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Production and optimization of Cellulases by thermophilic coprophilous fungus *Malbranchea cinnamomea*

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Abstract
The production of the Cellulase enzyme by the thermophilic coprophilous fungus *Malbranchea cinnamomea* was observed through Congo red method and optimization conditions of Cellulase production was done. Of the different media used, it is observed that theYeast Extract Starch Medium and czapek Dox was found to support maximum growth and production of cellobiohydrolase and endoglucanase. The optimum production of cellulases by the fungus required 9 days of incubation at pH 7.0 and a temperature of 45^0C. Among the various carbon sources tested, starch was found to induce maximum amount of both the cellulases, whereas yeast extract induced more amount of cellulases among the different nitrogen sources tested for cellulase production by *Malbranchea cinnamomea*.

Keywords: goat dung, temperature, endoglucanase, cellobiohydrolase, carbon and nitrogen sources

Introduction
Cellulose is a polysaccharide which is abundant in plant cell walls. In primary cell wall they are arranged randomly while in secondary wall they are arranged parallelly [1]. Cellulose is degraded by the enzymecellulase produced by fungi, bacteria and actinomycetes, since the fungi yield high quantity of Cellulase it has been commercially exploited [2, 4]. Further, thermophilic fungi are known to produce thermostable enzymes with activity at high temperatures, broad tolerance to pH variation and resistance to denaturing agents [5-6]. Cellulases are used in the textile industry for cotton softening and denim finishing, in the food industry for mashing and in the pulp and paper industries [7, 9]. Thermostable cellulase can increase the rate of reaction, decrease the amount of enzyme needed and has capability of longer half-life and decrease the possibility of microbial contamination [10, 11]. Engineered thermostable fungal cellulases have the ability in synergistic cellulose hydrolysing at elevated temperatures [12].

"The degradation of cellulose to glucose is achieved by the both enzymes endocellulases and exocellulases as synergistically cooperative action on cellulose is to produce cellooligosaccharides and cellobiose, which are subsequently cleaved by beta-glucosidase to glucose" [13-14]. Degradation of cellulose to its constituent monosaccharides has also attracted considerable attention for the production of food and biofuels [15, 17]. Thermophilic coprophilous fungi play an important role in recycling of organic substances including cellulose [18]. Thermophilic cellulases are the crucial enzymes for capable biomass degradation and their importance stems from the fact that cellulose swells at higher temperatures, thereby becoming easier to break down [5, 19, 22]. Thermophilic fungi which love to grow on dung called as thermophilic coprophilous fungi [23] these organisms are exploited as potential source of thermostable enzymes for biotechnological and industrial applications. In view of above facts, an opportunity was seized to investigate cellulase producing capacity of the thermophilic coprophilous fungus *Malbranchea cinnamomea* isolated from herbivore dung.

Materials and Methods
1. Isolation and identification of the fungus
Dung samples of different herbivore animals viz., cow, sheep, bear, rabbit, deer, elephant, horse, wild sheep, buffalo, ox and zoo dump were collected in sterilized polythene bags from Zoo Park, Warangal, Andhra Pradesh, India. The thermophilic fungi were isolated by dilution plate and paired plate techniques [24]. *Malbranchae cinnamomea* was isolated from goat dung and identified by morphological characteristics under the 40-x compound Microscope based on standard manuals, after all molecularly confirmed by 28SrRNA ITS1 and ITS4 PCR amplification [23].

2. Screening for Cellulase Production
The production of cellulase by the fungus was screened by inoculating onto CMC agar ($NH_4H_2PO_4$ - 1g; KCl - 0.2g; $MgSO_4$. $7H_2O$ - 1g; Yeast extract - 1g; Carboxymethyl cellulose - 26g; Agar - 3g and Distilled Water - 1000ml) and incubating at 45 - 50^0C for 1 week. The colonies obtained were stained with 1% Congo red dye for 30 min and destained with 1 M NaCl for 20 min. The plates were observed for clear zones around the fungal colonies [26].

3. Estimation of Cellulases
1. **Endogluconase** (C_X) (EC 3.2.1.4) activity was assayed viscometrically as suggested by Reese *et al.* (1950) [29].

Ostwald-Fenske viscometer made of corning glass is used for the experiment. The reaction mixture consisted of 15 ml of 0.5% CMC, 5 ml of enzyme and 1 ml of citrate buffer (pH 5.5). The loss in viscosity was measured for every 10 minutes over a period of 30 minutes. The reaction mixture with heat killed (inactivated) enzyme and water served as control. The percentage of loss of viscosity was calculated by using the following formula:

$$A_n \min = \frac{t_1 - t_a}{t_1 - t_o} \times 100$$

Where,
A_n min = Percentage of loss of viscosity
t_1 = Flow time of reaction mixture + inactive enzyme.
t_a = Flow time of reaction mixture + active enzyme.
to = Flow time of water + active enzyme at '0' time

The activity of endogluconase (C_X) was expressed in relative viscometric units (RVU).

Where,
t_{v50} = time required in min to reduce the viscosity of CMC to 50% of the initial viscosity.

2. **Cellobiohydrolase** (C_1) (EC 3.2.1.91) / exogluconase activity was determined by DNS methods as suggested by Miller (1959) [30]. The reaction mixture consisting of 3.5 ml of 0.5% cellulose powder solution, 1 ml of citrate buffer (pH 5.5), 0.5 ml of enzyme and a few drops of toluene was incubated at 30 $\pm$ 1°C for 6 hours. At the end of the incubation period 1 ml of aliquot of the reaction mixture was withdrawn into a test tube and 3 ml of DNS reagent was added and heated for 15 minutes in a boiling water bath. 2 ml of 20% Rochelle salt (Potassium sodium tartarate) was added while the contents were hot and then cooled under running tap water. Blank is prepared by replacing 1ml of enzyme with 1ml of distilled water. Intensity of the colour developed was read at 575 nm against the blank. C_1 enzyme activity was expressed as the increase in mg of reducing sugars (as glucose/ml) liberated in 6 hours.

4. Optimization of cellulases

The optimum activity of the cellulase produced by the fungus was tested by inoculating the fungus into different media [Yeast extract Starch medium (A), Glucose Yeast extract Medium (B) and Czapek dox Modified Medium (C) and each medium is supplemented with 1% and 5% CMC] and incubating at different temperatures and pH. Effect of incubation period and different carbon and nitrogen sources on mycelial growth and cellulase production was also studied.

Results and Discussion

Malbranchea cinnamomea which was isolated from goat dung and identified (Fig.1: A &B) confirmed by molecular studies (Fig.2). *M. Cinnamomea* was found to produce cellulase as observed by the clear zone around the colony by staining with Congo red dye (Fig.3 A &B).

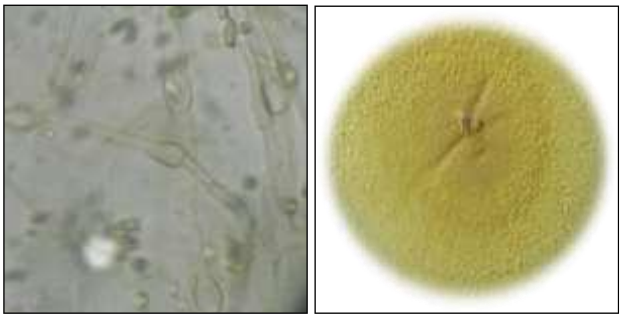

Fig 1: *M. cinnamomea*: a. Colony b. Morphology of fungi under compound microscope [Shanthipriya *et al.* 2019] [23]

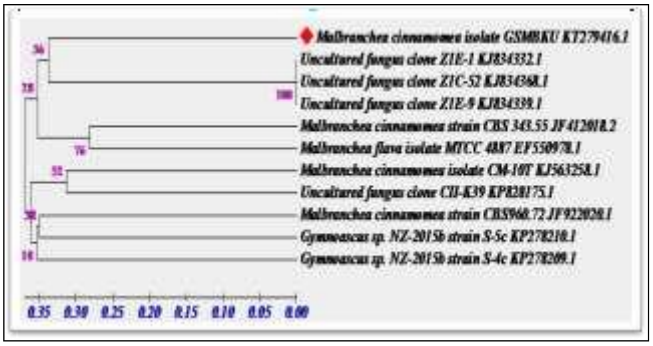

Fig 2: Phylogenetic tree of *Malbranchea cinnamomea* [Shanthipriya *et al.* 201] [31]

Fig 3: A. Control [without organism]

Fig 3: B. Zone around the colony

The amount of cellulase produced by the fungus varied on different media (graph. 1). Yeast Extract Starch Medium supplemented with 5% CMC supported maximum mycelial growth and cellobiohydrolase production, while Czapek Dox Modified Medium with 5% CMC was the next preferred one for vegetative growth. Glucose Yeast Extract Medium with 1% CMC was observed to be the primary medium for maximum production of endoglucanase, while Glucose Yeast Extract Medium with 5% CMC was next preferred for mycelial growth and production of cellobiohydrolase and endoglucanase (Table 1). Czapek dox Modified Medium was responsible for least production of

cellobiohydrolase and endoglucanase. Rest of the media supported intermediate growth and production of cellobiohydrolase and endoglucanase.

Note: Cellobiohydrolase activity expressed in µg/ml of Glucose liberated 6 hrs of incubation Endoglucanase activity expressed in relative viscometric units (RVU)

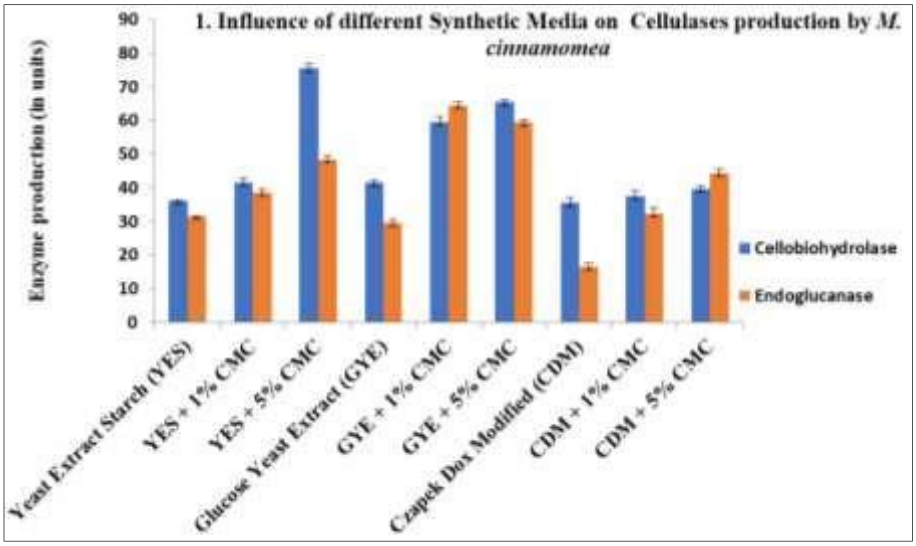

Fig 1

Effect of incubation period on mycelial growth and cellulase production by the fungus was studied and the results are presented in graph 2. The production of Cellulase was started from 3rd day of incubation and continued up to 15 days. The optimum incubation period for maximum growth and production of both cellobiohydrolase and endoglucanase by *Malbranchea cinnamomea* is nine days.

Effect of pH on mycelial growth and cellulase production by *Malbranchea cinnamomea* was studied and the results are presented in graph 3.The fungus could grow in the pH range of 5.0 to 9.0 and opted pH 7.0 for maximum mycelial growth and cellulase production followed by pH 8.0.pH 5.0 was responsible for poor growth and least production of cellobiohydrolase and endoglucanase by the fungus.

Effect of temperature on mycelial growth and cellulase production by *Malbranchea cinnamomea* was investigated and the results are summarized in graph 4. Production of biomass (dry weight) and cellobiohydrolase increased with increase in temperature up to 45˚C, whereas, production of endoglucanase was excellent at a temperature of 50˚C followed by 45˚C. The further increase in temperature decreased the biomass and cellulase production.

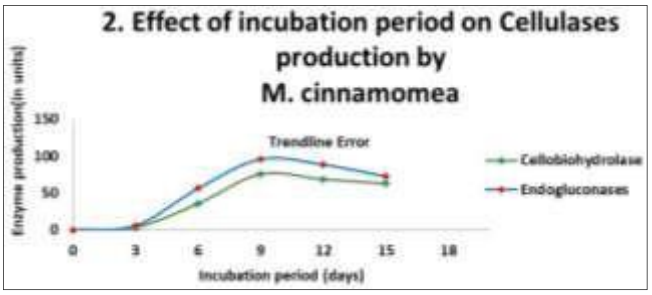

Fig 2

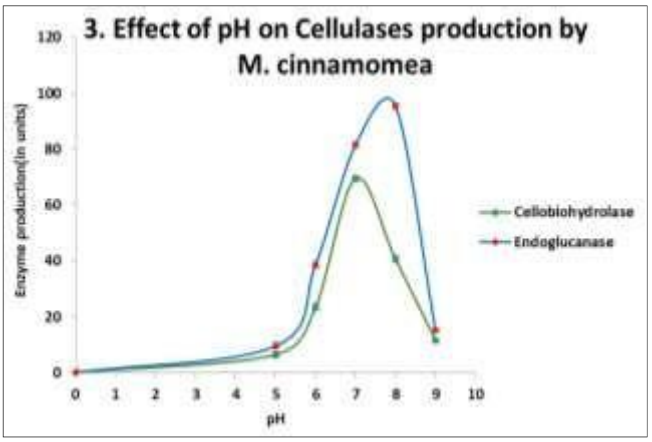

Fig 3

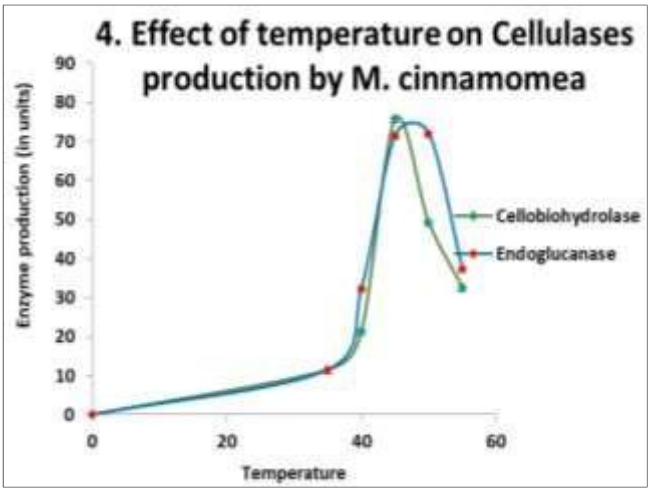

Fig 4

The influence of different carbon sources on mycelial growth and cellulase production (cellobiohydrolase and endoglucanase) by *Malbranchea cinnamomea* was studied and the results are presented in graph 5. *M. cinnamomea* could produce maximum mycelial growth and both the cellulases in starch containing medium. Lactose followed by D-Glucose were the next preferred carbon sources for cellobiohydrolase production and the reverse was found in the production of endoglucanase. D-Glucose followed by lactose were the next preferred carbon sources for mycelial growth of *M. cinnamomea*. Citric acid accomplished poor growth and cellulase production. Succinic acid totally inhibited the growth and cellulase production, while rest of the carbon sources supported intermediate growth and cellulase production by the fungus.

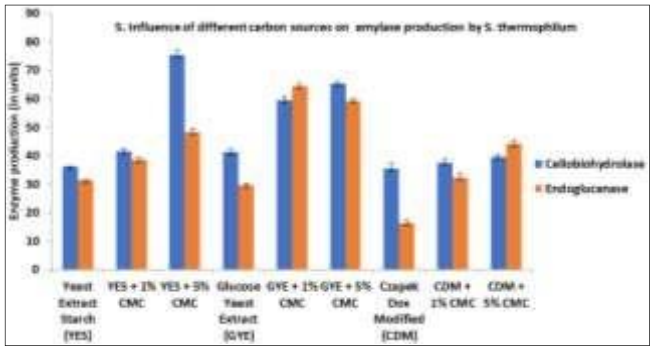

Fig 5

M. cinnamomea could secrete maximum mycelial growth and cellulases (cellobiohydrolase and endogluconase) in yeast extract supplemented media. Ammonium sulphate, L-tryptophan and L- tyrosine were next preferred nitrogen sources for mycelial growth (graph 6) whereas, Ammonium sulphate followed by L- tyrosine for cellobiohydrolase production and L- glutamine and ammonium sulphate were the next preferred nitrogen sources for endogluconase production by *M. cinnamomea*. Sodium nitrate was responsible for poor growth of *M. cinnamomea* and least production of cellobiohydrolase. L-methionine and urea were responsible for least endogluconase activity, while rest of the nitrogen sources induced intermediate growth and cellulase production.

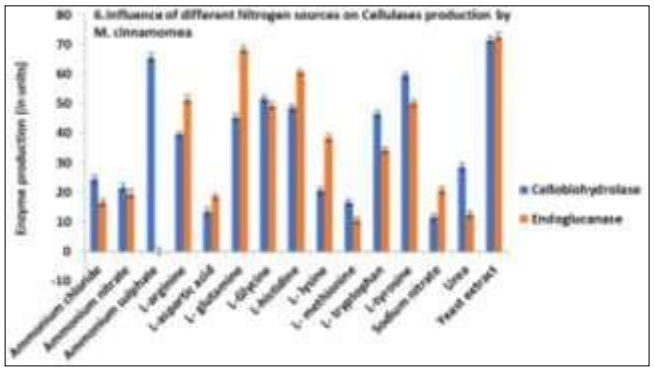

Fig 6

Conclusion

Malbranchea cinnamomea isolated from goat dung has been found to be a potential candidate for production of both endoglucanase and cellobiohydrolase in significant amounts under the optimized conditions of growth medium, temperature, pH and carbon, nitrogen sources and can be used for industrial production of cellulases.

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