SCREENING OF THE CELLULASE PRODUCING ENDOPHYTIC FUNGI FOR THE PRODUCTION OF CELLULASES

Dissertation

Submitted in the partial fulfillment of the requirement for the degree of

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Biotechnology

By

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July 2014

CERTIFICATE

This is to certify that dissertation entitled, **"Screening of cellulase producing endophytic fungi for the production of cellulases"** submitted by Mr. Ambuj in the partial fulfillment of the requirements for the award of M.Sc. in Biotechnology at Thapar University, Patiala is an authentic work carried out by him under our supervision and guidance. To the best of our knowledge, the matter embodied in this dissertation has not been submitted by any other university/ institute for award of any Degree or Diploma.

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DECLARATION

I hereby declare that the work being presented in the thesis entitled "Screening of cellulase producing endophytic fungi for the production of cellulases" in the partial fulfillment of requirements for the award of degree of M .Sc Biotechnology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of January 2014 to June 2014, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology (DBT), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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TABLE OF CONTENTS

S. NO.	CHAPTERS	PAGE NO.
	Executive summary	
1	Introduction	11-13
2	Review of literature	14-19
3	Aim of study	20
4	Materials and methods	21-24
5	Results and discussions	25-31
6	Conclusions	32
7	References	33-35

LIST OF GRAPHS

S.No.	Name of the graph	Page No.
1	showing FPase activity of #53AMSTYEL at varying temperature	30
2	showing CMCase activity of #53AMSTYEL at varying temperature	30
3	showing β- glucosidase activity of #53AMSTYEL at varying temperature	31
4	showing FPase activity of #53AMSTYEL at varying pH	31
5	showing CMCase activity of #53AMSTYEL at varying pH	31
6	showing β- glucosidase activity of #53AMSTYEL at varying pH	31

LIST OF FIGURES

S.No.	Name of the Figure	Page No.
1	Structure of Cellulase	14
2	Reaction shown by Cellulase	15
3	Showing zone of hydrolysis in primary and secondary screening	28
4	Showing zone of hydrolysis from culture filtrate	29

LIST OF TABLES

S.No.	Title	Page No.
1	List of microorganisms producing cellulases.	17
2	List of fungal endophytes used for screening of cellulolytic endophytic fungi.	26-27
3	Showing biomass produced by culture filtrates	28
4	Showing zone diameter (mm) produced by selected fungal culture filtrates	28
5	Showing Fpase, CMCase and β-glucosidase activity of selected four isolates	30

ABBREVIATIONS

CMC	Carboxymethyl Cellulose
PDA	Potato Dextrose Agar
CZDA	Czapek Dox Broth
CZDB	Czapek Dox Broth
PNGP	p-nitrophenyl –β- glucopyranoside
FPU	Filter Paper Unit
FPA	Filter Paper Assay
CMCase	Carboxymethyl Cellulase
rpm	revolution per minute
psi	pounds per square inch
O.D	Optical density
µg	microgram
nm	nanometer

ABSTRACT

Cellulose is most abundant natural bio-polymer on earth and major municipal and agricultural waste. In nature, hydrolysis of cellulose occurs as a result of the action of a cellulase complex produced by cellulolytic microorganisms. Most cellulolytic microorganisms comprised of eubacteria and fungi, although some anaerobic protozoa and slime molds are also able to degrade cellulose. Bacterial and fungal cellulases are extensively studied for the cellulose degradation. These enzymes have various novel industrial applications and now considered as one of the major group of industrial enzymes.

In the present study, 34 endophytic fungi isolated from medicinal plants were screened for cellulolytic activity on Czapek Dox agar plates containing cellulose. Four cultures viz. #53AMSTYEL, #6AMSTYEL, #1CCSTITD, #23(b) AMSTYEL after primary and secondary screening were selected for culture filtrate production, which was then screened for cellulase activity. #6AMSTYEL showed maximum zone of clearance i.e. 18mm followed by #53AMSTYEL. These selected culture filtrates were then subjected to Filter paper activity (FPase), Endoglucanase (CMCase) activity, β-glucosidase activity assays. #53AMSTYEL was showing maximum amount of product released which was directly correlated with cellulose degradation followed by #6AMSTYEL, #1CCSTITD in FPase, CMCase and β-glucosidase activity assays. Effect of temperature and pH variation on #53AMSTYEL celulolytic potential revealed that the cellulolytic activity increases with increase in temperature and but was poorest if pH drops down to 3. #53AMSTYEL exhibited maximum celluloytic activity at 35°C-40°C and pH 5.

Further studies on morphological and molecular identification of the potential endophytic fungi and cellulase purification, characterization, kinetics and improvement in production process needs to be done.

1. INTRODUCTION

Cellulase is the most prominent group of hydrolytic enzymes produced mainly by bacteria, fungi and protozoans that catalyze hydrolysis of β-1, 4-glycosidic bonds present in the cellulose. Cellulases break down the cellulose molecules into the simple monosaccharides such as glucose. Glucose produced from cellulosic substrate could be further used as substrate for subsequent fermentation or other processes which could yield valuable products like ethanol, methanol, amino acids, single-cell protein etc.

Cellulases are multi-enzyme system that consist of three major components: (1) endo-*β-1,4-glucanases* (EC 3.2.1.4), (2) *exo-β-1,4-glucanases* (EC 3.2.1.91), (3*) β-glucosidases* (EC 3.2.1.21). These three components work together synergistically to hydrolyze cellulose into the sugar. Endoglucanases hydrolyze internal β-1,4 linkages of cellulose to create new reducing and non-reducing ends. The exoglucanases acts on the ends of cellulose chain and releases β-cellobiose as the end product. The β-glucosidase acts specifically on the β-cellobiose disaccharides and produce glucose. Various forms of these enzymes are present in the different species to hydrolyze diverse forms of cellulose present in the nature.

The most important application of cellulase is in the production of bio-fuel from the cellulosic biomass. Every year, about 200 gigatons of carbon-dioxide are fixed from the earth and equivalent amount of organic material has to be degraded approximately 30% by plants and animals to 70% by microorganisms. On an average, cellulose accounts as 50% of the dry weight of plant biomass. Such plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity. Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited and inexpensive. Due to limited nature of fossil fuels reserves which has been depleted at an alarming rate has created lot of problems for civilized world. These concerns have been replaced to utilize renewable resources for the production of a greener energy replacement which can meet the high energy demand of the world. Cellulases are the enzymes which can hydrolyze these sources of cellulose. The cellulose is initially hydrolyzed into smaller sugar subunits like monomers and dimers. These sugar subunits can be further hydrolyzed to form biofuels or bioethanol.

Apart from the production of biofuel, the major industrial application of cellulases are in textile industry for bio polishing of fabrics and producing stonewashed looks of cloths, as well as household laundry for improving fabric brightness and softens dimers etc. These sugars can be

further be broken down and fermented into bio-ethanol. Besides, they are used in the animal food as feed additives; they are also used in the processing of fruit and vegetable juices. De-inking of paper is yet another exciting application of cellulases. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals. Application of these enzymes in the detergent, leather and paper industries demands identification of highly stable enzymes at extreme pH and temperature.

Today, the most prominent microbial sources of cellulases for industrial scale production are mesophiles grown in the temperature range of 30-35°C such as *Trichoderma reesi* and *Aspergillus niger*. The enzymes from thermophiles grown in the temperature range of 50-90°C are important for the harsh industrial processes, least prone to the contamination and have faster reaction rates. A few examples of such micro-organisms are *Clostridium thermocellum*, *Thermoanaerobacter* sp, and *Thermotoga maritime* (Gomes and Steiner, 2004). The enzymes isolated from pyschrophiles such as *Clostridium* sp, and *Pseudalteromonas haloplanktis* (Violot *et al*., which grow within the temperature range of 5-20°C have advantages in applications where temperature is detrimental to the product and also reduce the energy consumption. Hence, depending upon the suitability of desired product, an appropriate microbial producer of hydrolytic enzymes can be selected from a wide array present in the nature.

Among bacteria, *Acetobacter xylinum* is a well-known cellulase producer. Other bacteria such as *Aerobacter*, *Achromobacter*, *Agrobacterium*, *Alacaligenes*, *Azotobacter*, *pseudomonas*, *Rhizobium* and *Sarcina* are also known to synthesize cellulase. Cellulases have been commercially available for more than 30 years and theses enzymes have represented a target for both academic as well as industrial research. But the major limitation in all the microbial cellulase lies in the fact that low product yield and intrinsic properties like enzyme synthesis, secretion and lower activity profiling. So there exists need to shift to better natural sources that can overcome all the limitations existing in previous microbial models.

Endophytic fungi are one of the most common producers of cellulases. Endophytic fungi are those which live symbiotically with the majority of plants by entering their cells, are utilized as an indirect defense against herbivores (Lumyong and Hyde, 2004) The endophytic fungi uses plant carbohydrate as a source of energy, while in turn, fungus provides plants the benefits which includes increased water or nutrient uptake and protection from phytophagous insects. Endophytic fungi are the gold mines of novel bioactive compounds like antimicrobials,

antivirals, anti-oxidants and immunosuppressive agents. *Cryptosporiopsis quercina,* an endophyte from *Tripterigeum wilfordii*, produces a very potent anti-mycotic agent cryptocandin (Strobel *et al*., 1999). Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria as well as against the fungus *Helminthsporium sativum* (Zou *et al*., 2000). *Taxomyces andreanae* was discovered from *T. brevifolia* and found to produce the billion dollar anti-cancer agent Paclitaxel (Strobel *et al.*, 1993).

Endophytic fungi thus represent an underexplored biological resource for screening cellulase production and are the basis of the current study.

2. REVIEW OF LITERATURE:

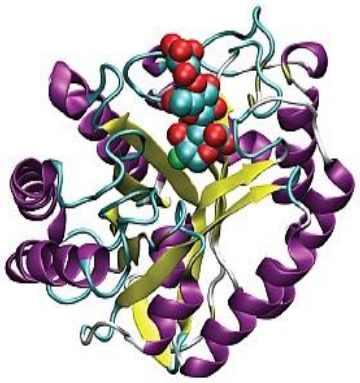


Figure 1 showing structure of cellulase.

Cellulose has occupied a unique place in the world of polymers. In 1838, Payen identified cellulose as a definitive substance and gave the term "cellulose" (Payen, 1838). Cellulose has been used as precursor for chemical modification even before its polymeric nature was discovered and well understood. The main source of cellulose is the plant material. Cellulose is a polymer of raw material used for two main purposes. For many years, it has served mankind as a construction material, mainly in the form of intact wood and textile fibers such as cotton or flax or in the form of paper and board. On the other hand, cellulose is a versatile starting material for chemical conversions, aiming at the production of artificial, cellulose based threads and films. In addition to plants, cellulose is also produced by various bacterial species in nature.

A wide variety of bacteria and fungi produce cellulolytic enzymes able to hydrolyze cellulose. However, relatively few fungi and bacteria produce high levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively (Bhat and Bhat, 1997).

2.1 Classification of cellulases:

Micro-organisms produced extracellular cellulases that are either free or cell associated to and anaerobic bacteria and fungi has been thoroughly reviewed during the past three decades. Cellulases are generally classified on the basis of their mode of action.

(1) Endoglucanases or Endo-1,4-β-D-Glucan Glucanohydrolases (EC 3.2.1.4); they cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharide of various lengths and consequently new chain ends.

(2) Exoglucanase or 1,4-β-D-Glucan cellobiohydrolases (EC 3.2.1.91); they act in a possessive manner on the reducing or nonreducing ends of the cellulose polysaccharide chain, releasing either glucose (glucanohydrolases) or cellobiose (cellobiohydrolases) as the major products. These enzymes are active against the crystalline substrate such as Avicel, amorphous cellulose

(3) β-glucosidases; they act specifically on the β-cellobiose disaccharides and produce glucose

The mechanism of cellulase degradation by aerobic bacteria and fungi is similar thus, it is clear that anaerobic bacteria operate in different manner to degrade the cellulose.

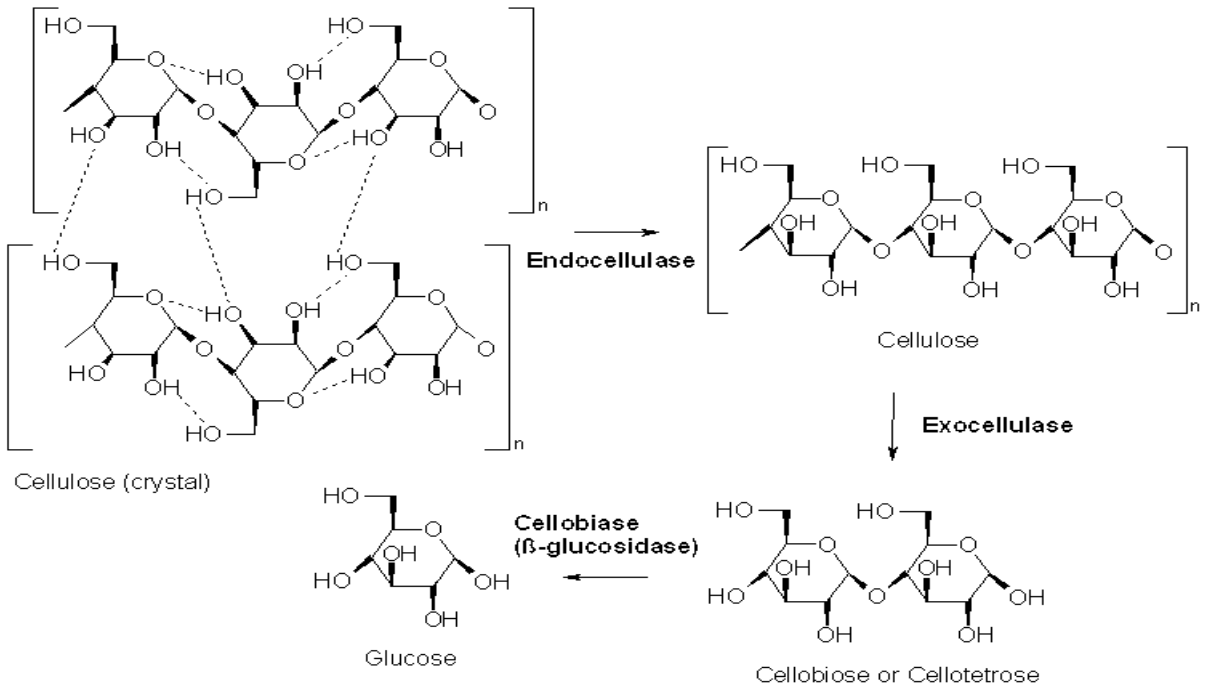


Figure 2 Reaction shown by cellulases: breakage of the non-covalent (endonuclease), Hydrolysis of chain ends (exoglucanase), Hydrolysis of disaccharide and tetrasaccharides into glucose.

2.3 Sources of cellulase

2.3.1 Fungus as a source of cellulases:

Since more than forty years scientists have worked on isolation, characterization, estimation and application of cellulases from fungal and bacterial species. The history of cellulose degradation starts with the understanding about the role of *Trichoderma* sp in cellulose degradation. *T. reesei* is most commonly employed by researchers to produce cellulases from fungus. It is one of best characterized fungal cellulase system. The fungus produces large amount of different cellulases that act synergistically for complete hydrolysis of cellulose to produce glucose. It produces at least three different types of endoglucanases, two different cellobiohyrolases and beta glucosidases (Dwey & Mandel, 1980).

The filamentous fungus *Trichoderma reesei* is today a paradigm for commercial scale production of different cellulases and hemicellulases and is well adapted to fermenter cultivation. Beside well-established applications of these enzymes in paper, pulp, food, feed or textile processing industries, these plant degrading enzymes are nowadays being used in

saccharification of cellulosic biomass to simple sugar for bio-fuel production (Bouws *et al*., 2008; Kumar *et al*.,2008).

The cellulase systems of the aerobic fungi *Trichoderma reesei, Trichoderma viride, Penicillium pinophilum, Phanerochaete chrysosporium (Sporotrichum pulverulentum), Fusarium solani, Talaromyces emersonii, Trichoderma koningii and Rhizopus oryzae* are well characterized (Bhat and Bhat, 1997, Murashima *et al*., 2002). Some thermophilic aerobic fungi and mesophilic anaerobic fungi (*Neocallimastix frontalis, Piromonas communis, Sphaeromonas communis*) (Bhat and Bhat, 1997) also produce cellulases.

2.3.2 Bacterial sources of cellulases:

The bacterial system has also been investigated for the production of cellulases. Both bacteria and fungi have been exploited for the production of a wide variety of cellulases and hemicellulases. However, fungi have been exploited the most because of their capability to produce large amounts of cellulolytic enzymes and often less complex than the bacterial cellulase and easy for extraction and purification. However, the isolation and characterization of novel cellulases from bacterial strain are now becoming widely popular. There are several reasons for this (i) bacteria have higher growth rate than fungi allowing for more recombinant production of enzymes. (ii) Bacterial enzymes are more complex and are in multi-enzyme complex providing increased function and synergy.(iii) Bacteria often inhabit a wide variety of environmental and industrial niches like thermophilic or psychrophilic, alkaliphilic or acidophilic and halophilic strains, which produce cellulolytic enzymes are highly resistant to harsh condition of environment. These strains can survive and produces cellulolytic enzymes which can withstand the harsh conditions of environment which can be used in the bioconversion process. This may increase rates of enzymatic hydrolysis, fermentation, and, product recovery.

Cellulose-decomposing aerobic and non-aerobic bacteria have been isolated from the sugar cane field soil. Single clones of cellulolytic organism were identified as a member of *Cellulomonas*.

2.3.3 Other Sources of cellulase

Apart from these conventional sources of enzymes, cellulases have also been found in various

insects like termites, nematodes, mollusks and plants. It has been found that several termites have cellulolytic potential as they use the lignocellulosic substrate as their source of energy. This ability makes them an optimal resource to prospect for novel cellulolytic enzymes (Martin, 1983). Several cellulase studies have been reported on mollusks, including snails (Parnas, 1961), a sea slug (Elyakova, Sova, & Vaskovsky, 1968) a periwinkle, and some bivalves (Yokoe & Yasumasu 1964)

Source	**Name of organism**	**Reference**
Fungi	*Pestalotia sp.*	Metha *et al*., 1975
	Stachy sp.	Mandels *et al*., 1976
	Botsysatra sp.	Shoemaker *et al*., 1978
	Polypores sp.	Hyashida *et al*., 1980
	Aspergillus niger	Ghokle *et al*., 1984
	Aspergillus flavus	Ojumu *et al*., 2003
	Trichoderma reesi	Muthuvelayudham R., 2006,
Bacteria	*Ruminococcus albus*	Varel, 1984
	Succinogens	Groleu *et al.*, 1981
	Cytophaga hutchinsonii	Clifford Louime *et al.*, 2006
	Branhamella	Ekperigin, M.M., *et al*., 2006
	Pseudomonas sp.	Yamane *et al*., 1970
	P. fluorescens	Yoshikawa *et al*., 1974
	Bacillus subtilis (CBTK 106)	Chundakkadu Krishna, 1999
	Clostridia	Bisaria *et al*., 1981

Table No. 1 showing list of micro-organisms producing cellulase

2.3.4 Endophytic fungi as a source of cellulase

Endophytic fungi have been widely explored for the production of cellulases and bioactive compounds. Endophytic fungi live in a symbiotic relationship with the majority of plants by entering their cells. There are reports demonstrating that many antitumor agents like taxol and antimicrobial agents can be produced by these endophytic fungi.

Strobel and Daisy (2003) demonstrated the phenomenon of bio prospecting endophytic microbes for the natural products. Accordingly endophytes are group of microbes that represents an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial arenas. The endophytic fungi that have been reported as cellulase producers include *Alternaria alternata*, *Hymenoscyphus ericae* and *Aspergillus terrus.* Endophytes represent a promising group of organisms, as they are mostly untapped reservoir of metabolic diversity. They are often able to degrade cellulose, and they can produce an extraordinary diversity of metabolites.

The filamentous fungal endophyte *Ascocoryne sarcoides* was shown to produce potential biofuel metabolites when grown on cellulose based medium. *Aspergillus niger* is another fungi which produces secondary metabolites like citric acids, cellulases and gluconic acid. According to fungal taxonomist, *A. niger* consist of a group of nine genera some of which have a great potential for cellulase production. For the production of cellulases various types of substrates including wheat bran, wheat straw, rice straw, corn cob, cotton flower shell, ground nut shell etc. have been used by the researchers. Wheat straw has been reported as best substrate for cellulase activity (Kang *et al.,* 2004; Yang *et al*.,2004).

2.4 Production of Secondary Metabolites

Fermentation is an anaerobic process which is carried out in the presence of nutrients and metabolites, which are required for growth and reproduction of microorganisms. It is a metabolic process which involves the breakdown of complex organic compounds into simpler products with the release of energy, which is further used to carry out many activities. This oxido-reduction process is mostly extracellular and involves the enzymes which are released by microorganisms.

2.5 Methods of cellulase activity

There are two basic approaches two determine the cellulase activity (1) measuring the individual cellulase (endoglucanase, exoglucanase, β-glucosidase) activities.

2.5.1 Endoglucanase Activity Assay

Endoglucanase activities can be measured on a reduction in substrate viscosity or by an increase

in reducing ends determined by reducing sugar assay. Because exoglucanase also increase the number of reducing ends thus, it is strongly recommended that endoglucanase activity can be measured by both the methods.

2.5.2 Exoglucanase Activity Assay

Avicel has been widely used for measuring the endoglucanase activity. During chromatographic fractionation of cellulase mixture, enzymes with low activity on CMC but showing relatively high activity on avicel are usually identified as exoglucanase.

2.5.3 β-D-Glucosidase Activity Assay

β-D-glucosidase are very amenable to a wide range of simple sensitivity assay methods, based on coloured or fluorescent products from p-nitrophenyl-β-D-glucopyranoside (strobe and Russel, 1987). Also, β-D-glucosidase activity can be measured using cellobiose, which is not hydrolyzed by endoglucanases and exoglucanases.

2.5.4 Total Cellulase Activity Assay

Total cellulase system comprises endoglucanases, exoglucanases and β-D-glucosidases. All of them hydrolyze crystalline cellulose synergistically. Total cellulase activity assays are always measured using insoluble substrates, including pure cellulosic substrates such as Whatman no.1 filter paper, cotton linter, microcrystalline cellulose, bacterial cellulose, fungal cellulose etc.

Therefore, the cellulase activity is generally expressed in terms of the substrate degraded by the microbes, for example: hydrolysis of carboxymethyl cellulose as carboxymethyl cellulase (CMCase), filter paper hydrolysis as filter paper cellulase units (FPU), Avicel cellulose digestion as Avicelase, cotton fiber hydrolysis as cotton cellulase, etc.

3. Aim of the study:

Aim of the current study includes:

1. Screening of cellulase producing endophytic fungi
2. Analysis of cellulase activities
3. Effect of variable temperature and pH on cellulase activity

4. Material and methods:

4.1 Maintenance and preservation of pure culture of Endophytic fungi

Potato Dextrose Agar (PDA) plates were used for the recovery of cultures from the main stock culture. Then, these cultures were maintained by sub culturing after regular interval and finally preserved on the PDA slants (Agarwal and Hasija, 1986).

4.1.1 Preparation of Potato Dextrose Agar (PDA) plates

39.0 g of PDA (Hi-Media) was dissolved in 1L of double distilled water, stirred thoroughly to ensure proper mixing and autoclaved at 121°C, 15 psi for 15 min. Aseptically pour 22.5 ml of autoclaved PDA in sterile 90 mm petri-plates and allowed to solidify at room temperature. The plates were kept at 26 ± 2°C overnight for quality check.

4.1.2 Sub- Culturing and preservation

34 endophytic fungi isolated from medicinally important plants viz. *Aegle marmelos, Cinnamomum malabaricum, Cinnamomum camphora, Cinnamomum zeylanicium, Raulwofia serpentina, Cathranthus roseus* and *Camellia sinensis* were aseptically sub cultured on to PDA plates and were incubated at 26 ± 2°C for 7-10 days until the profuse growth was seen. The loop full of the culture was aseptically inoculated on to PDA slants containing 10% glycerol for long term preservation.

4.2 Primary Screening for cellulolytic endophytic fungi

34 endophytic fungi were subjected for the cellulolytic activity on the basis of their ability to produce cellulase complex following the method of Teather and Wood (1982).

The spore suspension of 10^6 spores (Narasimha *et al*., 2006) was used for the screening of cellulolytic endophytic fungi on Czapek-Dox agar medium supplemented with 1% carboxymethyl cellulose and 1% Agar. The pH of the medium was adjusted to 5 and the media was autoclaved at 121°C, 15 psi for 15 min. After autoclaving, the media was poured into the sterile petri-plates and allowed to solidify. In each petri-plate, 5mm well was punched out with the help of sterile cork borer. To the well, 100µl of spore suspension of different endophytic fungi was dispensed and incubated at 28 ± 2ºC for 3days and then incubated at 50°C for 18 hours which is the optimum temperature for cellulase activity. Distilled water served as control. After

incubation is over, the plates were stained with 10 ml of 2% Congo-red dye and left for 15 min at 50 rev/min. The Congo red solution was then drained off and plates were destained by adding 1N NaOH followed by shaking at 15 rev/min for 15 min. Finally, the destain solution1N NaOH was discarded and the zone of hydrolysis was observed.

4.3 Secondary screening of cellulolytic endophytic fungi

The endophytic fungi which showed positive results in primary screening for cellulolytic activity were further subjected for secondary screening. The cultures were screened by following the same protocol used in primary screening. Each isolate was tested in triplicate and zone of hydrolysis of each isolate was noted. Mean and SD was calculated.

4.4 Production of culture filtrate of selected endophytic fungi

The selected enodphytic fungi were subjected to culture filtrate production. 5mm mycelia plug of 7 day old culture was inoculated in 100ml sterilized Czapek-Dox broth (pH- 5) supplemented with 1% cellulose in 250ml Erlenmeyer conical flasks. The flasks were incubated at 28°C, 120rpm for10 days. After the incubation is over, the fungal mycelium was separated from broth through filtration using Whatman filter paper No.1 followed by centrifugation at 12,000 rpm for 15 min to get cell free culture. The weight of biomass produced was noted. The filtrate was further used for testing cellulase activity i.e. endoglucanase activity, exoglucanase activity and β-D-glucosidase activity.

4.5 Cellulase Activity assay

The cell free filtrate of selected isolate were tested for cellulase activity on Czapek-Dox agar plates containing 1% carboxymethyl cellulose. The plates were divided into four equal sections and 5mm well was punched out in each section. In three sections, 40µl of culture filtrate was dispensed and in fourth section the un-inoculated Czapek dox broth with 1% cellulose was added which served as control. The plates were then incubated at 28 ± 2ºC for 3days followed by incubation at 50°C for 18 hours. After the incubation is over, the plates were stained with congo red for 15 min followed by counter destaining with 1N NaOH and plates were observed for the zone of cellulose hydrolysis. The zone diameter was noted in triplicate.

4.6 Filter paper Activity (FPase) assay

Filter paper activity of culture filtrates was determined according to the method proposed by Mandels & Webber, 1969. The 5mm discs of Whatman filter paper No.1 weighing about 50 mg were suspended in 1 ml of 0.05 M Sodium citrate buffer (pH 4.8) and incubated in a water bath at 50°C for 15min. 50µl of culture filtrate of selected isolates were added to the mixture and incubated at 50°C for 1h. After incubation is over, the 1 ml of 3, 5,-dinitrosalicylic acid was added to the mixture to estimate the amount of sugar liberated which is directly proportional to amount of cellulose digested. The absorbance of the mixture was measured at 540nm. One filter paper unit (FPU) was defined as the amount of enzyme releasing 1µmole of reducing sugar from filter paper/ml/hr.

4.7 Endoglucanase activity (CMCase) assay

Endoglucanase activity of the selected culture filtrates was quantified by using the carboxymethyl cellulose method (Ghosh 1987). To initiate the reaction, 1 ml of 1% CMC in 0.2 M acetate buffer (pH 5.0) was pre- incubated at 50°C in a water bath for 20 minutes followed by addition of 0.5 ml of selected culture filtrate and incubated at 50°C in the water bath for 1h. Control comprised of CZD broth instead of test sample. After incubation is over, the 1 ml of 3, 5,-dinitrosalicylic acid was added to the mixture to estimate the amount of reducing sugar liberated. The color developed in the reaction mixture was measured at 540 nm. One unit of endoglucanase activity was defined as the amount of enzyme releasing one µ mole of reducing sugar/ml/hr.

4.8 β-D-Glucosidase activity

For the determination of β-D- glucosidase activity, the test reaction mixture comprised of 0.2 ml of 5mM p–nitrophenyl-β-D-glucopyranoside (PNGP) in 0.05M citrate buffer (pH 4.8) and 0.2 ml of test culture filtrate. Control is same as that of test except that the CZD broth was added instead of test culture filtrate sample. The reaction mixture was incubated at 50°C for 30 min. After that, the reaction was stopped by the addition of 4 ml of 0.05M NaOH-glycine buffer (pH 10.6).The yellow color will develop due to formation of p-nitrophenol. The absorbance was measured at 420nm. One unit of β – glucosidase activity was defined as the amount of enzymes liberating 1 µ mole of p – nitro phenol/ml/h under standard assay condition.

4.9 Effect of Temperature on Cellulase Activity

Temperature is an important factor which has vital effect on the yield of product. The selected cultures were subjected to production of culture filtrate at varying temperature viz. 30ºC, 35ºC, 40ºC in CZD media supplemented with 1% cellulose. Each of the culture filtrate obtained were again screened for FPase, CMCase, β-glucosidase activity so as to check the optimum temperature at which maximum activity is observed

4.12 Effect of pH on cellulase activity

pH of the medium also play a very important role in cellulase production. To demonstrate the effects of varying pH value on cellulase synthesis, initial pH of CZD media were adjusted to 3, 4, 5, 6, 7 and 8. Each of the culture filtrate obtained were again screened for FPase, CMCase, β-glucosidase activity so as to check the optimum pH at which maximum activity is observed

5. Results and discussion

5.1 Re-culturing

34 Endophytic fungi were used in the present investigation for potential cellulolytic endophytic fungi. Each of the isolate was regularly sub-cultured on PDA plates and maintained at 28˚C. For long term preservation, all the isolates were transferred to PDA slants containing 10% glycerol and maintained at 28˚C.

In the present study, endophytic fungi were isolates of the medicinally important plants belonging to family Rutaceae (*Aegle marmelos*), Lauraceae (*Cinnamomum malabaricum, Cinnamomum zeylanicium, Cinnamomum camphora*), Apocyanaceae (*Catharanthus roseus, Tabernaemontana divaricata)* and Piperaceae *(Piper nigrum)* collected from the biodiversity hot spots of India.

Out of the 34 isolates, 12 were isolated from *Aegle marmelos*- 6 were from stem, 2 from stem internal tissue, 2 from leaves, 1 was from bark; 5 endophytic fungi were isolated from stem internal tissue of *Cinnamomum camphora*; 6 endophytic fungi were isolated from *Cinnamomum malabaricum*- 2 were from bark, 2 from stem, 2 from stem internal tissue; 2 were from stem of *Catharanthus roseus*; 7 endophytic fungi were isolated from *Raulwofia serpentina* - 2 were from stem, 2 from stem internal tissue; 3 from leaf; one endopyhyte was isolated from stem of *Tabernaemontana divaricata*, bark of *Cinnamomum zeylanicium* and Leaf of *Piper nigrum.*

5.2 Primary and secondary Screening for cellulolytic endophytic fungi

34 endophytic fungi were subjected for preliminary screening for their ability to degrade cellulose as their source of carbon and results in the formation of zones of hydrolysis on the carboxymethyl cellulose (CMC) agar plates. Table No. lists the fungal endophytes screened for the cellulolytic activity.

Out of the 34 cultures, 15 cultures were found to be positive for cellulolytic activity. These 15 cultures were further subjected for secondary screening. Each of selected isolate was tested in triplicate. Out of selected cultures, #6AMSTYEL, #23(b) AMSTYEL, #53AMSTYEL, and #1CCSTITD were found to possess potential cellulolytic activity. #6AMSTYEL was having the maximum activity with 20mm zone around the colony on CMC plates followed by #23(b)AMSTYEL, #53AMSTYEL, and #1CCSTITD with 14mm zone around colony.

S.no	Culture code	Name of plant	Plant part	Place of Sampling	Zone after 72h (mm)
1	#16AMLWLS	*Aegle marmelos*	Leaf	Wayand Wildlife Scantuary, Kerala	+
2	#1088AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayand Wildlife Scantuary, Kerala	+
3	#23(b) AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Kerala	++
4	#9(b) AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Kerala	-
5	#53 AMSTWLS	*Aegle marmelos*	Stem	Wayand Wildlife Scantuary, Kerala	++
6	#1 AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Kerala	-
7	#6 AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Kerala	+++
8	#1048 AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Kerala	+
9	#61AMLWLS	*Aegle marmelos*	Stem	Wayand Wildlife Scantuary, Kerala	-
10	#20 AMSTWLS	*Aegle marmelos*	Stem	Wayand Wildlife Scantuary, Kerala	-
11	#11 AMBAWLS	*Aegle marmelos*	Stem	Wayand Wildlife Scantuary, Kerala	-
12	#6(b) CCSTITD	*Cinnamomu camphora*	Stem internal tissue	Tiger hills, Darjeeling West Bengal	-
13	#2 CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Tiger hills, Darjeeling West Bengal	-
14	#36 CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Tiger hills, Darjeeling West Bengal	+
15	#1CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Tiger hills, Darjeeling West Bengal	++
16	#1639 CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Tiger hills, Darjeeling West Bengal	-
17	#55 CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar, Kerala	-
18	#12CMBABRT	*Cinnamomum malabaricum*	Bark	BRT Wildlife Scantury, Karnataka	+
19	#20 CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	++
20	#6610 CMSTITBRT	*Cinnamomum malabaricum*	Stem internal tissue	BRT Wildlife Scantury, Karnataka	-
21	#36CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-
22	#55 CMSTNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar, Kerala	++
23	#26 CRSTNEY	*Catheranthus reseus*	Stem	Neyyar, Kerala	+

24	#3T CRSTBRT	*Catheranthus reseus*	Stem	BRT Wildlife Scantury, Karnataka	-
25	#5 CZBAWLS	*Cinnamomum zeylanicum*	Bark	Wayand Wildlife Scantuary, Kerala	+
26	#130K TMDSTYEL	*Tabernemontana divaricate*	Stem	Yelandur, Kerala	+
27	#2PNLNEY	*Piper nigerum*	Leaf	Neyyar, Kerala	+
28	#4RSLBRT	*Raulwolfia serpentine*	Leaf	BRT Wildlife Scantury, Karnataka	-
29	#14RSBANEY	*Raulwolfia serpentine*	Bark	Neyyar, Kerala	-
30	#1RSBANEY	*Raulwolfia serpentine*	Bark	Neyyar, Kerala	-
31	#16RSLBRT	*Raulwolfia serpentine*	Bark	BRT Wildlife Scantury, Karnataka	+
32	#3RSSTNEY	*Raulwolfia serpentine*	Stem	Neyyar, Kerala	-
33	#23(a) RSSTNEY	*Raulwolfia serpentine*	Stem	Neyyar, Kerala	-
34	#1RSLBRT	*Raulwolfia serpentine*	Leaf	BRT Wildlife Scantury, Karnataka	-

Note: poor activity (+); average activity (++); Good activity (+++),No activity (-)

Table 2 lists the fungal endophytes used for Screening of cellulolytic endophytic fungi

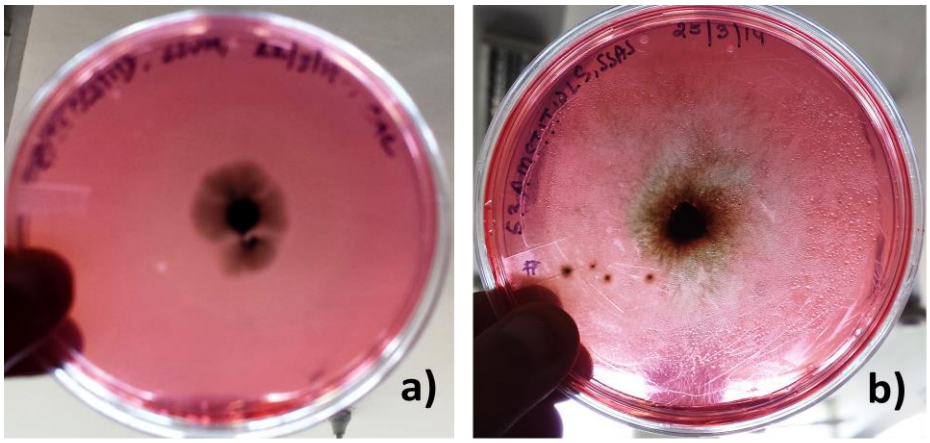


Figure 3 showing zone of hydrolysis of cellulose by endophytic fungi in primary and secondary screening a)1CCSTITD b) #53AMSTYEL

5.3 Production of culture filtrate of selected endophytic fungi

Among 34 cultures, only four culture viz. #6AMSTYEL, #53AMSTYEL, #23(b)AMSTYEL and #1CCSTITD were selected after screening. These cultures were subjected to submerged fermentation in Czapek Dox broth medium so as to qualitatively and quantitatively estimate the cellulase activity. #6AMSTYEL produced highest amount of biomass and also exhibited potential cellulase activity. #53AMSTYEL and #23(b) AMSTYEL produced very less amount of biomass (Table no.3). The culture filtrates were further screened for cellulase activity: endoglucanase activity, exoglucanase activity and β-D-glucosidase activity.

Culture Code	Wt. of Biomass (g)
#6AMSTYEL	3.57
#23(b)AMSTYEL	1.96
#53AMSTYEL	1.58
#1CCSTITD	2.7

Table 3 Showing biomass produced by culture filtrates

5.4 Cellulase activity assay:

The cell free filtrates of selected isolates were screened for cellulase activity on CMC agar plates. The zone of clearance was directly proportional to cellulose degradation. #6AMSTYEL showed maximum zone of 18mm followed by #53AMSTYEL, #23(b) AMSTYEL, #1CCSTITD with 14mm zone.

Culture Code	Zone diameter (mm)
#23(b)AMSTYEL	14 ± 0
#1 CCSTITD	14 ± 0
#6 AMSTYEL	18.33± 1
#53 AMSTYEL	14 ± 0

Table 4 showing the zone Diameter (in mm) produced by selected fungal culture filtrates

Control well containing un-inoculated broth did not result in any zone formation thus eliminating the risk of false results (Figure 4, Table 4).

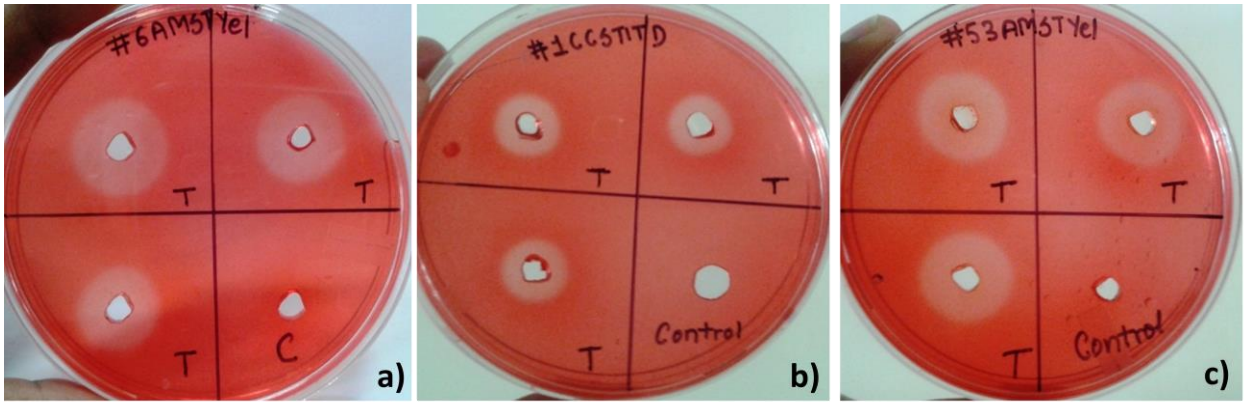


Figure 4 showing cellulase activity of culture filtrate (a) #6AMSTYEL (b) #1CCSTITD (c) #53AMSTYEL

5.5 Filter paper activity (FPase) assay

The four cultures were subjected to FPase activity assay. The amount of cellulose digested is directly proportional to amount of sugar liberated in the reaction mixture which is measured at 540nm. #53AMSTYEL was found to exhibit maximum FPase activity i.e. 20.88 U/ml/h followed by #1CCSTITD, #6AMSTYEL and #23(b) AMSTYEL. Control sample did not show any activity. Maximum FPase activity exhibited by #53AMSTYEL was correlated with amount of cellulose digested by culture filtrate and thus confirming it to be potential producer of cellulase.

5.6 Endoglucanase (CMCase) and β-glucosidase activity assay

In CMCase as well as β-glucosidase assay,#53AMSTYEL exhibited maximum activity followed by #1CCSTITD, #6AMSTYEL and #23(b)AMSTYEL. The amount of sugar and p-nitrophenol liberated in CMCase and β-glucosidase assay respectively was found to be maximum in #53AMSTYEL that is correlated with cellulase activity. Control was not showing any activity.

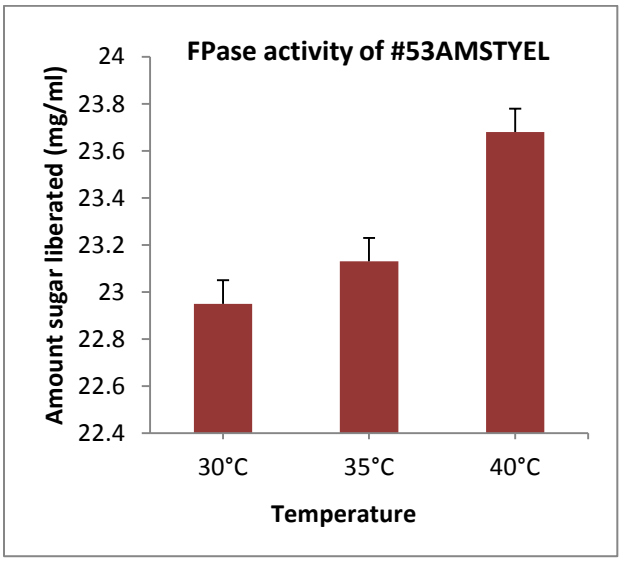
Filter paper activity, CMCase and β-glucosidase assay confirmed #53AMSTYEL to be potential producer of cellulase. (Table 5)

Culture Code	FPase (U/ml/h)	CMCase (U/ml/h)	β-glucosidase (U/ml/h)
Control	0.014	1.161	0.0320
#23(b) AMSTYEL	1.915	3.885	0.417
#1CCSTITD	10.32	6.950	0.496
#6AMSTYEL	5.055	4.936	0.408
#53AMSTYEL	20.88	8.5	0.501

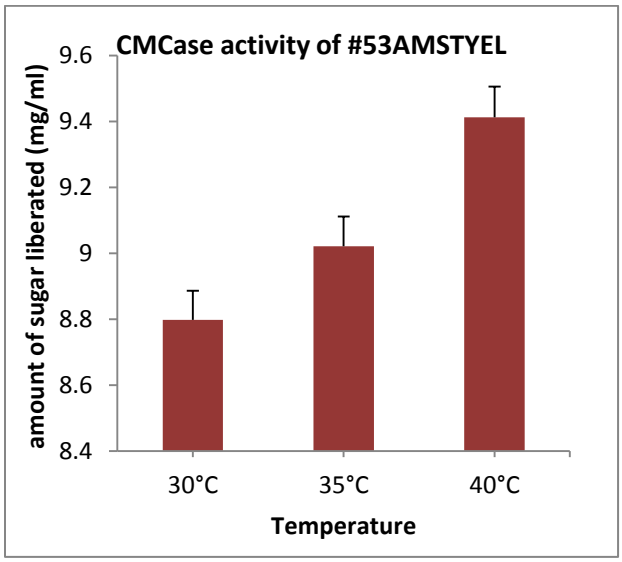
Table 5 showing FPase, CMCase and β-glucosidase activity of selected four isolates.

5.7 Effect of Temperature and pH on cellulase activity

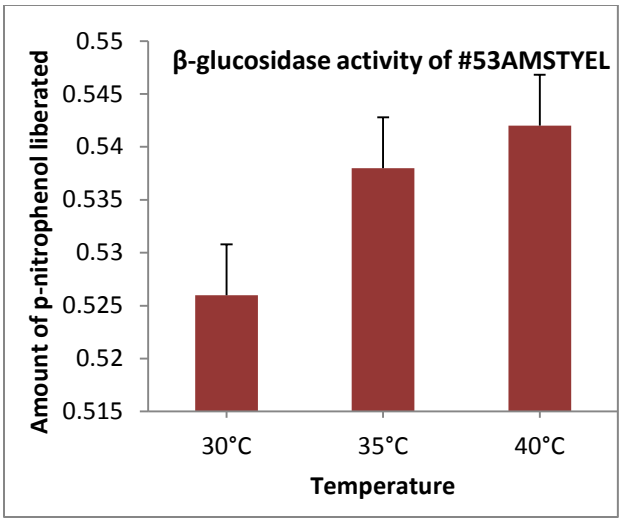
As evident from the Filter paper activity, CMCase, β-glucosidase activity assay, #53AMSTYEL was found to exhibit maximum cellulase activity. Further effect of varying pH and temperature on the cellulase activity viz. Filter paper activity, CMCase, β-glucosidase activity of #53AMSTYEL was studied. It was observed that cellulase activity increases with rise in temperature. The maximum cellulase activity was observed at 35°C -40°C and pH 5 (Graph1-6).



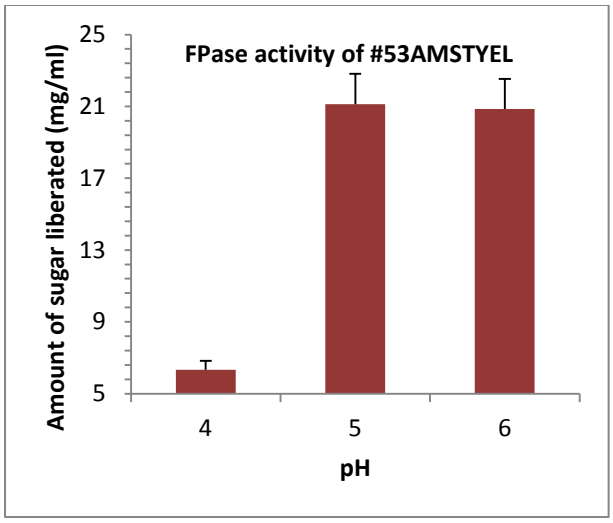
Graph 1 showing FPase activity of #53AMSTYEL at varying temperature



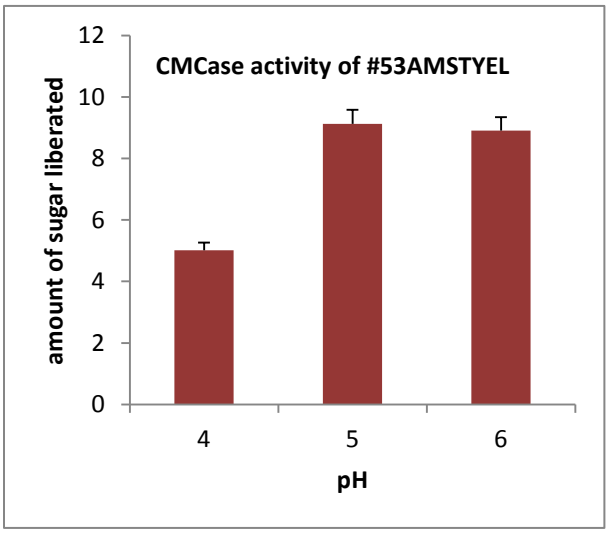
Graph 2 showing CMCase activity of #53AMSTYEL at varying temperature



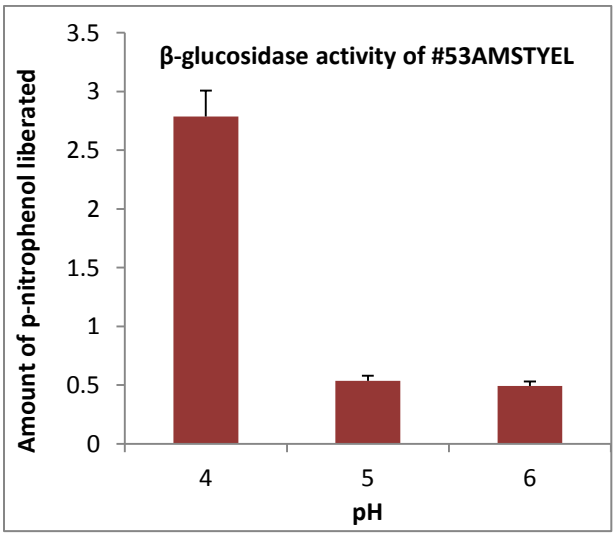
Graph 3 showing β- glucosidase activity of #53AMSTYEL at varying temperature



Graph 4 showing FPase activity of #53AMSTYEL at varying pH



Graph 5 showing CMCase activity of #53AMSTYEL at varying pH



Graph 6 showing β- glucosidase activity of #53AMSTYEL at varying pH

The maximum growth and cellulase production by *Trichoderma sp.* were found at 25- 35°C (Sun *et al*., 2009). The temperature maintained in the SSF system by *Trichoderma koningii*, in general, is the range of 27- 33°C depends upon the growth kinetics of the micro-organisms rather than the enzyme produced. While in case of bacteria like *Pseudomonas* sp. the maximum yield of enzyme activity was found within temperature range of 40 to 45 °C. It was observed that if the initial pH of the substrates varied from 5.0 to 6.0, no major difference was found in the cellulase yield. Optimal cellulase production in #53AMSTYEL was found at pH 5.0 and temperature 35°C -40°C and it may change during fermentation process.

CONCLUSION

The current study describes apart from bacteria, fungi, mollusks, actinomycetes and protozoa, endophytic fungi can be potential source of cellulase with high productivity and thermostability.

Out of 34 endophytic fungi screened for cellulolytic potential, #53AMSTYEL, an endophytic fungus isolated from Indian medicinal plant *Aegle marmelos* exhibited maximum cellulolytic activity in FPase, CMCase and β- glucosidase assays. The cellulolytic activity profiling was found to be maximum at 35ºC-40ºC, pH-5.

Further identification of potential endophytic fungi and cellulase purification, characterization and kinetic parameters needs to be done.

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