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Potential of Peanut Hulls as Substrates for Fungal Cellulase Bioproduction Through Solid State Fermentation

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Abstract

One of the main challenges of cellulosic bio-ethanol production is the cellulase enzyme. Cellulase is used in the hydrolysis of cellulose to sugars for the fermentation of bio-ethanol, but the commercial cellulase enzyme preparations are very expensive. This study has attempted to use peanut hulls as a substrate for cellulase production via solid-state fermentation (SSF) by white-rot fungus, *Ganoderma australe*. The effects of moisture content, culture temperature and initial pH value on cellulase biosynthesis were observed for optimal production in flask fermentors. The activities of different cellulase enzymes, namely filter paper activity (FPAse), carboxymethyl cellulase (CMCase) and β-Glucosidase (cellobiase) were carried out using filter paper, carboxymethylcellulose and cellobiose as the substrate, repectively. The optimal FPAse activity (0.062±0.017 IU/ml), CMCase activity (0.426±0.074 IU/ml) and cellobiase activity (0.035±0.007 IU/ml), were obtained after 9 days of cultivation with an initial 70% of moisture content, a temperature of 25°C and an initial pH of 5.5. These results suggest that the crude cellulase production under SSF using peanut hulls as a substrate could be an alternative choice for commercial enzyme preparations.

Keywords: cellulosic bio-ethanol, solid-state fermentation, cellulase, *Ganoderma australe*, peanut hulls

1. Introduction

In the context of green energy, one of the main challenges of cellulosic bio-ethanol production is the cellulase enzyme itself, which is used in the hydrolysis of cellulose to fermentable sugars for bio-ethanol production. The bioconversion of cellulose to fermentable sugars requires the synergistic action of the complete cellulase system, comprised of endoglucanases (EC 3.2.1.4), exoglucanases (cellobiohydrolases; EC 3.2.1.91) and β-glucosidases or cellobiase (EC 3.2.1.21) (1-3). However, production cost of the enzymes is very high and accounts for about 40–60% of the total production cost (4). In recent years, research efforts have been focused on lowering the cost of the enzymes. The use of agro-industrial waste and its byproducts as substrates in solid-state fermentation (SSF) is one of the alternative choices in reducing cellulase costs (5). The SSF process obtained higher yields in a shorter time period than the submerged fermentation (SmF) applications (6). Chahal *et al*. (7) had reported a higher yield of cellulase from *Trichoderma reesei* in SSF cultures, compared to SmF. Tengerdy *et al*. (8) compared

cellulase production in SmF and SSF systems and had indicated that there was about a 10-fold reduction in the production cost when SSF is employed for production. SSF are strongly recommended in systems for producing cellulase at a lower price, over SmF (9). There are several reports that have described the use of agro-industrial wastes for cellulase production, but there have still been very few reports on the utilization of peanut hulls. In Thailand, peanut hulls are by-products in the peanut processing industry and are abundant sources of less expensive forms of biomass (10). There have been no specific reports on cellulase production from peanut hulls. From this point of view, we developed a method for utilizing peanut hulls as a substrate for producing cellulase by white rot fungus. *Ganoderma australe* which was isolated and identified by our laboratory (11). This is the first report on production of cellulase enzymes by *G. australe* from peanut hulls.

The aim of the present study was to optimize various factors, including the level of moisture content, initial pH and temperature, for the maximum yield of cellulase in SSF using peanut hulls as a substrate, which is considered a value-added bio-product.

2. Materials and Methods

2.1 Microorganisms

The fungal strain, *G. australe* was isolated and identified by our laboratory and was cultivated on potato dextrose agar (PDA) plates containing 2.0% agar and incubated at ambient temperatures 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 (12). *G. australe* was grown on CMC agar containing (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethyl cellulose (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 Substrates and pretreatment

Peanut hulls used in this experiment were obtained from a local market in Chiang Mai. These materials were dried overnight in a hot air oven (60°C) before being finely crushed and stored in air-tight containers. The pretreatment of peanut hulls was then carried out separately by treating them with 0.5% (w/v) H_2SO_4 and 2.5% NaOH at 121°C for 15 min. The pretreated residues were washed extensively to the neutral pH (7.0) level and dried at 60°C in the oven.

2.4 Fiber analysis

To determine the lignocellulosic composition of peanut hulls, the cellulose, hemicellulose and lignin content were routinely established using the neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL), and then were analyzed by the method of Van Soest *et al*. (13). Cellulose content was estimated as the difference between ADF and ADL, hemicellulose content was estimated as being between NDF and ADF, and lignin content was estimated as the difference between ADL and the ash.

The components that were estimated by these tests is summarized below:

NDF = lignin and Acid Insoluble Ash + cellulose + hemicellulose

ADF = lignin and Acid Insoluble Ash + cellulose

ADL = lignin and Acid Insoluble Ash

2.5 Optimization of cellulase production under solid state fermentation (SSF)

Solid state fermentation was carried out in 250 ml erlenmeyer flasks, each having 2.0 g of dried pretreated peanut residue. The initial moisture was adjusted to 60, 70, 80 and 90% with the 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 allowed to cool at room temperature. Inoculation was performed using a sterile cork borer, in 7-day old mycelium agar disc (diameter, 0.6 cm.) and incubated at 25°C in an incubator for 12 days. The maximum levels of enzyme production were selected for further optimization of the SSF production process.

2.5.1 Optimization of fermentation time period and moisture content

To optimize conditions for fermentation, flasks containing 2.0 g of dried pretreated peanut residue were used with various levels of moisture content (60, 70, 80 and 90%), and periods of fermentation time (3, 6, 9 and 12 days). Other factors were constant at the initial pH value of 5.5, with a fermentation temperature of 25°C. Fermented products were harvested in triplicate at the specified fermentation times and analyzed for cellulase activities.

2.5.2 Optimization of temperature and initial pH

To determine the optimum temperature, triplicate flasks containing 2.0 g of dried pretreated peanut residue were adjusted to a constant initial pH value of 7.0, inoculated and subjected to fermentation at varying temperatures (20, 25 and 30°C). To investigate the effects of the initial pH value on enzyme production, pH values were varied (5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) with inoculation and fermentation being performed at 25°C for a specified time period of 9 days.

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 and the contents were shaken on a rotary shaker at 150 rpm for 60 min at room temperature. The contents 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 x g for 15 min at 4°C). The supernatant was saved and analyzed for its 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), carboxylmethyl cellulase (CMCase) and cellobiase) according to standard IUPAC procedures and were then expressed as international units (IU) by Ghose (14). Filter paper activity (FPase) was assayed by measuring the release of reducing sugars in a reaction mixture of Whatman's No. 1 filter paper (1.0 x 6.0 cm.) as a substrate in 50 mM sodium citrate buffer (pH 4.8) at 50°C, after 60-min of incubation. Carboxymethyl cellulase (CMCase) activity was assayed by measuring the release of reducing sugars in a reaction mixture containing 0.5 ml of crude enzyme and 0.5 ml of 2% (w/v) of CMC (Sigma) solution in 50 mM sodium citrate buffer (pH 4.8) incubated at 50°C for a period of 30 min. The liberated reducing sugars were measured using 3,5-dinitrosalicylic acid (DNS), according to the method of Miller (15). One international unit of FPAse and CMCase activity is the amount of enzyme that releases 1 μmol of glucose per min during the hydrolysis reaction. Cellobiase activity was determined using 15 mM cellobiose (Fluka) at 50°C after 30 min of incubation. 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/ml.

2.8 Data analysis

Lignocellulosic composition and the enzyme activity values are expressed as the mean ±S.D. of three replications calculated using MS Excel 2007.

3. Results and Discussion

3.1 Detection of microbial cellulase on agar plate

The fungal strain, *G. australe* was grown on CMC agar plates and checked for the cellulolytic activity by incubation at ambient temperatures for 3 days. The cellulase activity was indicated as a clear orange halo after being stained with 1% Congo red solution. *G. australe* showed a

clear zone with a diameter of 17 mm. This result indicated that *G. australe* had the potential to produce cellulolytic enzymes.

3.2 Fiber analysis

Table 1. shows the linocellulosic composition as NDF, ADF, and ADL, cellulose, hemicellulose, and lignin content of each composition before and after pretreatment of peanut hulls. Cellulose content in untreated peanut hulls and pretreated peanut residue were 38.7% and 33.8%, respectively. The acid-base pretreatment significantly hydrolyzed the hemicellulose and lignin contents by decreasing their percent values from 22.7 and 20.6 percent, to 7.8 and 8.6 percent, respectively.

3.3 Optimization of cellulase production under solid state fermentation (SSF)

In the present study, maximum enzyme production occurred 9 days of *G. australe* was best suited for FPase and CMCase revealing the yields 0.048±0.005 U/mL and 0.382±0.033 U/mL as depicted in Figure 2-3, and cellobiase yields of 0.028±0.005 U/mL (Figure 4) was observed with an initial 70% of moisture content. On further incubation, the enzyme yields declined gradually at the end of 12 days It might be due to the depletion of nutritions, accumulation of harmful by-products in the culture medium and proteolysis of enzyme. This finding is in accordance to the finding of Tsao *et al*. (16). During SSF, both high and low moisture contents affect enzyme activity (17).

Table 1. Lignocellulose composition of peanut hulls.

Sample	Untreated Peanut hulls	Pretreated-Peanut residue
%NDF	82.0207±0.5001	50.2533±2.6880
% ADF	59.3392±0.7176	42.4677±1.5874
% Hemicellulose	22.6815±0.9420	7.7856±2.5270
% Cellulose	38.7230±0.8968	33.8184±1.5723
% Lignin	20.6162±0.660	8.6493±1.9386
% Acid Insoluble Ash	1.2911±0.4888	1.1424±0.0631

Figure 1. *Ganoderma australe* showed a clear zone of hydrolysis, which indicates CMC degradation.

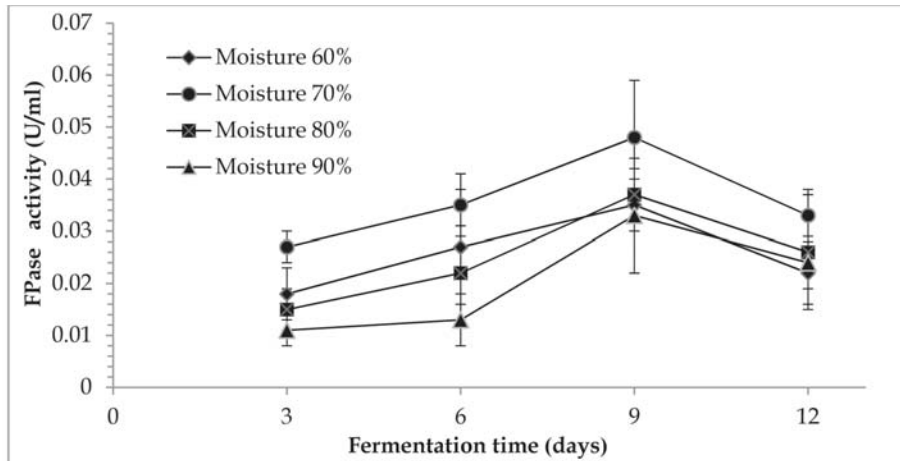

Figure 2. Fermentation time profiles of filter paper cellulase (FPAse) activity of peanut hulls.

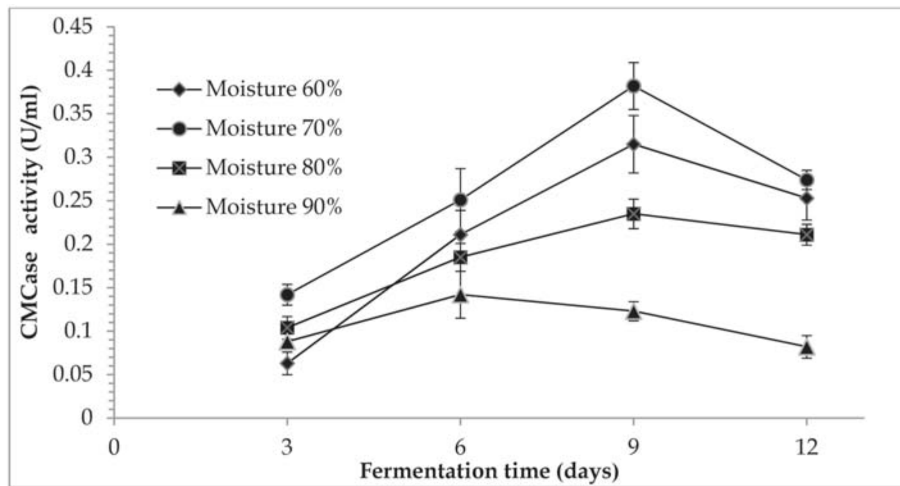

Figure 3. Fermentation time profiles of carboxymethyl cellulase (CMCase) activity of peanut hulls.

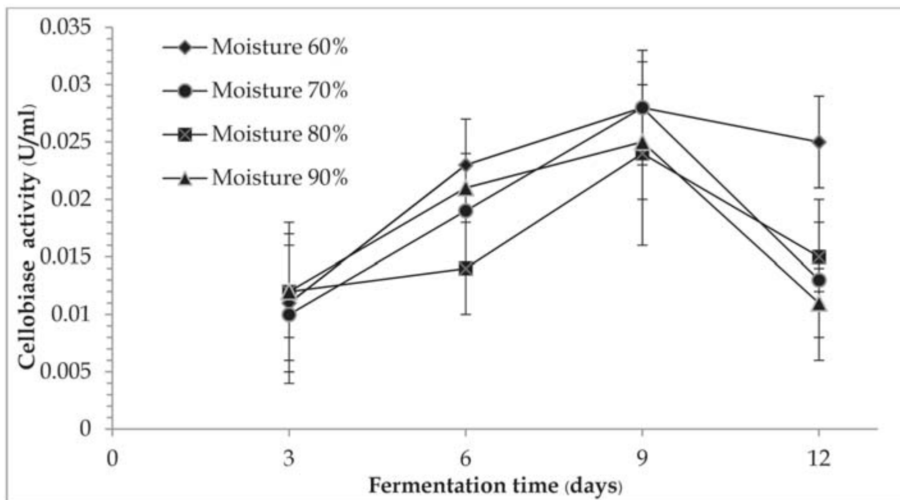

Figure 4. Fermentation time profiles of cellobiase activity of peanut hulls.

The temperature of the fermentation medium is one of the parameters that has a significant influence on the end product. Figure 5 illustrates that the enzyme activity increased with an initial increase in temperature to 25°C. When cultivated at 30°C, the activity of the enzymes decreased substantially. The highest yields of FPase (0.054±0.013 U/mL), CMCase (0.415±0.057 U/mL) and cellobiase (0.029±0.004 U/mL) were obtained at 25°C on Day 9, whereas the enzyme yield was reduced to 0.033±0.011, 0.372±0.031 and 0.023±0.003 U/mL, respectively, at 30°C of incubation with a significant reduction in the cellulase activity. It is a well-known fact that higher temperatures (above 30°C) alter the cell membrane composition and stimulate protein catabolism, causing cell death (18).

To study the effect of initial pH on cellulase production, the pH value of the moistening agent was adjusted to between 5.0 and 7.5. The production profiles of all the three components, as shown in (Figure 6), depict the highest FPase (0.062±0.017 U/mL), CMCase (0.426±0.074 U/mL) and cellobiase (0.035±0.007 U/mL) were observed at a pH value of 5.5. Any variation from this optimal pH value resulted in reduced enzyme activity. This might be due to the fact that cultivation of fungi at an unfavourable pH value may result in reduced enzyme activities by reducing accessibility of the substrate (19).

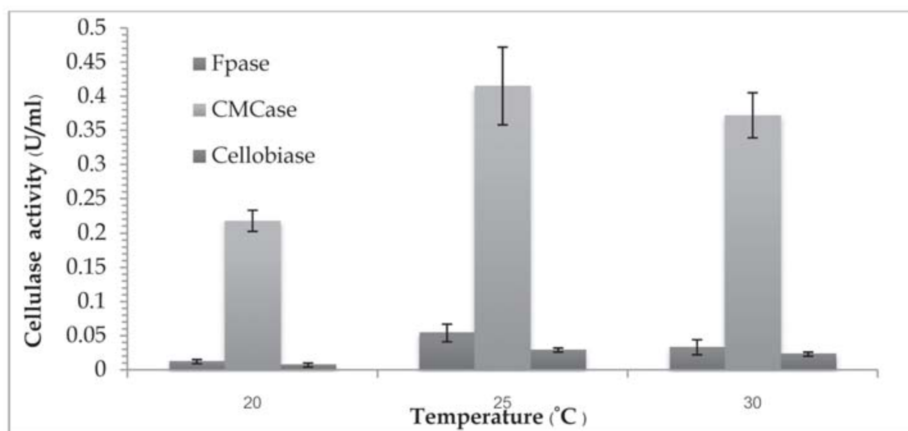

Figure 5. Cellulase activities at varying temperatures.

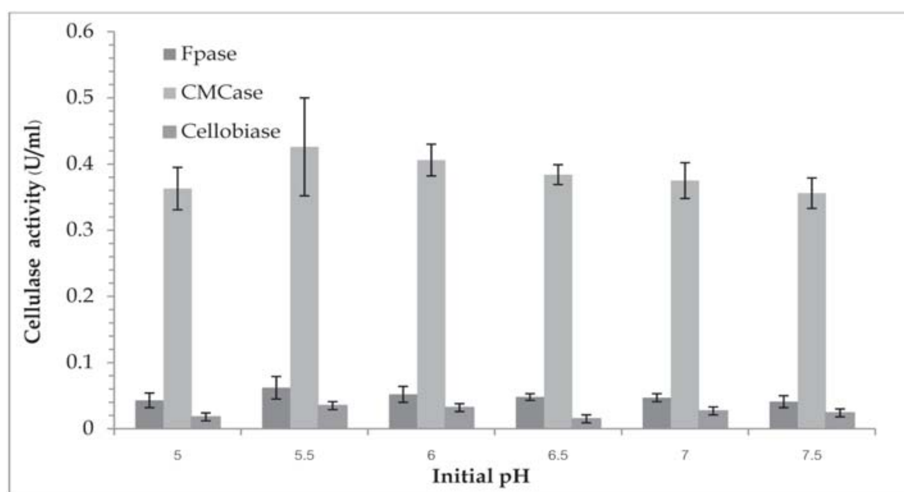

Figure 6. Cellulase activities at different pH levels.

4. Discussions

In this investigation, we studied the potential of utilizing peanut hulls as substrate for cellulase production under SSF by *G. australe* isolated and identified by our laboratory The use of abundantly available and cost-effective agro-industrial waste residues that were once considered useless, are presently being recognized as a potential raw material in achieving higher cellulase yields and reducing the overall cost of enzyme production (20). Cellulase production under SSF is gaining considerable interest because it is a cost effective form of technology that has been suggested to be capable of reducing production costs by 10 fold and has the ability to provide much higher yields, when compared to the submerged fermentation method (8,21). The present study demonstrated that the peanut residue could provided the maximum levels of cellulase, with yields of 0.062±0.017, 0.426±0.074, and 0.035±0.007 IU/ml for FPase, CMCase and cellobiase, respectively, after 9 days of cultivation with an initial 70% moisture content and an initial pH value of 5.5. The production of CMCase was always higher than FPase, and this phenomenon is in accordance with other reported findings (22-26). In the case of cellobiase, from Fig.4, it is obvious that cellobiase activity was the lowest. There were no significant differences for days 9 and 12. When cellobiase secretion is low, cellobiose accumulates instead of glucose (27). Cellobiose accumulation acts as a feedback inhibitor of cellulose depolymerization by endo and exoglucanases (28-29) which is a critical factor in the industrial scale conversion of cellulose to glucose. A study of the time course is of prime importance for the cellulase synthesis of fungi, the maximum enzyme production occurred at 9 days with the highest yields. In a similar study for cellulase production by *T. reesei*, cellulase yields remained fairly constant over the 3-5 days of fermentation, with a maximum yield observed at Day 4 (30). Some factors like moisture content is essential for microbial metabolism, and its depletion affects the diffusion of the solute, and the osmotic change (31). During SSF, both high and low moisture contents affect enzyme productivity. Furthermore, the effect of pH value on cellulase production was studied and it was found that the acidic pH range of 5.5 was optimal for enzyme production. The enzyme activity increased to the maximum level, followed by a slight decrease in activity. Das *et al*. (32) also observed that cellulase activity was optimum at a pH value of 4.8. The variation of pH from the optimum level causes denaturation of the enzymes and reduces enzyme synthesis ability. In a similar study by Dinis *et al*. (33) the production of ligninolytic enzymes during wheat straw its activity was very high, comparatively with CMCase. There were no significant differences for days 7, 14 and 21 and the maximum value observed was on *Trametes versicolor* on day 28 (0.03 U/ml). In the case of CMCase activity the most active producer appeared to be *Ganoderma applanatum*, with a maximum value (0.13 U/ml) on day 14, while in *Trametes versicolor* (0.07 U/ml), *Phlebia rufa* (0.06 U/ml) and *Bjerkandera adusta* (0.02 U/ml) was observed on day 7 While all fungi produced FPase activity was very low, comparatively with CMCase.

5. Conclusion

Successful attempts have been made to utilize peanut hulls, a highly abundant form of agro-industrial waste, as a substrate for the production of cellulase complex by *G. australe* under SSF, in order to develop a low cost production system. 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 novel inducer and scale up studies, need to be carried out in order to exploit these inexpensively produced commercial cellulase enzyme preparations in the second-generation biofuel

production process.

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