdery	Gray	Black
CMC Agar	+++	Powdery	Light gray	Nil
Cellulose Congo Red Agar	+++	Powdery	Gray	Black
Stanier's basal medium	+	Dry	Pale	Nil
Mcbeth medium	+++	Powdery	Light gray	Black
CSPY-ME medium	++	Powdery	White	Nil
ISP medium				
ISP 1	+++	Powdery	Light gray	Black

ISP 2	+++	Dry	Yellowish	Nil
ISP 3	+++	Powdery	Gray	
Black				
ISP 4	+++	Powdery	Gray	
Black				
ISP 5	++	Powdery	Gray	
Black				
ISP 6	+	Dry	Pale	Nil
ISP 7	+++	Powdery	Gray	
Black				
Carbohydrates				
Dextrose	++	Powdery	White	Nil
Fructose	++	Powdery	White	Nil
Inositol	+++	Powdery	White	Nil
Lactose	++	Powdery	White	Nil
Mannitol	+++	Powdery	White	Nil
Sucrose	-	-	-	-

Table.3 Physiological Characteristics of *Streptomyces matensis* strain St-5

Effect of pH	**Growth**	**Effect of temperature (˚C)**	**Growth**
3	-	4	++
5	+++	15	++
7	+++	26	+++
9	+++	37	+++
11	+++	45	+++
		50	+++
		55	+++
		60	+++

Table.4 Antibiotic susceptibility of *Streptomyces matensis* strain St-5 expressed as zone of inhibition (diameter in mm)

Amoxyllin	R
Chloremphenicol	R
Gentamicin	29
Nalidixic acid	08

(-) nil; (++) moderate; (+++) luxuriant; (R) Resistant

Table.5 Exoglucanase and endoglucanase activities and production of reducing sugar by *Streptomyces matensis* strain St-5

Name of the isolate	Exoglucanase activity (IU/mL)	Endoglucanase activity (IU/mL)	Reducing sugar (µg/mL)
Streptomyces matensis strain St-5	0.011	0.008	118

non-reducing end of the cellulose chain to generate cellobiose or glucose (Béguin & Aubert, 1994). Mandels and Weber (1969) reported that when the cellulases act on the cellulose, the more assessable amorphous portions are rapidly digested and the residue becomes increasingly resistant to enzyme attack, resulting in an increase in the proportion of more resistant crystalline cellulose (Howell and Mangat, 1978).

In the present investigation, the strain St-5 was found to possess more of the exoglucanase activity as compared to its endoglucanase activity which makes it a suitable candidate to be used as a cellulose degrader in the natural environment. Further, its thermophilic property and wide spectrum pH tolerance promote it to be suitably used in the Indian environment.

Effect of pH and temperature on production of reducing sugar

Investigations revealed that the variations in temperature as well as pH level played significant role in production of reducing sugar by the isolate St-5. The sugar production increased when the pH level increased from 5 to 7 but further increase in pH level from 9 onwards resulted in the decrease in sugar production. This trend was observed at all the specified temperatures. Further, it was also found that with increase in temperature, the production of reducing sugar initially decreased from 20 to 30 ˚C but increased afterwards from 40 to 50 ˚C. The optimum reducing sugar production was recorded at a combination of pH 7 and temperature 50 ˚C (62 µg/ mL) and the least at pH 11 and temperatures 20, 30 and 40 ˚C (47 µg/ mL). The sugar production at different combinations of various pH and temperatures are presented in Figure 5.

Effect of pH and temperature on endoglucanase enzyme activity

The endoglucanase enzyme activity was determined in the pH range of 5-11 at an incubation temperature of 50°C. The pH profile demonstrates that initially the enzyme activity increased with increase in pH value from 0.02035 IU/mL at pH 5 to the optimum 0.02072 IU/mL at pH 7, but with further increase in pH there was about 50% reduction in the enzyme activity (0.01887 IU/mL) at pH 9 and about 60% (0.0185 IU/ mL) at pH 11. The data are presented in Figure 6. Similar results are reported by Rahna and Ambili (2011); Immanuel et al. (2006); GoKhan Coral et al. (2002); Akiba et al., (1995); Prasetson and Doelle (1987); and Garcia-Martinez et al. (1980).

Regarding the effect of temperature on enzyme activity, there was stability in the endoglucanase activity between 20 and 40°C (0.01924 IU/mL) but it almost doubled at 50°C (0.01998 IU/mL), but further increase of temperature up to 60°C decreased the enzyme activity to 0.01887 IUmL. The data are presented in Figure 7.

The thermostable enzymes are advantageous in industrial processes (Kristjansson, 1989) than the thermolabile enzymes as the rate of enzyme reaction increases with increase in the temperature of the process. A 10 ~ increase in temperature approximately doubles the reaction rate, which in turn decreases the amount of enzyme needed (Haki and Rakslit, 2003). The thermostable enzymes are also able to tolerate higher temperatures, which give a longer half-life to the enzyme.

Isolation, identification and characterization of a novel thermophilic cellulolytic strain *Streptomyces matensis* strain St-5 (Accession No. KF553639) possessing tolerance to various pH and temperature as well as wide adaptability to different growth media and carbon sources may prove to be helpful in its mass production and utilization in biodegradation of cellulosic biomass.

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