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Enhanced Production of *A. Niger* Cellulase with Culture Based Strategies.

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ABSTRACT

Production of cellulase components, exoglucanase, endoglucanase and β-glucosidase by a local fungal isolate of A. niger in submerged fermentation on cellulose and lignocelluloses was studied. The effect of varying optimal cultural and nutritional parameters – incubation period, substrate concentration, pH, and temperature were evaluated for optimal production of enzymes. The growth of the fungus on the medium with 1% cellulose concentration, pH 5.0 at 30^0C after 5-day incubation yielded higher titers of cellulase. Control of pH during the course of the growth of the fungus enhanced enzyme production. Supplementation of culture medium with natural lignocellulose induced higher production of cellulase than with cellulose substrate.

Keywords: *A.niger*, Cellulase, Optimal conditions.

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INTRODUCTION

Cellulose is the most abundant available natural carbohydrate polymer found on earth. Utilization of this natural resource for production of useful materials by enzymatic means is a challenging task in the field of biotechnology. Cellulase is a complex enzyme which is Exo, Endo and β -glucosidases acts synergistically on cellulose to convert cellulose polymer into simple sugars and can be used to produce fuels and chemicals from plant biomass [1]. They also play a key role in increasing the yield of the fruit juices, oil extraction and in improving the nutritive quality of bakery products and animal feed [2] . The hydrolysis of lignocellulosic biomass with the application of cellulases in order to further convert the released fermentable sugars into ethanol has increased because of their worldwide demand for renewable fuels [3] . For fermentation processes, use of intensive raw material and process yield (product produced/substrate consumed), in addition to productivity are critical measures of performance and economic viability [4-5] . To enhance the rate of saccharification there is continuous search for highly efficient cellulolytic microorganism with secretion of copious amounts of cellulase. Production of Cellulase depends on a variety of factors like inoculum size, carbon source and cellulose quality, pH value, temperature, presence of inducers, medium additives, aeration, growth time, etc. [6-7]. Even though there were several studies in optimization of cellulase production by various fungi, there is scanty information in control of pH during entire fermentation. Therefore, this local isolate, *Aspergillus niger* with cellulolytic activity which was isolated from forest soils of tirumala has been focused on studying the cellulase production in an optimized environment and attention was also paid in pH control during fermentation time .

MATERIALS AND METHODS

Microorganism: A local isolate of *Aspergillus niger* was used in this study was isolated from Seshachala forest soils of Tirumala, Andhra Pradesh and was maintained on PDA slants.

Preparation of Fungal Inoculum: Inoculums was prepared by slanting the 7 day old slant with 3 ml of sterile distilled water having 0.01 % Triton -X 100 and shaked well on vortex for 5 min before incubating into experimental flasks.

Culture Conditions with substrate concentration and incubation time: All experiments were conducted in 250 ml conical flaks having 50ml Czapek- Dox medium embedded with 0.5% to 2% cellulose and were incubated with spores of *Aspergillus niger* with initial pH 5.0 and were incubated for 7 day time intervals at 30°C on a rotary shaker (180rpm).

Optimization of temperature

Optimization of temperature was carried out by incubating the fermentation medium at 25, 30 and 40°C, in orbital shaker incubator at 180 rpm. After regular intervals, enzyme assay was performed.

Optimization of pH

Optimization was carried out by adjusting the pH ranges from 4.0, 5.0 and 6.0 of the fermentation media and incubated as above said. The pH of the medium was adjusted by using 1 N HCl / NaOH.

pH Correction

Another set of experiment was conducted by adjusting the pH to optimal condition (5.0) throughout incubation in the fermentation medium and enzyme assays were carried out

Cellulase production on different carbon sources

Enzyme production was carried out on different carbon sources like wheat straw, sugarcane bagasse, corn cobs and groundnut shells 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 (Hi-Media) served as control. All these flasks were incubated at 30°C on a rotary shaker (180rpm) after inoculating with a spore suspension (2×10^6) of *A. niger* and allowed to

continue for 7 days. Samples were taken at regular intervals and the enzyme activity was checked using standard assays.

Enzyme Assays: Enzyme assays for filter paper activity (FPase), Carboxymethyl cellulase (Cmase) were performed according the method of [8]. β-glucosidase activity was determined according to method described by [9] .

Extra cellular protein determination: Total protein in the supernatant was measured according the method of [10].

Total soluble sugar content: Release of soluble sugar was estimated according to the method of [11].

Biomass estimation: Fungal biomass was estimated in terms of dry weight.

RESULTS

Optimization conditions for production of cellulase by *A. niger*

Substrate concentrations and incubation time: The soluble sugar and extra cellular protein contents and cellulase activities were improved at 5 days of incubation in liquid medium (1% cellulose) there after these activities declined. The saccharification process has also reached maximum at 5-day with 1.112mg/ml (Figure 1) and this saccharification of cellulose to soluble sugars for utilization improved the secretion of cellulolytic enzymes by *A.niger* with 17.75U/ml Fpase, 18.93U/ml and Cmcase and 0.87 U/ml β-glucosidase (Table 1). The secretion of FPase and CMCase were higher after 5 days of incubation, whereas β-glucosidase production was more at 6^{th} day incubation. The secreted extracellular protein was recorded as 0.99mg/ml (Figure 2). Biomass change was shown in Fig.3. There was drastic changes observed in pH also from initial pH 5.0 to 3.07 - 2.18 and there was a rise in a range of 5.12-5.64 subsequently at all cellulase levels within 7-days of incubation (Table 2).

Temperature: One of main factors influencing the production of enzymes is the incubation temperature of the fermentation medium. The activities of cellulase produced at 3 different temperatures i.e. at 25 ^{o}C, 30^{o}C and 40^{o}C was observed in this study. The secretion of three enzyme activities in terms of Fpase with 17.75U/ml, Cmcase 18.93U/ml and β- glucosidase with 0.85U/ml were maximum at 30^{o}C than to 25 and 40^{o}C (Table 3).

Initial pH: The pH has a marked effect on cellulase production. Effect of pH on enzyme production, analyzed and they were decreased at both pH 3.0 and pH 7.0, whereas maximum at 5.0 (Table 4).

PH correction: pH 5.0. was found to be optimum for cellulase production on 5^{th} day of fermentation. Even in other experiments pH changes occurred in the medium during the course of growth of *A.niger*. But another experiment was conducted in which pH was controlled during the course of incubation. The pH of the culture broth was only once adjusted to 5.0. by adding sterile diluted NaOH at 48 hours of incubation .

High activities of Fpase (19.87 U/ml), Cmcase (20.28 U/ml) and β-glucosidase (0.92 U/ml) were recorded (Table 5). In spite of once pH correction the culture broth underwent changes in pH, but severe pH drop was prevented. It was clear from this experiment that pH correction during the course of the growth was beneficial and enhanced cellulase production.

Natural lignocelluloses: Lignocelluloses at 1% level in Czapek-Dox medium for 7 days was compared to yields of cellulase by the *A. niger* on cellulose. Of all lignocelluloses used in this study, corn cob in the medium showed the highest titers of Fpase (23.85 U/ml), Cmcase (25.65 U/ml) and 0.97 U/ml of β- glucosidase were obtained (Table 6). wheat straw were less efficient of all lignocelluloses in terms of production of enzyme. Changes in the pH of the medium with lignocelluloses occurred within a range of 3.71-6.23 due to growth of *A.niger* for 7 days.

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Table 1: Cellulase production on cellulose by *Aspergillus niger*

Concentration	FPase (U/ml)						CMCase (U/ml)						β-glucosidase (U/ml)					
	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day
0.5 %	0.626	3.439	8.704	11.32	10.94	9.041	1.23	5.226	9.439	13.45	12.23	11.34	0.00	0.04	0.23	0.42	0.54	0.58
1 %	0.731	5.928	9.25	17.75	15.78	15.12	0.952	7.537	13.28	18.93	17.76	15.87	0.01	0.12	0.32	0.58	0.87	0.61
1.5%	0.436	3.187	6.78	10.40	8.65	7.63	0.594	4.116	9.187	11.23	13.45	12.56	0.00	0.07	0.19	0.36	0.40	0.47
2%	0.208	2.973	4.31	7.56	7.21	5.65	0.325	3.008	6.973	7.65	6.54	6.32	0.00	0.01	0.05	0.275	0.21	0.26

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

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

Table. 2: P^H changes in the medium upon the growth of *A. niger*

Substrate Concentration	Change in pH					
	2nd day	3rd day	4th day	5th day	6th day	7th day
0.5 %	3.07	2.18	3.62	3.95	4.06	5.73
1 %	3.23	2.94	3.98	4.69	4.89	5.12
1.5%	3.84	2.51	3.62	3.69	4.41	5.87
2%	3.85	3.01	3.78	4.76.	4.90	5.84

Table 3: Effect of temperature on cellulase production by *Aspergillus niger*

Temperature ^{0}C	*FPase (U/ml)*	CMCase (U/ml)	β-glucosidase (U/ml)
25	14.28	15.95	0.431
30	17.75	18.93	0.87
40	12.36	14.78	0.37

Table 4: Effect of initial pH on cellulase production by *A. niger*

pH	Fpase (U/ml)	CMCase (U/ml)	β-Glucosidase (U/ml)
3	9.28	9.95	0.131
5	17.75	18.93	0.85
7	14.36	15.78	0.57

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Table 5 : Cellulase production by *A. niger* on medium with pH regulation

Substrate Concen-tration	FPase (U/ml)						CMCase (U/ml)						β-glucosidase (U/ml					
	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	2nd Day	3rd Day	4th Day	5th	6th Day	7th Day
0.5 %	1.02	5.473	19.26	18.64	14.44	10.34	1.12	6.226	19.79	18.85	13.31	12.11	0.00	0.12	0.73	0.64	0.52	0.48
1 %	1.19	6.128	19.87	19.23	16.81	15.56	1.23	8.57	20.28	19.93	17.93	15.96	0.01	0.16	0.92	0.78	0.67	0.53
1.5%	0.96	4.824	16.00	17.12	10.23	9.43	0.94	6.66	11.45	12.24	14.14	13.78	0.00	0.07	0.49	0.41	0.37	0.37
2%	0.85	3.673	8.81	9.93	8.32	6.43	0.93	4.76	8.34	8.65	7.62	6.87	0.00	0.06	0.35	0.35	0.31	0.24

Table 6: Effect of supplementation of natural lignocellulose on cellulase production by *A. niger*

Lignocelluloses	Fpase	Cmcase	β-glucosidase
Cellulose	17.75	18.93	0.85
Saw-dust	19.00	20.42	0.88
Corncobs	23.85	25.65	0.97
Rice bran	22.85	19.58	0.84
Wheat straw	18.92	19.10	0.82

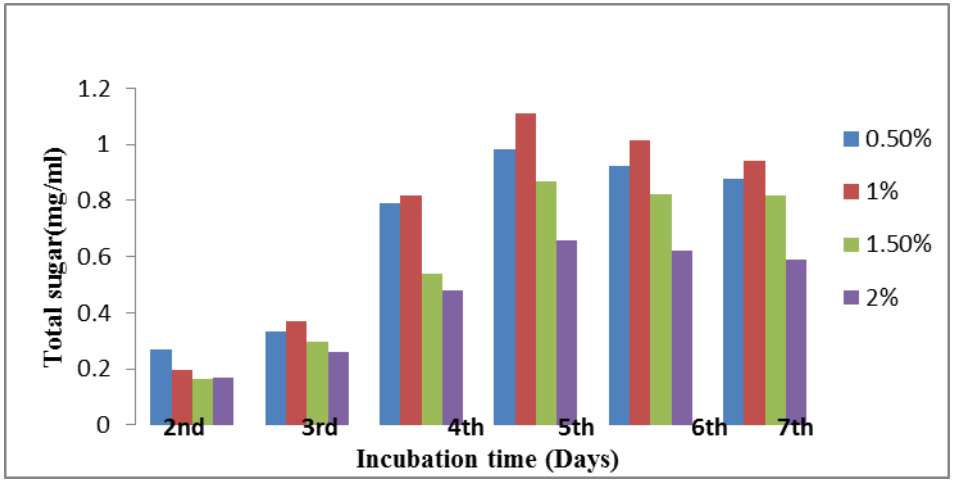

Figure 1: Total soluble sugar content in the culture broth of *A. niger* grown on cellulose

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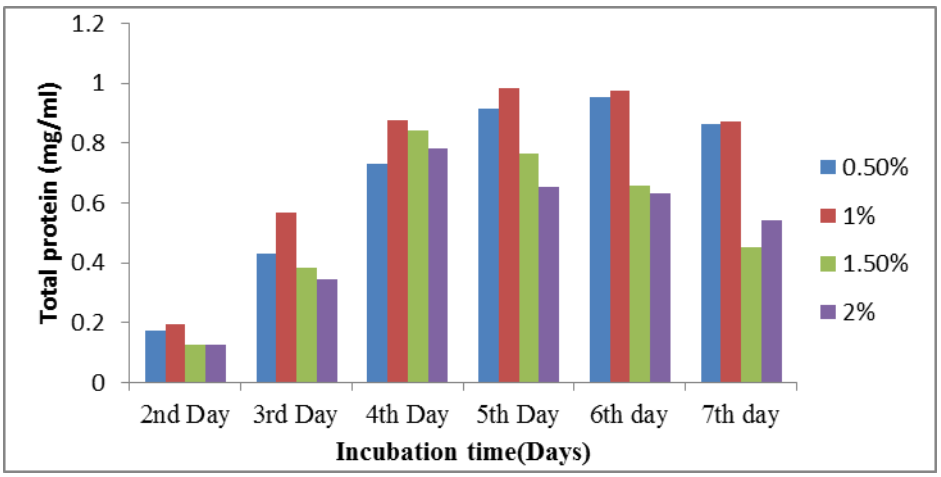

Figure 2: Total protein content in the culture broth of *A. niger* grown on cellulose

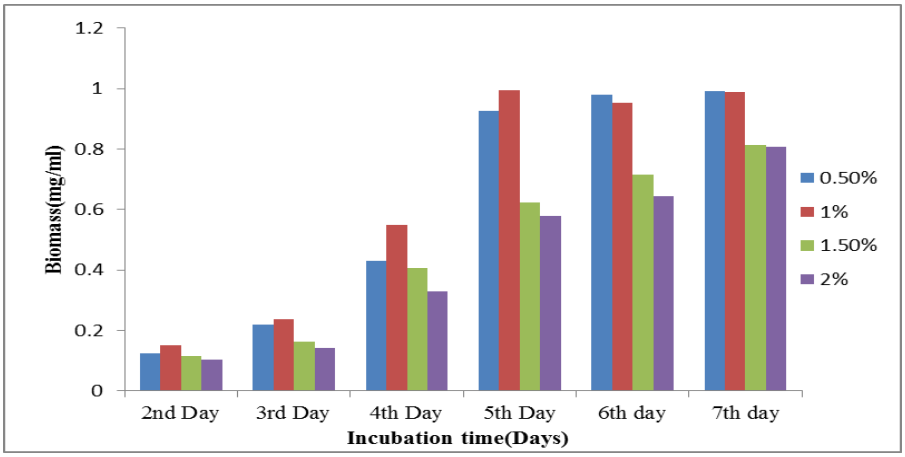

Figure 3: Biomass of *A.niger* grown on cellulose substrate

DISCUSSION

Cellulases are the third largest industrial enzymes and gaining interest throughout world due to their applications in lignocelluloses conversions to bioethanol and bioenergy. Species of *Aspergillus* and *Trichoderma* are known to be potential producers of cellulases. Major impediments to exploit the commercial potential of cellulases are the yield stability and cost of cellulase production. To meet this research should be carried by exploiting the commercial potential of existing and for new cellulases in nature [12]. A similar attempt was made in this study for maximum production of cellulase by optimizing culture conditions.

Fungal production of cellulolytic enzymes was conducted with either submerged or solid state fermentation methods. The fungal culture *Aspergillus niger was* used for cellulase production under submerged (SmF) fermentation in this study. The maximum production of cellulase was obtained after 5 days of incubation in Smf at 30^0C . Similarly *Trichoderma reesei* NRRL 11460 yielded maximum titers of FPase after 96 hours of growth [13]. A comparative study with two fungal cultures on wheat bran in solid state fermentation indicated that *T. reesei Rut C-30* relatively produced higher yields of FPase and endoglucanase whereas *A. niger* gave relatively higher titers of glucosidase after 5-days of incubation [14]. Yields of FPase and CMCase obtained with *Trichoderma viridae* in submerged fermentation were 1.5 and 1.0 U/mL [15]. Cultivation of *Trichoderma reesei ZU02* in deep trough fermentor for 5days in solid state fermentation generated 128 U/g of FPase [16].

The optimal conditions for maximum biosynthesis of cellulase by *A. niger* were shown to be at pH 5, temperature 30 °C in this study. Cellulases in general show optimum temperature between 30 to 55°C [17-18]. The results are in support with the studies of Ghori et al (2012), where temperature optimum for crude EXG

and EG was found to be 30°C [19]. Whereas Highest yield of enzyme was noted at 37ºC by *Aspergillus* sp. inoculated in synthetic medium containing cellulose [20]. Some investigators found maximum cellulase production at higher temperatures and others recorded highest yield at lower temperatures in comparison with our results. Enzymes have an optimum temperature at which their activity is maximum and at higher or lower temperatures, their activity decreases. Reports of Ali *et al* showed a maximum yield of cellulase from *A. terrus* at 40 °C on water hyacinth after 6 days [21]. Immanuel *et al* found high level of cellulase production at 40 °C (0.292 and 0.258 U/ml) by *Aspergillus fumigatus* and *A. niger* on coir waste [22].

The optimal pH for fungal cellulases varies from species to species though in most cases the optimum pH ranges from 3.0 to 6.0 [23-24]. pH requirement was determined at pH 5.0 in fungal species *Trichoderma harizianum* [25] and *Fusarium avenaceum* [26] etc. Bao et al. (1994) reported a strong influence of the pH value and the buffer system used on cellobiose dehydrogenase (CDH) production by *P. chrysosporium*, with lower pH values being harmful for enzyme production [27]. The results in xu et al and Peng et al studies were in agreement with the observation that stability of the fungal cellulases is commonly between pH 3.0 and pH 8.0]17-18]. The highest cellulases production by *A. niger* NS-2 was founded at pH 7.0 [28].

Cellulase enzyme production accounts for 40% of cost in bioethanol synthesis. To reduce cost of production and for the prevention of environmental pollution the lignocellulosic Substrates are used instead of synthetic cellulase due to their reasonable cost, high Enzyme production capacity etc. The reduction in cost paves an economically easy way Production of ethanol. Likewise Corn cob is a suitable lignocellulosic bio wastes for the production of cellulase Enzyme. From the present study, it could understand that corn cob is most suitable Substrate for cellulase production when compared to that of bagasse or cotton stems [29] as it gives highest yield of enzyme. There are several reports describing the use of lignocellulosic agro industrial wastes for the production of cellulases [30,31]. The study of Itelima was also found that ethanol was produced from a cheap commonly available agricultural waste such as corn cob with aid of a co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* [32].

CONCLUSION

From the above results it can be concluded that the medium selected in this study Czapek-Dox liquid medium with 1% Cellulose concentration yielded maximum cellulase production by *A. niger* at 5th day of incubation. Culturing of *A. niger* with spores gave better yields of cellulase than culturing with vegetative mycelium. Temperature at 30 ^{0}C induced the cellulase activity Czapek-Dox medium with initial pH of 5.0 was optimal for higher yields of cellulase control of pH during the course of growth improved the cellulase production by *A. niger.* Significant research, therefore, have to be directed In addition to identify efficient cellulase systems process conditions are also important , besides those aimed at the biochemical and genetic improvement of existing organisms utilized in the process. Natural lignocelluloses induced higher production of cellulase than chemical based cellulose. Based on the our reported that utilization of natural wastes for enzyme production is inexpensive and ecofriendly method.

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