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Utilization of pretreated corn cobs for cellulase production by *Pycnoporus coccineus*

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Abstract

This research study was conducted with the greater aim of understanding the potentially efficient fungal cellulase production from low cost lignocellulosic substrate. This study has attempted to use pretreated corn cobs as a substrate for cellulase production via solid-state fermentation (SSF) by white rot fungus, *Pycnoporus coccineus*. The effects of moisture content, incubation temperature, initial pH value and the nitrogen source on cellulase biosynthesis were observed for optimal production in flask fermentors. The optimal filter paper activity (FPase; 10.303±0.353 U/gds), carboxymethyl cellulase (CMCase; 14.812±0.360 U/gds) and cellobiase (1.118±0.054 U/gds), were obtained after 9 days of fermentation with an initial moisture content of 70%, initial pH value of 6.0, incubation temperature of 30 °C. Additionally, yeast extract has been determined to be a good nitrogen source. These results suggest that the crude cellulase production under SSF using pretreated corn cobs as a substrate could be an alternative choice for commercial enzyme preparations.

Keywords : *cellulosic bioethanol, solid-state fermentation, cellulase, Pycnoporus coccineus, corn cobs*

1. Introduction

In the context of green energy, one of the major challenges of second generation bioethanol production is the cellulase enzyme, which is used in the hydrolysis of cellulose to fermentable sugars for bioethanol production. The bioconversion of cellulose to fermentable sugars requires the synergistic action of complete cellulase system comprised of endoglucanases (EC 3.2.1.4) which act randomly on soluble and insoluble cellulose chains, exoglucanases (cellobiohydrolases; EC 3.2.1.91) which liberate cellobiose from the reducing and non-reducing ends of cellulose chains and β-glucosidases or cellobiases (EC 3.2.1.21) which liberate glucose from cellobiose (1-3). The total costs of cellulase account for more than 40% of the total processing costs (4). The ability to obtain cellulase at a reduced cost could be one solution to improve the cost of commercial bioethanol production. Therefore, the uses of low-cost technology as well as the development of a low cost substrate for cellulase production

or two significant are the highly desirable objectives. Normally, fungal cellulase production can be produced through solid-state fermentation (SSF) when carried out with the use of different agricultural waste substances. A corn cob is the central core of a maize ear and has become an abundant form of agricultural residue in parts of the world. It typically contains 32.3-45.6%, 39.8% and 6.7-13.9% of cellulose, hemicelluloses and lignin, respectively (5). From this point, we have developed a method for utilization of pretreated corn cobs as a substrate to produce cellulase from white rot fungus, *Pycnoporus coccineus*. The objective of the present study was to optimize various factors including the level of moisture content, initial pH value and temperature for the high activity and yield of cellulase production.

2. Materials and Methods

2.1 Microorganism

The fungal strain, *P. coccineus* was cultivated on potato dextrose agar (PDA) plates containing 2.0% agar and was then incubated at 25-27 ºC for 7 days.

2.2 Detection of microbial cellulase on agar plate

A preliminary qualitative analysis for cellulolytic activity was conducted using Congo red dye (6). *P. coccineus* was grown on CMC agar containing 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose (CMC) sodium salt, 0.02% peptone, and 1.7% agar. Plates were incubated at ambient temperatures for 3 days. The agar medium was flooded with 0.1% Congo red dye for 15 to 20 minutes, and then de-stained with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation.

2.3 Raw materials and their pretreatment

Corn cobs were obtained locally. These raw materials were first dried and chopped into small pieces by a chopper, then ground into smaller particles in a hammer mill (Armfield, England) and finally separated by a 20-mesh sieve. The pretreatment of corn cobs was carried out separately with 0.5% (w/v) H_2SO_4 and 2.5% NaOH at 121 °C for 15 minutes. The pretreated residues were washed extensively to the neutral pH and dried at 60 °C in an oven.

2.4 Cellulase production under solid-state fermentation

Solid-state fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 2.0 g of dry corn cobs. The initial moisture content (60, 70, 80 and 90%) were adjusted with mineral salt solution [$(NH_4)_2SO_4$, 0.5 gl^{-1}; KH_2PO_4, 0.5 $gl^{-1;}$ $MgSO_4$, 0.5 gl^{-1} and pH 5.5]. The flasks were sterilized by autoclaving at 121 °C (15 psi), and afterward were cooled to room temperature. One week-old-mycelium was taken from the agar plate using a cork borer (5 disc, 0.6 cm. diameter.) and then was used as an inoculum. The contents of the flasks were mixed well and incubated at 25 ºC in an incubator for 3, 6, 9 and 12 days. All the experiments were conducted in triplicates and analyzed for cellulase activities. The maximum levels of enzyme production were selected for further optimization of the solid-state fermentation process.

2.5 Optimization of cellulase production

The effect of various parameters such as initial pH (5.0–7.0), incubation temperature (25–37 °C) and different

nitrogen sources (0.5 gl^{-1}; $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, yeast extract and peptone) were investigated on the production profile of the cellulase system using pretreated corn cobs in SSF under optimal time and moisture content.

2.6 Enzyme extraction

The enzymes were extracted by adding 15 ml of 50 mM citrate buffer (pH 4.8) to the solid- state cultures, thereafter they were shaken on a rotary shaker at 150 rpm for 60 minutes at room temperature. The fermented substrates in the flasks were then filtered through a metallic sieve and the solid residue was pressed to remove any remaining liquid, followed by centrifugation (10000×g for 15 minutes at 4 ^oC). The supernatant was used as crude enzymes for analysis of enzyme activities.

2.7 Enzyme activity assays

Samples were collected every 3 days during the fermentation process for the determination of cellulase activity (Filter paper activity (FPase), carboxymethyl cellulase (CMCase) and cellobiase) were determined in accordance with the International Union of Pure and Applied Chemistry procedures as reported by Ghose (7). The liberated reducing sugars were measured using 3, 5-dinitrosalicylic acid (DNS), according to the method of Miller (8). One international unit of FPase and CMCase activity is equivalent to the amount of enzyme that releases 1 μmol of glucose per min during the hydrolysis reaction. One international unit of cellobiase activity is the amount of enzyme that forms 2 μmol of glucose per min from cellobiose. The values of enzymatic activity were expressed as U/gds (international units per gram dry substrate).

2.8 Data analysis

Enzyme activity values were expressed as means±S.D. Analysis of variance was performed by ANOVA procedures. SPSS software version.11.5 was used for statistical analysis. The results with $P<0.05$ were regarded as being statistically significant.

3. Results

3.1 Detection of microbial cellulase on agar plate

The fungal strain, *P. coccineus* was grown on CMC agar plates and checked for cellulolytic activity by incubation at ambient temperatures for 3 days. The cellulase activity was indicated as a clear orange halo after stained with 1% congo red solution. *P. coccineus* showed a clear zone with a diameter of 28 mm. This result indicates that *P. coccineus* had definitely potential for production of cellulolytic enzymes.

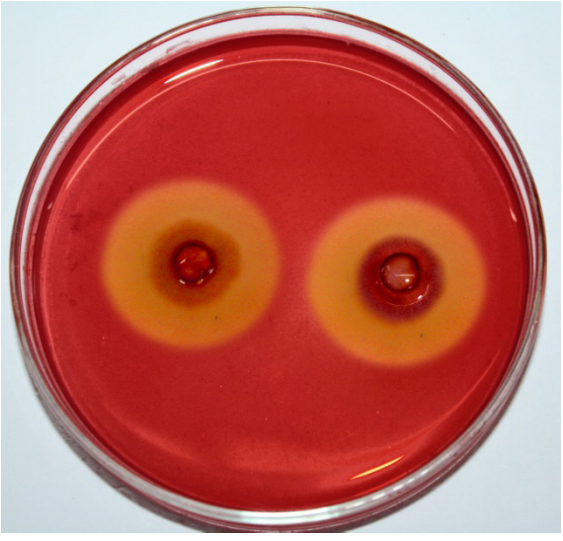

Figure 1. P. coccineus showed a clear zone of CMC hydrolysis, which indicates CMC degradation.

3.2 Cellulase production under solid-state fermentation (SSF)

In the present study, the maximum production of FPase, CMCase and cellobiase occurred after 9 days of *P. coccineus* fermentation (Figure 2-4) with the yields of 4.188±0.066 U/gds, 12.039±0.161 U/gds and 1.173±0.040 U/gds, respectively. However, the activity of these enzymes declined after 9 days of fermentation.

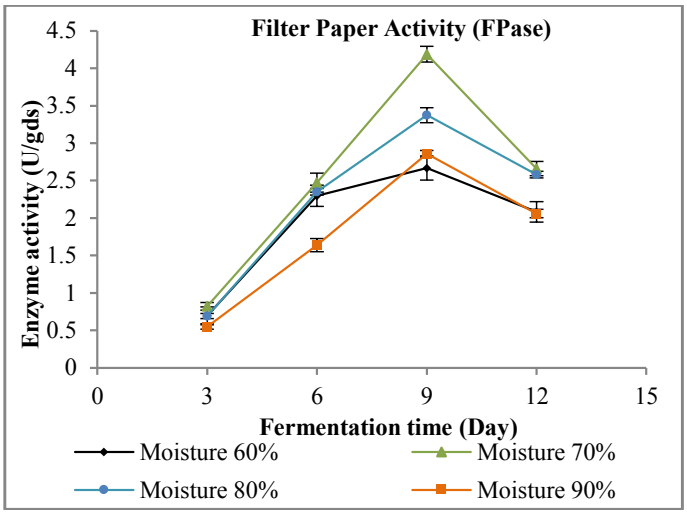

Figure 2. Time course of filter paper activity (FPase) production by SSF at different initial moisture content and initial pH 5.5

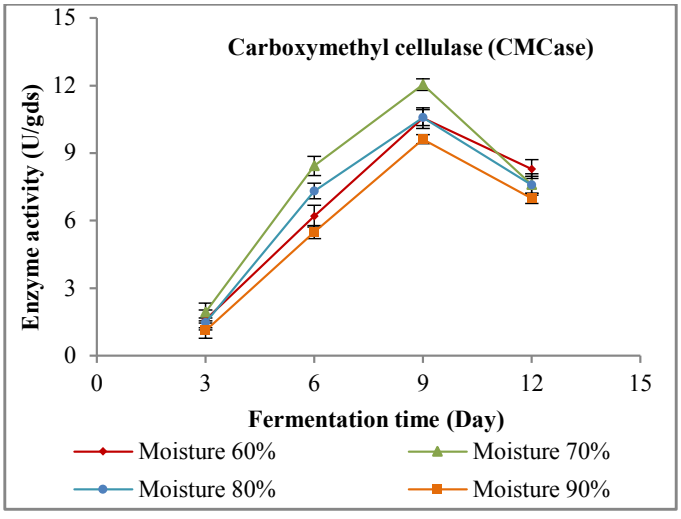

Figure 3. Time course of carboxymethyl cellulase (CMCase) by SSF at different initial moisture content and initial pH 5.5

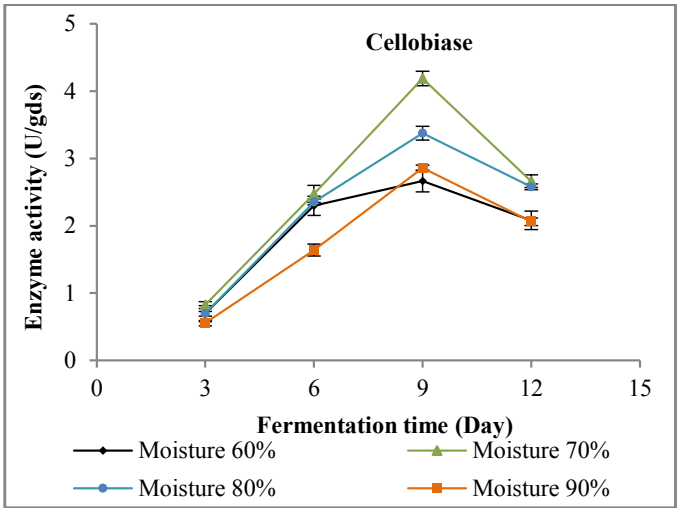

Figure 4. Time course of cellobiase production by SSF at different initial moisture content and initial pH 5.5

3.3 The effect of initial pH on cellulose production

To study the effect of initial pH value on cellulase production, the pH value was adjusted to 5.0 and 7.0. The production profiles of all three enzyme-components are shown in Figure 5. The highest FPase (7.081±0.245 U/gds), CMCase (13.808±0.277 U/gds) and cellobiase (1.026±0.108 U/gds) activities were observed at a pH value of 6.0

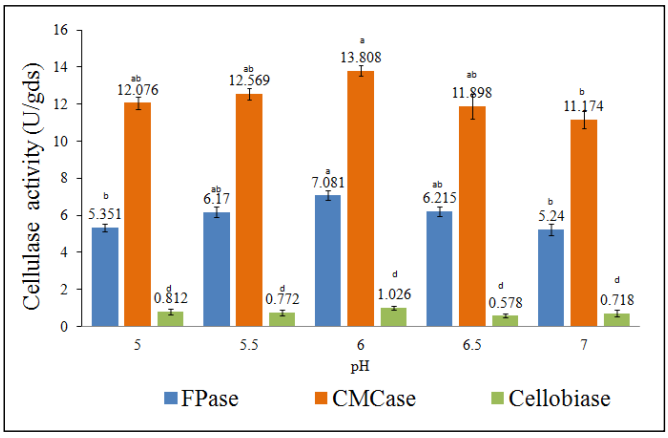

Figure 5. Cellulase activities at different initial pH levels. a-d indicates significant differences among means ($P < 0.05$).

3.4 The effect of incubation temperature on cellulase production

The temperature of the fermentation medium is one of the parameters that has a significant influence on the end-product. The highest yields of FPase (7.085±0.239 U/gds), CMCase (13.397±0.223 U/gds) and cellobiase (0.830±0.105 U/gds) were obtained at 30 °C on Day 9 as shown in Figure 6. Whereas, the enzyme yield decreased significantly (*P<0.05*) when incubated at temperature of 37 °C particularly, FPase and CMCase.

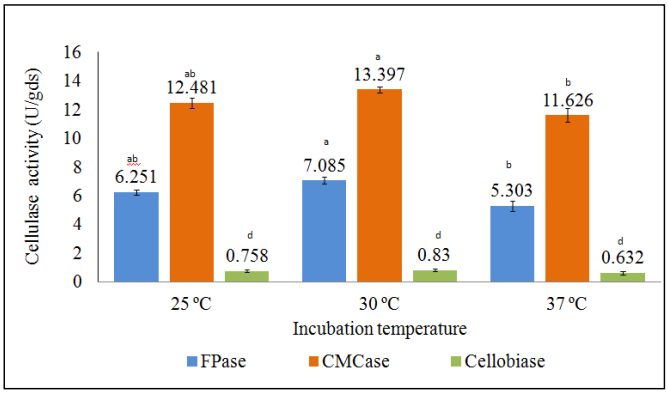

Figure 6. Cellulase activities at various different temperatures. a-d indicates significant differences among means ($P<0.05$).

3.5 The effect of nitrogen source on cellulase production

Organic nitrogen showed higher cellulolytic enzyme activities relative to other nitrogen sources (Figure 7). The maximal cellulase activities were obtained when yeast extract was added which gave the maximal activities of FPase, CMCase and cellobiase as 10.303±0.353, 14.812±0.360, and 1.118±0.054 U/gds, respectively.

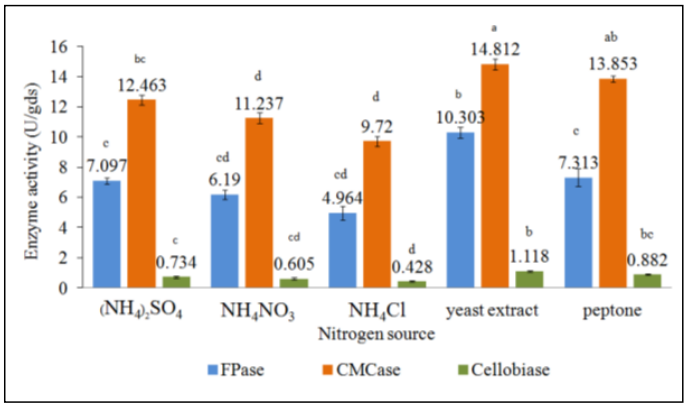

Figure 7. Cellulase activities at different nitrogen sources. a-d indicates significant differences among means ($P < 0.05$).

4. Discussions

In the present study, corn cobs were chosen as a substrate for cellulase production under SSF using the fungus, *P. coccineus*. Thermochemical pretreatment with diluted acid and alkaline hydrolysis were carried out. The effective results of cellulase production such as FPase (10.303±0.353 U/gds), CMCase (14.812±0.360 U/gds), and cellobiase (1.118±0.054 U/gds) was achieved from 9 days of fermentation with an initial moisture content of 70%, an initial pH value of 6.0 and the temperature at 30 ºC using yeast extract as a nitrogen source. The combination of dilute acid and alkaline pretreatment was effective for the removal of hemicellulose and lignin, resulting in high cellulose content using as substrate (9). The moisture content of the medium was also one of the critical factors in the production of cellulase. Fungi were incubated on a moist environment not only for their growth but also for the secretion of enzymes. The results (Figure 2-4) revealed a positive relationship between moisture content and production of cellulase. Lee *et al*.(10) reported that the maximum FPase yield of 2.3 U/gds in *Aspergillus niger* was also found under 70% moisture content. Liu and Yang (11) showed that at high moisture levels (50-70%) of substrate prevents the oxygen penetration, whereas lower moisture content inhibits growth, enzyme activity, and accessibility to nutrients. Furthermore, the effect of initial pH values on cellulase production was found that the acidic pH range of 6.0 was optimal for enzyme production. At this level, the enzyme activity increased to the maximum level followed by a slight decrease in activity between pH 5.0-5.5 and 6.5-7.0. Sivakumar (12) also observed that cellulase production showed a good result at pH 6.0, a variation of the pH values from the optimum range caused denaturation of the enzymes and reduced the enzyme synthesis ability. Incubation temperature is an important factor affecting to enzyme production. The maximum yields of this study were obtained at 30 ºC. Haq *et al.* (13) reported that a temperature at 30 ºC was found to be optimal temperature for the best growth of *A. niger* and *Trichoderma viride.* The effect of different nitrogen sources were verified.

The maximum enzyme yield was obtained when the yeast extract was used, followed by peptone. However, the inorganic nitrogen sources did not exhibit any significant effects in terms of increasing enzyme production. Other studies have shown that the yeast extract was confirmed as the best nitrogen source for cellulase production by *Penicillium* sp. (14), whereas peptone was found to be the best nitrogen source for cellulase production by *A. niger*1433 (15).

5. Conclusion

Successful attempts have been made to utilize corncobs, a highly abundant form of agro-industrial waste, as a substrate for the production of cellulase complex by *P. coccineus* under SSF, in order to develop a low cost production system. In this study, fairly good amounts of FPase, CMCase, and cellobiase were obtained. This process highlighted the potential of these raw materials for enzyme production, thereby reducing the cost of cellulase production. Further utilization, in terms of a novel inducer and scale-up studies need to be carried out in order to exploit these inexpensive commercial cellulase enzyme preparations for the second-generation of bioethanol production process.

6. Acknowledgements

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