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Isolation, Optimization and Production of Cellulase by *Aspergillus niger* from Agricultural Waste

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Cellulases are the group of hydrolytic enzymes such as endoglucanase (CMCase), exoglucanase, β-glucosidase (BGL) and FPase which are responsible for release of sugars in the bioconversion of the cellulosic biomass into a variety of value-added products. The cellulase producing fungi were isolated from various agriculture fields. Total 21 isolates were obtained on Czapek's Dox agar medium. *Aspergillus niger* was selected as most efficient enzyme producer by screening technique. Optimization of some nutritional and environmental factors like nitrogen source, temperature, pH and fermentation time were studied under submerged culture condition for cellulolytic enzyme production. Different agriculture waste material was used as carbon source. Maximum cellulolytic activity was observed in 4.2 pH media at 28°C after 96 hours in submerge condition. Wheat straw showed maximum activity of CMCase, exoglucanase, β-glucosidase and FPase were 8.38 IU/ml, 5.21 IU/ml, 0.30 IU/ml and 8.08 IU/ml, respectively followed by baggase.

Keywords: *Aspergillus niger*, Cellulase, Lignocellulose, Wheat straw, Rice burn, Banana waste.

The importance of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial importance[1]. It is the primary product of photosynthesis in terrestrial environments and the most abundant organic substrate (100 billion dry tons/year) on earth for the production of glucose, for fuel and as chemical feed stock[2-3]. Cellulase enzymes, which can hydrolyze cellulose forming glucose and other commodity chemicals, can be divided into three categories: endoglucanase (EC 3.2.1.4); exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21)[4-5]. Scientists have strong interests in cellulases because of their various applications include starch processing, animal food production, alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, fuel, pulp and paper industry, waste management, protoplast production, genetic engineering and pollution treatment medical/pharmaceutical industry and textile industry[6-9].

The cost of production and low yields of cellulase enzymes are the major problems for industrial application. Therefore, investigations on the ability of the lignocellulose hydrolyzing microbial strains to utilize inexpensive substrate have been done[10]. The lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin that are strongly intermeshed and chemically bonded by noncovalent interactions and by covalent crosslinkages[11]. Because of the use of

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machinery in agriculture, the livestock population with farmer decreases and resulted in the addition of these agro-wastes in the biosphere leading to environmental pollution in the form of carbon dioxide, methane etc[12-13].

Every part of plant such as leaves, straws, stems, stalks, corncobs, bran, baggase, etc., comes under plant biomass and it was decomposed by microorganisms such as bacteria, actinomycetes and fungi[14]. Bacteria, actinomycetes and fungi have been found to produce cell-bound enzymes and multi-protein complexes expressing cellulases and hemicellulases called cellulosomes. The cellulosome was first discovered in 1983 from *Clostridium thermocellum* which is anaerobic and thermophilic spore-forming bacteria[15]. The production of cellulase generally depends on variety of growth parameters which includes pH value, temperature, time, pre-treatments lignocellulosic material and medium nutrients[16].

This work focuses on different factors relevant to improvement of enzymatic hydrolysis of lignocellulosic materials such as wheat straw, rice straw, baggase and banana agro-waste. To understand the biochemistry of cellulose degrading fungi, it is needed to optimize various conditions.

MATERIALS AND METHODS

Isolation and maintenance of cellulase producing fungi

The soil and agro-waste samples were collected from various farm including banana, rice, wheat and sugarcane near Anand, Gujrat, INDIA. Crushed agro-waste samples were used for preparing suspension. The samples were sprinkled over the Czapek's Dox agar plates and incubated for 7 days at 28±0.5°C. The growth of fungal colonies was observed and individual colony were isolated and restreaked on the same agar. The isolate was identified based on of their colony characteristics, microscopic examination and morphological observation. Isolated fungi were maintained on modified Czapek Dox agar slant & PDA slant and stored at 4°C.

Screening for cellulose producing fungi

Plate screening using cellulose as carbon source

After 7 days of growth on modified Czapek's Dox agar medium, plates were sprayed with iodine solution. It was allowed to stand at room temperature for 5 minutes.

Plate screening using carboxymethylcellulose as carbon source

Spores from one week old PDA plates were suspended in sterile D/W. A small well created in the middle of the carboxymethylcellulose agar medium and spores of each isolates were inoculated into the well. Plates were incubated at 28±0.5 ºC for three days followed by 18 hours at 50ºC. Plates were stained with 0.1% Congo red dye for 0.5-1 hour and distained with 1 M NaCl solution for 15-20 min[17].

Production of cellulase enzyme by submerge fermentation process

Media composition described by Mandels & Weber[18] was used under fermentation process for enzyme production. The composition of media was: $(NH_4)_2SO_4$-1.4 gm, KH_2PO_4-2.0 gm, $CaCl_2$-0.3 gm, Urea*#-0.3 gm, $MgSO_4.7H_2O$*-0.3 gm, $FeSO_4.7H_2O$*-5.0 mg, $MnSO_4.7H_2O$*-1.6 mg, $ZnSO_4.7H_2O$*-1.4 mg, $COCl_2$-2.0 mg, Proteose peptone-1.0 gm, Tween 80-1.0 ml, Cellulose-10.0 gm, Distilled water-1000 ml, pH-5.0±0.2. (*Components were added after autoclaving, #Urea was sterilized by filtration).

For preparation of inoculum, those isolates showed a maximum zone of hydrolysis in screening were used. 1.0 ml of a spore suspension (~10^8 spores/ml) was inoculated into production medium and kept at 28±0.5 °C in orbital shaker incubator at 110-120 rpm.

Cellulase enzyme activity assay

Culture was harvested at 24 hours interval and homogenized at 8,000 rpm at 4°C for 10 min. The supernatant was used as the crude extracellular enzyme source for enzyme assay. Isolates which showed maximum cellulase production was used for the further study.

Enzyme activity was assayed according to the DNSA (3,5-dinitrosalicylic acid) methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission of Biotechnology[19]. One unit of enzyme activity is defined as 1 µmol glucose equivalents released per minute (µmol/ml/min).

DNSA method for Endoglucanase assay

Endoglucanase activity was determined by incubating 0.5 ml of supernatant with 0.5 ml of

1% carboxymethylcellulose (CMC) in 0.05M citrate buffer (pH 4.8) at 50°C for 30 minute. Appropriate control without of enzyme was simultaneously run. The reaction was terminated by addition of 3 ml dinitrosalicylic acid reagent. The tubes were placed in boiling waterbath for 10 minutes & cooled at room temperature. Absorbance was read at 540 nm.

FPase assay

Filter paper activity was determined by adding 0.5 ml of culture supernatant with 1 ml of 0.05 M citrate buffer (pH 4.8) containing a 1cm×6cm strip (50 mg) of Whatman No.1 filter paper, and incubated for 1 hour at 50°C[20]. Reaming steps was followed as per endoglucanase assay.

Exoglucanase assay

Exoglucanase activity was determined by adding 1.0 ml of culture supernatant with 1.0 ml of 0.1 M citrate buffer (pH 4.8) containing 50 mg of absorbent cotton incubated for 24 hour at 50°C. Reaming steps was followed as per endoglucanase assay.

β-glucosidase assay

β-glucosidase activity was determined by incubating 1 ml of culture supernatant with 10 mg salicin in 1 ml 0.05 M citrate buffer (pH 4.8) at 50°C for 30 minute[21]. Reaming steps was followed as per endoglucanase assay.

Protein estimation

Protein estimation was carried out using folin lowery's method using Bovine serum albumin as standard[22].

Optimization of pH, temperature and incubation period

To select the optimum pH, temperature, incubation period for fermentative production of the enzyme the selected fungal strain were cultivated with varying pH ranges 4.2, 5.2, 6.2, 7.2 and 8.2, temperatures of 20°C, 28°C, 37°C, 55°C and 75°C, incubation period range of 24 to 144 hours, by keeping all other parameters constant. Cellulose used as carbon source in fermentation media. The flaks were inoculated with 1.0 ml of spores and incubated at 28±0.5 °C in shaker incubator at 110-120 rpm. Enzyme assays were carried out at regular intervals.

Optimization of nitrogen source

Different types of nitrogen sources (Peptone, $(NH_4)_2SO_4$ and Urea) were used for optimization. Peptone was used from 0.05, 0.075, 0.1, 0.125 and 0.15 gm%. $(NH_4)_2SO_4$ was used from 0.1, 0.12, 0.14, 0.16 and 0.18 gm% and Urea was used in range from 0.01 to 0.05 gm% in cellulose containing fermentation medium. The flaks were inoculated with 1.0 ml of spore and incubated it at 28±0.5 °C in shaker incubator at 110-120 rpm. Enzyme assays were carried out at regular intervals.

Optimization of carbon source

Beside cellulose, different types of agriculture material were used as carbon sources which included wheat straw powder*, rice straw powder*, baggase powder* and banana agro waste powder* without and with pre-treatment (1N NaOH) (*Particle size: 180 & B.S.S. Mesh No. 85). The flaks were inoculated with 1 % carbon source and 1.0 ml of spores into modified production medium and incubated at 28±0.5 °C in shaker incubator at 110-120 rpm. Enzyme assays were carried out at regular intervals.

RESULTS AND DISCUSSION

Isolation and screening for cellulase producing fungi

Total 21 isolates were obtained on Czapek's Dox agar medium, from which 10 fungi that showed clear zone on addition of Iodine solution (Figure 1 & 2). Further these fungi were grown on the CMC agar plates. After 5 days of incubation, the appearance of the clear zone around

Table 1. Comparison of cellulose and wheat straw for the production of cellulase enzyme

	Endoglucanase		Exoglucanase		β-glucosidase		FPase	
Carbon source	Unit activity IU/ml	Specific activity U/mg	Unit activity IU/ml	Specific activity U/mg	Unit activity IU/ml	Specific activity U/mg	Unit activity IU/ml	Specific activity U/mg
Cellulose	0.24	1.12	0.03	0.12	0.21	1.10	0.21	0.98
Wheat	8.38	10.58	5.21	6.57	0.30	1.18	8.08	10.20

Fig. 1. Cellulase positive 10 isolated fungi on Czapek's Dox agar medium

Fig. 2. Clear zone on Czapek's Dox agar medium

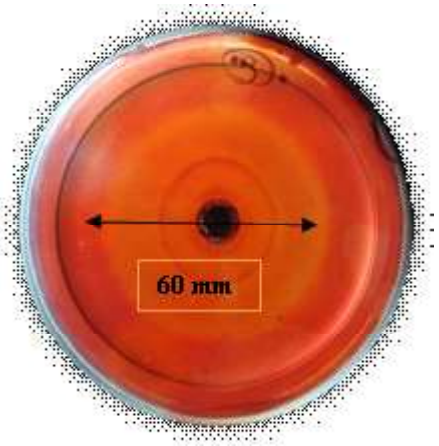

Fig. 3. Clear zone on CMC agar medium

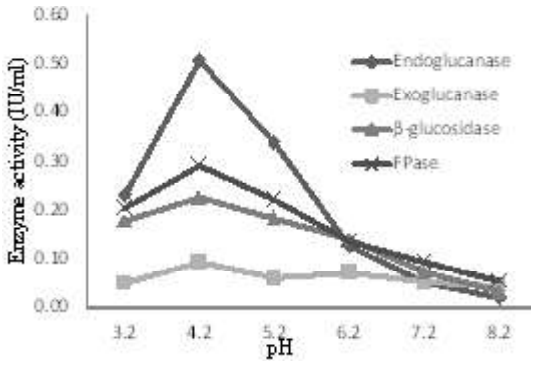

Fig. 4. Effect of pH on *Aspergillus niger* cellulase activity using cellulose substrate

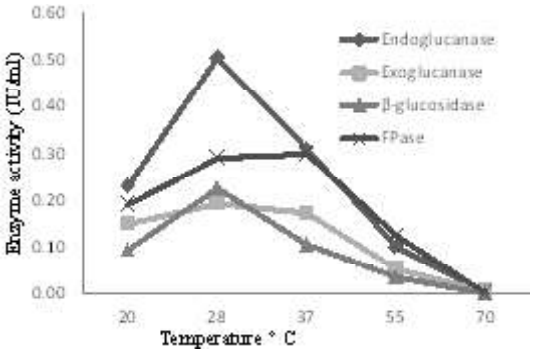

Fig. 5. Effect of temperature on *Aspergillus niger* cellulase activity using cellulose substrate

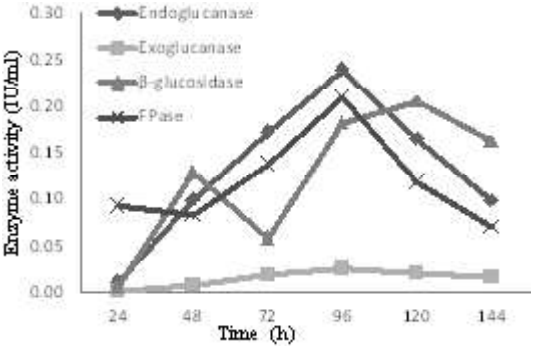

Fig. 6. Effect of time on *Aspergillus niger* cellulase activity using cellulose substrate

the surrounding the small well when added Congo Red solution was strong evidence that the fungi produced cellulase in order to degrade cellulose. The clearing zone diameter was measured and maximum zone of clearance producing fungus was selected for further studies (Figure 3).

Cellulase enzyme activity assay

The protein concentration in crude samples was determined with bovine serum albumin (BSA) as standard. The enzyme unit (IU/ml) of crude enzyme was determined by using DNSA method and their specific activity U/mg was calculated. In which isolate No. 3 showed higher endoglucanase (0.2389 IU/ml), exoglucanase (0.0255 IU/ml), β-glucosidase (0.2059 IU/ml) and FPase activity (0.2100 IU/ml) among 10 isolates. On the basis of enzyme activity, specific activity, microscopic examination and morphological observation isolate no. 3 was *Aspergillus niger.*

Optimization of pH, temperature and incubation period

The maximum cellulase activity was reported at pH 4.2-5.2. The enzyme activities of CMCase, exoglucanase, β-glucosidase, FPase were 0.51 IU/ml, 0.19 IU/ml, 0.23 IU/ml and 0.29 IU/ml, respectively (Figure 4). Maximum cellulase activity was observed at 28°C, the activity of CMCase, exoglucanase, β-glucosidase, FPase was 0.51 IU/ml, 0.19 IU/ml, 0.23 IU/ml and 0.29 IU/ml, respectively (Figure 5) and Endoglucanase, exoglucanase and FPase activity was found maximum 0.24 IU/ml, 0.03 IU/ml, 0.21 IU/ml, respectively at 96 hour while β-glucosidase activity was observed maximum (0.21 IU/ml) at 96 hour (Figure 6).

Similar finding was observed when rice bran and orange peel used as substrates and highest cellulase activity reported at 4.0 pH with

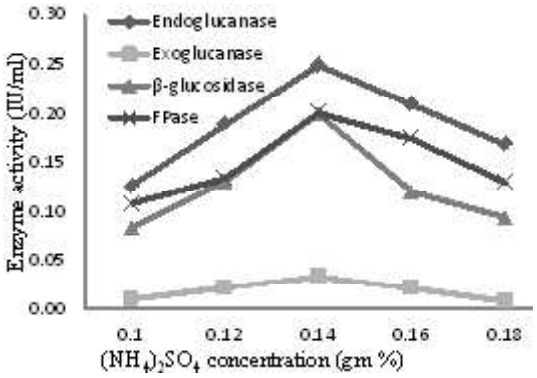

Fig. 7. Effect of $(NH_4)_2SO_4$ on *Aspergillus niger* cellulase activity using cellulose substrate

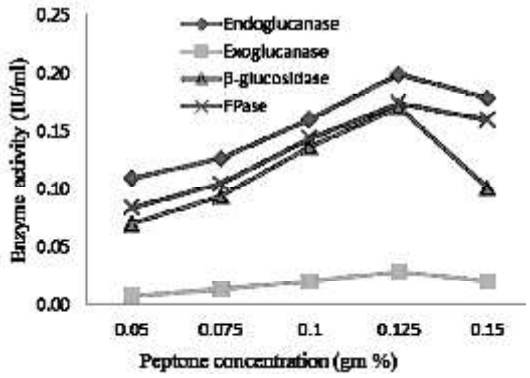

Fig. 8. Effect of peptone on *Aspergillus niger* cellulase activity using cellulose substrate

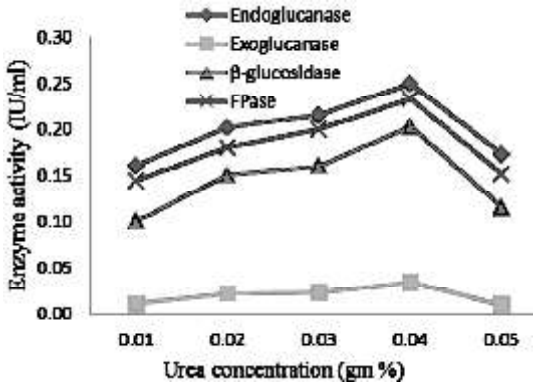

Fig. 9. Effect of urea on *Aspergillus niger* cellulase activity using cellulose substrate

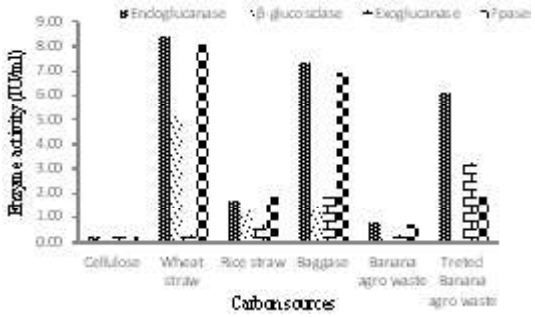

Fig. 10. Effect of different lignocellulose material on *Aspergillus niger* cellulase activity with cellulose substrate as standard

by *Aspergillus niger*[23]. A highest level of β-glucosidase was obtained at pH 4.0, which decreased drastically with an increase in pH of the medium to 5 by *Aspergillus niger* [24]. When media was cultured with pineapple peel, orange peel and CMC using *Aspergillus niger* at pH 4 gave maximum cellulase activities of 0.270, 0.200 and 0.173 mg/ml, respectively[25]. Optimum temperature for maximum enzyme production was recorded at 30ºC under SmF and SSF for *Aspergillus spp.*[26]. Maximum endoglucanase activity was reported at 28°C when *Aspergillus niger* using wheat straw:wheat bran as carbon source[27]. Rice bran produced maximum cellulase activity at 45 °C with value of 20.35µmol/min[23]. Maximum activity of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass was reported after 96 hours[10]. Secretion of maximum activity of cellulase enzymes was reported at after 7 days by *Aspergillus niger*[13]. Highest production of CMCase by *Aspergillus niger* using plant wastes as substrate was reported after 144 hours[12].

Optimization of nitrogen source

The maximum CMCase activity 0.25 IU/ml, exoglucanase 0.03 IU/ml, â-glucosidase 0.20 IU/ml, FPase 0.20 IU/ml were observed after 96 hours, When Ammonium sulphate applied at 0.14 gm% (Figure 7).

Peptone at 0.125 gm% was showed maximum CMCase activity 0.20 IU/ml, exoglucanase 0.03 IU/ml, β-glucosidase 0.17 IU/ml, FPase 0.17 IU/ml after 96 hours (Figure 8).

Urea at 0.04 gm% was observed maximum CMCase activity 0.25 IU/ml, exoglucanase 0.03 IU/ml, β-glucosidase 0.20 IU/ml, FPase 0.23 IU/ml after 96 hours (Figure 9).

According to previous studies at 0.03% urea, peptone and $NaNO_3$ used as nitrogen source, the activity of cellulase were obtained 0.824, 0.421 and 0.401 IU/ml, respectively[28]. Cellulase activities were obtained 0.1196, 0.1528 and 0.1528 IU/ml using peptone (0.125%), $(NH_4)_2SO_4$ (0.15%) and urea (0.03), respectively using saw dust as substrate[29].

Optimization of different carbon source

Different agricultural wastes such as wheat straw, rice straw, baggase and banana agro-waste were tested for the production of enzyme. Of all the substrates tested, wheat straw was found to the best substrates for the production of cellulase followed by baggase. The other substrates gave comparatively less enzyme production. The highest CMCase (8.39 IU/ml), exoglucanase (5.21 IU/ml), β-glucosidase (0.30 IU/ml), FPase (8.08 IU/ml) activities were reported in lignocellulosic material wheat straw (Figure 10) as well as specific activity were also higher compare to cellulose as standard carbon source (Table 1).

A result was agreement with previously finding that *Aspergillus fumigateus* grown on wheat straw gave maximum β-glucosidase (0.1320 IU/ml) and CMCase (0.225 IU/ml) only after 24 hrs[30]. 0.72 IU/ml CMCase and 0.43 U/ml FPase under submerged condition when *Aspergillus niger* grow on wheat bran[28]. The maximum CMCase activity of 0.499 IU/ml was achieved with 4% wheat bran concentration[14]. Maximum titers of FPase, CMCase and BGL obtained on ricebran + Wheatbran combination were 2.632, 2.478 and 2.984 IU/ml in submerged condition[15]. 14.88 IU/L CMCase activity was reported in wheat bran by *A. awamori*[31].

Present study was aimed at isolation of promising cellulase producing fungus and its identification, optimization of cultural conditions for production of cellulolytic enzymes. Fungal culture was initially identified as species of the genera of *Aspergillus niger* based on cultural, morphological and microscopic characteristics. The cellulolytic activity of the culture was studied by standard CMC and Congo Red plate assay method. Cellulase production with *Aspergillus spp.* in liquid state fermentation was highest at temperature 28°C, pH-4.2, incubation time (4 days), 0.04 % urea as nitrogen source and in presence of carbon substrates (Wheat straw). This study revealed that the successful use of lignocellulosic material as carbon source is dependent on the development of economically feasible process for cellulases production.

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