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RESEARCH ARTICLE

FACTORS INFLUENCING THE RAMIE CELLULASE SYNTHESIS IN *ASPERGILLUS FLAVUS* BY ASSESSMENT OF DECORTICATION AND DEGUMMING

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ABSTRACT

Ramie (Boehmeria nivea Gaud) a member of Utricacease, yields fibres which are longest and toughest. These fibres showed highest resistant to the action of water and are use industrial point of view in paper and textile industry. A total of 20 fungal strains were isolated from Ramie canes. Of the 20 there are six strains were identified as *Aspergillus* species. Among the Six strains Aspergillus flavus showed maximum colony count at 72 hours of incubation. In the present study deals with the isolation and screening of freshly isolated potent fungal strain as *Aspergillus flavus* for the production of cellulase enzyme consisting of the endoglucanase. Assessment of Decortication and degumming also recorded in cellulase enzyme production by *Aspergillus flavus*. Further the studies were undertaken on the optimization of physiological and nutritional culture conditions in respect of pH, incubation time, and temperature. Maximum cellulase production was recorded with 9 days of incubation time, optimum pH 7.0 and 35^0 C temperature. Synthesis of maximum endoglucanase 77.8% viscosity loss at 12 days of age. *Aspergillus flavus* showed the production of higher activity of cellulase enzyme consisting of endoglucanase yielded higher cellulase enzyme activity.

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INTRODUCTION

Cellulose occurs in native fibres in close interaction with lignin and hemi cellulose. Cellulose represents the most abundant substance in the environment. In the enzyme world, Cellulose is the major component, and cellulose mainly appears on plant cell wall and the most abundant renewable biological resource in the biosphere (Gruno *et al*., 2004). Cellulose is a bio-polymer of glucose units linked by β1,4- glycosidic bonds. Cellulose a readily available renewable energy resource in environment utilized properly. Extensive studies on Cellulolytic enzymes produced by microorganisms like bacteria and fungi shoed the maximum cellulase production. Cellulose is a potentially valuable resource it may available in many forms like fibre, fuel and feed. The cellulosic systems of most fungi are inducible by cellulose and its derivatives and are repressed, almost generally, by glucose, sucrose, fructose, lactose and maltose. These carbon sources greatly influenced the cellulase enzyme production. This regulation is controlled by various parameters such incubation periods, pH and temperature.

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Many fungal strains secrete higher amounts of cellulases than bacterial ones, when compared with *Aspergillus strains* as the leading one (Takashima *et al*., 1997). Other fungal species were shown to be highest cellulase producers, such as *Trichoderma* and *Humicola* (Enari., 1983). A few of unstudied strains could reveal some difference of cellulases since much still to be known on this class of hydrolytic enzymes. The cellulolytic enzymes are composed of three main activities: endoglucanase, exoglucanase and β- glucosidase, are wide spread among bacterial and fungal strains even though the exoglucanases are rare in the bacterial generations. From the available literature a huge information on fungal cellulases. There are more than hundred of endo and exoglucanase sequences are known (Bourne *et al*., 2002). Endo β-gluconases produce nicks in the cellulose polymer exposing reducing and non-reducing ends, cellobiohydrolases acts upon the reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose and β-glucosidase cleave the cellobiose to liberate glucose completing the hydrolysis (Sukumaran *et al.,* 2009). The process of saccharification of lignocellulosic biomass, cellooligosaccharides, hydrolytic enzymes and cellobiose are often produced by the limited hydrolysis of cellulosic materials, something that leads to inefficient ethanol fermentation, whereas their hydrolytic product, glucose, is the best and suitable substrate for ethanol

production was revealed by many authors (Nakata *et al*. 2006) by yeast (Takeshi *et al*., 2008; Tokuhiro *et al*., 2008; Yu *et al*., 2008). Ramie plant is extremely hardy and grow in any soils, preference of sandy loam soils. Ramie shows the maximum fibre yields on moist atmosphere. Ramie contains about 66% of the cellulose. The fibre of the Ramie as the longest (40-200 mm) toughest and most silky of all the vegetables. There was much information regarding the enzyme cellulose particularly. But no information on this important Ramie cellulose from *Aspergillus* species. The production of cellulases by this strain has been analyzed and its cellulase activity characterized. Our results show that the strain *Aspergillus flavus* produces the maximum enzymes with hydrolytic activity on cellulose.

MATERIALS AND METHODS

Isolation of *Aspergillus flavus:* Ramie canes were cut into small pieces with appropriate lengths (2"- 4") and were surface sterilized. The pieces were buried at a depth of 3" in the soil of coconut in holes bored with sterilized troves. The pieces were removed after desired interval and washed in gentle stream of water to remove excess soil. The canes were then placed on Glucosenitrate Agar, cellulose peptone Agar and incubated 96 hours and at 35^0 C temperatures. After incubation the pure colonies were picked and maintained for further studies. Six species of *Aspergillus* were isolated from soil surrounding Ramie canes. *Aspergillus flavus* gave highest colony counts after 72 hours of incubation period.

Preliminary identification of *Aspergillus flavus:* Cellulase producing fungal strains were identified based on colony characteristics (colony size, colony color, colony shape, appearance and pigment production) and micro morphological (mycelium, conidiophores and conidia) characteristics (Jong and Devis, 1976: Butler and Mann, 1959). The isolates were mounted in lacto phenol using tape preparations and examined under microscope.

Growth and morphogenesis of *Aspergillus flavus:* Ramie stem pieces and ribbons (fresh and dried) collected from fields from four months old plants were cut into small pieces and prepared spore suspension. Observations were made for the growth (OD at 610 nm) and Decortication and degumming. Use of NaOH treated ramie cellulose didn't alter this trend significantly. Fibres obtained were of fine quality and bleached.

Optimization studies for enhanced Cellulase production: *Aspergillus flavus* was inoculated into 200 ml of the liquid medium taken into 500 ml Erlenmeyer flask and incubated at room temperature. The impact of incubation period on fungal growth and its enzyme production was studied by incubating the inoculated broth for 6 to 12 days. Influence of pH was determined by cultivating the fungus in culture broth at various levels of pH (5.0 - 10.0) with optimized incubation period. Effect of temperature was investigated by inoculating the strain in CD broth and incubated at different temperatures ((15^0 - 45^0 C) with optimized pH and incubation period.

Cellulase Synthesis

Synthesis of Endoglucanase: The endoglucanase activity was determined by using carboxy methylcellulose (CMC), as follows: 0.5 ml of 10 mg/ ml CMC was incubated at 50^0 C for 30 min in presence of 0.5 ml of crude enzyme preparation, diluted with citrate buffer with slight modifications (Ali Gargouri *et al* ., 2016). DNS (Di nitro Salicylic Acid) reagent (3 ml) was added and the solution was boiled for 10 min. Finally, 20 ml of distilled water was added and the absorbance was measured at 550 nm. One unit of enzymatic activity was determined as the amount of enzyme required to liberate one micromole of reducing sugar (RS) per minute under the assay conditions. The ''auto zero'' was set using heat denatured enzymatic preparation and substrate in order to eliminate any potential interference by using spectrophotometer measurements (Tanguchi *et al*., 2005, Kalogeris *et al*., 2003).

Assessment of Decortication and Degumming: Two different methods were employed in assessing the Decortication and degumming.

Use of LAWN cultures: Ramie canes were collected from four month old ramie plants and 4" long and cut into the pieces. After sterilization stem pieces were rolled on the sporulating petriplate culture of test fungi. It was then transferred aseptically and incubated for 6-12 days at room temperature. Finally the observations were made for decorticating and degumming of fibres at regular intervals.

Spore suspensions of individual fungi: Ramie stem pieces prepared as previously mentioned that the pieces were dipped in standard spore suspension selected fungi and incubated in petriplate moist chambers. After regular intervals stem pieces were removed from moist chambers and observations for Decortication and degumming. The growth of the microorganisms was observed by using spectrophotometer at 610 nm.

Statistical Analysis

Triplicates were maintained for each treatment. Statistical analysis of the data was performed using SPSS software (version 20). ANOVA Two way test was carried out and results were considered to be significant with $P<0.05$.

RESULTS AND DISCUSSION

Isolation of fungi: For the successful isolation of ramie canes fungi, for the months of July to September a period of heavy rain falls yielded a 20 fungi on ramie canes. The cellulase production ability of these strains on CMC-agar plate was observed after primary screening based on the clear zone diameter following 0.1% Congo red staining. Of the twenty strains only 6 were identified as *Aspergillus* strains on the preliminary identification. Among the *Aspergillus* strains *Aspergillus flavus* showed the maximum colony count and showed best colony characteristics. So this strain was selected for the further studies like optimization and assessment studies were recorded. Colonial morphology and microscopic examinations of the various isolates of pure cultures were used to determine the reproductive and vegetative structures. Consequently, identification was done using Onion AHS *et al.,* (1981).

Growth and morphogenesis of *Aspergillus flavus:* Out of 6 *Aspergillus* strains there are only one strain *Aspergillus flavus* which showed the maximum colony count on GNA plates after 72 hours of incubation. The colonies were quite low in all cases.

Effect of incubation period: Cellulase production by Aspergillus flavus was observed form 6 to 12 days of incubation peiod (Table-1). Maximum enzyme production was observed at 9 days of incubation period with 1.23 U/ml. Enzyme production was started initially at 6 days of incubation period. Increasing incubation period up to 9 enzyme production also increased. Above 10 adys of incubation period the enzyme production decreased. Maximum production was observed after 10 days and beyond this, the enzyme production substantially decreased, probably due to the depletion of essential nutrients in the media and/ or accumulation of toxic secondary metabolites produced by the fungus itself (Gautam *et al*., 2010).

Table 1. Effect of incubation period

incubation period (Days)	Cellulase production (U/ml)
6	0.22
7	0.35
8	0.89
9	1.23
10	0.91
11	0.45
12	0.11

Effect of pH: Maximum enzyme production was observed at neutral pH with 1.23 U/ml. Lowest enzyme production was observed with the acidic pH (Table-2). Enzyme production was observed in different pH like 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. Increasing pH above 7.0 the enzyme production was decreased. Acidic and alkaline pH may affect the growth of the microorganism. Maximum specific activity of cellulase (0.393.mol/min/mg) was achieved when the pH of basal medium was kept a 6.5 (Akinyele et al., 2011). some environmental factors are also influenced the growth of the organisms as well as maximum production of enzymes will be at certain optimum temperature, pH, time duration and so on (Immanuel *et al*., 2006). Reports of Dharam datt and kumar (2012) Aspergillus strains showed maximal CMCase activities of *A. flavus* AT-2 and *A. niger* AT-3 were of 9.50 and 12.73 IU/mL at pH 4.8 and 5.3, respectively. FPase activities were also found to be maximum (1.4 and 1.73 IU/mL) at the same pH values for both strains *A. flavus* AT-2 and *A. niger* AT-3, respectively.

Table 2. Effect of pH

pH	Cellulase production (U/ml)
5	0.15
6	0.62
7	1.23
8	0.78
9	0.52
10	0.14

Effect of temperature: For the effect of different temperatures on Aspergillus flavus, our investigation reveals that the 35^0 C temperature was optimum 1.23 U/ml of cellulase enzyme production. Cellulase enzyme production was recorded in 15^0 to 45^0 C temperatures (Table-3). Further increase in temperatures above 35^0 C the enzyme production decreased. A little production of enzyme was also observed in high temperatures like 45^0 C. Temperature and pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion and the cellulase enzyme activity was maximum (0.198 U/ ml of substrate) at temperature 30°C (Panda *et al*., 2012).

Table 3. Effect of temperature

Temperature (^{0}C)	Cellulase production (U/ml)
15	0.05
20	0.23
25	0.68
30	0.92
35	1.23
40	1.10
45	0.60

Synthesis of Endoglucanase (EG) activity on cellulose by *Aspergillus flavus:* Results showed in Table-4, the maximum endoglucanase activity 77.8% at 12 days. The endoglucanase activity was varied from 2 to 20 days. There is a irregular manner of Endoglucanse production was recorded. The activity of EG2 was stimulated in the presence of some solvents (25 % v/v) such as n-hexane (132.75 % ± 3) and acetone (116 % ± 3) and stabilized in the presence of most organic solvents (25 % v/v) up to 4 h at room temperature was reported by Ali Gargouri., (2016).

Table 4. Synthesis of Endoglucanase activity on cellulose by *Aspergillus flavus*

Sl.no	Age of C.F. (Days)	Endoglucanase activity EG (% viscosity loss)
1	2	72.5
2	4	44.4
3	8	26.3
4	10	58.8
5	12	77.8
6	14	69.2
7	20	75.0

Conclusion

Ramie Cellulase enzyme production by Aspergillus species are rare. In the present study cellulase production by ramie canes. This cellulase may useful for industrial purpose. The optimization conditions like incubation time, pH and temperature of the medium are one of the most critical parameters that affect the mycelia growth and enzyme production. The cellulase enzyme thus produced with higher activity level may use in improving Decortication and degumming process respectively.

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