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Production of Cellulase by *Aspergillus niger* NCFT 4263.10 using Agro waste as a Substrate

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Abstract
Aspergillus niger NCFT 4263.10 was evaluated for the production of cellulase by liquid static surface fermentation (LSSF), Liquid shaking fermentation (LShF) and solid state fermentation (SSF). Higher cellulase and protein secretion was observed when fermentation carried out in LSSF condition as compared to ShF and SSF. Maximum cellulase production was observed in LSSF (393.5±3.41U/ml) when wheat bran was used as the substrate followed by banana peels and saw dust. Immobilized spores of *A. niger* applied for fermentation up to five cycle and study revealed that when wheat bran was used as the carbon source, the activity of cellulase was found to be maximum. Highest Cellulase activity observed at second cycle (415.4±6.5U/ml) followed by third cycle (379.24 ± 2.65U/ml), fourth cycle (340.07± 2.6U/ml) and fifth cycle (204.25± 1.71U/ml).

Keywords: *Aspergillus niger*, banana peels, fermentation, wheat bran

1. Introduction
Many agricultural by products from agricultural activities and agro based processing litter the environment and constitute waste problems [1]. Large amounts of these agricultural residues are also frequently burnt in the fields causing severe environmental pollution by increase in the CO_2 level [2]. Efficient utilization and microbial biodegradation of, large quantities of agro-industrial wastes and crop residues will lead to several processes of great economic value [3]. These agro wastes are mainly composed of lignocellulosic material which gained considerable interest as an abundant renewable bio energy resource because of their possible use in secondary fermentation process for the production of food, fuel and chemicals. For biodegradation and bioconversion of these cellulosic materials into useful product application of microbial cellulase is used as an essential tool for several researchers all over the world. Most of the bacteria and fungi degrade this cellulosic material by producing enzyme called cellulase [4]. Cellulase is the enzyme that hydrolyses the β-1, 4-glycosidic bonds in the polymer to release glucose units and are among the industrially important hydrolytic enzymes of great significance in present day biotechnology [4].
The use of agro wastes as the basis for the cultivation media is a matter of great interest today, aiming to decrease the costs of energy production and meeting the increase in awareness on energy conservation and recycling [1]. Agro wastes such as rice straw, wheat bran, banana peels, corn stover, sugar cane bagasse, pomace, corn cobs etc are used as substrate in solid state fermentation [5]. Cellulase production using these agro wastes by different organisms in submerged fermentation state has received more attention and is found to be cost prohibitive because of high cost of process engineering [6, 7]. More than 14,000 species of fungi have been found to be active in cellulose degradation. Among several hypercellulolytic fungal strains, filamentous fungi such as *Trichoderma* and *Aspergillus* sp. are considered as the major microbial sources for production of cellulose-degrading enzymes [8, 9]. *Trichoderma* sp. is a well-known commercial cellulase producer but lacks sufficient β-glucosidase activity [10]. In contrary, *Aspergillus* sp. produces high amounts of β-glucosidase in the extracellular medium and is commonly used for commercial production of this enzyme.
The present research investigation deals with studies on the production cellulase from *Aspergillus niger* using different agro waste as a substrate.

2. Materials and Methods
2.1. Sample collection and inoculums preparation
Fungal strains, such as, *Aspergillus niger* (NCFT 4263.10) was used as a microbial strain. Banana peels (BP), saw dust (SD) and wheat bran (WB) were collected from local area. These agro-substrates were dried at 60°C for 48 h or more until the moisture content was reduced. Cooled samples were later grounded in a blender and kept in sterile containers until required [11]. Spore suspension (1 ml) having spore concentration of about 1×10^7 cells ml^{-1} from 7 days old culture was used as inoculum in the experiments.

2.2. Growth medium condition for cellulase production
In 150 ml capacity Erlenmeyer flasks, 50 ml of sterilized fermentation medium having individually BP, SD, WB and commercial carboxymethyl cellulose was inoculated with 1.0×10^7 cells ml^{-1} from 7 days old cultures of *A. niger* and incubated at 30 ± 2°C at liquid static surface fermentation, solid static fermentation and by shaking condition at 100 rpm. Samples were processed at 24 h of regular interval up to 120 h for recovery of cellulase and its activity assay. The fermented broth (50ml) cultures were centrifuged at 10,000 rpm, at 4°C for 10 min to obtain the crude supernatant. Further, the clear crude supernatant was passed through Whatman No. 1 filter paper before cellulase activity assay. The remaining crude enzyme was preserved at -20°C for further use.

2.3. Estimation of Cellulase activity

The activity of cellulase was assayed by incubating 1 ml of reaction mixture consisting of 0.5 ml of 1% CMC in 0.02 M Sodium acetate buffer, pH 5.2 and 0.5 ml of suitably diluted enzyme solution incubated at 40°C for 1 h. Enzyme and reagent blanks were incubated maintaining the same condition simultaneously. The amount of reducing sugar released was determined by dinitrosalicylic acid (DNS) method [12]. One Unit (U) of enzyme activity for cellulase was defined as the amount of enzyme releasing 1 µmol of reducing sugar from CMC per minute per ml. The specific activity determined as the number of units of enzyme activity per milligram of enzyme protein.

Total protein content present in crude enzyme was determined according to Lowery *et al.* [13] method taking Bovine Serum Albumin (BSA) as the standard.

2.4. Immobilization of Whole Cells in sodium alginate

The alginate entrapment of fungal cells was carried out as per the method of Sharma and Satyanarayana [14]. Sodium alginate solution (3%) was prepared by dissolving 3gm sodium alginate in 100 ml boiling water and autoclaved at 121°C for 15 minutes. Both alginate slurry and cell suspension (10^5 spores/ml) were mixed well i.e. in a ratio of 1:1 for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into chilled 0.2 M $CaCl_2$ solution from 5-cm height and kept for curing at 4°C for 1 h. The calcium alginate beads thus formed were cured in 0.2 M $CaCl_2$ for 2 h at 4 °C, and thereafter, beads were washed with sterile distilled water and used for the production of cellulase.

2.5. Production of cellulase by repeated batch process

Conidia immobilized in beads of calcium alginate were inoculated in fermentation medium (50 beads/100ml) by inoculating to sterilized fermentation broth (BP, SD and WB) and incubated at 30°C for 96h. Finally, the fermented media were filtered on Whatman No. 1 paper and beads were washed twice with sterile saline solution (0.8% NaCl) and kept in phosphate buffer (1M, pH 7.0). Then, the beads were again introduced into the fresh medium. After attaining maximum production of enzymes, the spent medium was replaced with fresh production medium (50 ml) and the process was repeated for five batches until the beads/blocks started disintegrating. The enzyme titers and cell leakage of each cycle were determined.

3. Results and Discussion

In the present study cellulase production from *Aspergillus niger* was carried out by liquid static surface fermentation (LSSF), solid state fermentation (SSF) and shaking fermentation (ShF) taking BP, WB, SD as a substrate up to 120 h of incubation period. Higher cellulase and protein secretion was observed after 96 h (data not showed) when fermentation carried out in LSSF in comparison to ShF and SSF (Fig.1). Maximum cellulase production was observed in LSSF (393.5 ± 3.41 U/ml) when WB was used as a substrate followed by BP (295.8 6 ± 2.04 U/ml) and SD (33.66 ± 2.2 U/ml). The specific activity of cellulase produced by *A. niger* was also evaluated and found that maximum specific activity was observed (526.77 U/mg protein) when wheat bran was used as the sole source of carbon (Table 1). The present investigation revealed that wheat bran is the best substrate for the production of cellulase as compared to other agro waste used in the above fermentation process (Figure 1). Haq *et al.* [15] and Abo-State *et al.* [16] also reported that wheat bran as best source of carbon and nitrogen for cellulase production. It might be due to the fact that wheat bran contains adequate amount of nutrient like proteins 1.32%, carbohydrates 69%, fats 1.9%, fibre 2.6%, ash 1.8%, Ca 0.05%, Mg 0.17%, Po 35%, K 0.45%, S 0.12%, various amino acids and porosity for oxygen supply. All these nutrients are necessary for the production of enzyme as well as biomass formation [17]. Our findings is higher than the findings of Padmavathi *et al.* [1] who reported cellulase production of 213.3 IU/ml and 206 IU/ml by two marine fungal species such as *Aspergillus terreus* and *Mucor plumbeus* respectively by liquid static surface fermentation. Cellulase production of 50.33 U/ml using fruit bunch fibre as a substrate by *Aspergillus sp.* was also reported by Shahriarinour *et al.* [18]. But the present findings is lower than the findings of Irshad *et al.* [19] who reported maximum cellulase production of 655 ± 5.5 U/ml by a fungal sp. *Trichoderma viride* in SSF using orange peels as an agro waste. Higher cellulase production of 487 U/ml by the strain *Aspergillus* MAM-F35 and 309 U/ml by the strain *Aspergillus* MAM-F23 was reported previously by Abo-state *et al.* [16]. Similar to the present investigation least amount of cellulase production by saw dust (SD) in comparison to other agro wastes was also reported [20, 21, 22].

A number of studies revealed that *Aspergillus sp.* produce relatively large quantities of endoglucanase and β-glucosidase, but low levels of exoglucanase, together with high levels of protein make it an ideal organism for industrial applications [23, 24]. The total protein content during cellulase production was found to be maximum (869.27 ± 6.15 µg/ml) when shaking fermentation was carried out at 100 rpm using wheat bran as a substrate followed by LSSF of WB (747.11 ± 9.7) (Fig. 1). Reddy *et al.* [25] reported maximum protein secretion (1400 µg/ml) by *A. niger* using rice bran as a substrate and wheat bran was the second best substrate that supported secretion of extracellular protein in to the broth.

Immobilization of enzymes on a carrier offers significant cost benefits for industrial processes, because it facilitates enzyme recycling, enables improvements in thermo-stability (thereby reducing enzyme inactivation), and allows for greater control of enzyme activity [26, 27]. In addition, immobilization eliminates the need to separate an enzyme from the product solution and allow these expensive compounds to be reused [28]. In the present investigation, the spores of *A. niger* was immobilized (10^5 spore/ml) using alginate for the production of cellulase and its reusability was evaluated. Immobilized cells/spores of *A. niger* was aseptically inoculated to fermentation flasks in 5 repeated cycles in the presence of banana peels, wheat bran and saw dust as the carbon sources (Fig. 2). When wheat bran was used as the carbon source, the activity of cellulase was found to be maximum (415.4 ± 6.5 U/ml) followed by third cycle (379.24 ± 2.65U/ml), fourth cycle (340.07± 2.6U/ml) and fifth cycle (204.25± 1.71U/ml). As compared to free cell, cellulase production was found to be higher in immobilized cell. The present finding is accords to Ahmed *et al.* [29] who reported maximum cellulase production in sponge immobilized cell of *A. niger* than the free cell of *A. niger*. Gupta *et al.* [30] also observed maximum enzyme production by *F. solani F7* in immobilized cell than in free cell. The secretion of protein content was noticed at every

cycle and continued up to 5th cycle displaying a peak in activity at 4th cycle (980.55 ± 2.1) when wheat bran used as the substrate **(Fig. 2)**. After the successful completion of fermentation, the crude cellulase and biomass were separated. The yield factors were calculated and presented in the **Table 2**. It was observed that the highest specific growth rate (μ_{max}) of *A. niger* observed during the fermentation was with wheat bran i.e. 15.08 $mgl^{-1}h^{-1}$ (Table 2). Similarly, the enzyme accumulation in the culture medium per hour (dP/dt) was observed and found to be maximum when wheat bran was used as the substrate where as the growth-independent coefficient of enzyme production (β) was optimum when banana peels was used as the substrate.

Table 1: Specific activity of cellulase produced by *A. niger*

Substrates	Cellulase activity (U/ml)	Total protein (mg/ml)	Specific Activity (U/mg)
Sawdust	33.66±2.22	0.185±17.93	181.94
Banana peels	295.86±2.04	0.641±0.04	461.56
Wheat bran	393.5±3.41	0.747±9.74	526.77

Table 2: Fermentation kinetics of the cellulase produced by *A. niger*

Substrates	$Y_{e/s}$ (U/g)	$Y_{x/s}$ (g/g)	μ (h^{-1})	β (U/g/h)	dx/dt (g/L/h)	dP/dt (U/ml/h)	x (mg/ml)	μ_{max} (mg/L/h)
Sawdust	336.64	0.014	0.735	3.51	0.014	0.35	0.73	14.71
Banana peels	757.49	0.096	5.013	7.89	0.10	3.05	0.50	10.03
Wheat bran	378.55	0.014	0.753	3.94	0.015	4.09	0.75	15.08

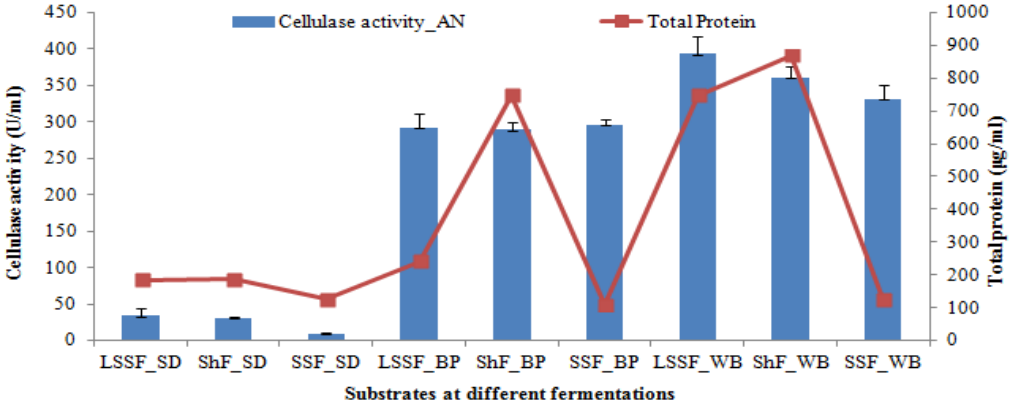

Fig 1: Production of cellulase and total protein content in liquid static surface fermentation (LSSF), liquid shaking fermentation (ShF) and solid state fermentation (SSF) individually using saw dust (SD), banana peel (BP) and wheat bran (WB) as substrates by *A. niger* at 30°C after 96 h

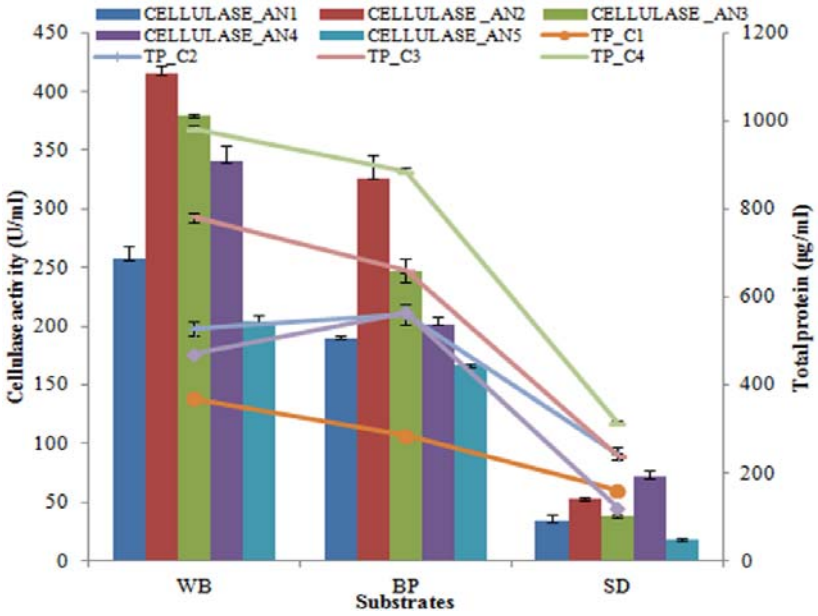

Fig 2: Production of cellulase using calcium alginate beads entrapping the spores of *A. niger* using repeated batch cultures performed at 30°C for 96h (CEL: cellulose; BP: banana peels; WB: wheat bran; SD: saw dust) (Cellulase_ AN1, Cellulase_ AN2 Cellulase_ AN3 Cellulase_ AN4 Cellulase_ AN5: number of cycle for cellulase production) (C1,C2, C3, C4, C5: number of cycles, TP: Total protein content)

4. Conclusion

Production of cellulases on agro wastes such as wheat bran, banana peel and saw dust under different fermentation conditions were carried out by *A. niger*. Among the three different substrates used, wheat bran gave best enzyme activity of 393.5U/ ml in liquid static surface fermentation whereas the total protein content was found maximum (869.27 ± 6.15 µg/ml) when shaking fermentation was carried out at 100 rpm using wheat bran as a substrate. Again fermentation was carried out with alginate immobilized spores of *A. niger* in 5 repeated cycles in the presence of banana peels, wheat bran and saw dust as the carbon sources. Cellulase production and protein secretion were found to be increased with maximum production of 415.4 U/ml and 980.55 ± 2.1 µg/ml respectively with wheat bran used as a substrate. On the basis of the above study it was concluded that, the selected fungal strains have the ability to degrade the agricultural wastes and wheat bran, which is an example of domestic and industrial agro-wastes, produce large amounts of cellulase enzymes when hydrolyzed by *A. niger* that could have potential application for wide range of industries. Hence Wheat bran is a cheap residue instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce cellulase which can reduce the cost of enzyme production.

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