SCREENING OF PARAMETERS FOR SOLID-STATE FERMENTATION OF *ASPERGILLUS NIGER* PRODUCING CELLULASE USING SUGARCANE BAGASSE AS SUBSTRATE

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ABSTRACT: **The utilization of agroindustry wastes such as sugarcane bagasse (SCB) for cellulase production could help to reduce the problem of lignocellulosic wastes. Thus, this study aimed to use the sugarcane bagasse as a substrate in the production of fungal cellulases via solid-state fermentation of *Aspergillus niger*. The variables of solid-state fermentation condition of *A. niger* such as sugarcane bagasse particle size (400 and 600 µm), inoculum size (2% (v/v) and 5% (v/v), medium pH (5 and 7), and fermentation time (5 and 15 days) were screened using two-level factorial design (Design expert software, Stat-Ease Inc., Version 8.0). Filter paper activity (FPA) was determined to quantify the produced enzymes activity. The observation on the structure and physicochemical changes of SCB before and after SSF using scanning electron microscopy (SEM) and optical microscope was also conducted. Analysis of variance (ANOVA) shows that the significant parameters of SSF that affected the cellulose production were particle size of SCB and inoculum size–pH interaction.**

KEYWORDS: *Sugarcane bagasse (SCB); Aspergillus niger; Cellulase; 2-level factorial design.*

1. INTRODUCTION

A complex enzyme, cellulase which comprises three groups of enzymes; endo-(1,4)-*β*-D-glucanase (EC 3.2.1.4) exo-(1,4)-*β*-D-glucanase (EC 3.2.1.91), and *β*-glucosidases (EC 3.2.1.21) is one of the microbial enzymes that offers many applications in industries, (Kuhad *et al.*, 2011). This inducible enzyme can be produced by both bacteria and fungi, however, the latter produce noncomplex cellulase compared to the former, (Zhang *et al.*, 2006). Currently, *Trichoderma* and *Aspergillus* species are the main commercial producers of cellulose, (Zhang *et al.*, 2006).

Extensive studies have been carried out in improving the production of cellulase using both bacteria and fungi and lignocellulosic materials such as rice straw, corn cob, wheat bran, and sugarcane bagasse as substrates. However, pretreatment of the lignocellulosic materials are required prior to its use as a substrate. This step absolutely adds more costs not only on the production process but also in the treatment of wastes generated from the pretreatment process. Only a few studies have been carried out on the use of untreated agroindustry wastes as substrates in producing microbial cellulases. Therefore, it is necessary to use low-cost technologies and employ cheap substrate to produce microbial cellulases with more reasonable overall costs. Thus, this study attempts to investigate the production of cellulase via solid-state fermentation using *A. niger* and untreated sugarcane bagasse as a substrate. Solid-state fermentation (SSF) is often used in the cultivation of filamentous fungi due to its simplicity, low capital investment, and energy requirement as well as less water output and better product recovery, (Souza & Magalhães 2010 and Zeng & Chen 2009). Sugarcane bagasse (SCB) is one of the bulk agroindustry wastes generated from sugar industries in Malaysia and widely used in fuel and energy production. On a dry weight basis, it consists of celluloses (43.6%), hemicelluloses (33.8%), lignin (18.1%), ash (2.3%), and wax (0.8%), (Sun *et al.*, 2004). Therefore, this type of agrowaste is used as a substrate in this study due to its high content of celluloses. The culture conditions of SSF with respect to pH, incubation time, particle size of SCB, and inoculum size were screened with the aid of Design expert version 8.0 by employing two-level factorial design. Filter paper cellulase (FPase) was determined to quantify the produced enzymes activity. The observation on the structure and physicochemical changes of SCB before and after SSF using scanning electron microscopy (SEM) and optical microscope was also performed.

2. MATERIALS AND METHODS

2.1 Preparation of Lignocellulosic Substrate: Sugarcane Bagasse (SCB)

Sugarcane bagasse (SCB) was washed and rinsed with tap water to remove all impurities, e.g., dirt, stones, and sand. SCB was then cut into smaller size with the length of 1 cm prior to the drying process. Then, the dried SCB was ground into powder and sieved into two different sizes (400 and 600 µm). The ground SCB was autoclaved at 121°C for 15 mins and subsequently stored in an airtight container at room temperature prior to usage.

2.2 Preparation of Inoculum

A culture of *A. niger* was used as inoculum for the solid-state fermentation of SCB. The culture was grown on potato dextrose agar (PDA) solid medium for 5 days.

2.3 Fungal Pretreatment by *Aspergillus niger* (Solid-State Fermentation)

The solid-state fermentation of SCB was performed as described by Keera and co-authors (2014). Two grams of sterilized SCB was placed in a 250 mL Erlenmeyer flask. Then, the substrate was moistened with 5 mL of sterilized distilled water and left overnight in a fume hood prior to the inoculation. Ten milliliters of basal medium pH 5 (1:5 ratio of substrate-to-medium) was added followed by 2% (v/v) of inoculum suspension. The flasks were incubated at 37 °C for 5 or 15 days. Basalt medium (modified) was used to support the growth of the inoculums by giving 5.0% (w/w) of

the culture medium with the following compositions (g/L): yeast extract (2.0), $NaNO_3$ (5.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), and $FeCl_3$ (0.001), (Oyeleke *et al.*, 2012). At the end of the fermentation process, sodium citrate buffer solution (pH 4.8, 50 mM) was used to extract the enzyme and the mixture was centrifuged at 10,000 rpm for 10 mins to separate the extracted enzyme with the pellet.

2.4 Enzyme Assays

Filter paper activity (FPase) was determined using filter paper as described by Ghose (1987). Cellulase activity was calculated and expressed in International Units (IU). One unit of cellulase corresponds to the amount of filter paper to form 1 mg of glucose per min at 50°C.

2.5 Optical Microscopy

The pretreated SCB was filtered using 5 µm pore size filter paper and washed repeatedly using sterilized distilled water until all the fungus was removed and the washed water was clear. After that, the suspension was dispersed in petri dishes in a fume hood and left to air-dry for 5 days before further analysis. The suspension was placed on the glass slide and covered with a glass slip. Then, this glass slide was placed on the stage of an optical microscope (Chemopharm ILLINOS).

2.6 Scanning Electron Microscopy (SEM)

The surface morphology of the dried crude SCB and pellet samples after SSF was observed using an environmental scanning electron microscopy (ESEM, Quanta Feg 450 FEI) with the voltage of 5 kV. All dried sample were placed on the holder with the 1 × 1 cm double-sided cellophane tape.

2.7 Experimental Design and Data Analysis

A statistical two-level factorial design was employed with the aid of Design Expert software (Stat-Ease Inc., version 8.0) to screen the most significant factors in the solid-state fermentation of SCB with *A. niger*. Four operating factors which were pH, incubation time, inoculum size, and particle size were chosen to yield 16 different experimental runs. The four parameters were designated as X1 (particle size), X2 (inoculum size), X3 (pH), and X4 (incubation time). Table 1 shows the details of the design with the filter paper activity (FPase) as the response.

3. RESULTS AND DISCUSSION

3.1 Evaluation of Factors in Solid-State Fermentation of *Aspergillus niger* Affecting the Cellulase Production using Two-Level Factorial Design

As can be seen from the Pareto chart analysis in Figure 1, the particle size was the most significant parameter followed by inoculum size–pH interaction on the basis of FPase response. The coefficients with *t*-value of effect above Bonferroni line are designated as definitely significant coefficients, the coefficients with *t*-value of the effect between Bonferroni line and *t*-limit are termed as likely to be significant, and the coefficients below the *t*-limit line is statistically insignificant. Inoculum size, pH, and incubation time were not significant as the effects of the parameters were below the *t*-limit line (2.446). These results show that the increased of particle size would increase the FPase response.

Table 1: Two-level factorial design with filter paper activity (FPase) as response.

Run	Particle Size (µm)	Inoculum Size (%)	pH	Incubation Time (Days)	FPase (IU/mL)
1	600	2	5	5	0.258
2	600	2	5	15	0.240
3	600	2	7	5	0.212
4	600	2	7	15	0.198
5	600	5	5	5	0.196
6	600	5	5	15	0.176
7	**600**	**5**	**7**	**5**	**0.290**
8	600	5	7	15	0.260
9	400	2	5	5	0.134
10	400	2	5	15	0.147
11	400	2	7	5	0.149
12	400	2	7	15	0.109
13	400	5	5	5	0.085
14	400	5	5	15	0.127
15	400	5	7	5	0.241
16	400	5	7	15	0.230

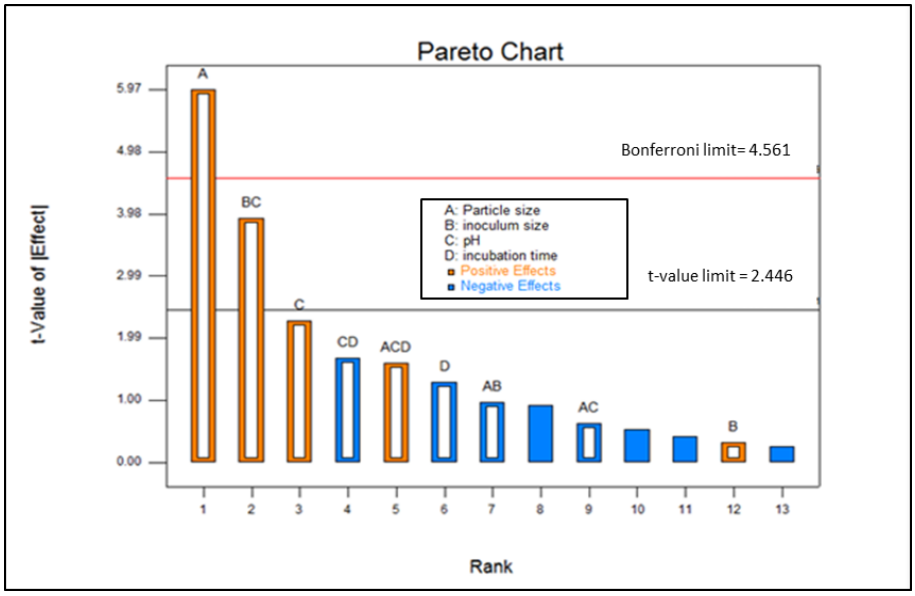

Figure 1: Pareto chart of four parameters and their interaction effects on FPase; the orange-colored bar indicates positive effect; the blue-colored bar indicates negative effect.

From the analysis of variance (ANOVA) in Table 2, the model was highly significant ($p < 0.01$) which indicates 99.74% confidence level. Particle size (A) and inoculum size–pH (BC) were the significant model terms as their values of p-value > F were less than 0.05.

The model *F*-value of 13.28 implies the model was significant and the lack of fit *F*-value of 0.46 implies the lack of fit is insignificant. This suggests that there was a good fit of factorial model. The high values of R^2 (95.2%) and adjusted R^2 (88.05%) indicate a high dependence and relationship between the experimental and predicted values of response.

As shown in Figure 2, the FPase value for the particle size of 600 μm with 2% (v/v) of inoculum size was slightly higher compared to the same particle size but with 5% (v/v) of inoculum size. The FPase value further decreased to less than 0.16 IU/mL when the particle size was 300 μm at different pH values. A similar pattern was observed by Wan *et al.* (2010). This result showed that 600 μm of particle size gave a high cellulase activity. It might be due to the increased accessible surface-area-to-volume ratio which determines the fraction of the substrate, (Chandra *et al.*, 2010). Another significant variable was the interaction of inoculum size with pH.

As can be seen in Figure 3, the FPase value was low (0.194 IU/mL) at 2% (v/v) of inoculum size even though at different pH values. However, the FPase value increased when the inoculum size was increased to 5% (v/v)at pH 7 compared to that of pH 5. A study reported that pH affects the permeability of cells as well as stability of the produced enzyme, (Mase *et al.*, 1996). Furthermore, it depends on the type of solid substrate used in the culture. Small inoculum size may need a longer time to grow due to less multiplication of fungus and substrate consumption and results in less amount of the desired enzyme. On the other hand, a rapid proliferation of fungal biomass might occur if large inoculum size is used and yields a high enzyme activity, (Sherief *et al.*, 2010).

3.2 Morphology observation of SCB after SSF

The crude SCB and the samples after SSF were observed under an optical microscope (Chemopharm ILLINOS) and the images are shown in Figure 4. The distinct structure of the crude SCB before (Figures 4a and 4b) and after SSF (Figures 4c and 4d) at different magnifications can be clearly seen. The original SCB structure is compact, rough, and has thick-walled fiber cells. The fibers were intact and organized by parallel stripes. On the other hand, after SSF, the lignocellulosic components of SCB were ruptured and loosely aggregated (Figures 4c and 4d). These aggregates were the separated fibers from pith and loosening of the fibrous network (Figure 4d). The appearance of individual and thinned cells were widely spread. This observation was more visible in the SEM images in Figure 5. These results show that due to SSF, native cellulose was able to be digested indirectly attributing to the degradation of noncellulosic materials (lignin and hemicellulose).

The morphology of crude SCB is shown in Figure 5(a) where the surface is smooth rigid, nofine fiber is seen along the walls, and less pores were observed. However, after SSF, the fibers structure were partially ruptured and had irregular shape compared to that of the crude SCB. A study showed that less enzyme activity (0.22 U/mL) was produced by *A. niger* MTCC 7956 in SSF using untreated SCB as a substrate compared to that using the treated SCB, (Sukumaran *et al.*, 2009). Another study reported that pretreated substrate showed high cellulase yields compared to that of the untreated substrate, (Bansal *et al.*, 2012). They described that the higher lignin

Table 2: Analysis of variance (ANOVA) for selected factorial model obtained from experimental design.

Source	Sum of squares	*F*-value	*p*-value>*F*
Model	0.053	13.28	0.0026
A-particle size	0.016	35.7	0.0010
B-inoculum size	4.33×10^{-5}	0.099	0.7642
C-pH	2.280×10^{-3}	5.18	0.0631
D-incubation time	7.322×10^{-4}	1.68	0.2444
AB	4.061×10^{-4}	0.92	0.3737
AC	1.758×10^{-4}	0.40	0.5505
BC	6.742×10^{-3}	15.33	0.0078
Lack of fit	1.264×10^{-3}	0.46	
Std. Dev.	0.021	R^2	0.9522
Mean	0.19	Adj. R^2	0.8805
C.V	10.99	Pred. R^2	0.7619
PRESS	0.013		

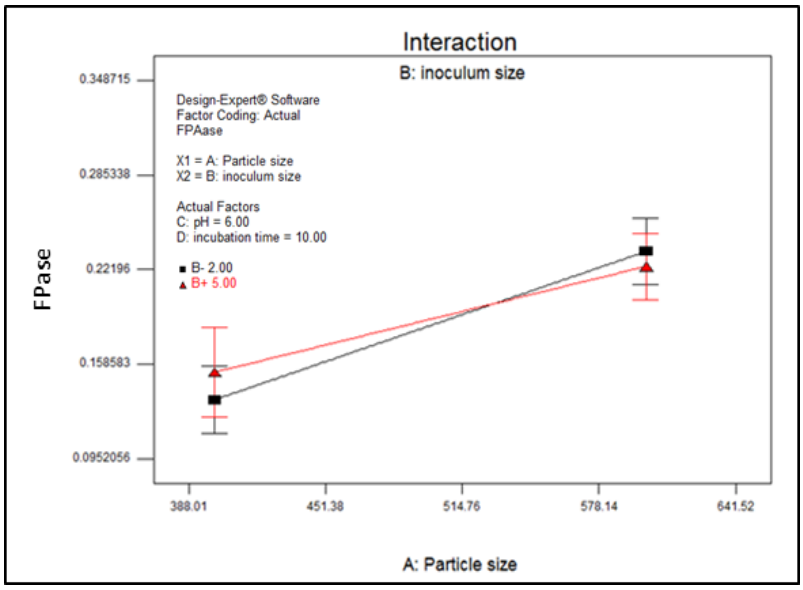

Figure 2: Effect of interaction between inoculum size and particle size on FPase.

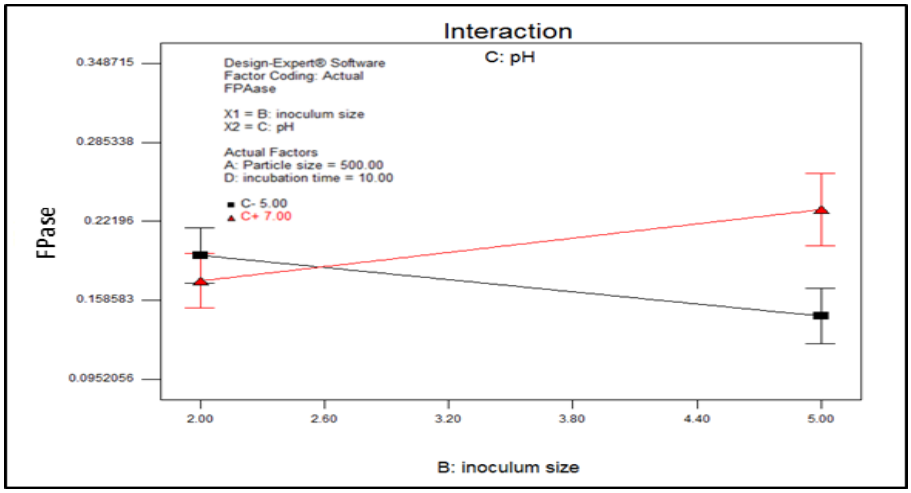

Figure 3: Effect of interaction between inoculum size and pH on FPase.

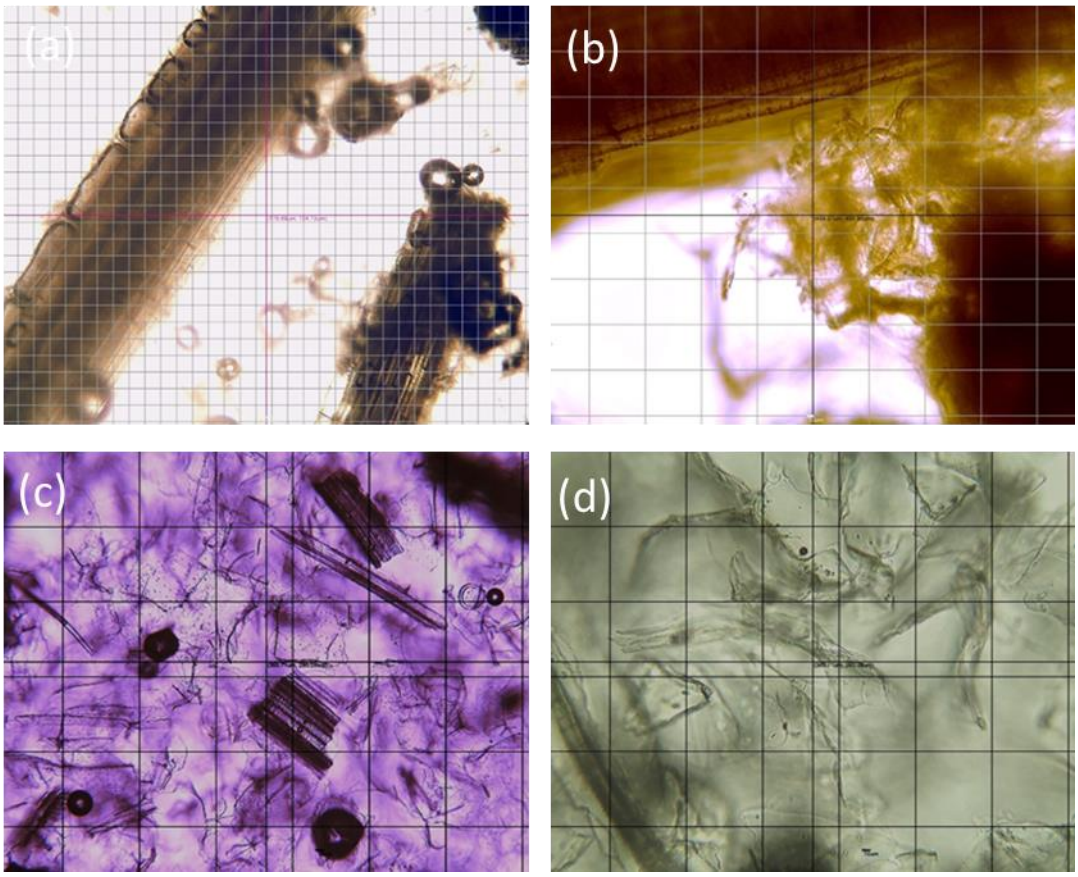

Figure 4: Optical microscope image of sugarcane bagasse with (a) 100× and (b) 400× magnifications before pretreatment (control) and (c) 100× and (d) 400× magnifications after SSF.

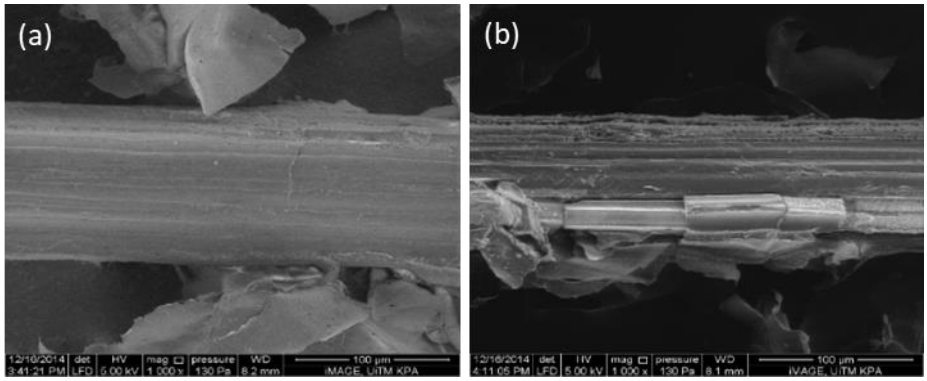

Figure 5: FESEM micrographs at 1000× magnification of (a) crude sugarcane bagasse and (b) sugarcane bagasse after SSF.

content and firm binding of the untreated substrate caused it to be less accessible to the microorganism. Contrast to the chemically treated substrate where its noncellulosic constituents were released causing the crystalline region of cellulose easily to solubilize and modify. The best FPase level obtained in this study was 0.29 IU/mL and this result was slightly the same as the reported study. Therefore, to enhance cellulase activity, it is a necessity to treat the lignocellulosic material before being used as a substrate.

4. CONCLUSION

The particle size of substrate (SCB) and inoculum size–pH interaction were the statistically significant ($p < 0.010$) parameters in the solid-state fermentation of *A. niger* for high FPase response. Based on the morphology observation, a slight change in the SCB fiber structure after SSF possibly contributed to the best FPase (0.29 IU/mL).

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