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Article

Higher Production of Cellulase and Amylase by Novel *Aspergillus* sp. in Solid-State Fermentation

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ABSTRACT

A potentially novel *Aspergillus* sp. as a producer of multienzymes is exploited in fungal fermentation on lignocellulosic substrates. High-level production of extracellular cellulase (exoglucanase, endoglucanase, and β-glucosidase) and amylase has been achieved. Three different types of "waste" solids such as sugarcane bagasse, sawdust, and tea residue have been used in solid-state fermentation (SSF). Among the three different lignocellulosic substrates used in this study, sugarcane bagasse was the excellent solid substrate for higher production of cellulase and amylase enzymes. Sugarcane bagasse supported remarkable production of filter paperase (15.42 FPU/g of substrate), carboxymethyl cellulase (CMCase) (17.89 U/g of substrate) and β-glucosidase (2.43 U/g of substrate), and amylase activity (15.12 U/g of substrate) in SSF. Significant secretion of protein (45.75 mg/g of substrate) on sugarcane bagasse was noticed. Though, producing sustainable biofuels from lignocellulosic substrates in a cost-effective manner, it is an important challenge in the commercialization of the production process.

Key words: Lignocellulosic substrates, *Aspergillus* sp., Exoglucanase, Endoglucanase, β-Glucosidase, Amylase, Solid-state fermentation.

1. INTRODUCTION

Lignocellulosic material is the most rich renewable and abundantly available resource [1,2] frequently refilled by photosynthetic decrease of carbon dioxide by sunlight energy [3]. Lignocelluloses comprise a major division of agroresidues and forest wastes [4]. Therefore, they are the most gifted feedstock for the production of energy, food, and chemicals [5,4] and their consumption could allow self-sustainable processes and products. The bioconversion of lignocellulosic materials at economic rate will lead to the expansion of large-scale strategies useful to humankind [6]. Liberation of soluble sugars from cellulose in lignocellulosic substrates depends on the coordinated action of individual components including exoglucanase or filter paperase (FPase), endoglucanase or carboxymethyl cellulase (CMCase), and β-glucosidase in cellulase enzymes [7]. Starch hydrolytic amylase enzymes are more vital in industries with vast applications in food, fermentation, textile, and paper [8].

Microorganisms have become progressively very vital as a producer of hydrolytic enzymes. Due to their biochemical assortment and the enzyme concentrations may be enhanced by environmental and genetic manipulation, efforts are now being made to substitute enzymes, which conventionally have been isolated from complex eukaryotes [9]. The selection of novel microorganism for the production of enzymes is very imperative. The fungi selected must have relatively stable characteristics and the ability to grow rapidly and vigorously [10]. The most important characteristic to search for the selection of the microorganism is its ability to degrade the lignocellulosic substrates. This will lead to the production of high-level titers of the desired enzymes. The selection of a particular strain of microorganism, however, remains a dreary task, particularly when commercially capable enzyme yields are to be attained [11,12]. In the present study, the production of cellulase and amylase enzymes by a new isolate of *Aspergillus* sp. in solid-state fermentation (SSF) is reported.

2. MATERIALS AND METHODS

2.1. Isolation of Hypercellulase and Amylase-Producing Fungi

Three different soil samples were collected from Botanical Garden, Yogi Vemana University, Kadapa, Andhra Pradesh. Samples were collected from the top 1–15 inches, subjected into sterile polythene bags, and transferred to the laboratory for subsequent experiments.

In each collection, a homogenized sample was prepared by careful mixing of equal quantities of soil. Single colonies were augmented by subjecting to serial dilution and suitable dilutions (10^{-2}–10^{-5}) were plated on Czapek Dox agar medium added with 0.5% cellulose and streptomycin. The inoculated plates were incubated at 28°C for 3–7 days.

2.2. Identification of the Fungal Isolate

Fungi were isolated as monocultures on potato dextrose agar medium and Czapek Dox agar medium, and every pure culture was inoculated individually onto PDA slants and stored at 4°C. A little amount of mycelia mat was picked with a sterile needle, kept on a glass slide, and stained with lactophenol cotton blue and examined under microscope for mycelia, conidiophores/fruiting bodies, and conidia.

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2.2.1. Pure culture and inoculum preparation

Aspergillus sp. isolated from the soils of Botanical Garden, Yogi Vemana University, Kadapa, Andhra Pradesh, was maintained on Czapek Dox agar medium and spore inoculum was prepared from 7-day grown culture slants by addition ample amount of sterile distilled water with Tween-20 (0.2%,v/v). The culture was preserved on Czapek Dox agar medium.

2.2.2. Lignocellulosic substrates

Substrates, i.e., sugarcane bagasse, tea residue, and sawdust were selected as solid substrates for SSF due to their relative copiousness in the local region. The substrates were separately sieved through a 2 mm screen to give consistent particle size.

2.2.3. SSF

SSF was performed in 250 ml Erlenmeyer flasks. Ten grams of various lignocellulosic substrates were used. One liter of Czapek Dox liquid medium comprised $NaNO_3$ (2.0 g), K_2HPO_4 (1.0 g), $MgSO_4$ $7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4$ $7H_2O$ (0.01 g), sucrose (30.0 g), and cellulose (5.0 g). The various lignocellulosic substrates required different volumes of water (10–15 ml) for 50% moisturization of 10.0 g substrate. Each flask was covered with cotton plug and autoclaved at 121°C for 15 min. Sterile solid culture medium was inoculated with *Aspergillus* sp. spores at 2 × 10^6 spores/flask and incubated at ambient temperature (30 ± 2°C). The samples were taken at daily intervals for processing. Fermented substrate was mixed by distilled water, the slurry filtered with muslin cloth, and the filtrate was centrifuged at 9000 g for 20 min at 4°C. The supernatant was used for protein estimation and enzyme activity [13].

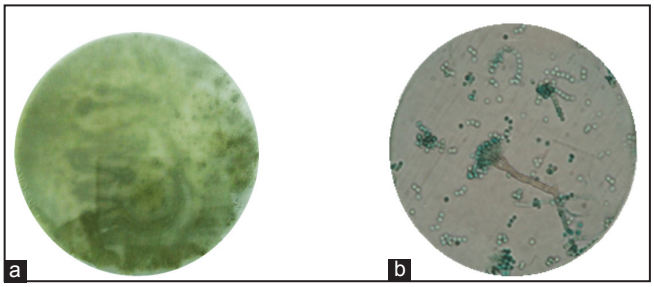

Figure 1: Isolation of hypercellulase and amylase-producing fungi. (a) Growth of *Aspergillus* sp. on Czapek Dox agar medium. (b) Microscopic observation of the culture.

2.2.4. Cellulase assay

Every sample filtrate was examined for FPase, CMCase, and β-glucosidase activity. The filter paper assay method [14] was used to measure total cellulase activity. The activity of cellulase was articulated in filter paper units. One unit of filter paperase activity was distinct as the amount of enzyme releasing 1 µmole of reducing sugar per minute. Activity of endoglucanase was quantified by the carboxymethyl cellulase method [15]. One unit of endoglucanase activity was expressed as the amount of enzyme releasing 1 µmole of reducing sugar per minute. β-Glucosidase activity in the culture filtrate of *Aspergillus* sp. was determined according to Herr [16]. Activities of FPase, CMCase, and β-glucosidase were calculated on substrate – filter paper, CMC, and p-Nitrophenyl β-D-glucopyranoside, correspondingly with suitable controls.

2.2.5. Amylase assay

Activity of amylase in the culture filtrate was estimated according to the method of Manning and Campbell [17]. Amylase activity was calculated in assay blend comprising 1 ml of starch dissolved in 0.05 M citrate buffer (pH 4.8) and 0.1 ml of diluted enzyme solution with suitable controls. Appropriate aliquots of enzyme source with/without dilution were added to the above blend and incubated for 30 min at 50°C. Enzyme blank (without enzyme) was run concurrently in the same method as specified above. After incubation, the addition of 3, 5-dinitrosalicylic acid was made and the contents were mixed. All samples, enzyme blanks, and glucose standards were vigorously boiled for exactly 5 min in a boiling water bath. After cooling, color developed in tubes was read at 540 nm in UV spectrophotometer.

2.2.6. Protein determination

Aliquots of *Aspergillus* culture filtrates with proper dilution were used to calculate protein content according to Lowry *et al.* [18].

2.3. Statistical Analysis

All the experiments were carried out in triplicate. The statistical analysis for standard deviation was performed using Instat+ v3.3 and SPSS 10.0 software.

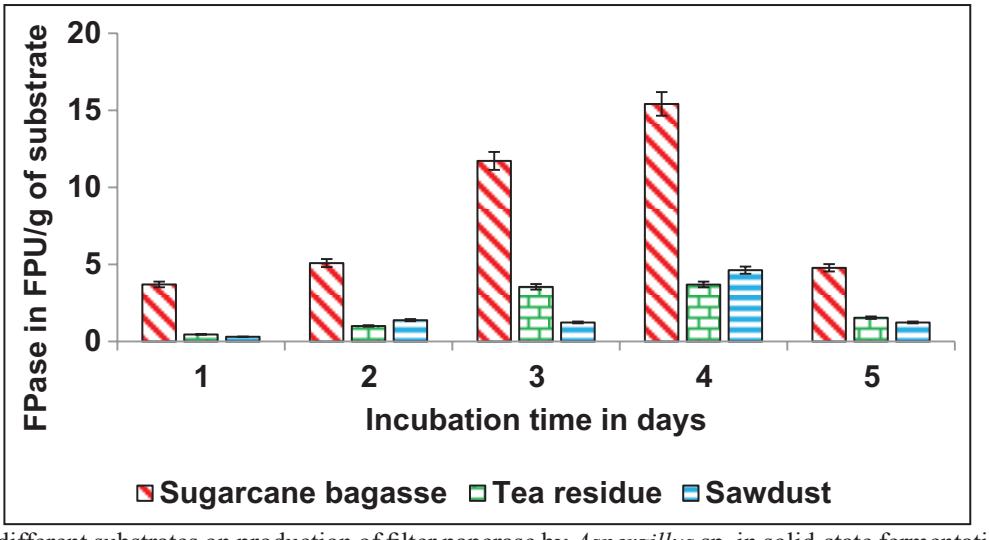

Figure 2: Effect of different substrates on production of filter paperase by *Aspergillus* sp. in solid-state fermentation. Data presented are the averages of three replicates. Statistical analysis for all data was carried out by standard error.

3. RESULTS AND DISCUSSION

3.1. Isolation of Hypercellulase and Amylase-Producing Fungi

The soil samples were diluted in sterile distilled water and spread onto Czapek Dox agar plates containing cellulose (0.5%). The plates were incubated at 30°C for 7 days. Based on morphological and microscopic studies, the isolated strain was tentatively identified as *Aspergillus* sp. (Figure 1a and b).

3.2. Effect of Solid Substrates on Production of Exoglucanase in SSF

Maximum exoglucanase activity was noticed on the 4th day of incubation on every untreated lignocellulosic substrates and activity was declined on the 5th day of incubation (Figure 2). Among three different lignocellulosic substrates tested, sugarcane bagasse was found to be the best substrate (15.42 U/g of substrate) followed by tea residue (4.6 U/g of substrate) and sawdust (3.7 U/g of substrate) for exoglucanase activity at the 4th day of incubation. Thus, sugarcane bagasse is the most appropriate substrate for higher exoglucanase production followed by tea residue.

3.3. Effect of Solid Substrates on Production of Endoglucanase in SSF

Higher yields of endoglucanase were obtained on the 3rd day of incubation on sugarcane bagasse and sawdust, as against tea residue on the 5th day of incubation (Figure 3). Sugarcane bagasse yielded highest titers of endoglucanase with 17.89 U/g of substrate in SSF on the 3rd day of incubation was observed. The second highest endoglucanase activity of 6.94 U/g of substrate was recovered on sawdust on the 3rd day of incubation, whereas tea residue was poor producer of endoglucanase reflected by the recovery of 6.39 U/g of substrate on the 5th day of incubation. Sugarcane bagasse is the most appropriate substrate for endoglucanase production.

3.4. Effect of Solid Substrates on Production of β-glucosidase in SSF

Maximum titer of β-glucosidase was observed on the 5th day of incubation with all substrates apart from tea residue (Figure 4). Maximum titer was observed on the 5th and 4th days of incubation. The production of β-glucosidase was primarily low or undetectable on the 1st and 2nd days of incubation in all substrates and production of β-glucosidase was enhanced from the 3rd day onward on all

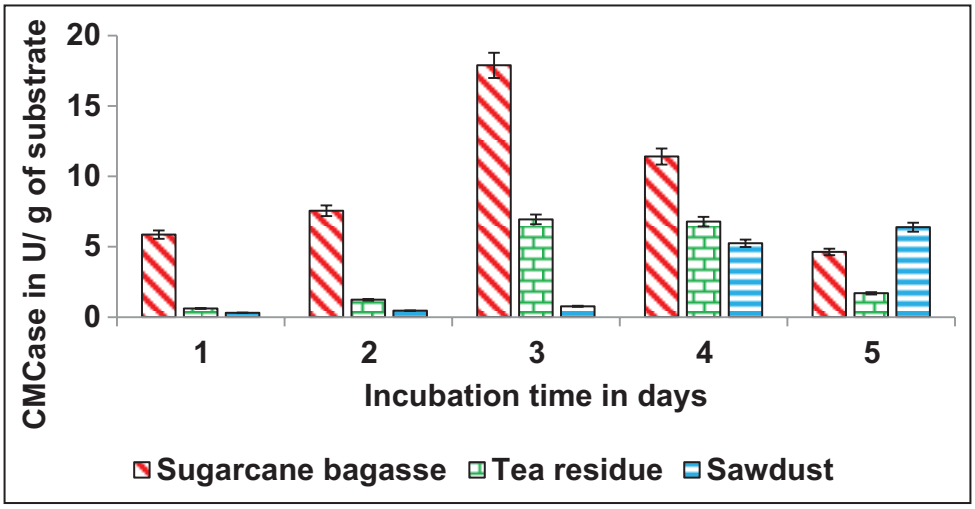

Figure 3: Effect of different substrates on production of CMCase by *Aspergillus* sp. in solid-state fermentation. Data presented are the averages of three replicates. Statistical analysis for all data was carried out by standard error.

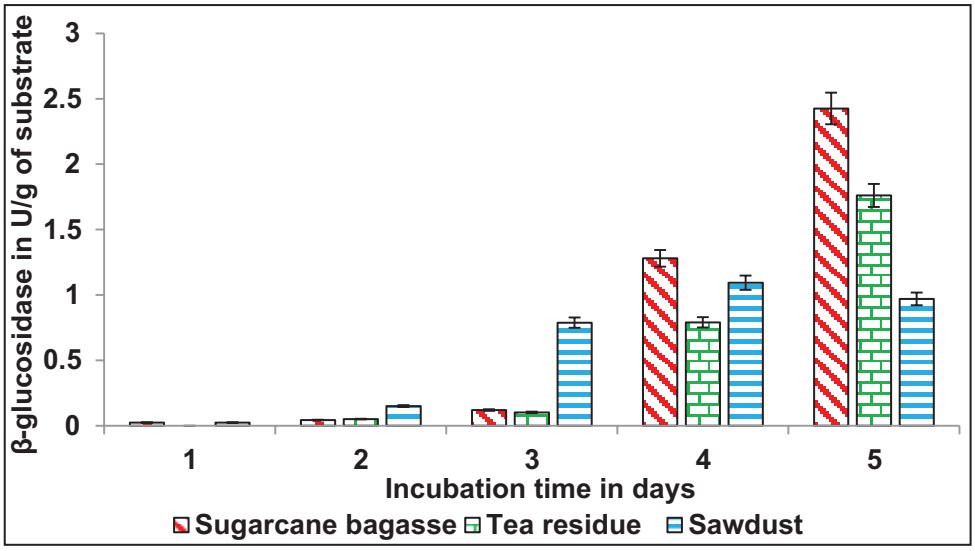

Figure 4: Effect of different substrates on production of β-glucosidase by *Aspergillus* sp. in solid-state fermentation. Data presented are the averages of three replicates. Statistical analysis for all data was carried out by standard error.

substrates. Maximum titers (2.43 U/g of substrate) of β-glucosidase were recovered on the 5th day from sugarcane bagasse. The second maximum titers (1.76 U/g of substrate) of β-glucosidase were recovered on sawdust on the 5th day. Tea residue was supported poorly for the production of β-glucosidase as reflected by the recovery of 1.09 U/g of substrate on the 4th day. Therefore, sugarcane bagasse was the right substrate for maximum production of β-glucosidase followed by sawdust.

3.5. Effect of Solid Substrates on Production of Amylase in SSF

Maximum yield of amylase (15.12 U/g of substrate) was noticed on the 3rd day when sugarcane bagasse used as solid substrate, while very less yield of amylase (5.86 U/g of substrate) was recorded on the 3rd day when sawdust was used as solid substrate (Figure 5). Tea residue yielded amylase activity of 7.41 U/g of substrate on the 4th day and was considered as the second highest producer of amylase. Therefore, sugarcane bagasse was the most apt substrate for higher production of amylase followed by tea residue.

3.6. Effect of Solid Substrates on Secretion of Extracellular Protein in SSF

The maximum protein content was obtained on the 1st day of incubation in all the three solid substrates (Figure 6). The maximum protein content with 78 mg/g of substrate was noticed on sawdust as the substrate. Whereas, the low protein content with 23.25 mg/g of substrate was found on tea residue as the substrate.

Lignocellulose has a composite structure, which obstructs enzymes and as a result, stops the degradation of the lignocellulosic biomass into soluble sugars. Though, producing sustainable biofuels from lignocellulosic substrates in a cost-effective manner is a important challenge in the commercialization of the production process [19]. In addition, chemical hydrolysis of biomass results in the accumulation of toxic components that are unsafe to the environment. Microbes are recognized to produce enzyme complexes that hydrolysis plant biomass, contribution an efficient, and cost-effective choice [20]. The most frequently used microbes for the production of hydrolytic enzymes belong to the genera *Aspergillus*, *Trichoderma*, and *Penicillium* [21]. A modern study noticed that some of the white-rot fungi are the most gifted fungi in terms of biomass degradation and delignification as their ability to synthesize hydrolytic enzymes [22].

The yields of cellulase production in the present study were higher when compared to the results in the recent studies of Muniswaran and Charyulu, Suresh *et al.*, and Shruthi *et al.* [23-25]. Even the amylase production was also higher than in the studies [26-29]. Pre-treatment

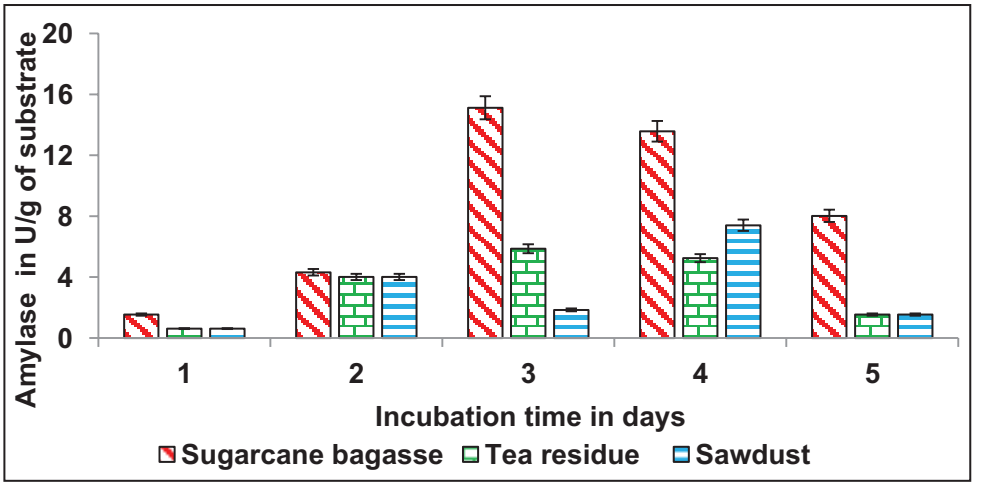

Figure 5: Effect of different substrates on production of amylase enzyme by *Aspergillus* sp. in solid-state fermentation. Data presented are the averages of three replicates. Statistical analysis for all data was carried out by standard error.

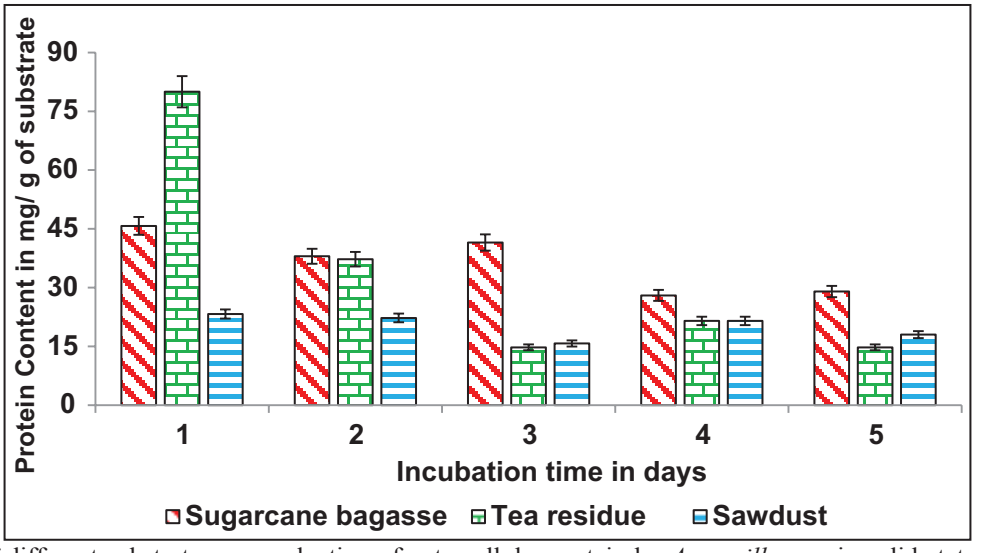

Figure 6: Effect of different substrates on production of extracellular protein by *Aspergillus* sp. in solid-state fermentation. Data presented are the averages of three replicates. Statistical analysis for all data was carried out by standard error.

process may additional enhancement of the enzyme production by utilization of solid substrates by microorganisms in SSF [30]. In the present study, only untreated lignocellulosic substrates were used. The use of pretreated substrates and optimization of nutritional parameters may further improve the yields of cellulase and amylase enzymes in SSF and needs to be further explored.

4. CONCLUSIONS

The higher production of hydrolytic enzymes that cut various β-1,4-glycosidic bonds still remains a challenge and is the main troubleshoot for the lignocellulosic alteration. In particular, the discovery of multienzymes producing organisms which would permit the advancement of more vigorous processes is a key goal in the field of industrial microbiology. It can be concluded from the present study, sugarcane bagasse (untreated) served as the best solid substrate for high-level production of cellulase and amylase enzymes by a novel isolate of *Aspergillus* sp. This study represents an approach to scaling up studies by selecting highly efficient organisms, which can produce higher level of extracellular cellulolytic enzymes and amylase enzymes.

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