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RESEARCH ARTICLE

EXPLORATION OF SEDIMENT DERIVED *STREPTOMYCES* SP. FOR THE PRODUCTION OF ENDOGLUCANASE ENZYME

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ABSTRACT

Among the cellulases, which break down the polymeric cellulose, are endoglucanase that acts synergistically for subsequent hydrolytic reactions. In the present investigation, sediment derived isolates of Actinomycetes were screened for endoglucanase enzyme and the potential isolate, Streptomyces sp. was used further for the isolation of enzyme. In microbial enzyme screening experiment, Streptomyces sp. showed the production of endoglucanase enzyme. The Endoglucanase activity was found to be 2.75U/ml. From among the three media used Vogel's medium showed a higher activity for Endoglucanase with 1% rice straw (0.2U/ml). The enzyme was highly stable in a broad range of buffers for the test incubation period (1 h). More than 40% of initial activity was retained after 5 h incubation at pH 6.0. At acidic pH of 3 the enzyme showed about 86% of activity after which the activity was completely lost due to precipitation of enzyme. 10 – 15 % of concentrated Endoglucanase showed good results in liquefaction. These results highlight the importance of this isolate for the production of potential endoglucanase enzyme.

INTRODUCTION

Bacteria within the order Actinomycetales (that is, the actinomycetes) are common soil inhabitants with an unprecedented ability to produce clinically useful antibiotics. Though more than 50% of the microbial antibiotics discovered so far originate from actinomycete bacteria, only a few select soil-derived genera (*Streptomyces* and *Micromonospora*) account for most of these compounds (Bérdy, 2005). Sediment derived Actinomycetes are investigated not only for their morphology and physiology but also for their metabolites. Actinomycetes are saprophytic soil bacteria, and a rich source of industrial enzymes. Actinomycetes, particularly *Streptomyces* spp. have been a widely exploited group of microorganisms in the production of secondary metabolites and enzymes of commercial importance in medical and agricultural applications (Kumar and Gupta 2006). There has been considerable interest since last few years in the enzymatic degradation of lignocellulosic biomass – a renewable, abundant and inexpensive resource. The applications of such enzymes varied, which included potential applications in waste treatment, fuel production, oxychemical production, textile industry for biopolishing of fabrics and producing stonewashed look of denims; and more recently, the pulp and paper industry (Kasana *et al*., 2008, Noe *et al*., 1986). As reported earlier actinomycetes are potential cellulase-producers and help considerably in recycling nutrients in the biosphere and are thought to be involved in the primary degradation of organic matter in compost and related materials (Jang and Chen 2003). Since cellulose is the major component of plant biomass and potentially utilizable source of glucose, therefore, the process of microbial degradation of cellulose can be considered as financially viable and seems to be the wise choice (Prasad *et al*., 2013). Actinomycete cellulases are inducible extracellular enzymes that can be produced during their growth on cellulosic materials (Ibrahim and El-diwany, 2007). In the present investigation, sediment derived Actinomycete) *Streptomyces* sp. investigated for cellulase enzyme with special emphasis on endo-glucanase enzyme.

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MATERIALS AND METHODS

Sample collection

Sediment samples were collected from mangrove areas of Ratnagiri (West coast of India) at low tide using a plastic spatula and immediately poured into a plastic bag and carried to laboratory in an icebox. Sediment sample were stored at 4^0C for further use.

Isolation and culture of Actinomycetes strains

To isolate the microorganisms from these samples, 1 g of each sample was crushed using a sterile mortar and pestle and suspended in 10 ml of sterile distilled water by vortexing for 2 min on maximum speed. A 10^{-3} serial dilution of the suspension was prepared. This dilution of the sample was spread onto Actinomycete isolation agar, GYM Streptomyces medium and Glycerol Yeast extract agar using the standard spread plate technique. Rifampicin was used for the inhibition of bacterial growth while cycloheximide was used for the inhibition of fungal growth.

Screening for endo-glucanase enzyme

All cultures were spot inoculated on Carboxymethyl cellulose agar plates. The media was then incubated at room temperature for 48 hours. The plates were stained with 0.1% Congo red staining solution. After 10- 15 minutes the congo red solution was drained out and the plates were flooded with 1M NaCl destaining solution for 5 minutes to remove the excess unbound Congo red. Glucose formed during the reaction stains yellow with Congo red while the unused Carboxymethyl cellulose stains red. Diameter of clear yellow zones on red background were measured.

Production of endo-glucanase by isolate

The culture was grown for 48h on IGB agar slants. The cells were then inoculated in 50ml IGB seed medium and grown at 37^0C for 24h. Production media was prepared by adding 1% Rice straw in Vogel's minimal media. Rice straw was used as a carbon source. It was washed dried and ground to fine powder before using in the production medium. Cells were harvested from the 120h to 144h grown culture. Cell harvestation was done by centrifugation method. The cells are centrifuged at 10000 rpm for 10 min at 4^0C. The culture supernatant was collected separately and the residual cell pellet was discarded. The cell free supernatant was then filtered through 0.3µm glass fiber filter using vacuum pressure to remove the suspended particles. The micro-filtered sample was collected in a separate container and then processed further.

Strain improvement for enhanced production of cellulases

The isolated strain was grown in IGB broth for 24 h at 40^0C. From the broth, 100µl of grown culture sample was transferred aseptically onto a sterile IGA agar plate. Simultaneously, several more agar plates were prepared as above and each plate was spread with 100µl of grown culture. In order to obtain strain mutations, these plates were exposed to UV light for a specific time interval. The distance between the agar plates and the UV light was maintained approximately 10 cm to have a direct effect of UV light on the microbial cells. The time intervals used for UV exposure were control, 15sec, 30sec, 45sec, 60sec, 5min, 15min, 30min, 45min and 60min, respectively. After each time interval, the UV treated agar plates were incubated at 40^0C for a period of 48h to allow growth. The stability of UV mutation on the microbial strain was studied by subculturing the UV treated microbial strain for successive generations for a period of 2 months. The final ammonium sulfate precipitated cellulase enzyme concentrate was subjected to SDS-PAGE to check the variations of cellulase production in different UV treated samples. For SDS-PAGE equal amount of protein sample (30µg/lane) was loaded and zymogram analysis was performed. For zymogram analysis, the SDS-PAGE gel was washed with 25% IPA solution for 30 min to remove the loosly bound SDS from the gel. Then the gel was incubated further for 1h with two washes of 50mM Sodium citrate buffer. The gel was then stained with 0.5% congo red for 5 min and destained with 1M NaCl till the clear cellulase bands are observed.

Preparation of seed inoculum media

The microbial colonies from the agar plates were transferred aseptically to IGA slants and further incubated the flasks for 48h at 40°C. Further, the production of endoglucanase enzyme was tested in liquid culture broth. For culture broth preparation, IGB medium was used. IGB medium contains the following composition: 0.5% peptone, 0.5% yeast extract, 0.5% malt extract and 0.5% glycerol, 3% 10X mineral medium. Simultaneously, fresh agar slants were prepared using Bennet agar medium. The composition of Bennet agar medium is as follows: Maltose, 1%; NZ-Amine, 0.2%; Beef extract, 0.1% and yeast extract, 0.1%. From 48 h grown Bennet agar slants, cells were inoculated into 50ml IGB production media and further incubated at 40^0C for at least 24h.

Optimization of seed inoculums: Different concentrations of seed inoculums were tested in the production media for endoglucanase production. The tested concentrations were 1%, 2%, 4%, 6%, 8% and 10%, respectively.

Preparation of production media: endoglucanase production was tested using different production media compositions. These include M13 mineral media, Mandels mineral media, Czapek media and Vogels mineral media.

Preparation of Carbon substrate for endoglucanase production: The carbon substrates RS, CP, SW, RH, WH, CW and NW were cut into 2-3 cm with scissors and then grounded to 0.5 to 1 cm size by a conventional motor grinder, separately. The above grounded substrates were washed thoroughly with water and then dried in oven at 60^0C for overnight. This was followed by hydrothermal treatment with autoclaving at 121^0C for 30 min. For Tea powder waste, the carbon waste was directly washed with water without any grinding and then dried in oven at 60^0C overnight. This was followed by hydrothermal treatment with autoclaving at 121^0C for 30 min. For walsath cellulose (WC) substrate, the WC was prepared using commercial cellulose powder and then pre-treated with 83% phosphoric acid for 25h at 40^0C. The precipitate obtained after filtration process was washed twice with 70% ethanol and then neutralized to pH 7.0. Finally, the precipitate is dehydrated with acetone and vaccum dried to form walsath cellulose powder.

Inducers for endoglucanase production: Several inducers were tested in order to enhance the enzyme production. The tested inducers were glucose, lactose, xylose, and cellulose powder.

Enzyme stability studies: To analyze the pH stability, the endoglucanase protein that was obtained from the TFF filtration method as previously described was treated with the

test pH buffers for a minimum period of 30 min to a maximum of 60 min, respectively. The test buffer systems used in the present experiment were as follows: 50mM glycine-HCl, pH 2.0, 50mM sodium citrate, pH 5.0, pH sodium phosphate, pH 7.0, 50mM Tris-HCl, pH 9.0, and 50mM glycine-NaOH, pH 11.0.

Analyses of the temperature stability of the endoglucanase enzyme: To analyze the temperature stability, the cellulase (endoglucanase) protein samples were incubated at different test temperatures for a minimum of 30 min to a maximum of 60 min, respectively. The test temperatures used in the present experiment were 80^0C, 90^0C and 100^0C, respectively. After every 15 min incubation period, the enzyme sample was taken and assayed for CMCase activity.

Analyses of the pH optima of the endoglucanase enzyme: The pH optima of the endoglucanase enzyme analyzed similarly to the temperature optima where the CMCase activity was measured in the each test pH assay buffer solutions separately at a constant assay temperature of 40^0C. The test assay buffer solutions used in the present experiment were as follows: 50mM glycine-HCl, pH 2.0, 50mM sodium citrate, pH 5.0, pH sodium phosphate, pH 7.0, 50mM Tris-HCl, pH 9.0, and 50mM glycine-NaOH, pH 11.0.

Analyses of the temperature optima of the endoglucanase enzyme: Temperature optima of the enzyme were measured at varying assay temperatures ranging from 40^0C to 100^0C. A difference of 10^0C is maintained for each experiment. The CMCase assay was measured by standard DNSA assay method as stated above. The pH of the assay mixture was maintained using 50mM sodium citrate, pH 5.0.

SDS PAGE and Zymography of endoglucanase enzyme: SDS-PAGE separation gel was cast with 0.1% Carboxymethyl cellulose. 30 microliter of TFF concentrated sample and 1:100 diluted sigma cellulase was loaded in the gel using loading dye without boiling. All the samples along with the protein marker were run in duplicate. The gel was run at 100 volt in cold conditions of 4˚C.

Phylogenetic analysis of enzyme producing Actinomycetes

Phylogenetic analysis: Molecular phylogeny of actinomycete was determined by amplifying genomic 16S rRNA region. Two primers specific to 16S rRNA region used in this study were 235F and 878R in order to amplify approx. 643bp sequence of actinomycete 16srRNA gene. DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505) by following the protocol of the manufacturer. The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of actinomycetes. Altogether twenty sequences, including sample were used to generate phylogenetic tree (Figure 1). The tree was constructed by using MEGA 5 software (Felsenstein, 1985; Saitou and Nei., 1987 and Tamura *et al*., 2011).

RESULTS AND DISCUSSION

A total of nearly 13 isolates were collected and screened based on the formation of clear zones around the microbial colonies (Fig. 1). Among these nearly 4 cultures of them showed positive reactions to qualitative test of cellulase production as indicated by the formation of clear zone (halo) in CMC agar medium. The second step of screening showed that cellulolytic indexes (in mm) were ranging from 5 to 34.0 mm (Table 1). Based on the halo formation on CMC agar plates, four cultures (ACT1, ACT5, ACT6 and ACT13) showed highest cellulolytic index values. Out of these four positive isolates ACT13 was taken further for enzyme production and analysis.

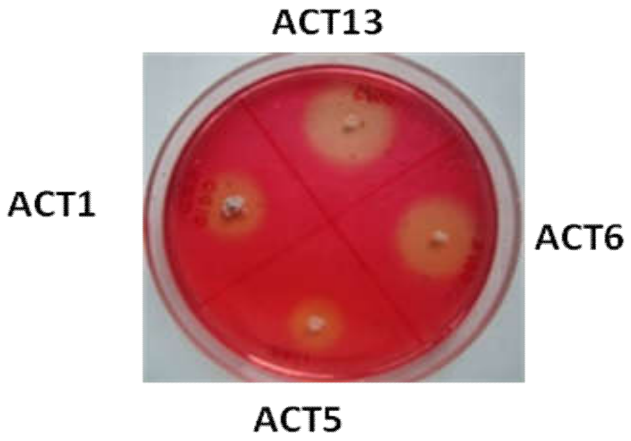

Fig. 1. Positive Actinomycetes isolates showing cellulase enzyme production

Table 1. Selected microbial cultures for endoglucanase production. Based on the cellulolytic index value, the strains were selected and ranked in ascending order

S.No	Strain Number	Cellulolytic index (mm)
1	ACT1	15.0
2	ACT2	7.0
3	ACT3	-
4	ACT4	-
5	ACT5	18.0
6	ACT6	30.0
7	ACT7	5.0
8	ACT8	-
9	ACT9	-
10	ACT10	--
11	ACT11	9.0
12	ACT12	-
13	ACT13	34.0

Strain improvement for enzyme production

After UV treatment, the cells which were resistant to UV light were only able to grow and are visualized as microbial colonies on the agar plates after 48 h incubation. The UV-treated and untreated cells showed similar growth in cellulase production media. One of the most intriguing features of this microbial strain is that the cells were found to be highly resistant to UV light even after exposure for a period of 1 h, indicating COD0040 is a highly UV resistant strain. In nature, very few microbes can survive under such harsh conditions. Above all, we observed that the UV treatment is stable for more than 20 generations even after 2 months. The microbial strain is highly active and found to produce similar amount of cellulase when compared to the first generation microbial strain, indicating the effect of UV on the microbe is highly stable. Interestingly, zymogram analysis showed that the UV light treatment had a positive effect on cellulase production when compared to the control un-treated cells (Fig. 2). The UV treated cells with an exposure time of 5 min and 30 min showed significant enhancement in cellulase activity (Table 2).

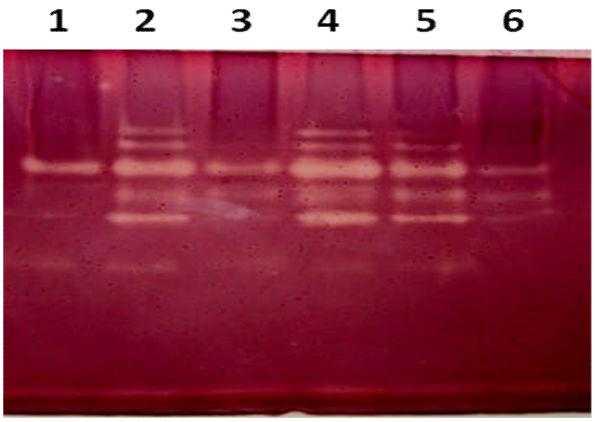

Figure 2. Zymogram analysis of cellulase proteins isolated from UV treated microbial cells. Cells were treated with UV light for different time periods. Lane 1: 0 min; Lane2: 5 min; Lane 3: 15 min; Lane 4: 30 min; Lane 5: 45 min; and Lane 6: 60 min

Table 2. Cellulase activity comparison with and without UV treatment

UV treatment time	CMCase activity (mg/ml)	CBase activity (mg/ml)	Protein (mg/ml)
Control	2.84	15.6	0.081
15 sec	3.2	12.0	0.062
30 sec	2.84	11.2	0.074
45 sec	2.8	4.8	0.064
60 sec	2.32	16.0	0.079
5 min	3.88	17.6	0.096
15 min	3.76	12.0	0.097
30 min	4.4	10.4	0.109
45 min	3.32	12.8	0.115
60 min	3.08	11.6	0.086

Optimization of enzyme production

Among the above tested production media, Vogels mineral media was found to be the best production media for endoglucanase production (Figure 3).

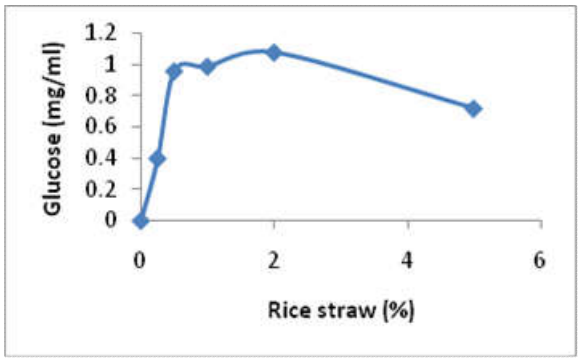

Figure 3. Effect of different concentration of rice straw in the production media on endoglucanase production

All the production media were incubated at 40^{0}C and 130 to 160 rpm for different time periods. We found that endoglucanase production is maximum within 48 h incubation when grown in 50 ml volumes, whereas, the incubation times are prolonged with the production volume. In 0.5L production volume, maximum endoglucanase production was observed between 96 h to 120 h. Maximum growth was observed when 4% seed inoculum was used as the pre-culture. While, at lower seed inoculum concentrations, the growth of the microbe is found to be slow and reaches similar growth under prolonged incubation. Thereafter, the effect of seed inoculum above 4% does not have any significant effect. Thus 4% inoculum was considered to be optimum for endoglucanase production. Among these tested carbon sources, Rice straw was found to be the best carbon source for enzyme production. Different concentrations of rice straw (0.25%, 0.5%, 0.75%, 1%, 2%, and 5%) were tested for optimum enzyme production. Cellulase production was found to be increased with increasing rice straw concentration till 1% final concentration and thereafter did not find significant difference in protein production. Thus, 1% Rice straw was selected as the suitable concentration for cellulase production. Xylose and lactose were found to be good inducers for cellulase production while glucose inhibits the cellulase production. Different concentrations (1mM, 2mM, 5mM and 10mM) of xylose and lactose were tested and found that 10mM xylose and 10mM lactose together or separately showed the similar effect in inducing cellulase production (Table 3). Cellulase activity was determined in the cell free supernatant solution. This indicates that the cellulase protein is secreted into the culture medium and is extracellular in nature. Studies were also performed to detect the cell bound cellulase. For cell bound cellulase isolation, the harvested cells were washed twice with buffer containing 0.1% Tween 20 detegent and extracted the supernatant. Our studies showed that there was no significant cell bound cellulase associated with the organism. Both the ammonium sulfate precipitated sample and the ultrafiltration concentrated sample contains cellulase protein. While, the ammonium sulphate precipitated sample is used for protein stability assays, the ultrafiltered sample is stored at 4^{0}C for comparison studies. In all the above experiments, the cellulase protein was detected by zymogram analysis.

Table 3. Effect of Inducer on endoglucanase enzyme activity

Sample Name	Effector		CMC activity (mg/ml)
Cell free supernatant	Xylose	Lactose	
Control	--	--	1.12
Test 1	10Mm	--	3.4
Test 2	--	10mM	3.6
Test 3	10mM	10mM	3.8

Enzyme stability studies

Based on the endoglucanase activities at a specific pH, the enzymes are categorized into three groups namely: acidic, basic and neutral, respectively. Intriguingly, the cellulase(s) isolated from the actinomycetes strain in this report instead of a single pH optima, has a dual two pH optima with a maximum activity at pH 5.0 and pH 9.0 (Fig. 4).

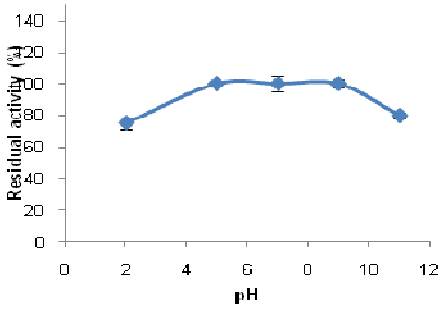

Figure 4. Enzyme activity at different pH

This indicates that the isolated endoglucanase is distinct from the known reported proteins and cannot be grouped into a specific above mentioned category, rather can be regarded as a

broad pH cellulase protein. To our information, there are no reports on the broad pH cellulase proteins till date. This type of cellulase proteins have multiple applications, where a single protein can be used in a range of applications thus reducing the overall cost of the protein, where either acidic or basic or neutral cellulase proteins need to be supplemented for specific application, rather a single cellulase enzyme cocktail is preferred over other individual cellulase proteins. Additionally, the cellulase protein also has a broad pH stability (described in later sections) to suffice the above applications. The optimum temperature for the endoglucanase activity was found to be 65±5^{0}C, respectively (Fig. 5). Most of the cellulases reported are highly thermostable in nature. Our experiments also complies with this nature of thermostability (Fig. 5).

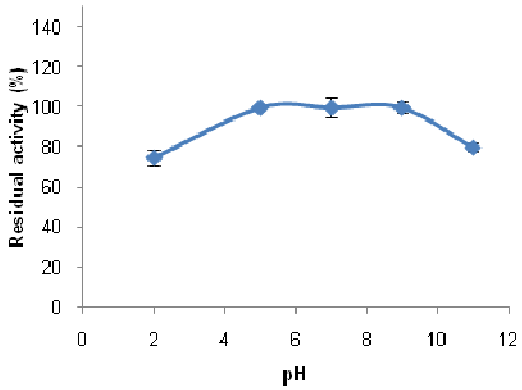

Fig. 5. Optimum temperature for endoglucanase enzyme assay. The test temperatures used for the assay were from 40^{0}C to 100^{0}C with a difference of 10^{0}C for each experiment. All the assays were performed using 50mM sodium citrate pH 5.0 buffer

identified on the gel with cellulase activity with varying molecular weight indicating that there are different forms of cellulases induced when grown on rice straw as carbon substrate. The cellulases are assigned with numbers as shown in the given Figure 6.

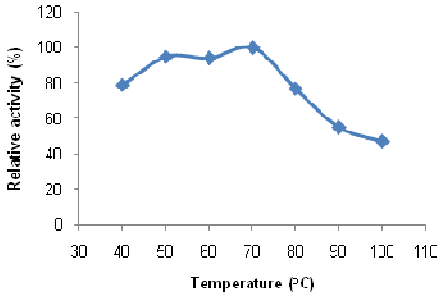

Fig 6. Endoglucanase stability at varying temperatures for a period of 1 h incubation. The test temperatures used in this experiment are 80^{o}C, 90^{o}C and 100^{o}C, respectively. The cellulase assay was measured at a constant 40^{o}C using 50mM sodium citrate buffer, pH 5.0

Phylogenetic analysis

The 16S rDNA sequence analysis revealed that ACT13 strain belongs to actinomycetes group and the high identity (98%) with *Streptomycetes* sps (Table 3). The top hits from the Blast analysis was given in Table 2. Phylogenetic analysis of the top blast sequences showed that the strain has closely relative to *Streptomyces violaceolatus* (Fig. 7). The strain ACT13 was identified and belongs to the actinomycetes group and shows more than 98% similarity with the *Streptomyces violaceolatus* based on the 16S rRNA gene sequencing analysis.

Table 4. Top list of Blast sequences from NCBI Blast using ACT13 16S rDNA sequence as template

Description	Max score	Query cover	E value	Max ident	Accession
Streptomyces violaceolatus strain KCTC 9772	660	93%	0.0	98%	AY999822.1
Streptomyces somaliensis strain G1-8-20	652	93%	0.0	98%	KC494310.1
Streptomyces sp. SY007	652	93%	0.0	98%	AB794692.1
Streptomyces rochei strain AM5-16	652	93%	0.0	98%	KC511808.1
Streptomyces spiralis strain AM2-7	652	93%	0.0	98%	KC511806.1
Streptomyces sp. M1B1-2009	652	93%	0.0	98%	KC469955.1
Streptomyces lividans strain TCA20046	652	93%	0.0	98%	KC462527.1
Streptomyces sp. NEAU-BS1	652	93%	0.0	98%	KC417352.1
Streptomyces lividans strain FMA_277	652	93%	0.0	98%	JX860385.1
Streptomyces sampsonii strain FMA_205	652	93%	0.0	98%	JX860373.1
Streptomyces sp. R97-2	652	93%	0.0	98%	KC329482.1
Streptomyces sp. ANR	652	93%	0.0	98%	KC512242.1
Streptomyces sp. SA	652	93%	0.0	98%	KC512241.1
Streptomyces sp. CBE	652	93%	0.0	98%	KC512240.1
Streptomyces sp. MDU	652	93%	0.0	98%	KC512239.1
Streptomyces sp. PDK	652	93%	0.0	98%	KC512238.1
Streptomyces ambofaciens	652	93%	0.0	98%	HF585526.1
Streptomyces sp. PT2	652	93%	0.0	98%	KC414013.1
Streptomyces sp. CA2	652	93%	0.0	98%	KC414006.1

However, the enzyme was found to be highly stable at 80^{0}C and 90^{0}C even after 60 min. After 60 min incubation, there was no significant loss of activity at 80^{0}C whereas 60% initial activity was retained at 90^{o}C. Nearly 50% initial activity was lost after 30 min incubation at 100^{0}C. Thereafter, the activity is decreased gradually and complete loss of activity was observed after 60 min incubation. This indicates that the endoglucanase enzyme isolated in this present study is highly thermostable.

Zymography of enzyme: Endoglucanase enzyme band was detected by zymogram analysis. Four (04) protein bands were

Based on the biochemical and molecular analysis, the strain was found to be producing extracellular endoglucanase protein with high cellulolytic index value. The tested microbial strain COD0040 has been improved for enhanced production of cellulase by UV treatment and moreover UV light acts as an inducer for cellulase production in this strain. The main reason attributed to this positive effect could be due to the change in the cellulase gene by UV light and the strain modification at the genetic level. The cellulase is an extracellular protein and there is no cell-bound or intracellular cellulase protein in the studied micro-organism. The crude cellulase protein can be prepared by both ammonium sulphate and ultrafiltration

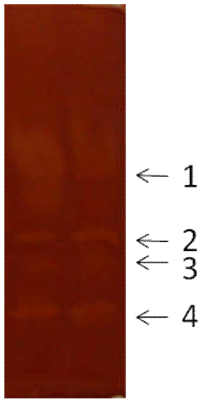

Figure 7. Zymogram analysis for endoglucanase detection. Four (04) protein bands were identified on the gel with endoglucanase activity with varying molecular weight indicating that there are different forms of cellulases induced when grown on rice straw as carbon substrate. The cellulases are assigned with numbers as shown in the given figure

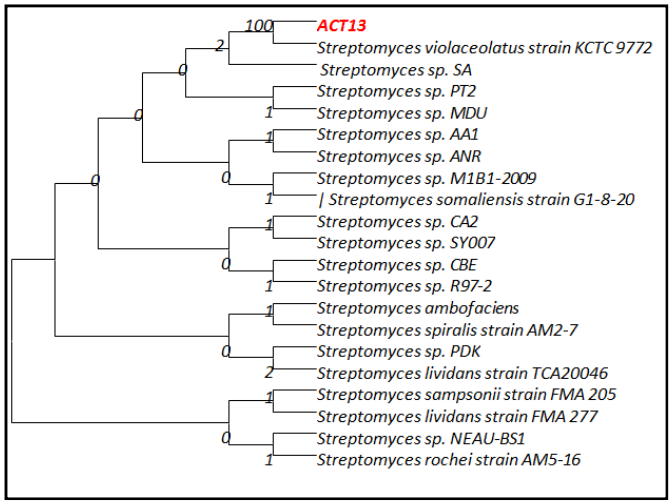

Figure 8. Phylogenetic tree analysis of the Blast sequences

methods. Our experimental analysis showed that the endoglucanase enzyme isolated from the actinomycetes strain from the present study is a highly thermostable and also highly stable in a broad range of pH solutions.

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