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Full Length Research Paper

Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate

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Cellulases are a group of hydrolytic enzymes and are capable of degrading lignocellulosic materials. Cellulases have wide range of applications. This work focuses on factors relevant for improvement of enzymatic hydrolysis of saw dust by using *Aspergillus niger*. Different cultural conditions were examined to assess their effect in optimizing enzyme production. Alkaline pretreated (2 N NaOH) saw dust at 9.6% concentration gave 0.1813 IU/mL cellulase activity. Optimum pH for cellulase production was between 4.0 and 4.5. Submerged fermentation at 120 rpm at 28°C gave higher yields of cellulase compared to static condition. Several other parameters like inoculum size, time duration, nitrogen source and its concentration were also optimized for the cellulase production by using saw dust as substrate.

Key words: Cellulase, *Aspergillus niger*, lignocelluloses, saw dust.

INTRODUCTION

The recent thrust in bioconversion of agricultural and industrial wastes to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by fungi and bacteria (Baig et al., 2004). Large quantities of lignocellulosic wastes are generated through forestry, agricultural practices and industrial processes, particularly from agro-allied industries such as breweries, paper-pulp, textile and timber industries. These wastes generally accumulate in the environment thereby causing pollution problem (Abu et al., 2000). Lignocellulose is a major renewable natural resource of the world and represents a major source of renewable organic matter. The plant biomass regarded as “wastes” are biodegradable and can be converted into valuable products such as biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients (Howard et al., 2003).

Lignocelluloses are the most abundant natural materials present on the earth. The polysaccharide component includes cellulose and hemicelluloses, which amounts to 60 - 80% of the total system. In addition to cellulose, hemicelluloses and lignin plant cell wall contains extraneous components including extractives and non-extractives. Extractives consist of fats, waxes, tannins, resins, etc. The non-extractives of extraneous components mainly consist of inorganic components such as silica, carbonates, oxalates, etc (Kodali and Pogaku, 2006). Cellulose has enormous potential as a renewable source of energy (Coral et al., 2002). A great variety of fungi and bacteria can fragment these macromolecules by using hydrolytic or oxidative enzymes and use as a carbon source. Cellulose is an unbranched glucose polymer composed of an β-1,4 glucose units linked by a β-1,4-D-glycosidic bond (Gielkens et al., 1999; Han et al., 1995).

Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Chellapandi and Jani, 2008). The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo-(1,4)-β-D-glucanase (endoglucanase, endocellulase, CMCase [EC 3.2.1.4]), which cleaves β-linkages at random, commonly in the amorphous parts of cellulose, exo-(1,4)-β-D-glucanase (cellobiohydrolase, exocellulase, microcrystalline cellulase, avicelase [EC 3.2.1.91]), which releases cellobiose from nonreducing or reducing end, generally from the crystalline parts of cellulose and β-glucosidase (cellobiase [EC 3.2.1.21]),

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which releases glucose from cellobiose and short-chain cellooligosaccharides (Bhat and Bhat, 1997).

Cellulases have a wide range of applications. Potential applications are in food, animal feed, textile, fuel, chemical industries, paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Tarek and Nagwa, 2007; Beguin and Anbert, 1993; Coughlan, 1985; Mandels, 1985). This work focuses on factors relevant for improvement of enzymatic hydrolysis of lignocellulosic material saw dust. To understand the biochemistry of lignocellulose degrading fungi, it is needed to optimize various conditions.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Media and chemicals used in this study were purchased from HiMedia, Qualigen, Nice and SD Fine Chemicals, India.

Cellulosic materials

Saw dust was collected from Saw Mill, Chiloda, Gandhinagar, Gujarat, India. It was sieved by Jayant Test Sieves (B. S. S. Mesh No. 60 and 0.250 mm) to make uniform particle size and to obtain the respective fine powder which was used for the study.

Organism and culture condition

The experimental organism *Aspergillus niger* was isolated from soil, near paper manufacturing industry, Sadra, Gandhinagar, Gujarat, India, by using CMC agar. The culture was maintained on PDA slant at 4°C and sub-cultured on fresh sterile PDA slant and incubated for 72 - 120 h.

Media preparation and enzyme production

Medium composition described by Mandles and Weber was used for fermentation. The media contained (per liter of distilled water): Urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, $MgSO4{\cdot}7H_2O$ 0.3 g, protease peptone 1.0 g, $FeSO_4{\cdot}7H_2O$ 5.0 mg, $MnSO_4{\cdot}7H_2O$ 1.6 mg, $ZnSO_4{\cdot}7H_2O$ 1.4 mg, $CoCl_2$ 2.0 mg. The pH of media was adjusted to 5.0 ± 0.2. Then, 100 ml of the liquid medium was placed in 250 ml Erlenmeyer flask and sterilized by autoclaving 121°C for 15 min. This was cooled and inoculated with 10 discs of 8 mm diameter of the organism from PDA culture plates using sterile cup borer. Cultures were harvested at 24 h intervals by centrifugation at 5000 rpm for 10 min at 4°C using refrigerated ultracentrifuge (REMI, k-70) over a period of 96 h. The supernatants were used as the crude extracellular enzyme source.

Cellulase assay

Cellulase activity was determined at 40°C by using carboxymethyl cellulose (Sodium salt, HiMedia, India) as a substrate. A reactive mixture contains 0.5 ml of 1% (w/v) substrate in 0.1 M citrate buffer (pH 4.8) and 0.5 ml of culture supernatant. The mixture was incubated at 40°C for 30 min. The reducing sugar released was measured using 3,5-dinitrosalicyclic acid (DNSA) (Miller, 1959). Control was prepared with 10 min boiled enzyme. One unit of endoglucanase activity was expressed as the amount of enzyme required to release 1 µmol reducing sugars per ml under the above assay condition by using glucose as a standard curve.

Optimization of pretreatment of saw dust with NaOH

Alkaline pretreatment was given to saw dust, with different normality of NaOH. There was 1, 2, 3, 4 and 5 N solution of NaOH prepared. In these solution 100 g of saw dust was mixed and incubated at room temperature for 12 h. This mixture was washed with water to neutralize. This pretreated saw dust was stored at 4°C in refrigerator and used as carbon source in fermentation media.

Optimization of substrate concentration

The pretreated saw dust used from 2.4, 4.8, 7.2, 9.6 and 12% in wet weight condition, in 100 ml fermentation media, in 250 ml Erlenmeyer flasks. The flask were inoculated with 10 discs of 8 mm size of *A. niger* and incubated at 28 ± 2°C at 120 rpm in orbital shaker-incubator.

Optimization of pH

Optimization was carried out by adjusting the pH ranges from 4.0, 4.5, 5.0, 5.5 and 6.0 of the saw dust containing fermentation media. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. After inoculation with 10 discs of *A. niger* of 8 mm size, the flasks were kept in orbital shaker-incubator at 28 ± 2°C at 120 rpm.

Optimization of temperature

Optimization of temperature was carried out by incubating the saw dust containing fermentation medium at 23, 28 and 37°C, in orbital-shaker incubator at 120 rpm. After regular intervals, enzyme assay was performed.

Optimization of inoculum size

The inoculum size was optimized by preparing the inoculum on PDA plate containing *A. niger*, by using sterile cup borer of 8 mm size. The saw dust containing fermentation media inoculated with 5, 10, 15 and 20 discs of *A. niger* aseptically. After inoculation the flasks were incubated in orbital shaker-incubator at 28 ± 2°C at 120 rpm. At regular intervals, enzyme assay was performed.

Optimization of fermentation time

Fermentation time was optimized by putting various flasks, which have saw dust in fermentation medium, at from 24 to 192 h, at 28 ± 2°C in to orbital shaker-incubator at 120 rpm. Enzyme assay was carried out at regular intervals.

Optimization of nitrogen source and its concentration

For optimization, different nitrogen sources used were peptone, $(NH_4)_2SO_4$ and urea. These all sources were used in different concentration. Peptone was used in range from 0.05, 0.075, 0.1, 0.125 and 0.15%. The $(NH_4)_2SO_4$ was used in range from 0.1, 0.12, 0.14, 0.16 and 0.18%. While urea was used in range from 0.01, 0.02, 0.03, 0.04 and 0.05% in saw dust containing fermentation me-

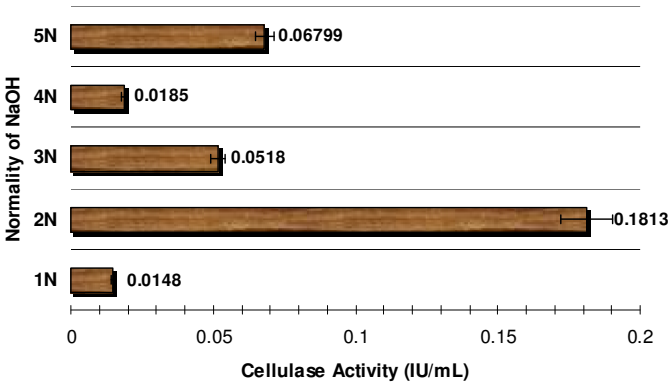

Figure 1. Effect of NaOH pretreatment on *Aspergillus niger* cellulase activity using saw dust as substrate.

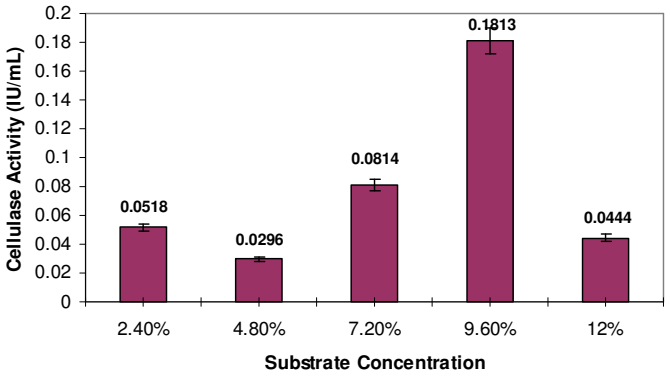

Figure 2. Effect of substrate concentration on *Aspergillus niger* cellulase activity using saw dust as substrate.

dium. All the flasks were incubated at 28 ± 2°C in orbital shaker-incubator at 120 rpm. At regular intervals, enzyme assay was performed.

Effect of static and agitated condition

There were two sets prepared, to check the effect of static and agitated condition on enzyme activity. In both sets, all the conditions (pH, temperature, inoculum size, substrate concentration) applied were kept similar. One set was put in orbital-shaker incubator at 120 rpm, while other set was kept in static condition. After regular intervals, enzyme assay was carried out.

RESULTS AND DISCUSSION

Optimization of pretreatment of saw dust with NaOH

Alkaline pretreatment was given to saw dust with 1, 2, 3, 4 and 5 N NaOH solutions. 2 N NaOH gives maximum cellulase activity 0.1813 IU/mL (Figure 1). Ojumu et al. (2003) reported that *A. flavus* grown on saw dust gave the highest cellulase activity of 0.0743 IU/mL, at about 12 h. Thereafter the cellulase activity was decreased between 20th and 30th h. In their experiement, Ojumu et al. gave treatment of 1% NaOH (w/v), at a ratio of 1:10 (substrate : solution) for 2 h at room temperature, after it was washed and autoclaved at 121°C (15 psi g steam) for 1 h. At same condition applied by Solomon et al. (1999) using bagasse, 0.056425 IU/mL cellulase activity was obtained.

Optimization of substrate saw dust

2 N NaOH pretreated saw dust, was used for further study. As shown in Figure 2, various substrate concentrations of saw dust, 2.4, 4.8, 7.2, 9.6 and 12% in wet weight condition were used and cellulase activities of 0.0518, 0.0296, 0.0814, 0.1813 and 0.0444 IU/mL were found, respectively. Thus, 9.6% substrate gave maximum cellulase activity (0.1813 U/mL). Ojumu et al. (2003) reported high cellulase activity when 3% pretreated saw dust substrate was used (0.0743 IU/mL) while 0.0573 and 0.05 IU/mL were obtained for bagasse and corn cob, respectively.

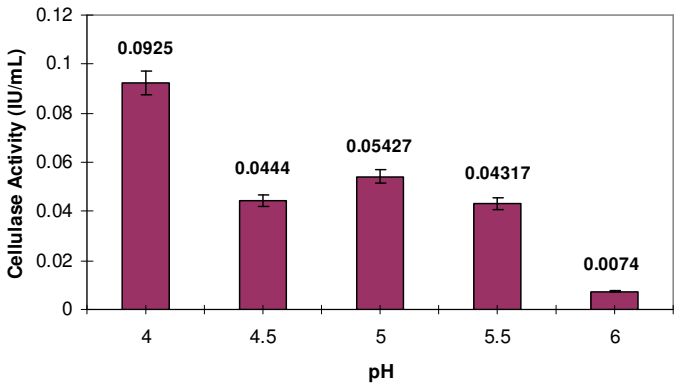

Figure 3. Effect of pH on *Aspergillus niger* cellulase activity using saw dust as substrate.

Optimization of pH

As shown in Figure 3, effect of pH on cellulase production was determined at pH values of 4.0, 4.5, 5.0, 5.5 and 6.0; and cellulase activity obtained were 0.0925, 0.0444, 0.05427, 0.04317 and 0.0074 IU/mL, respectively. Optimum pH for cellulase activity was between 4.0 and 4.5. Maximum cellulase activity was 0.0925 IU/mL found at pH 4.0. It was observed that the cellulase activity has a broad pH range between 3.0 and 9.0. Two major activity peaks were obtained at 4.5 pH and 7.5 (Coral et al., 2002). It was reported that the optimal pH for a cellulase from *A. niger* was between 6.0 and 7.0 (Akiba et al., 1995) and in another report the optimal pH activity of *A. niger* cellulase was found to be between 4.0 and 4.5 (McCleary and Glennie-Holmes, 1985). Such different

Table 1. Optimization of temperature.

Hours	23°C	28°C	37°C
	Cellulase activity IU/mL	Cellulase activity IU/mL	Cellulase activity IU/mL
48	0.03454	0.0629	0.0333
72	0.0555	0.0777	0.03947
96	0.03207	0.0925	0.02713

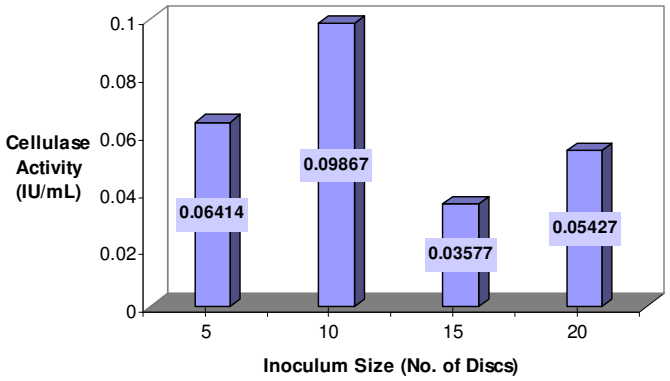

Figure 4. Effect of inoculum size on *Aspergillus niger* cellulase activity using saw dust as substrate.

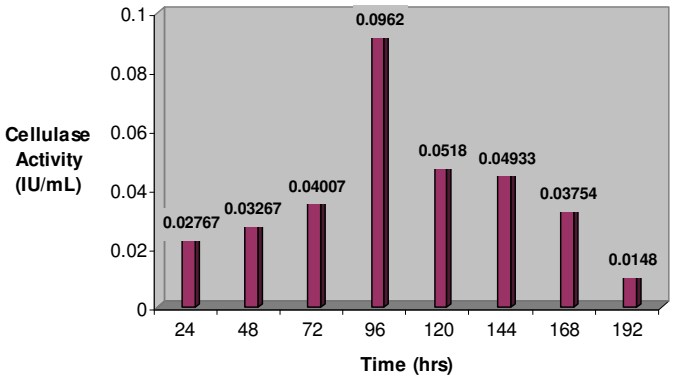

Figure 5. Effect of time duration on *Aspergillus niger* cellulase activity using saw dust as substrate.

results may appear because of differences within the same genus.

Optimization of temperature

Optimization was carried out by incubating the fermentation flask at 23, 28 and 37°C and the cellulase activities were 0.03207, 0.0925 and 0.02173 IU/mL, respectively. Thus, as shown in Table 1, maximum degradation of saw dust was observed at 28°C. The reduction of sugar conversion as well as enzyme production was observed at the temperature above and below 28°C. Narasimha et al. (2006) used saw dust for production of cellulase by *A. niger* with 0.775 IU/mL cellulase activity obtained at 28°C.

Optimization of inoculum size

Inoculum size also affects the maximum cellulase enzyme production. To check the effect of inoculum size, as shown in Figure 4, we used 5, 10, 15 and 20 discs of 8 mm size and cellulase activities were 0.06414, 0.09867, 0.03577 and 0.05427 IU/mL, respectively. Thus, by using 10 discs of 8 mm size of *A. niger* maximum cellulase activity of 0.09867 IU/mL was found. When high fungal mass was inoculated, initially cellulase activity and reducing sugar production decreased. But when low load of fungal mass was inoculated, increase in sugar production and cellulase activity was continuously observed up to 96 h.

Optimization of fermentation time

As seen in Figure 5, the flasks were incubated at different time duration; 24, 48, 72, 96, 120, 144, 168 and 192 h and cellulase activities of 0.02767, 0.03267, 0.04007, 0.0962, 0.0518, 0.04933, 0.03754 and 0.0148 IU/mL were obtained, respectively. Thus, at 96 h maximum degradation was observed. The highest cellulase level of 1.88, 1.53 and 2.40 IU/mL of CMCase activity was achieved on the 4^{th} day of the fermentation period by *Trichoderma harzianam*, *Trichoderma spp.* and *Phanerochaete chrysosporium*, respectively (Khan et al., 2007). Ojumu et al. (2003) found that the highest level of cellulase activity occurred at the 12^{th} h of fermentation by *A. flavus*. It was noticed that a high concentration of reducing sugar was released on the 4^{th} day of the fermentation (Khan et al., 2007).

Optimization of nitrogen source and its concentration

In this study peptone, $(NH_4)_2SO_4$ and urea were used to optimize nitrogen sources for maximum enzyme production. As seen in Figures 6, 7 and 8, after 96 h, 0.1196, 0.15281 and 0.1528 IU/mL cellulase activities were obtained using peptone, $(NH_4)_2SO_4$ and urea, respectively. At 0.125% peptone concentration, maximum cellulase activity (0.1196 IU/mL) was observed. According to studies of Narasimha et al. (2006), at 0.03% urea, peptone and $NaNO_3$ used as nitrogen source, the activity of cellulase obtained were 0.824, 0.421 and 0.401 IU/mL,

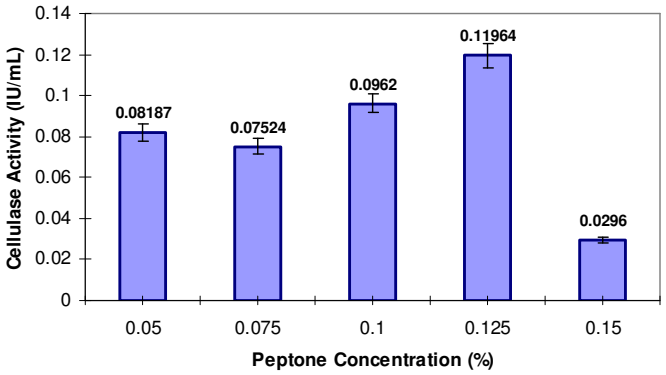

Figure 6. Effect of peptone on *Aspergillus niger* cellulase activity using saw dust as substrate.

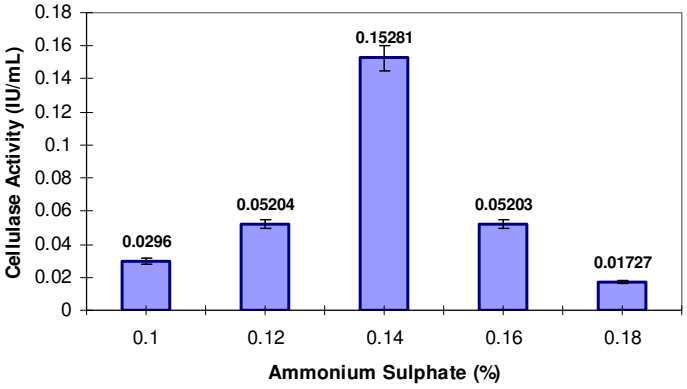

Figure 7. Effect of $(NH_4)_2SO_4$ on *Aspergillus niger* cellulase activity using saw dust as substrate.

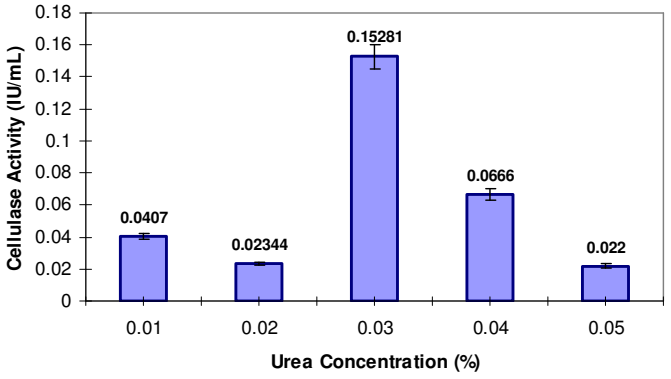

Figure 8. Effect of urea on *Aspergillus niger* cellulase activity using saw dust as substrate.

respectively.

Effect of static and agitated condition

Maximum activity of cellulase enzyme was observed at

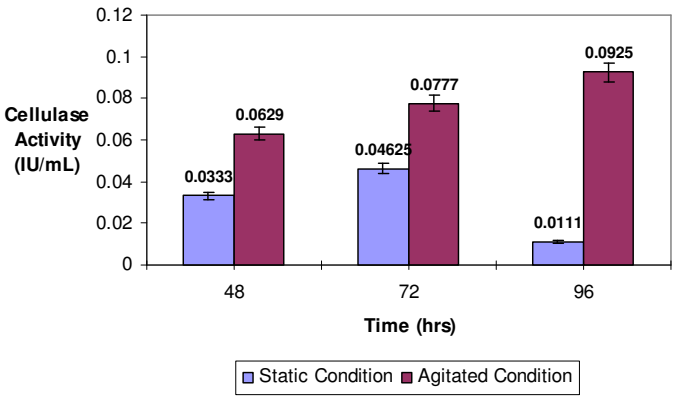

Figure 9. Effect of static and agitated conditions on *Aspergillus niger* cellulase activity using saw dust as substrate.

agitated condition. As seen in Figure 9, cellulase activity was around 0.0925 IU/mL at 120 rpm after 96 h incubation period. But in static condition, maximum cellulase activity was observed at 72 h which was 0.04625 IU/mL. This was around half of the maximum activity than agitation condition. When Ojumu et al. (2003) proceeded fermentation at agitation of rate 200 rpm and 35°C, the maximum activity was 0.0743 IU/mL.

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