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Production of Cellulase by Filamentous Fungi with Sorghum as Substrates

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***Abstract*: The aim of present work was focused on the cellulase production by filamentous fungi using sorghum waste as a substrate. Sorghum is an important crop grown in most of the countries and is one of the most abundant sources for grain production. These agricultural and industrial residues are cheap raw material for cellulase production. The maximum production of cellulase (in terms of glucose production) was obtained after 5 days of incubation period in solid state fermentation and 5 days of incubation in submerged fermentation. The physical and nutritional parameters like pH, temperature, moisture content, and incubation period and nitrogen sources are optimized. The optimal condition for maximum biosynthesis of cellulose by the isolated fungi were shown to be at pH 6.0, temperature 25°C, moisture ratio 1:1.5 (w/v), incubation period 5 days and in NH_4NO_2 as nitrogen source. The maximum yield was 13.5U/ml in SmF and 7.9 U/ml in SSF. The yield was maximum at the pH 6.0, and the optimal temperature was 30°C and the optimal fermentation period was 120 hours. The production in the SmF was 1.7 times greater than the SSF.**

Key Words: fermentation, cellulase, sorghum, solid state fermentation, submerged fermentation, industrial microbiology, enzyme, enzyme production

I. Introduction

Sorghum is a genus of flowering plants found in the grass family Poaceae. Seventeen of the twenty-five species are endemic Australia, with the range of some extending to Africa, Asia, Mesoamerica, and certain islands in the Pacific Oceans and India. These species are grown for grain and as a fodder crop and are cultivated in warm climates worldwide. Sorghum is in the subfamily Panicoideae and belongs to the tribe Andropogoneae.

Sorghum is an important crop used for food and as grain.it is also been used for the production of sorghum syrup or molasses and also an animal fodder, the production of alcoholic beverages, and ethanol. These varieties form important components of pastures in many tropical regions. S. bicolor is used as an important food crop in most countries of the world including Africa and Central America and is the fifth-most important cereal crop grown in the entire world. Many species of the crop are tolerant to drought- and are also heat-tolerant, and are especially important in arid regions where rainfall is low, where the grain is one of the staples for poor and rural people in many parts of the world.

Cellulases can break down the cellulose molecule into monosaccharide's ("simple sugars") such as beta-glucose, or shorter polysaccharides and oligosaccharides. The breakdown of cellulose molecules have considerable economic importance, because it makes a major constituent of plants available for consumption and use in chemical reactions. The reaction involved is the hydrolysis of the 1, 4-beta-D-glycosidic linkages in cellulose, hemicelluloses, lignin, and cereal beta-D-glucans. Because of the strong binding of cellulose molecule to each other, the lysis of cellulose is relatively difficult compared to the breakdown of other polysaccharides such as starch in comparison. Cellulase is any of several enzymes produced chiefly by fungi, bacteria, and protozoans and actinomycetes that catalyse cellulolysis, the decomposition of cellulose and of some related polysaccharides. Most mammals cannot digest dietary fibres such as cellulose by themselves. In many herbivorous animals such as ruminants like cattle and sheep and hindgut fomenters like horses, celluloses are produced by symbiotic bacteria present in their gut. Celluloses are produced by a few types of animals, for example some termites.

8% of the worldwide industrial enzyme demands cellulose. The major industrial application of cellulases are in textile industry for processes like bio-polishing of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness. Besides, they are also used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juice and in baking, de-inking of paper is also another emerging application. Celluloses also have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals. Application of this enzyme in detergent, leather and paper industries demands identification of highly stable enzymes active in extreme pH and temperature.

Industrially important enzymes like cellulolytic enzymes have mostly been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. However, solid state fermentation (SSF) technique can improve the yield and reduces the cost of enzyme production in comparison with submerged fermentation. Filamentous fungi are the most commonly used microorganisms in solid state fermentation because they are able to grow on solid materials with low water contents. There are many reports describing use of agro industrial residues for the production of cellulose such as wheat straw, wheat bran and rice straw as substrates. The other advantages of solid state fermentation include high productivity; low capital investment, requires less energy and less water output, better product recovery and lack of foam build up, relatively cheap and reported to be most appropriate process for developing countries. Since the production of cellulose enzyme is a major factor in hydrolysis of cellulosic material, it is important to make the process economically

viable; this study therefore investigated on the bioconversion of sorghum into a more useful product (cellulose) using fungi.

II. Materials and Methods

A. Microorganism

The fungus was isolated from sorghum fields of Coimbatore north sub district, Tamil Nadu, India. The samples were serially diluted and spread plated in potato dextrose agar medium. The isolated fungal colony was sub-cultured and maintained on Potato dextrose agar slants and stored at 4 °C in a refrigerator, until needed.

B. Screening of Isolates

Cellulase producing fungi were isolated from soil by the dilution pour plate or spread plate method using carboxymethyl cellulose (CMC) agar media. The plates were incubated at room temperature for 3 days. To visualize the zones of hydrolysis the plates were flooded with an aqueous solution of 0.1 % Congo-red for 15 min and washed with 1M NaCl. To indicate the cellulose activity of the organisms, diameter of the clear zone around colonies on CMC agar was measured.

C. Pre-Treatment of Substrates

The procured cellulosic substrate such as leaves and stalk of sorghum plant were ground to fine powder and the substrates were individually treated with 1% (w/v) NaOH solution in the ratio of 1:10 (substrate: solution) for 1h and was brought to neutral pH by washing thoroughly with distilled water and dried at room temperature. The treated substrates were autoclaved at 121°C for 1 h (18).

D. Preparation of Inoculum

The inoculum was prepared by growing the organism in 250 ml Erlenmeyer flask with 100 ml of Sabouraud dextrose broth containing g/l of dextrose 40g; peptone 10g; distilled water 1000ml. The medium was inoculated from the potato dextrose agar slants and incubated at 30 °C for 3 days in a shaker (200 rpm) before it was used for the fermentation process.

E. Submerged Fermentation (Smf)

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium. The composition of the medium contained the following g/l of distilled water. L-Glutamic acid, 0.3; NH4NO2, 1.4; K_2HPO_4, 2.0; $CaCl_2$, 2.0; $MgSO_4$, 0.3; protease peptone, 7.5; $FeSO_4$, 5.0; $MnSO_4$, 1.6; $ZnSO_4$, 1.4; tween 20, 20 % (v/v); sorghum substrate, 30. The medium was sterilized by autoclaving at 121°C for 15 min. Each flask was inoculated with 1ml of the above said inoculum. The cultures were incubated on a rotary shaker (120 rpm) at 30°C for 120h.

F. Solid State Fermentation (Ssf)

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks that contained 10 g of coir waste and 15 ml of distilled water (moistening agent). The flasks were sterilized at 121°C for 15 min and cooled to room temperature. About 1ml of inoculum was added, mixed well and incubated at 30°C in a humidified incubator for 120 h. The flasks were periodically mixed by gentle shaking.

G. Enzyme Extraction

At the end of the fermentation the culture broth from submerged fermentation was centrifuged at 6000 rpm for 15 min and the supernatant was used as a source of extracellular enzyme. In solid state fermentation (SSF) the enzyme was extracted from the coir waste by mixing homogenously the entire waste with (1:10 w/v) distilled water and agitated on a rotary shaker (120 rpm) at 30 °C with a contact time of 1h. Dampened cheese cloth was used to filter the extract and pooled extracts were centrifuged at 6000 rpm for 15min and the clear supernatant was used as a source of extracellular enzyme.

H. Enzyme Assay

Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method of Hankin and Anagnostakis. Aliquots of approximately diluted culture filtrate as enzyme source was added to whatman no. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in one millilitre of 0.05 M Sodium citrate buffer of pH 5.0. After incubation at 50 ± 2°C for 1 hrs, the reducing sugar released was determined by dinitrosalicylic acid (DNS) method. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min. Endoglucanase activity (CMCase) was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.5 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50 ± 2°C for 1 h and the reducing sugar produced was determined by DNS method. One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µ mole of reducing sugar per min.

I. Carboxymethyal cellulose Assay:

0.5 ml of the enzyme solution was added into test tubes. The enzyme and substrate solution were equilibrated at 50°C. 0.5 ml of the CMC solution was taken into the test tubes and mixed well. Incubated at 50°C for 30 min. 3.0 ml of DNS solution was added and mixed well, boiled for exactly 5.0 min in vigorously boiling water. Place the tubes in an ice-cooled water bath to quench the reaction. Add 20 ml of distilled water. Mix by inverting the tubes several times. Absorbance was taken at 540 nm. Enzyme activity is expressed as IU/ml/min.

III. Optimization of Process Parameter

A. Effect of pH

Solid state fermentation and submerged fermentation was investigated the effect of pH on cellulose enzyme production by adjusting pH of basal salt solution to 4.0, 5.0, 6.0, 7.0 and 8.0. The substrate was then incubated for 5 days at room temperature.

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B. *Effect of Temperature*

The effect of temperature on cellulose enzyme production was investigated by solid state fermentation and submerged fermentation in sorghum substrate and incubated at 20°C, 25°C, and 30°C, 35°C, 40°C at pH 6.0 for 5 days.

C. *Effect of Incubation Period*

The effect of incubation period on cellulose enzyme production was investigated by checking the enzyme activity from 3-6 days of incubation at pH 6.0 at room temperature.

D. *Effect of Moisture Content*

Solid state fermentation investigated the effect of moisture content on cellulose enzyme production by varying the volume of distilled water to 5mL, 10mL, 15mL, and 20mL.The substrate was then incubated for 5 days at room temperature.

E. *Effect of Nitrogen Sources*

The effect of nitrogen sources on cellulose enzyme production was studied by replacing the nitrogen source NH_4NO_2, in basal salt solution at pH 7.0 with 0.2g of $NaNO_3$, NH_4Cl, NH_4NO_3, K_2HPO_4, $(NH_4)_2SO_4$ and incubated for 5 days at room temperature.

F. *Effect of Carbon Sources*

No carbon source has been used because of using the sorghum substrate as sole carbon source.

G. *Thermal Stability of Enzymes*

For thermal stability study enzymes were incubated at 50°C for the time period (60 mins). Then determine enzymatic activity by DNS method.

IV. Results and Discussion

A. *Isolation and Screening of Cellulolytic Fungi*

Fig. 1. Screening of cellulolytic activity by CMC agar

The soil sample collected from the sorghum fields of the Coimbatore north sub district and was serially diluted in sterile distilled water and was spread on to the carboxymethyl cellulose (CMC) agar plates. The plates were incubated at 30ºC for 3-5 days. Cellulase producing fungal colonies was selected after flooding the plates with Congo red (0.1% w/v), these stained plates were de-stained with NaCl (0.1 M). The colony that showed largest halo forming zone was selected for the cellulase production. This is shown in the Fig-1.,

B. *Temperature*

Incubation temperature plays an important role in the metabolic activities of a microorganism. In the present study the optimum temperature for maximum enzyme production was recorded at 30ºC under SmF and SSF. About 82.9 % of cellulase production was observed at 35ºC. Whereas 69.6% was observed in 40°C, 60.7% was in 25°C and about 49.6% in the 20°C in the submerged fermentation and 85% of the cellulase production was observed in 35°C, 46.8% was in 40°C, 84.8% was observed in the 25°C and 44.3% production in the 20°C in the solid state fermentation. This was similar to the results of other works related to this type of cellulase production. The results are displayed in the Table-I. The effect of temperature is shown in the Fig-2.

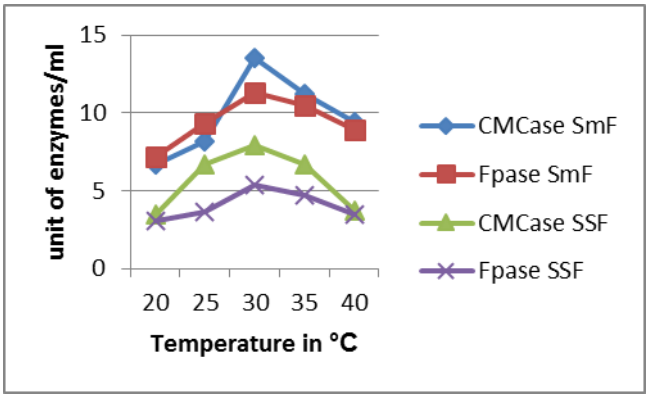

Fig. 2. Graph showing the temperature optimization

C. *pH*

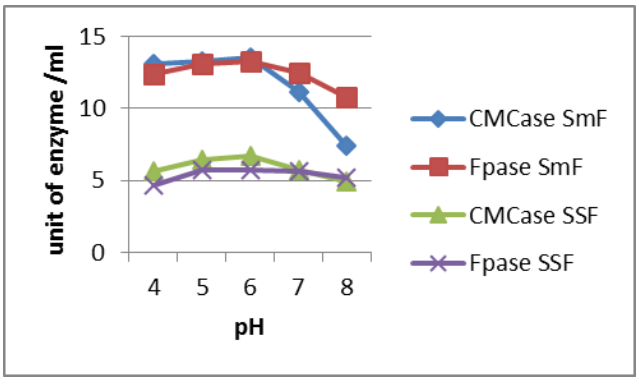

Fig. 3. Effect of pH optimization

pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion which is one of the most important physical parameters. The pH change observed during the growth of microbes also affects product stability in the medium. Optimum pH for maximum production of cellulase was 6.0

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when grown in SmF and SSF. The results are in the table 1. The results are in Fig-3.

D. Fermentation Period

Effect of fermentation period on the production of the cellulase in SmF and SSF is shown in the Table-I. In the present study cellulase activity increased steadily and reached maximum at 120 h of incubation when grown in SmF. the results are in Fig-4.

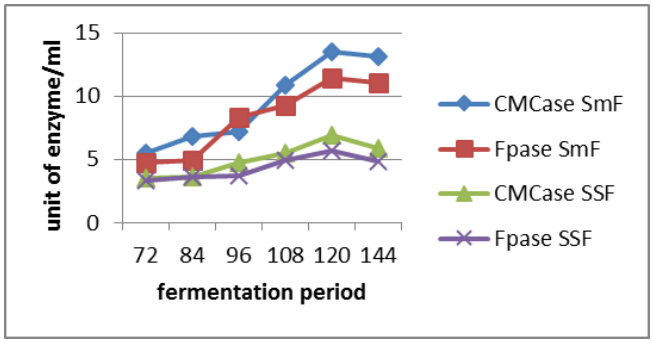

Fig. 4. Effect of fermentation period

E. Moisture Content

The amount of moisture content also influences the enzyme production the maximum production of the enzyme was reported in the sample that has 10g of substrate and 15 ml of sterile distilled water any increase or decrease in the moisture ratio cause decrease in the production of enzyme. The results are shown in the Table-I. The results are shown in Fig-5.

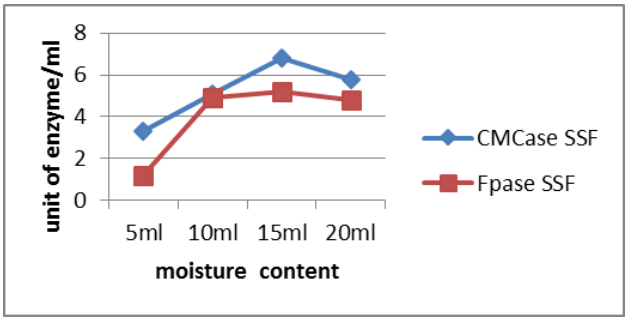

Fig. 5. Effect of moisture content

F. Enzyme Yields

Under the optimum conditions, the strain produced 13.5 units of cellulase per ml of culture broth in SmF and 7.9 units of cellulase per gram of dry mycelial bran in SSF.

G. Comparative Evaluation of SmF and SSF System for Enzyme Titres

The method adopted for comparison of submerged and solid state fermentation is the same as reported by Soma Mrudula et al. for cellulase production from *Aspergillus niger* by dividing the yield obtained from SSF in U/g DMB with the yield from SmF in U/ml culture broth. With this method, cellulase production the in SSF and SmF using sorghum as substrate were compared in terms of their extracellular enzyme production in U/g DMB and U/ml, respectively. When comparison made between SmF and SSF, production of total cellulase by SmF was 1.7 fold higher than that of SSF.

TABLE I
SUBMERGED AND SOLID STATE FERMENTATION

PARAMETER	SUBMERGED FERMENTATION		SOLID STATE FERMENTATION	
	CMCase	FPase	CMCase	FPase
TEMPERATURE				
20	6.7	7.2	3.5	3.1
25	8.2	9.3	6.7	3.6
30	13.5	11.3	7.9	5.4
35	11.2	10.5	6.7	4.7
40	9.4	8.9	3.7	3.5
pH				
4.0	13.1	12.4	5.6	4.7
5.0	13.5	13.3	6.7	5.7
6.0	13.3	13.1	6.4	5.7
7.0	11.1	12.5	5.7	5.6
8.0	7.4	10.8	4.9	5.2
FERMENTATION PERIOD				
72	5.5	4.7	3.5	3.3
84	6.8	4.9	3.6	3.6
96	7.2	8.3	4.7	3.7
108	10.9	9.3	5.5	4.9
120	13.5	11.4	6.9	5.7
144	13.1	11.1	5.9	4.8
MOISTURE CONTENT				
5ML	-	-	3.3	1.2
10ML	-	-	5.1	4.9
15ML	-	-	6.8	5.2
20ML	-	-	5.8	4.8

V. CONCLUSION

In conclusion the result of the present study clearly indicates the potential of sorghum substrate for the production of cellulolytic enzymes. Where in India sorghum is abundant and is not been properly utilized but by the above work, we are clearly seeing an economical application of the sorghum.

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