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Production of cellulases through Solid State Fermentation (SSF) using agricultural waste biomass as solid substrates by *Aspergillus niger*

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***Abstract*—** A study was conducted to appraise the potential of using agricultural lignocellulosic wastes for cellulase production by *Aspergillus niger* through Solid State Fermentation (SSF) technique. In the present study, agricultural waste biomass like Banana Peels (BP), Cotton Stalks and Leaves (CSL), Green Pea Shell (GPS), Soybean Leaves and Stalks (SLS), Sugarcane Bagasse (SB), Tur Leaves (TL) and Wheat Straw (WS) were used as substrates. These untreated substrates were found to be well suited for the organism's growth, producing good amounts of cellulases. It was observed that GPS, SB, TL, BP served as better substrates than others for fungal growth. These four substrates were thus selected for cellulase production study. They were supplemented with nitrogen source in the form peptone, casein, sodium nitrate and urea. This comparative study for the cellulase production via SSF indicated that cellulase activity produced by *A. niger* was higher in GPS supplemented with casein at p^H 5 and incubation temperature of 50°C when incubated for 60 min.

Keywords- *Aspergillus niger, Agricultural waste biomass, Cellulases, Solid State Fermentation (SSF)*

I. INTRODUCTION

Sustainability, abundant availability at almost zero cost of agricultural waste biomass has led to increased interest in their bioconversion into value-added products (including biofuels) [1,2,3]. Lignocelluloses are the structural polysaccharides of plants that composed cellulose as their major component followed by hemicellulose and lignin [4,5]. Complete hydrolysis of cellulose into basic subunit, glucose requires synergistic action of complete cellulase system encompassing three enzymes endoglucanases (EC 3.2.1.4), exoglucanases (cellobiohydrolases; EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) [6,7,8]. Several microorganisms have found to be capable of producing cellulases including bacteria, actinomycetes and fungi but later are of great interest as they excrete copious amounts of cellulases and hemicellulases enzymes extracellularly [9]. Commercially *Trichoderma reesei* is popular fungal strain for cellulase production as they produce high amount of both endo- and exo-glucanases, but does not produce sufficient amount of β-glucosidases [10,7,11]. And therefore *Aspergillus* strains have been recently exploited considering its ability to produce all the three components of cellulase [12].

Although cellulase is produced via submerged fermentation (SmF) commercially, solid state fermentation (SSF) is mostly employed because of its advantages over prior as it is cheap, requires lower energy, a simple fermentation medium, has high enzyme yield, superior productivity, an easy control of bacterial contamination and lower costs of downstream processing [13,14,15]. The present work aims at studying the potential of using various agricultural waste biomass for growth of *A. niger* as well as cellulase production.

Thus the present work focuses on the evaluation of various agricultural wastes for their ability to support growth of *A. niger* as well as production of cellulases by them via solid state fermentation.

Section I of the paper contains brief introduction of the topic, Section II contain the methodology used, Section III is about results and discussion and Section IV concludes research work with future directions.

II. METHODOLOGY

Microorganism

A. niger used in the present study was isolated from garden soil of DSM College, Parbhani and maintained on a Potato Dextrose Agar (PDA) as slants.

Inoculum preparation

Inoculum in the form of spore suspension used in the present study was prepared by adding 10 ml of acetate buffer of P^H 4 containing few drops of tween 80.

Substrate Preparation

Various agro-waste residues like Banana Peels (BP), Cotton Stalks and Leaves (CSL), Green Pea Shell (GPS), Soybean Leaves and Stalks (SLS), Sugarcane Bagasse (SB), Tur Leaves (TL) and Wheat Straw (WS) were collected locally. These were sun dried separately till moisture was completely removed, before being finely crushed into powder using

kitchen blender. Powder was then stored in air tight containers until further use.

Qualitative growth analysis of *A. niger* on different agricultural wastes

Each substrate (BP, CSL, GPS, SLS, SB, TL and WS) was taken 10 g into the separate flasks and inoculated with 3 ml inoculum. Media was then poured into Petri dishes and incubated at 30ºC for 12 days under static conditions.

Cellulase production

After 12 days four substrates exhibiting maximum *A. niger* growth were selected and supplemented with nitrogen sources such as peptone, casein, urea, sodium nitrate in order to find best optimized media in terms of nitrogen source for *A. niger* growth and enzyme production. 10 gram of GPS, SB, TL and BP powder was taken into the respective flasks and moistened with 4 ml distilled water. Flasks were autoclaved at 121°C for 20 minutes. After cooling, media was poured into sterile Petri plate. It was then inoculated with 2 ml inoculum followed by addition of 3 ml of 2% of each of the selected sterile nitrogen source. Plates were incubated at 30°C for 10 days under static conditions.

Enzyme extraction

After 10 days of fermentation the content of each dish was transferred into Erlenmeyer flasks containing 50 ml of acetate buffer (0.1 M, P^H 4.8) and then stirred in a rotary shaker (150 rpm, 30 min at room temperature). The contents of the flasks were then filtered through a metallic sieve and filtrate was centrifuged at 8000 rpm for 15 min. The supernatant was collected as crude enzyme extract for the determination of cellulolytic activities.

Enzyme Assay and Enzyme Unit

Filter paper assay (FPase) method: Whatman No. 1 filter paper strips of 50 mg (1 × 6 cm) were inserted into a test tube containing of 1 ml of 0.05 M citrate buffer (pH 4.8) in which 1 ml of crude enzyme was added. The test tubes were incubated in a water bath for 60 min at 50°C. After incubation, the reaction was terminated by the addition of 3 ml of dinitrosalicylic acid (DNS) reagent. The tubes were boiled for 15 min in a boiling water bath and cooled. After cooling to room temperature, optical density (OD) was measured at 540 nm. The amount of reducing sugars produced was calculated from a glucose standard curve [16].
One filter paper unit (FPU) was defined as the amount of enzyme releasing 1 mole of reducing sugar from filter paper per ml /min.

Evaluation of Enzyme Activity at Different Conditions of P^H, Temperature and Incubation time

Crude enzyme extract from substrate supplemented with nitrogen source exhibiting maximum enzyme activity was selected for further study. Its activity was checked at various parameters like pH, temperature and incubation time.

Effect of different P^H

In this experiment, 1 ml of 0.05 M citrate buffer of varying P^H 3.0, 4.0, 5.0 and 6.0 was used and cellulase activity was calculated by FPase assay.

Effect of different temperature

To evaluate cellulase activity at different temperature, extracted crude cellulase was incubated at various assay incubation temperatures of 30°, 40°, 50° and 60°.

Effect of different assay incubation time

In order to check effect of varying incubation time on cellulase activity, it was incubated for 60, 120, 180, and 240 min. and FPase activity was evaluated.

III. RESULTS AND DISCUSSION

Qualitative growth analysis of *A. niger* on different agricultural wastes

Table 1: Qualitative growth analysis of *A. niger* on different agricultural wastes

Substrate	Incubation days					
	2nd	*4th*	*6th*	*8th*	*10th*	*12th*
SB	-	+	++	++	+++	+++
GPS	-	-	+	+	++	+++
CSL	-	-	-	+	+	++
TL	-	+	+	++	++	+++
SLS	-	-	-	+	+	++
BP	-	+	++	++	+++	+++
WS	-	-	-	-	-	-

(+) Moderate growth; (++) Good growth; (+++) Very good growth.

The effect of eight different agricultural wastes viz., BP, CSL, GPS, SLS, SB, TL and WS on the qualitative growth of *A. niger*, were studied and the results are given in Table 1. Less growth on nearly all substrate was recorded at initial period of incubation. On further incubation more growth of *A. niger* were recorded on BP, GPS, SB and TL. Comparatively less growth recorded on CSL, SLS and no growth was recorded on WS.
And therefore, substrates showing maximum growth of fungi BP, TL, SB and GPS were selected for further study.

Cellulase production on nitrogen supplemented media

The result of this investigation shows that the organic nitrogen sources, peptone and casein supports better biomass of *A. niger* yield as compared to the inorganic nitrogen substrates tested (Table 2).
FPase activity of the respective samples is as mentioned in fig 1. Highest FPase activity i.e. 0.34 IU/ml is produced by GPS on casein nitrogen source. While on peptone, urea, and sodium nitrate it is found to be 0.10, 0.15, 0.06 respectively. TL shows maximum enzyme production on sodium nitrate,

0.25 IU/ml. SB produced highest cellulose on peptone i.e.0.13 and 0.009, 0.022, 0.005 on casein, urea, and sodium nitrate respectively. Compared to other nitrogen sources, sodium nitrate is found to be less efficient as nitrogen source for cellulase production.

Table 2: Comparative analysis of *A. niger* growth on agricultural substrate

Substrate	Nitrogen source	Incubation days				
		2nd	*4th*	*6th*	*8th*	*10th*
SB	Peptone	-	++	++	+++	+++
	Casein	-	+	+	++	++
	Urea	-	++	+++	+++	+++
	Sodium nitrate	-	-	+	+	+
BP	Peptone	-	-	-	+	+
	Casein	-	-	-	+	+
	Urea	-	++	++	++	++
	Sodium nitrate	-	-	-	-	-
GPS	Peptone	-	++	++	+++	+++
	Casein	-	++	++	+++	+++
	Urea	-	+	+	++	++
	Sodium nitrate	-	-	-	-	+
TL	Peptone	-	+	++	++	++
	Casein	-	+	++	++	++
	Urea	-	+++	+++	+++	+++
	Sodium nitrate	-	-	+	++	++

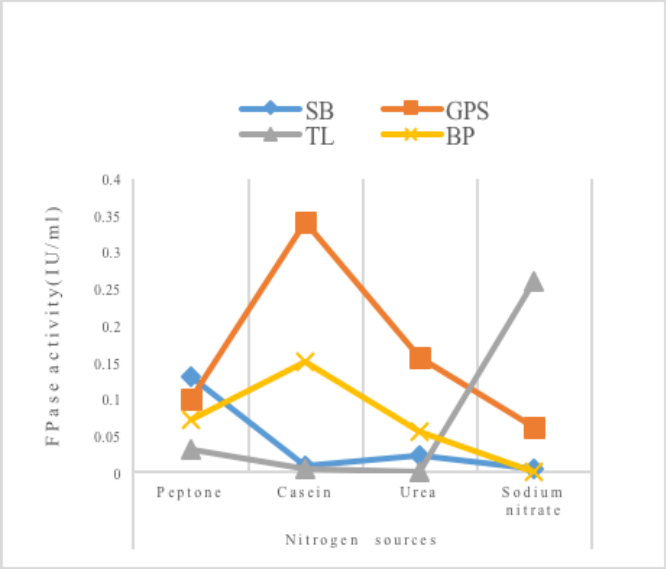

Figure 1 Cellulase activity using A. niger on nitrogen supplemented media.

Evaluation of Enzyme Activity at Different Conditions of P^H, Temperature and incubation time

Enzyme characterization of enzyme is necessary to understand their behaviour at various physical and chemical parameters. In this work, maximum enzyme activity was obtained of GPS on casein nitrogen source. Therefore, crude enzyme extracted from it was subjected to study at various pH, temperature and incubation time.

Effect of P^H

The P^H of a medium plays an important role in the production of a cellulase that uses natural catalyst i.e fungi. Cellulase activity of GPS from casein as nitrogen source was evaluated at P^H 3.0, 4.0, 5.0 and 6.0. It is inferred in Fig. 2 that P^H 5 is found to be the optimal P^H for cellulase production by *A. niger* where cellulase activity is maximum (0.25 IU/ml).

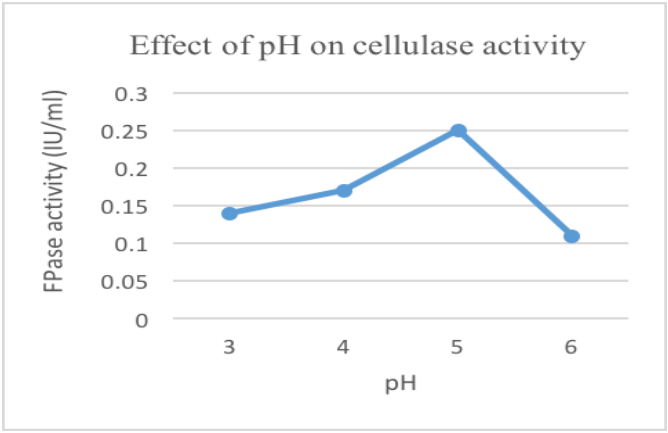

Figure 2 Cellulase activity by A. niger measured at different assay P^H using GPS (Casein as nitrogen source) as substrate.

Effect of temperature

In addition to P^H, the temperature is the second important parameter that affects the activity of an enzyme. Maximum enzyme activity by *A. niger* on GPS on casein nitrogen source as substrate is found at 50°C. FPase activity at 50°C is 0.35 IU/ml. Incubation at higher temperature reflected lower enzyme activity.

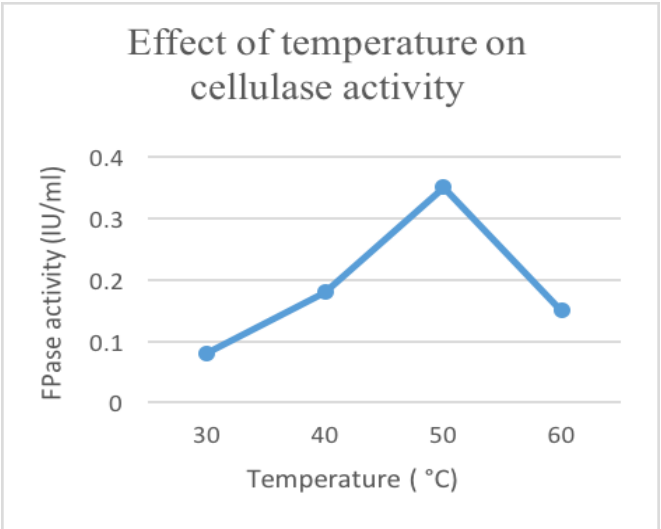

Figure 3 Cellulase activity by A. niger measured at different assay temperature using GPS (Casein as nitrogen source) as substrate.

Effect of incubation time

The effect of incubation time is studied to examine the maximum activity of cellulase. As shown in figure 4, the highest (0.35 IU/ml) enzyme activity of *A. niger* on GPS (Casein) is found at 60 min. later on as the incubation time increased enzyme activity is found to be decreased.

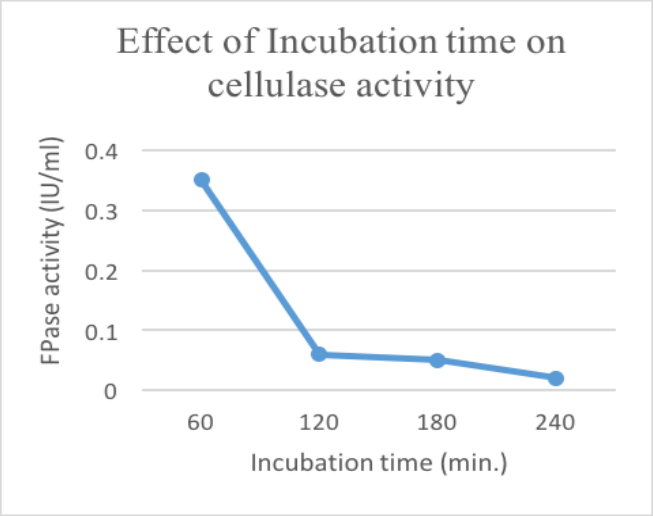

Figure 4 Cellulase activity by A. niger measured at different assay incubation time using GPS (Casein as nitrogen source) as substrate.

IV. Conclusion and Future Scope

Successful attempts have been made to make use of various agro-waste residues as substrates for the production of complete cellulase complex by *A. niger* via solid state fermentation, with a view to developing a low cost production system.

This study establishes that agricultural waste biomass could serve as an ideal substrate for production of cellulases. That will assuage both environmental and economic crisis along with reliving waste-management problem.

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