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Cellulolytic Activity of *Streptomyces Clavifer* TBG-MNR13 (MTCC 4150), Isolated From Neyyar Wild Life Sanctuary of Kerala, India

Divya Balakrishnan, Neethu Rajendran Sudhamani, Pradeep Nediyaparambu Sukumaran and Shiburaj Sugathan*
Division of Microbiology,
Jawaharlal Nehru Tropical Botanic Garden and Research Institute,
Palode, Karimancode P.O, Thiruvananthapuram, Kerala, India,
*Pin- 695562. *drshiburaj@gmail.com*

Abstract

Cellulose is regarded as the most abundant and biologically renewable resource found in nature. Cellulases are enzymes which break down the cellulose molecule into shorter polysaccharides, oligosaccharides and finally to monosaccharides ("simple sugars") such as beta-glucose. This study focuses on the isolation of efficient cellulase producing microbes from Western Ghats, a global biodiversity hotspot. A *Streptomyces* strain TBG-MNR13 isolated from the soil sample of Neyyar Wild Life Sanctuary, Kerala was found to be an efficient cellulase producer. The strain was identified as *Streptomyces clavifer* based on conventional morphological, micro-morphological and 16S rDNA sequence analysis. Different physiological parameters like incubation time, pH and temperature were optimized for maximum production of endoglucanase under submerged conditions. The highest activity of 12.6 U/mL was attained at seventh day of incubation in CMC broth with 2% Carboxymethyl cellulose as carbon source, pH 6.5 at 32°C. The optimized media showed an increase in activity from 8.70 U/mL to 12.6 U/mL. Endoglucanase (CMCase) was purified using Sephadex G200 and Sephadex G50 chromatography to 21.8 folds with 10.4% activity recovery. The molecular weight of purified enzyme was found to be 68 kDa by SDS-PAGE analysis and was subsequently characterized.

Keywords: Cellulose**,** *Streptomyces clavifer*, 16S rDNA, CMCase

Introduction

Cellulose is the most abundant biopolymer in nature, made up of glucose monomers that are linked by β 1-4 glycosidic bonds. The cell wall of plants is mostly made of

cellulose and provides structural support to the cell. This renewable source can hydrolyze to reducing sugar molecules through microbial process and subsequently can be converted to useful molecules [1]. There has been considerable interest over the past years in the enzymatic degradation of lignocellulosic biomass due to its potential applications. Cellulases are a multienzyme system which comprises (i) endocellulases (EC 3.1.1.4), (ii) exocellulases (cellobiohydrolases EC 3.2.1.91 and glucanohydrolases EC 3.2.1.74) and (iii) beta-glucosidases (β-D-glucoside glucohydrolase EC 3.2.1.21), leads to the degradation of cellulose into glucose [2]. Endocellulases and exocellulases act synergistically on cellulose to produce cellooligosaccharides and cellobiose, which are then cleaved ` by β-glucosidase to glucose [3]. Among these the endoglucanse is a well recognized component and have important industrial applications, ranging from waste treatment, production of biofuel, oxychemical and biopolishing of fabrics and stoning of denims in textile industry and in pulp and paper industry [4, 5].

A wide variety of microorganisms are known for their ability to produce cellulases. Most commonly studied cellulolytic organisms include fungal spp. like *Trichoderma, Humicola, Penicillium, Aspergillus* and bacteria like *Bacillus, Pseudomonas* and *Cellulomonas* [1]. Actinomycetes genus *Streptomyces* is also one of the best known enzyme producers [6]. *Streptomyces lividens* [7], *S. reticuli* [8], *S. albaduncus* [9] have already reported to produce extracellular endoglucanase significantly. Present study focused on isolation and characterization of a potential cellulase producing *Streptomyces* strain from soil samples of Neyyar Wild Life Sanctuary, Kerala, India and improvement of enzyme productivity by optimization of production conditions, purification and partial characterization of the enzyme.

Methods

Isolation and screening of cellulase producing *Streptomyces*

Soil and litter samples collected from the forest areas of Neyyar Wild Life Sanctuary were used for the isolation of *Streptomyces* following the dilution plate technique using Bennett's agar medium supplemented with 1% (w/w) cellulose [10]. Colonies showing morphological similarity with *Streptomyces* species were selected, purified by repeated subculturing and maintained on agar slants containing (g/l): malt extract, 10; yeast extract, 4; glucose, 4; and agar, 15 [11].

The *Streptomyces* isolates were individually transferred to CMC agar plates which contained (g/l): CMC 10; $(NH_4)_2SO_4$ 4; Na_2HPO_4 6; Yeast extract 1; $MgSO_4.7H_2O$ 0.2; agar 15 [12] and incubated at 30°C for 4 days. After incubation the plates were flooded with 1 % Congo red for 15 min and washed with 1M NaCl. The solubilization of cellulose is indicated by clear zone around the colonies. The isolate TBG-MNR13 showed maximum zone of clearance and was selected for further studies.

Cellulase Production Under Submerged Condition:

1ml cell suspension ($1x10^6$) of *Streptomyces* TBG-MNR13 was transferred into 500ml Erlenmeyer flasks containing 100 ml sterilized CMC medium containing (g/l):

KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; NaCl 0.5; $FeSO_4.7H_2O$ 0.01; $MnSO_4.H_2O$ 0.01; NH_4NO_3 0.3; and CMC 20.0; pH 6.0. The flasks were incubated on a shaker at 200 rpm for 12 days at 28^0C. The culture broth was collected at 24 hrs interval and centrifuged at 10,000 rpm for 15 minutes at 4°C. Supernatant thus collected was used as crude enzyme and subjected to CMCase assay.

CMCase Assay

An aliquot of 0.5 ml culture filtrate was added to 0.5 ml of substrate (1% CMC in acetate buffer pH 6.0) and incubated at 37°C for 30 min. The reducing sugar produced in the reaction mixture was determined by Di-Nitro Salicylic acid (DNS) method. 1 ml of DNS reagent was added to the reaction mixture and incubated at 100°C for 15 min. The mixture was cooled to room temperature and the absorbance was read at 540nm. One unit of cellulase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar per minute [13]. The protein content was determined by Bradford method [14]. The dry cell weight/ml of culture broth was determined for biomass quantification.

Taxonomic Characterization of Potential Strain

Various cultural, physiological and biochemical characteristics of the isolate showing potential cellulase activity was studied according to the methods described in International *Streptomyces* Project [11] and Bergey's manual [15]. Micro-morphology was determined by phase contrast microscopy following cover slip culture method on ISP-2 agar medium [16].

Genomic DNA was extracted following the method of Murray and Thompson [17] with adequate modifications. The 16S rDNA fragment was amplified with pair of primers 8-27F, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1495R 5'-ACGGCTACCTTGTTACGA-3' which were modified from primer pair fD1 and rP2 respectively of Weisburg *et al.*, [18]. PCR assay was carried out in 25µl reaction mixture containing 70 ng of genomic DNA, 10 pmol of both forward and reverse primers, 10x Taq buffer, 10 mM dNTP, and 2U DNA polymerase. Bio-Rad S1000 thermal cycler was used for amplification with the following PCR programme: an initial denaturation for 3min at 94°C, followed by 34 cycles of 1min at 94°C, 1min at 58°C and 1min at 72°C and a final extension at 72°C for 10min. The amplified products were resolved in 1.2% agarose gel containing 0.5 mg/ml ethidium bromide - and visualized under UV (Gel-Doc IT ^{TM}). Approximately 1500bp PCR product was cleaned up by QIAEX II Gel Extraction Kit (Qiagen, USA) and sequenced in an automated sequencer (ABI Prism). The sequence obtained was compared with already available sequences in the NCBI Genebank using the "BLAST" program (ncbi.nlm.nih.gov) and the highest matching sequences downloaded. The sequence of 16S rRNA were aligned with the similar sequences retrieved from databases using CLUSTAL W, and a phylogenetic tree was constructed using the neighbor-joining algorithm (MEGA version 4.0) with the bootstrap analysis of 1000 replicates [19]. The evolutionary distances were computed using the Maximum Composite Likelihood method.

Optimization of Endoglucanase (CMCase) Production:
Various physicochemical parameters under batch culture were analyzed to optimize cellulase production viz, incubation time (up to 10 days), incubation temperature (25 to 35^{o}C) and pH (4.0 to 8.0). After incubation cellulase activity was determined by DNS method as described above.

Purification of The Enzyme
After incubation the culture broth was centrifuged at 10,000 rpm for 15 minutes at 4°C to remove cells and crude enzyme was precipitated by 70% saturation of ammonium sulphate. The precipitate was centrifuged at 10,000 rpm for 15 min and dissolved in a minimum volume (100 ml) of 0.1 M citrate phosphate buffer pH 5.0 and dialyzed against the same buffer for 24 h at 4 ºC. Sephadex G-100 slurry was packed into a column (18 x 2 cm) and equilibrated with 50 ml buffer. The dialyzed fractions were loaded onto the column and fractions (5 ml) were collected at a flow rate of 20 ml/h. The eluted fractions were monitored for protein concentration at 280 nm and were also assayed for enzyme activity. The most active fractions were pooled, concentrated by freeze-drying and loaded (25 ml) onto DEAE-cellulose column. The bound proteins were eluted using a linear gradient of 0 - 0.8 M NaCl at a flow rate of 10 ml/h and 5 ml fractions were collected and dialyzed to remove Na^{+} and Cl^{-}.

Determination of Molecular Weight
The molecular weight of the purified cellulase was determined by sodium dodecyl sulphate-polyacryamide gel electrophoresis (SDS-PAGE) following Laemmli [20]. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250 and destained in acetic acid: methanol: water (1:4:5 v/v). The molecular weight of the purified cellulase was determined in comparison to standard protein marker (Bio-rad, USA) run in parallel to the enzyme.

Results

Isolation and Screening of Cellulase Producing Isolates
Sixty one actinomycete strains were isolated from the forest soil and litter of Neyyar Sanctaury by the standard dilution plate technique and screened for cellulase production by inoculating onto CMC agar plates. After incubation, appearance of the clear zone around the colony with the addition of Congo red solution was added [21] suggests that the fungi are able to produce cellulase to degrade cellulose. Among the 20 isolates which produced clear zone of cellulose hydrolysis the isolate TBG-MNR13 showed maximum zone of 23mm diameter (fig.1) and hence selected for the further studies.

Taxonomic identification of TBGMNR13
Culture characterization of the isolate TBGMNR13 was done on the basis of observations made on different ISP media after 7 and 14 days of incubation at 28^{o}C. The colony morphology, aerial mass colour, reverse colony colour and pigmentation

were noticed following International *Streptomyces* Project (ISP). The morphology of mycelia and spore bearing structures were observed under a phase contrast microscope (Nikon Optiphot-II). The morpho-physiological characters of the strain was summarized in table-I. The cultural observations suggest that the isolate TBG-MNR13 grew well on ISP-2, ISP-5 and ISP-6 media forming leathery grey to white, elevated colonies.

The microscopic observation reveals that the isolate is forming separate aerial and vegetative mycelium with long spore chains (conidial chains) on aerial mycelia (Fig. 2). The spore chain morphology is considered as one of the key characteristics in the identification of *Streptomyces* and it greatly varies among the species. On cell wall analysis LL-Diaminopimilic acid (LL-DAP) was observed as major cell wall peptidoglycan in this isolates. Based on the above characters the isolate was identified as *Streptomyces* spp.

Genomic DNA was extracted and 16S rDNA region was amplified by PCR. Approximately 1500 bp sequence obtained was sequenced and compared with already available sequences in NCBI GenBank using BLAST search programme. The similarity search showed 100% similarity with *Streptomyces clavifer*. The sequence with highest matches were downloaded and aligned using CLUSTALW multiple alignment tool. The Neighbour-joining tree (Fig.3) showing the relationship with selected isolates, retrieved from GenBank databases. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. 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. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 990 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

Optimization of physico-chemical parameters for maximum cellulase production

Optimization of incubation period was done by inoculating *Streptomyces* strain TBG-MNR13 in CMC medium and incubated at room temperature for 10 days. The isolate showed maximum cellulase activity of 8.70 U/mL at seventh day of incubation with a specific activity of 2.20 U/mg^{-1} (fig.4). Maximum cell growth (biomass) was recorded at the eight day of incubation with 836.66 mg/100mL.

The effect of pH on cellulase production by *Streptomyces* TBGMNR13 was determined by varying initial medium pH from 5.0 to 9.0. The Maximum cellulase activity of 10.775 UmL^{-1} (specific activity 2.167 Umg^{-1}) was achieved at pH 6.5 (fig. 5). However highest biomass was recorded high at pH 7.

The effect of temperature on CMCase production was determined by incubating the culture at various temperatures ranging from 26 to 36°C at pH 6.5. The strain showed maximum enzyme production at 30 to 34°C with maximum activity of 12.6 U/mL^{-1} at 32°C (fig. 6). The optimized media showed an increase in CMCase activity from 8.70 U/mL to 12.6 U/mL^{-1}

Purification And Molecular Weight Determination of Cellulase:
Summary of purification procedures of cellulase is presented in Table 2. Cellulase purification was performed at 4^{o}C by ammonium sulfate precipitation, Sephadex G-200 gel filtration chromatography followed by gel filtration on Spehadex G-50 column. The crude culture filtrate was subjected to partial purification, using 70% ammonium sulfate. Active fraction was applied on Sephadex G-200 column. After Sephadex G-200 chromatography, specific activity of CMCase was increased about 4.79 fold compared with the crude preparations. Elutes showing maximum CMCase activities were further purified by Sephadex G-50 chromatography and a 21.8 fold purified fraction was obtained after final purification. A single protein band (68 kDa) was observed on SDS-PAGE (Fig. 7), indicating that the major endoglucanase had been purified to homogeneity.

Discussions

Lignocellulosic pools are a renewable source of feedstock for biofuel production. Cellulose is regarded as the most abundant and biologically renewable resource for bio-conversion to produce glucose and other soluble sugars [22]. Since cellulose is the large proportion of vegetation added to soil, decomposition of cellulose has significance in the biological carbon cycle [23]. The bioconversion of cellulose occurs by the action of cellulolytic enzyme i.e., cellulase. Cellulases are the enzymes responsible for the cleavage of the β–1, 4–glycosidic linkages in cellulose. They are members of the glycoside hydrolase families of enzymes that hydrolyze oligosaccharides and/or polysaccharides.

In the present study efficient cellulose degraders are isolated from Neyyar Wild Life Sanctuary, Kerala, India. This area is a part of Southern Western Ghats, a global biodiversity hotspot and a home of ecological and biological diversity. Chellapandi and Jani [10] isolated cellulolytic organisms from similar sites like litter and soil samples containing decaying wood, decomposing leaves and twigs. Among the 20 isolates screened, the actinomycete isolate TBG-MNR13 showed potential CMCase activity and selected for further studies. Among the family actinomycetaceae, genus *Streptomyces* is represented in nature by the largest number of species and varieties [24]. The genus *Streptomyces* is determined based on the morphological and biochemical criteria, resulting in the arrangement of strains into cluster groups [25]. Based on conventional morphological and 16S rDNA sequence similarity analysis the isolate TBG-MNR13 was identified as *Streptomyces clavifer*.

Culture parameters like incubation time, pH and temperature were optimized to attain maximum cellulase production. Incubation time was optimized by inoculating the isolate in CMC media for 12 days. Maximum activity of 8.70 U/mL (specific activity 2.20 U/mg^{-1}) was attained at seventh day of incubation. A further increase in incubation time resulted in decreased activity. Similar results were also obtained for Azzeddine *et al.* [26] with *Streptomyces* sp. B-PNG23, where maximum yield of endoglucanase (1.12 U/ml) activity was obtained after 7 days of incubation. According to Arunachalam *et al.* [27] prolonged incubation period of 7 days were required for maximum enzyme production by *Streptomyces*. The decrease in CMCase

activity after attaining its maximum peak period is due to catabolic repression of cellulases by products viz., cellobiose and glucose [28].

The strain showed maximum CMCase activity of 10.775 UmL^{-1} (specific activity 2.167 Umg^{-1}) at an initial medium pH 6.5. Similar results were also observed in *Streptomyces* sp. NEAE-D [28] and *Streptomyces* C188 [29] with a maximum activity at pH 6.5. Studies also reveal that maximum CMCase production by *Streptomyces* spp was attained at an initial medium pH from 6.5 to 7.0 [9, 30-32]. But optimum pH for endo-glucanase from a strain of *Streptomyces lividans* was 5.5 [33] and pH 5 for *Streptomyces sp* S7 [34]. According to Liang *et al*., [35] initial medium pH affects enzymatic reactions by influencing the transport of chemical products and enzymes across the cell membrane.

Temperature is another important factor controlling the metabolite production under fermentation conditions. The strain showed maximum enzyme activity at a temperature range of 30-34^{o}C with optimum activity at 32^{o}C. Similar results were also observed by Naggar *et al.* [28] for *Streptomyces* sp. NEAE-D with maximum enzyme production occurred between 20 to 40°C and an optimum activity at 35°C.

The crude endoglucanase (CMCase) was purified to homogeneity by Sephadex G200 and Sephadex G50 chromatography. The purified enzyme had 10.4% activity recovery and approximately 21.8 fold purification. The purified enzyme showed a molecular mass ~68 kDa on SDS-PAGE. Vinha *et al*., [36] observed that cellulase enzyme produced by *S. Viridobrunneus* was 119 kDa, while CMCase from *S. longispororuber* strain C188 was estimated with a MW of about 42 kDa [29] and from *S. lividans* of 36 kDa [37].

Conclusion

A potent cellulase producing isolate TBG-MNR13 was isolated from the Neyyar wild life sanctuary, Kerala. The isolate was identified as *Streptomyces clavifer* based on conventional taxonomic and molecular techniques using 16S rDNA sequence homology. The enzyme production was improved by modifying the culture conditions and nutritional parameters of the fermentation medium. Maximum activity of 12.6 U/mL^{-1} was obtained at the seventh day of incubation, pH 6.5, and at 32°C. Purified enzyme showed a single band of 68kDa on SDS-PAGE.

Acknowledgments

The authors are thankful to Kerala State Council for Science Technology & Environment (KSCSTE), Govt. of Kerala for the financial support and Director, JNTBGRI for providing facilities.

Table 1: Morphological and Physiological Characters of Isolate TBG-MNR13

	TBG-MNR13
Aerial mycelia colour	Grey to white
Vegetative Mycelia Colour	Yellow Brown
Soluble pigments	Absent
Spore chain type	*Rectus-flexibilis*
No of spores/chain	>20
Sporangia	Absent
Cell wall Peptidoglycan	LL-DAP
Melanin production	Negative
Growth temperature	10 to 36^0C
Carbon Sources Utilized	L-Arabinose D-Fructose D-Glucose Inositol D-Mannose L-Rhamnose Sucrose D-Xylose

Table 2: Summary of purification of CMCase from *Streptomyces clavifer* TBG-MNR13

Purification step	CMCase Activity (UmL^{-1})	Specific Activity (Umg^{-1})	Yield (%)	Purification (fold)
Culture Filtrate	12.6	2.85	100	1
$(NH_4)_2SO_4$ precipitation	6.1	3.03	55.2	1.34
Gel Filtration on Sephadex G-200	22.47	10.24	19.2	4.79
Gel Filtration on Sephadex G-50	38.74	44.65	10.4	21.8

Fig 1. The isolate TBGMNR13 produced a large clear zone of hydrolysis on CMC agar. The isolate TBG-MNR13 was grown on CMC agar plates which contain 1% Carboxy methyl cellulose and incubated at 30°C for 4 days. Plates were flooded with 1 % Congo red for 15 min and washed with 1M NaCl. The solubilization of cellulose is indicated by clear zone around the colony.

Fig. 2: Phase contrast microphotograph of 7 days old culture of TBG-MNR13. The culture of TBG-MNR13 was prepared following cover slip culture method on ISP-2 agar for 7 days and photographed using a Nikon (Eclipse TS100) in phase contrast mode. The microphotograph is showing mycelia and spore chain (SPC).

Fig. 3: Neighbour-joining tree constructed using TREECON software, based on 16S rDNA sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the

branches. 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. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

Fig. 4: Effect of incubation time on CMCase activity of TBG-MNR13. *Streptomyces* strain TBG-MNR13 in CMC medium and incubating at room temperature for 10 days. The cellulase activity , total protein content and biomass were recorded at 24 hrs interval.

Fig 5: Effect of initial pH on CMCase activity of the isolate TBG-MNR13. The effect of pH on cellulase production by *Streptomyces* TBGMNR13 was determined by varying initial medium pH from 5.0 to 9.0. The cellulase activity, total protein content and biomass were recorded 7th day of incubation.

Fig. 6: Effect of temperature on CMCase activity of TBG-MNR13. The effect of temperature on CMCase production was determined by incubating the culture at various temperatures ranging from 26 to 36°C at pH 6.5. The cellulase activity, total protein content and biomass were recorded 7th day of incubation.

Fig.7: SDS-PAGE (12%) showing the crude and purified endoglucanse produced from *Streptomyces clavifer* TBG-MNR13. The SDS-PAGE was stained with Coomassie Brilliant blue. The line-1 shows the standard protein marker, 2-Crude protein from TBG-MNR13 and 3- the purified fraction.

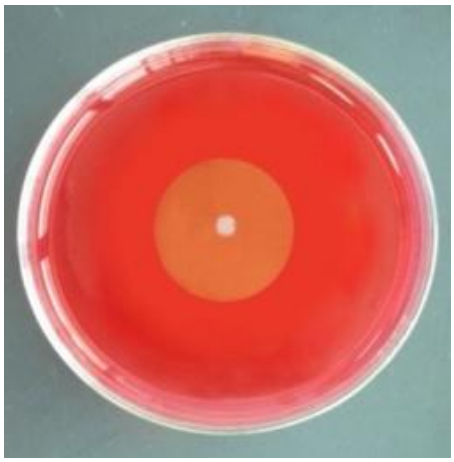

Figure 1: The isolate TBGMNR13 produced a large clear zone of hydrolysis on CMC agar.

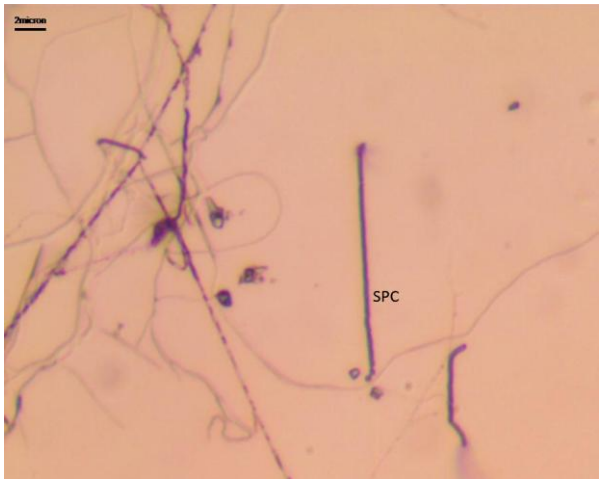

Figure 2: Phase contrast microphotograph of 7 days old culture of TBG-MNR13 grown on ISP2 Media, showing mycelia and spore chain (SPC).

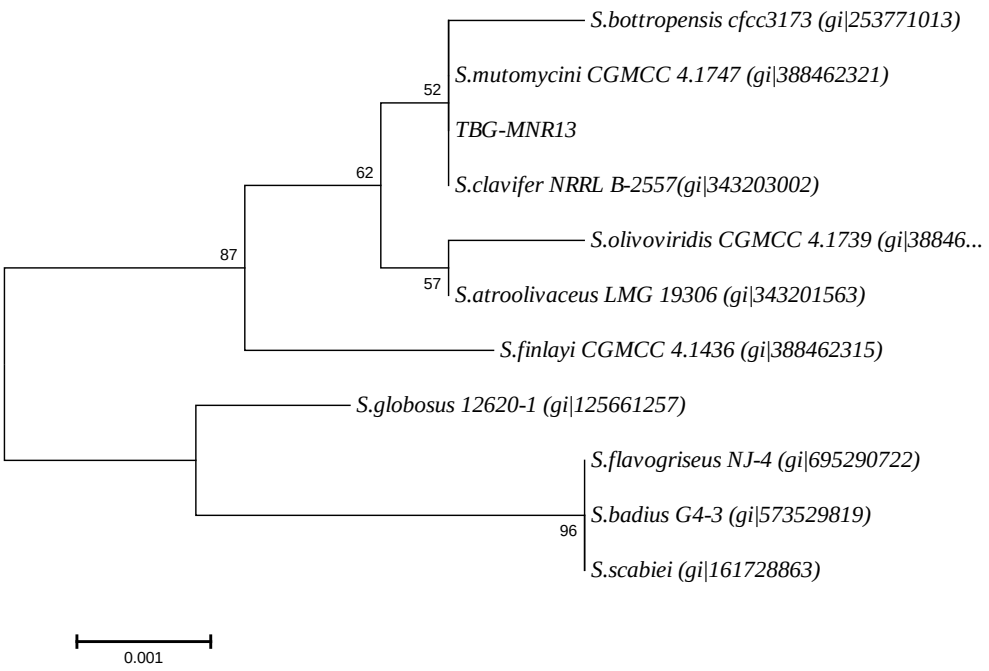

Figure 3: Neighbour- joining tree constructed using TREECON software, based on 16S rDNA sequences

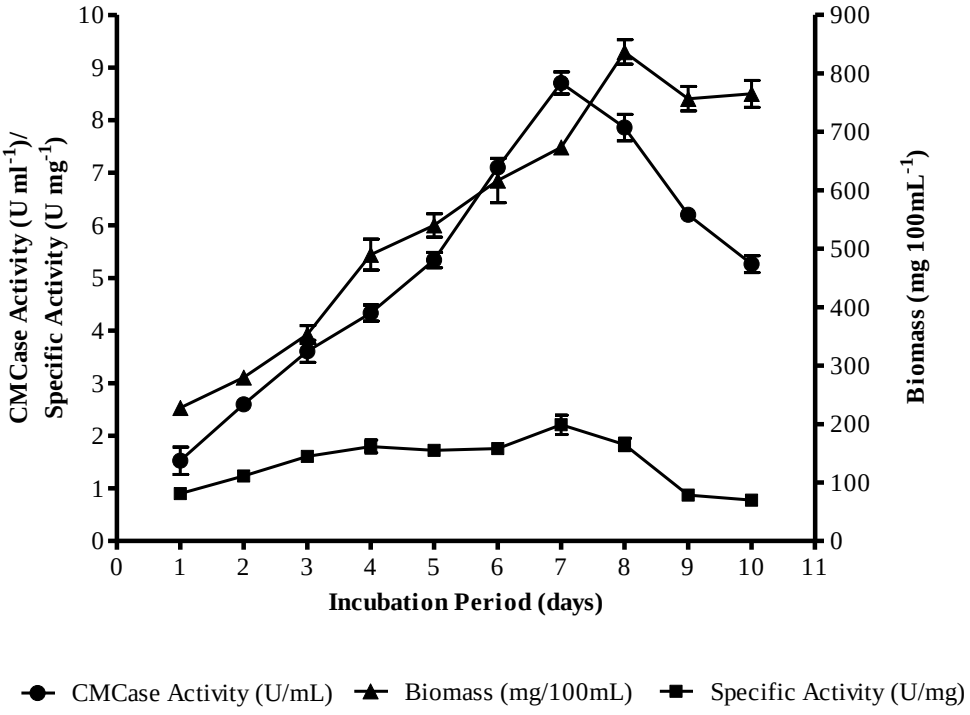

Figure 4: Effect of incubation time on CMCase activity of TBG-MNR13

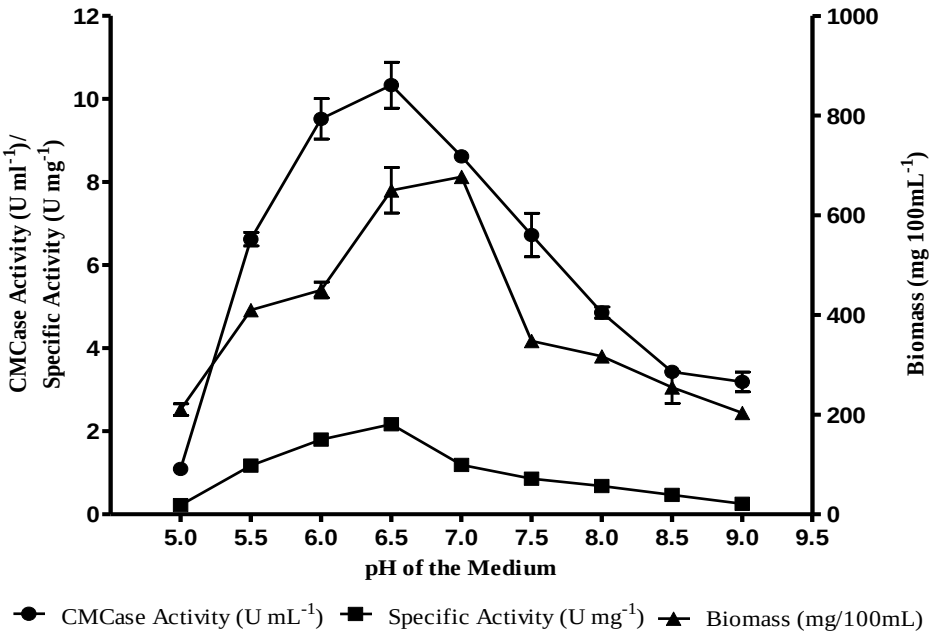

Figure 5: Effect of initial pH on CMCase activity of the isolate TBG-MNR13

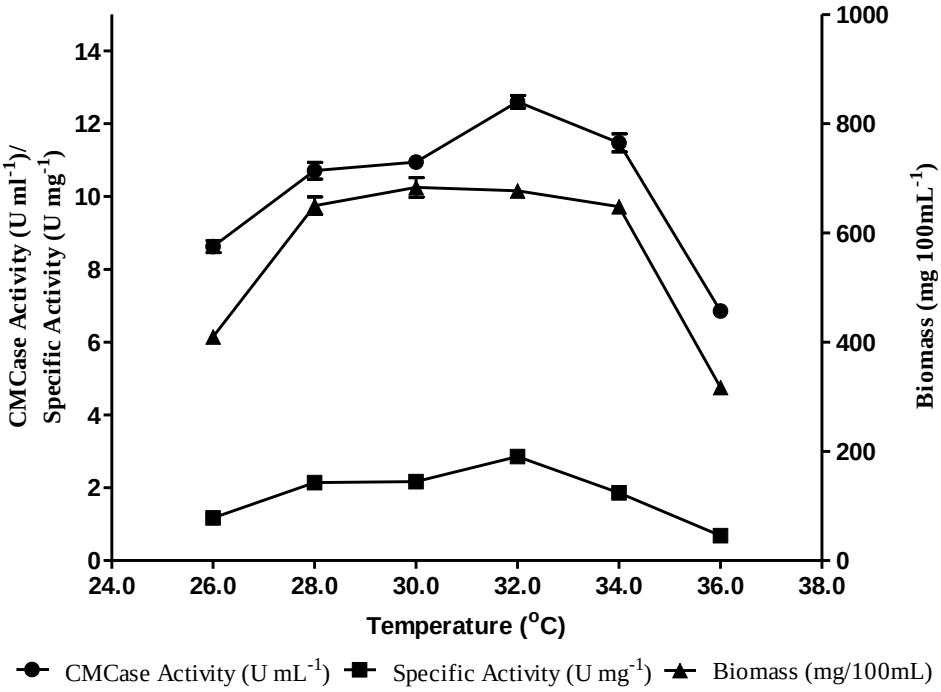

Figure 6: Effect of temperature on CMCase activity of TBG-MNR13

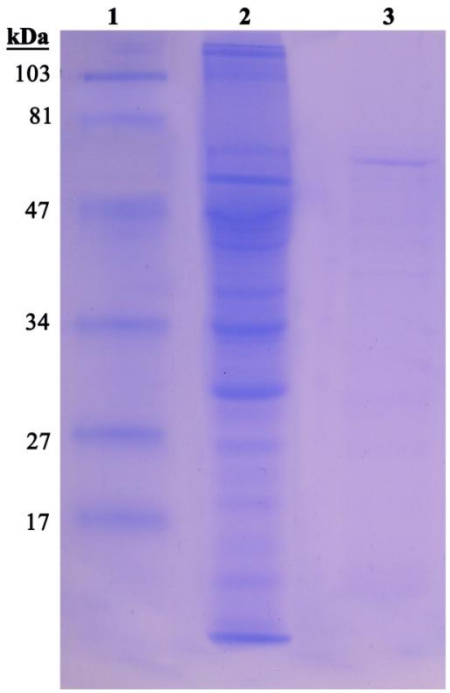

Figure 7: SDS- PAGE (12%) showing the crude and purified endoglucanse produced from *Streptomyces clavifer* TBG-MNR13.

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