Production of Cellulase by Aspergillus niger on natural and pretreated lignocellulosic wastes

A Sridevi, G Narasimha, B Rajasekhar Reddy

Citation

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Abstract

Growth of Aspergillus niger on Czapek-dox medium supplemented with native lignocellulosics like Sawdust, wheat straw, Sugarcane baggage and Ricebran used for the production of cellulase by A.niger. Of these Czapek-dox medium with 0.5% Wheat straw had yielded more cellulase, FPase (2.9 U/ml), CMCase (2.9 U/ml) and -glucosidase (0.93 U/ml) after 14-days of incubation. The lignocellulosics after pre-treatment improved cellulase production. Among the treated substrates, Czapek-dox medium with 0.5% saw dust had yielded FPase (6.3 U/ml), CMCase (7.2 U/ml) after 7-days of incubation and the medium with treated rice bran produced -glucosidase of 1.04 U/ml after 21-days of incubation.

INTRODUCTION

Cellulose is the major polysaccharide constituent of plant cell wall and one of the most abundant available organic compounds in the biosphere and its estimated synthesis rate of 10^{10} tones per year (Schlesinger, 1991; Singh and Hayashi, 1995; Lynd et al., 2002). Cellulose-rich plant biomass is one of the foreseeable and sustainable sources of fuel, animal feed and feed stock for chemical synthesis (Bhat, 2000). The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages (Kuhad et al., 1997; Gong et al., 1999). Cellulose serves as a vast reservoir of glucose residues linked by -1, 4 glycosidic bonds. The conversion of cellulosic mass to fermentable sugars by saccharification through biocatalyst cellulase derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution (Dale, 1999; Lynd et al., 1999). In addition to this process, the cellulolytic enzymes have also been exploited for commercial applications like malting, wood processing, preparation of denim fabrics in textile industries, maceration of protoplasts from plant tissues and de-inking process in recycling of printed papers. But the saccharification process has not reached to the level of commercialization in certain applications pertaining to production of biofuels. The major obstacle to the exploitation of cellulase is its high cost of production and include other factors like complexity of cellulose structure, the type and source of cellulose employed for production and low amounts of cellulases production by cellulolytic organisms due to catabolite repression influence economics of cellulase production. One effective approach to reduce the cost of enzyme production is to replace pure cellulose by relatively cheaper substrates such as lignocelluloses materials. The development of biorefineries to produce fuels and commodity chemicals from lignocellulosic biomass is viewed as a potential alternative to current reliance on nonrenewable resources. The so-called ‘‘sugar platform,’‘ involving enzymatic hydrolysis of the cellulose component to glucose, followed by fermentation to fuel-grade ethanol, is a focus of current attention. One of the primary challenges for process commercialization is the development of cost-effective pretreatment technologies for lignocellulosic feedstock’s (Lynd et al., 2002; Mosier et al., 2005; Wyman et al., 2005). Pretreatment is necessary to increase the accessibility of cellulose in lignocellulosic biomass to facilitate enzymatic hydrolysis. Unlike traditional sources of fermentable sugar, such as starch and sucrose, the cellulose component of lignocellulose is a structural polymer and is protected against enzymatic attack by the surrounding matrix of lignin and hemi cellulose.

In view of biotechnological importance of cellulase, this study was focused on the production of cellulase on natural and pretreated cheap locally available lignocelluloses in submerged fermentation in a laboratory scale by the local isolate of Aspergillus niger isolated from cotton ginning mill

effluents.

MATERIALS AND METHODS

MICROORGANISM

A local isolate of Aspergillus niger used in the study was isolated from soil contaminated with effluents of cotton ginning mill by Narasimha et al (1999). This culture was maintained on potato dextrose agar slants.

COLLECTION OF LIGNOCELLULOSES

Different native lignocelluloses like saw dust, wheat straw, sugarcane bagasse and rice bran were collected from the industries located around in Nandyal, Andhra Pradesh, India. They were sorted and then washed under running tap water to remove sand and other dirt particles. All these samples were sun-dried for about two weeks and then milled into a powder form.

PRODUCTION OF CELLULASE ON NATIVE LIGNOCELLULOSES

The different native lignocelluloses (saw dust, wheat straw, sugarcane bagasse and rice bran) were added at the rate of 0.5 %(W/V) level to each 250 ml Erlenmeyer conical flasks in which 50ml of Czapek-Dox liquid medium was distributed. Flasks containing Czapek-Dox liquid medium amended with 0.5% (W/V) cellulose instead of lignocellulose served as control. All these flasks were incubated at 30°C on a rotary shaker (180rpm) under axenic conditions after inoculating with a spore suspension (2 x 10^{8}) of A. niger which was prepared by flooding the 6 day old slant with 2ml of sterile distilled water.

PRETREATMENT OF LIGNOCELLULOSES

Removal of components of lignin and hemicellulose may improve the production of cellulolytic enzymes. Therefore these components were removed from these lignocelluloses by two selected pretreatment processes. The lignocelluloses (saw dust, wheat straw, sugarcane bagasse and rice bran) used in this study were pretreated by two different pretreatment processes, NaOH method and H_2O_2 methods.

In NaOH method, each lignocelluloses were treated with 50ml NaOH (1%W/Vof distilled water) for 1 hr in an autoclave at 121 ^{o} C and filtered through Whattman No.1filter paper and washed with water until washings turned neutral. The treated samples were dried at 110 ^{o} C overnight in oven, where as in H_2O_2 method, 1g of each sample was treated with alkaline peroxide in 50ml of distilled water containing H_2O_2 1%(W/V). The suspension was adjusted to pH 11.5 with NaOH and stirred gently at room temperature (25 ^{o} C) for 20 hr. The insoluble residue was collected by filtration, washed with distilled water until neutrality. The treated samples were dried at 110 ^{o} C overnight (Gould, 1984).

PRODUCTION OF CELLULASE ON PRETREATED LIGNOCELLULOSES

Fifty milliliters of Czapek-Dox liquid medium were distributed into 250 ml Erlenmeyer conical flasks. The treated lignocelluloses were added to these flasks at 0.5%(w/v) level. Flasks containing Czapek-Dox liquid medium amended with untreated lignocelluloses at 0.5%(W/V) served as controls. All flasks were inoculated with spores of A. niger and incubated for growth in the manner as specified above.

DRY WEIGHT DETERMINATION

Dry weight of the samples was determined by drying them in a hot air oven at 70°C for 24hr.

ESTIMATION OF EXTRACELLULAR PROTEIN CONTENT

Soluble protein contents were determined in the filterate by the method of Lowry et al.,(1951) using bovine serum albumin as protein standard.

ESIMATION OF TOTAL SOLUBLE SUGAR CONTENT

The estimation of total soluble sugar content was according to the method of Miller (1959) using glucose was as sugar standard.

PH MEASUREMENT

pH was measured in the filtrate after desired intervals by using pH meter(ELICO).

ENZYME ASSAYS

The filtrate obtained after removal of mycelial mat by filtration through Whatman Filter paper No.1 was used as an enzyme source. Flasks with the growing culture of A. niger were withdrawn at every 7-day interval for processing.

Activities of individual enzyme components of cellulase system secreted into the culture medium of A. niger, were estimated in accordance with methods listed by Wood and Bhat (1988).

FILTER PAPER ASSAY

Filter paper activity of the culture filtrate of A. niger was

determined according to the method of Mandels and Weber (1969). Whatman filter paper strips containing 50mg weight was suspended in one ml of 0.05 M sodium citrate buffer (pH 4.8) at 50°C in a water bath. Suitable aliquots of enzyme source with/without dilution was added to the above mixture and incubated for 60 minutes at 50°C. After incubation, the liberated reducing sugars were estimated by the addition of 3,5-Dinitrosalicylic acid (DNS). After cooling colour developed in tubes were read at 540 nm in a Spectrophotometer (ELICO SL 164). Appropriate control without of enzyme were simultaneously run.Activity of cellulase was expressed in filter paper units. One unit of filter paper unit (FPU) was defined as the amount of enzyme releasing µ mole of reducing sugar from filter paper per min.

ENDOGLUCANASE ASSAY

Activity of endoglucanase in the culture filtrate was quantified by carboxymethyl cellulase method (Ghosh, 1987).

The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50°C in a water bath for 20 minutes. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50°C in water bath for one hr. Appropriate control without of enzyme were simultaneously run. The reducing sugar produced in the reaction mixture was determined by dinitro salicylic acid (DNS) method (Miller, 1959). 3,5-Dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540 nm in a Spectrophotometer(ELICO-SL164). One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per minute.

-D-GLUCOSIDASE ASSAY

Activity of -glucosidase activity in the culture filtrate was based on the method of Herr (1979). For the determination of -D-glucosidase activity the assay mixture contained 0.2 ml of 5 mM p-nitrophenyl -D-gluco pyranoside (PNPG, Merck) in 0.05 M citrate buffer pH 4.8 and 0.2 ml of diluted enzyme solution with appropriate controls. After incubation for 30 min at 50°C, the reaction was stopped by adding 4 ml of 0.05 M NaOH-glycine buffer (pH 10.6)and the yellow coloured p-nitrophenol liberated was determined in Spectrophotometer (ELICO-SL164) at 420 nm. One unit of -glucosidase activity was defined as the amount of enzyme liberating 1 µmole of p-nitrophenol per min under standard assay conditions.

RESULTS

COMPARISON OF DIFFERENT LIGNOCELLULOSES FOR CELLULASE PRODUCTION FROM A. NIGER

Four different natural lignocelluloses were used for cellulase production from A. niger in order to compare the production of cellulase with that of control in which cellulose is amended instead of lignocellulose(Table 1). Of all , wheat straw served as best source for cellulose.

Figure 1

Table 1. Cellulase production by on natural lignocelluloses

Natural lignocellulose at 0.5% concentration	FPase (U/ml)[a]			CMCase (U/ml)[b]			β-glucosidase (U/ml)[c]		
	7th Day	14th Day	21st Day	7th Day	14th Day	21st Day	7th Day	14th Day	21st Day
Sawdust	2.4	2.1	1.6	2.8	2.1	1.6	0.6	0.8	0.7
Wheat straw	2.8	2.9	2.0	2.6	2.9	2.1	0.90	0.93	0.6
Sugarcane bagasse	2.0	1.8	0.7	2.5	1.9	1.4	0.71	0.64	0.56
Ricebran	1.8	1.3	0.7	1.5	0.9	1.0	0.53	0.58	0.6
Control[d]	1.5	1.7	1.2	0.7	0.9	0.8	0.9	1.3	1.2

Values represented in the Table are averages of results of two experiments.

a. Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1µmole of reducing sugar from filter paper per min.

b. Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.

c. One unit of -glucosidase activity is defined as the amount of enzyme liberating 1µmole of p-nitrophenol per min.

d. Contained cellulose at 0.5% (w/v) instead of lignocellulose.

COMPARISION OF PH CHANGES IN THE MEDIUM DURING THE INCUBATION PERIOD OF

The production of acidic metabolites changed the pH of the medium from 2.59 – 4.64.

Figure 2

Table 2. pH changes in the medium upon the growth of on natural lignocelluloses

Natural lignocelluloses at 0.5% concentration	pH of culture broth on		
	7th Day	14th Day	21st Day
Sawdust	3.07	3.76	4.64
Wheat straw	2.93	4.14	3.56
Sugarcane bagasse	2.64	2.98	3.01
Rice bran	2.59	3.51	4.13
Control[a]	3.01	4.18	4.44

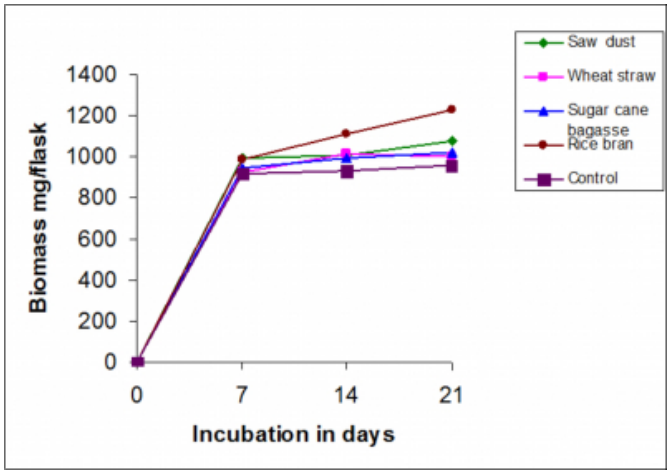
a Contained cellulose at 0.5% (w/v) instead of lignocellulose.

EFFECT OF NATURAL LIGNOCELLULOSES ON THE PRODUCTION OF BIOMASS OF

Figure 1 showed that the biomass was reached to maximum in the medium with wheat straw after 14 days of incubation, but after 21 days of incubation biomass was more in medium with rice bran as substrate.

Figure 3

Figure 1: Production of biomass of on natural lignocelluloses

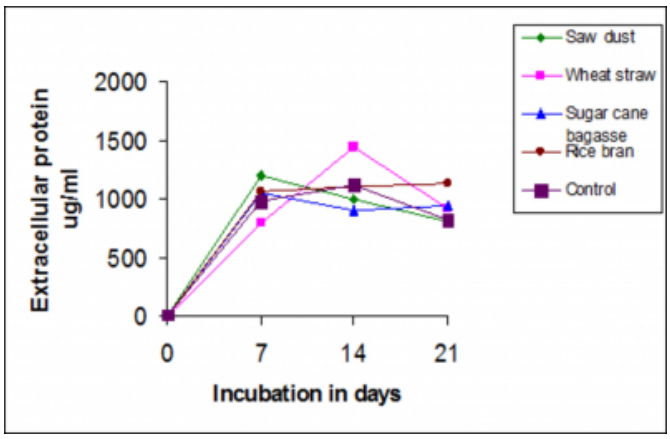


Effect of natural lignocelluloses on the Secretion of extracellular protein by A. niger

High amount of extracellular protein was secreted in wheat straw supported medium after 14 days of incubation(Figure 2)

Figure 4

Figure 2: Secretion of extracellular protein by during cultivation on natural lignocelluloses

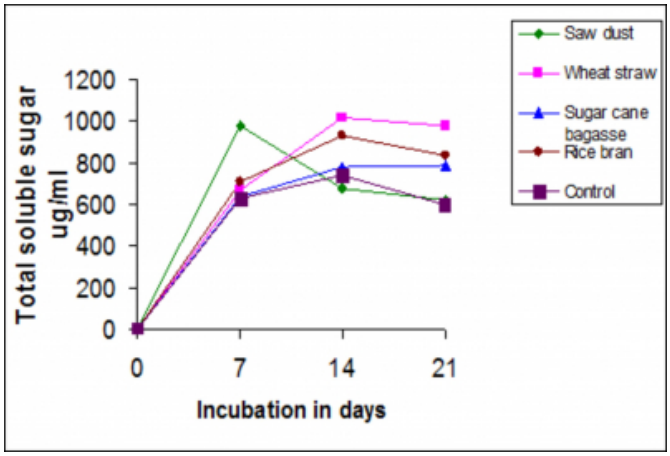


EFFECT OF NATURAL LIGNOCELLULOSES ON THE RELEASE OF TOTAL SOLUBLE SUGAR BY

Figure 3 showed that high amount of soluble sugar was released in medium with wheat straw after 14 days of incubation.

Figure 5

Figure 3: Total soluble sugar content by on cultivation on natural lignocelluloses

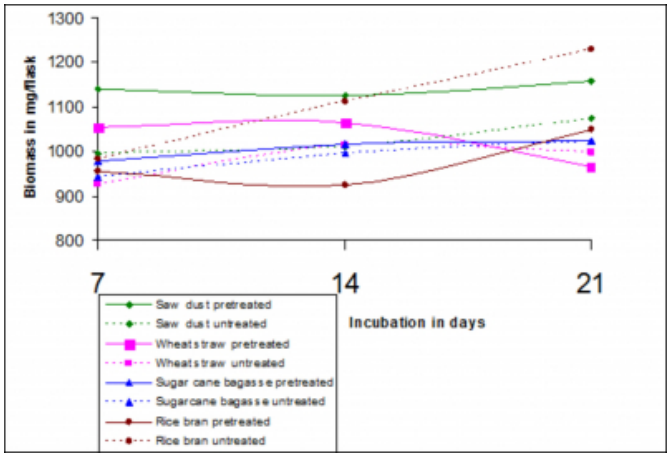


EFFECT OF NATURAL AND PRETREATED LIGNOCELLULOSES ON THE PRODUCTION OF BIOMASS OF

More biomass was achieved in medium with treated sawdust after 21 days of incubation, but less than that of the medium with native rice bran in the same incubation period(Figure 4).

Figure 6

Figure 4: Production of biomass of on pretreated lignocelluloses

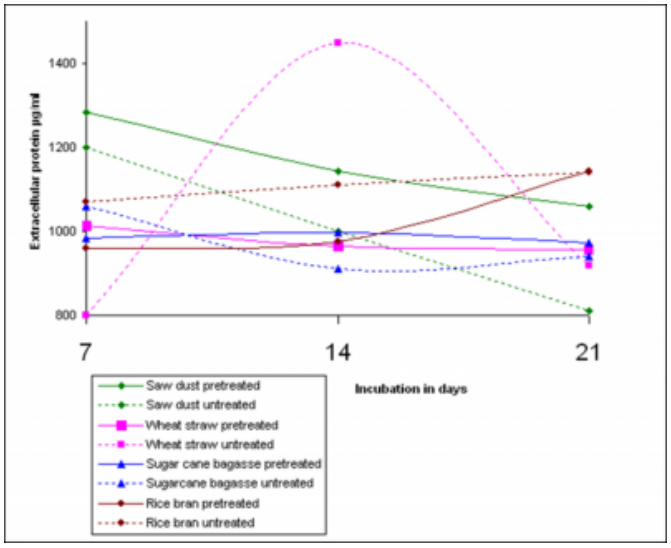


EFFECT OF NATURAL AND PRETREATED LIGNOCELLULOSES ON THE SECRETION OF EXTRACELLULAR PROTEIN BY

Secretion of extracellular protein was high in medium with native wheat straw after 14 days of incubation(Figure 5)

Figure 7

Figure 5: Secretion of extracellular protein by on untreated and pretreared lignocelluloses

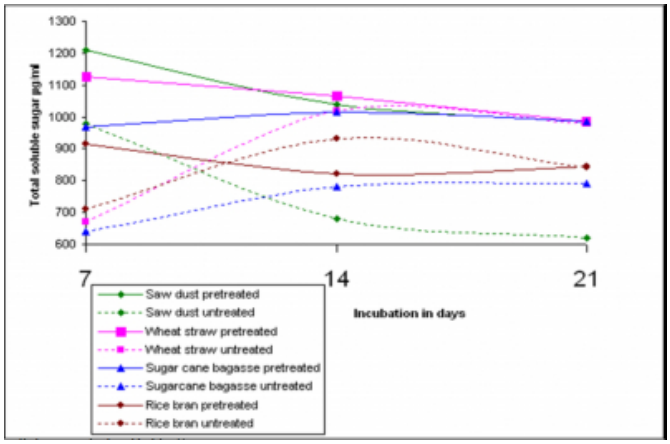


EFFECT OF NATURAL AND PRETREATED LIGNOCELLULOSES ON THE RELEASE OF TOTAL SOLUBLE SUGAR BY

Figure 6 showed that release of soluble sugar was more in medium with treated wheat straw after 14 days of incubation.

Figure 8

Figure 6: Total soluble sugar content in the culture broth of on untreated and pretreated lignocelluloses



COMPARISON OF DIFFERENT NATIVE AND PRETREATED LIGNOCELLULOSES FOR CELLULASE PRODUCTION FROM A. NIGER

Of all native and pretreated lignocelluloses, treated sawdust yielded maximum cellulase production(Table 3).

Figure 9

Table 3. Cellulase production on pretreated lignocelluloses by

Lignocelluloses at 0.5% concentration		FPase (U/ml)[a]			CMCase (U/ml)[b]			β-glucosidase(U/ml)[c]		
		7th Day	14th Day	21st Day	7th Day	14th Day	21st Day	7th Day	14th Day	21st Day
Sawdust	Untreated	2.4	2.1	1.6	2.8	2.1	1.6	0.6	0.8	0.7
	Pretreated	6.3	4.9	3.2	7.2	5.2	3.3	0.30	0.35	0.53
Wheat straw	Untreated	2.8	2.9	2.0	2.6	2.9	2.1	0.90	0.93	0.60
	Pretreated	5.8	4.7	3.4	6.6	4.4	3.6	0.24	0.54	0.50
ugarcane bagass	Untreated	2.0	1.8	0.7	2.5	1.9	1.4	0.71	0.64	0.56
	Pretreated	6.6	3.5	3.1	6.0	3.0	2.3	0.50	0.35	0.43
Rice bran	Untreated	1.8	1.3	0.7	1.5	0.9	1.0	0.53	0.58	0.60
	Pretreated	3.9	2.7	3.0	3.7	2.7	2.3	0.35	0.50	0.32

Values represented in the Table are averages of results of two experiments.

a. Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.

b. Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.

c. One unit of -glucosidase activity is defined as the amount of enzyme liberating 1µmole of p-nitrophenol per min.

COMPARISION OF PH CHANGES IN THE MEDIUM DURING THE INCUBATION PERIOD OF

The production of acidic metabolites changed the pH of the medium from 2.59 – 4.98(Table 4).

Figure 10

Table 4. pH changes in the medium upon the growth of on pretreated lignocelluloses

lignocelluloses		pH of the culture medium on		
		7th Day	14th Day	21st Day
Sawdust	Untreated	3.07	3.76	4.64
	Pretreated	3.94	4.21	4.98
Wheat straw	Untreated	2.93	4.14	3.56
	Pretreated	2.80	3.82	3.91
Sugarcane bagasse	Untreated	2.64	2.98	3.01
	Pretreated	3.12	4.01	4.12
Rice bran	Untreated	2.59	3.51	4.13
	Pretreated	3.43	3.96	3.99

DISCUSSION

A. niger isolated from cotton ginning mill effluents was produced more cellulase on natural lignocelluloses than to control. It has been reported that A. nigerMSK-7, T. viridae MSK-10 in Eggins & Pugh salt medium produced high cellulolytic and hemicellulolytic enzymes after 48 hr when 1% wheat bran was used as a substrate at pH 5.0 (Ikram-ul-Haq et al., 2006). In other study by Darmawal and Gaur, 1991 observed that basal medium containing 1% rice straw yielded high Fpase titres of 0.129 and 0.142 U/ml in Aspergillus awamori and Scerituyn rikfsuu, respectively. In the present study, along with enzyme production, biomass of the culture, extracellular protein, release of soluble sugar were recovered higher in all 4 lignocelluloses used in this study in comparison to control. Changes in the pH of the medium with lignocellulose occurred within a range of 2.59-4.64 and in cellulose amended medium 3.01 – 4.44. Enzyme production was still higher on pretreated lignocelluloses to natural lignocelluloses. The production of cellulase by A. niger is high on pretreated lignocelluloses was observed may be due to separation of the maximum cellulose from their other components of lignocellulosics and so that availability of more cellulose for enzymatic attack.. Highest titers of Fpase (2.9U/ml), CMCase (2.9U/ml) and 0.93U/ml of -glucosidase was obtained after 14-days of incubation on natural lignocelluloses, where as on pretreated lignocelluloses , enzyme activities were Fpase (6.3 U/ml), CMCase (7.2U/ml) and 0.53U/ml of - glucosidase after 7-days of incubation. This observation was in aggrement with the other works.

It has been reported that, of the pretreated bagasse, corncob and sawdust with caustic soda, sawdust gave the best result with an enzyme activity value of 0.0743 IU/ml of Fpase, while bagasse and corncob gave 0.0573IU/ml after 12hrs of incubation in the fermentation studies (Ojumu et al., 2003). Among the newspaper sludge, office paper sludge and steam exploded woods treated in various ways, the steam exploded wood showed the best properties for cellulase production (4.29 IU/ml) and (2.48 IU/ml) in paper sludge (Shin et al., 2000). Similar results have also been reported in instances where pretreated substrates were used for enzyme production.

Sugarcane bagasse and rice bran were less efficient of all natural lignocelluloses in terms of production of enzyme in this study. Of the treated lignocelluloses, other three were less efficient in terms of production of enzyme than treated sawdust, but have produced high enzyme yields than untreated lignocelluloses.

The medium with native rice bran produced higher growth with biomass of 1228 mg/flask after 21 days of incubation where as the growth of the culture was 1141 mg/flask maximum at 0.5% level of treated sawdust at 7- day interval and slightly declined to 1124 mg/flask at 14-day interval. Biomass of culture was further reached to maximum of 1159mg/flask at 21-day interval . Medium amended with native wheat straw had produced 1450 µg/ml of extracellular protein content after 14 – days of incubation and among the treated ones the maximum amount of extra cellular protein (1284 µg/ml) was secreted in 0.5% treated sawdust level at 7-day interval compared to other treated lignocelluloses. Growth of Aspergillus niger on medium containing native wheat straw had produced 1020 µg/ml of total soluble sugar content after 14 days of incubation where as treated sawdust yielded soluble sugar in large amounts to the tune of 1210µg/ml at 7- day interval.

CONCLUSION

In the present study, native and pretreated lignocelluloses were used for the maximum production of cellulase from A.niger. Production of cellulase was maximum in treated sawdust than native lignocelluloses. This study shows that waste substrates can be used for cellulase production by cheap methods and can be used for industrial purpose.

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Author Information

A. Sridevi
Dept. of Industrial Biotechnology School of Engineering & Technology Sri Padmavati Mahila university Tirupati.

G. Narasimha
Dept. of Virology, Sri Venkateswara university Tirupati.

B. Rajasekhar Reddy
Dept. of Microbiology Sri Krishnadevaraya University Anatapur.