Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Screening Cellulolytic Fungi Isolated From Malaysia Cocoa Pod Husk and Its Culture Conditions for Cellulases Production

Dzun N.Jimat*, Nur S.Talha, Nur F.D. Che Husin, Azlin S.Azmi, Raha A.Raus

Biotechnology Engineering Department, Kulliyyah of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728, Kuala Lumpur, Malaysia.

Available online 15 June 2015

Keywords:
Cocoa pod husk, cellulase, FPase, CMCase, two-level factorial design, *Rhizopus sp*.

⊠*Corresponding author:
Dr. Dzun Noraini Jimat, Biotechnology Engineering Department, Kulliyyah of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728, Kuala Lumpur, Malaysia.
Email: jnoraini@iium.edu.my

Abstract

The aim of this study was to screen few fungal isolates from local cocoa pod husks (CPH) which able to secrete cellulases. The isolates were plated on carboxymethyl cellulose (CMC) agar plates which then incubated for two days at 28ºC. Then, these plates were stained with congo red dye for 0.5-1 h followed by destaining with 1 M NaCl solution for 15-20 minutes to observe its cellulolytic activity. One isolates which exposed the largest cellulolytic zone on CMC agar plate was selected for further study. In this study, culture conditions with respect to pH, incubation time, amount of substrate (CPH) and temperature were screened using Design expert @version 8.0 by employing two-level factorial design. The selected fungus isolate was cultured in shake flask at 37°C with agitation of 200 rpm for 5 days in incubator shaker. During fermentation period, samples were collected every day for fungal-cellulases activity of filter paper activity (FPase) and carboxymethyl cellulase (CMCase) activity. Analysis of variance (ANOVA) of this study showed that the most significant parameters that affects the production of cellulases from the selected fungi isolates were the amount of substrate (CPH) used followed by the interaction of amount of substrate with pH ($p< 0.05$). It showed that the cellulases activity was high when the pH 9 with more amount of substrate used. However, it was observed that less significant changes of celllulases activity when different amount of substrate was used at same pH of 3. Based on the microscopic observation of isolate, it morphology was closed to *Rhizopus sp*.. In conclusion, it is suggested to optimize the selected culture parameters obtained in this study in order to maximize the activity of cellulases from the selected isolates.

1. Introduction

Cellulases are a complex inducible enzyme system consisting of β-glucosidase, endoglucanase, and exoglucanase (or cellobiohydrolase) which can be synthesized by variety microorganism such as *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Trichoderma* and *Aspergillus*. These enzymes involved in the hydrolysis of cellulose, the major polysaccharide of plant cell walls (Bhat M.K, 2000). They are also used in textile, detergent, beverage, juice extraction, animal feed and pulp and paper industries and recently in saccharification of agriculture wastes for biofuel production. Its capability to convert lignocellulose, the most abundant and renewable source of energy on earth to value added products could be one of the alternative ways to solve dispose agroindustrial wastes. Many works have been done on the production of cellulases from lignocellulosic materials via microbial fermentation. The bioconversion of various complex cellulosic waste materials such as mushroom compost (Chandel et al., 2013), agriculture waste i.e wheat straw, rice straw, wheat bran and corn corb (Abo-State et al., 2010, Ojumuet al., 2003), saw dust (Solomon et al., 1999; Immanuel et al., 2007; Qurat-ul-ain et al., 2012), and rice husk (Milala et al., 2005; Qurat-ul-ain et al., 2012) have been reported. Those studies showed

ISSN Number: 2289-3946

that the cellulosic waste materials can be utilized as substrate in producing microbial cellulases. Thus, in present study, the fungi-producing-cellulase were isolated from the rotten cocoa pod husks (CPHs) and used to produce cellulase. The cocoa pod wastes were used as a substrate to produce cellulase from the selected isolate fungi. Cocoa pod husks (CPHs) are the main byproduct of cocoa or chocolate industry. In Malaysia, these husks are still underexploited and commonly left to rot on the cocoa plantation. Nevertheless, some studies have been carried out to utilize these waste as food antioxidants, dietary fibers, and animal feed.These husks represents between 70 to 75 % of the whole weight of the cocoa fruit, means if per each ton of cocoa fruit there will be between 700 to 750 kg of waste. Thus, if these agricultural wastes are left in cocoa plantation it can cause environmental problems such as generating foul odors and propagate diseases (i.e. black pod rot). Therefore, it is essential to utilize these agrowastes for some useful applications indirectly provide alternative solutions for global environmental problems. Culture conditions with respect to pH, incubation time, amount of substrate (CPHs) and temperature were screened using Design expert @version 8.0 by employing two-level factorial design. Filter paper cellulase (FPase), endoglucanase activity and reducing sugar concentration were determined to quantify the produced enzymes activity.

2. Materials and Methods

2.1. Culture Isolation/Identification

The fungal strain was locally isolated from the rotten cocoa pod husks using a sterile dissecting forceps, mouldy cocoa pods were aseptically picked and placed on sterile Potato Dextrose Agar (PDA) and incubated at 37°C for 6 days. The fungi cultures were further subculture until pure colonies were obtained by successive hypha tip transfers. The cultures were examined under a microscope to determine the common fungi present.

2.2. Plate Screening

For plate screening, Caboxymethylcellulose-Agar (CMC-Agar) medium was used as described by Quratul-Ain et.al., 2012. The CMC-agar was sterilized at 1210C for 15 min prior used. Isolates fungal from one week old PDA plates were suspended in sterile distilled water. A small well created in the middle of the plates and 100 □l of isolates fungal suspension was inoculated into the wells. Plates were incubated at 28ºC for two days. For cellulolytic activity observations, plates were stained with 1% CongoRed dye for 0.5-1 h followed by destaining with 1 M NaCl solution for 15-20 min. (Onsori et al., 2005, Quratul-Ain et.al., 2012).

2.3. Preparation of Lignocellulosic Substrate

Cocoa pod wastes were collected from Lembaga Koko Malaysia (LKM), Jengka, Pahang. These cocoa pod husks were dried and weight until obtaining consistent weight. Then, they were cut into small strips with razor blade. They were cleaned with water and dried in the oven at 700C. The cocoa pod husks were cut into small pieces (1-3cm) then grinded (1-2mm). The ground cocoa pod husks were stored in air tight containers at room temperature before use.

2.4. Fermentation and Enzyme Production

5ml of selected fungi spore suspension were inoculated into 250ml Erlenmeyer flasks which contained 100ml of Vogel's medium. The Vogel's medium consists of (g/100ml): trisodium citrate, 0.25; KH2HPO4, 0.50; NH4NO3, 0.20; (NH4)2SO4, 0.40; MgSO4.7H2O, 0.02; Peptone, 0.10; yeast extract, 0.20; and 2 g of the cocoa pod husk powder as substrate. The pH of culture medium was adjusted to initial pH 7.0 by 1M NaOH and 1M HCl. Culture medium and cocoa pod husk powder were sterilized separately at 1210C for 15 min prior used. The inoculated flasks were incubated at the temperature of 37°C on rotary shaker at 200 rpm under aerobic condition for 5 days (Quratu-ul-ain, Baig & Saleem, 2012). Each day, 10ml of culture will be collected and centrifuged. The supernatant were used to determine the enzyme activities. As a control, the same procedures were performed except the component of CPH was replaced with carboxymethyl cellulose (CMC).

2.5. Enzyme Assays

During fermentation period, samples were withdrawn for analysis of cellulase activity at every 24 h until enzyme activity peaks off. Filter paper activity (FPA) was determined for both the substrates by using filter paper as the substrate as proposed by Ghose (1987). It was assayed by incubating 0.5 ml of each culture supernatant with a rolled '1 by 6 cm' filter

ISSN Number: 2289-3946

paper strip (Whatman No. 1) in one millilitre (1 ml) of 0.05 M citrate buffer (pH 4.8) contained in test tube at 50ºC for 30 min. 3.0 ml of DNS reagent was added mixed well, boiled for exactly 5.0 min. in a vigorously boiling water bath. The color formed, is measured against the spectro zero at 540 nm. Cellulase activity was calculated and expressed in International Units (IU). One unit of cellulase corresponded to the amount necessary to form 1 milligram (1 mg) of glucose per minute at 50ºC.

Endoglucanase activity was measured as described previously (Ghose, 1987). 0.5 ml of sample was pipetted into a test tube, 0.5 ml of 1% carboxymethylcellulose (CMC) substrate was added to the test tube, and tube was covered with aluminium foil and incubated at 50ºC for 30 min. 3.0 ml DNS was added, mixed well, boiled for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. After boiling, it was transferred immediately to a cold water bath and measured the absorbance at 540 nm.

For determination of reducing sugar concentration 1 ml of culture filtrate was taken in a test tube and 3 ml of DNS reagent was added. Test tubes were placed in boiling water for 10 minutes. Cooled to room temperature and absorbance was measured at 540 nm (Quratul-ain et.al, 2012).

Table 1: The full factorial design for screening culture conditions in cellulases production

Factors	Low level (-1)	High level (+1)
pH	3	9
Incubation time (Days)	2	5
Amount of substrate (g)	2	5
Temperature (^{0}C)	27	40

2.6. Experimental Design and Data Analysis

A statistical 24-factorial design was employed using Design expert software (Stat-Ease Inc., Version 8.0) for the experimental design of this study to screen the culture conditions of isolate fungi producing cellulases (Table 1). Four operating factors which are pH, incubation time, amount of substrate and temperature were chosen to yield 16 different experiment runs. Table 2 shows the details of the design. The four parameters chosen were designated as X1 (pH), X2 (incubation time), X3 (amount of substrate) and X4 (temperature). Table 2 shows the filter paper activity (FPAse) and endo-13-1,4-glucanase (CMCase) as experimental results of average values of triplicate runs.

Table 2: Full2^4-factorial design with cellulases activitiesan (FPAse and CMCase).

Standard	Run	X1: pH	X2: Incubation day	X3: Substrate (g)	X4: Temperature (0C)	FPAse (U/ml)	CMCase (U/ml)
5	1	3	2	5	27	0.101	0.249
10	2	9	2	2	40	0.08	0.162
8	3	9	5	5	27	0.109	0.315
7	4	3	5	5	27	0.111	0.207
13	5	3	2	5	40	0.166	0.377
2	6	9	2	2	27	0.067	0.139
6	7	9	2	5	27	0.223	0.409
15	8	3	5	5	40	0.096	0.219
4	9	9	5	2	27	0.017	0.155
9	10	3	2	2	40	0.052	0.145
3	11	3	5	2	27	0.112	0.173
11	12	3	5	2	40	0.051	0.148
12	13	9	5	2	40	0.022	0.106
16	14	9	5	5	40	0.079	0.241
1	15	3	2	2	27	0.081	0.161
14	16	9	2	5	40	0.295	0.609

ISSN Number: 2289-3946

3. Results and Discussion

3.1. Isolation and Screening of a Potent Cellulases Producing Fungi

Based on cultural and morphological characteristics (Fig. 1), it was suspected that the isolated strain from mouldy cocoa pod (*Theobroma cacao L.*) was the *Rhizopus sp*. The microscopic images of the isolates are as shown in Fig. 2.

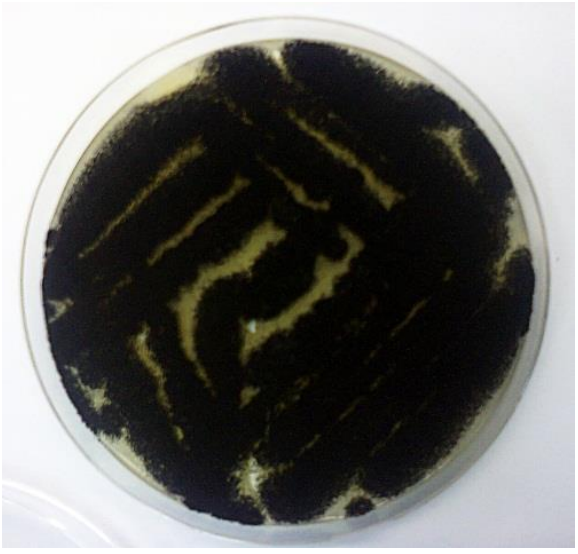


Figure 1: The isolated-cocoa pod fungi (expected as *Rhizopus sp.*)

Figure 2: Screening of cellulolytic fungal cultures on CMC agar plate

Also, fungal strain *Aspergillus* sp. was successfully screened for cellulase enzyme production by existent of a clear zone (Fig. 2). Since the cellulose in the CMC agar was the sole carbon source present, thus the result of the test was strongly shown that the cellulase was produced for degradation of cellulose.

3.2. Evaluation of culture conditions affecting the cellulases production using 24 factorial design.

Four culture condition parameters namely temperature, incubation time, pH and amount of substrate used were investigated by 24 factorial design for cellulases production from the isolated fungi. In the Pareto chart (Fig. 4), the upper portion indicated the maximal effect and then progress down indicated the minimal effect. Based on the Pareto chart, the critical "t" calculated was 3.8 and only amount of substrate (CPH) for both response variables (Figure 4a and b) was statistically significant ($p<0.10$) as the calculated "t" value for this parameter was higher than the critical "t". It indicates that the amount of substrate (CPH) significantly influenced ($p<0.10$) (out of other parameters pH, incubation time and temperature) both CMCase and FPAse activities.

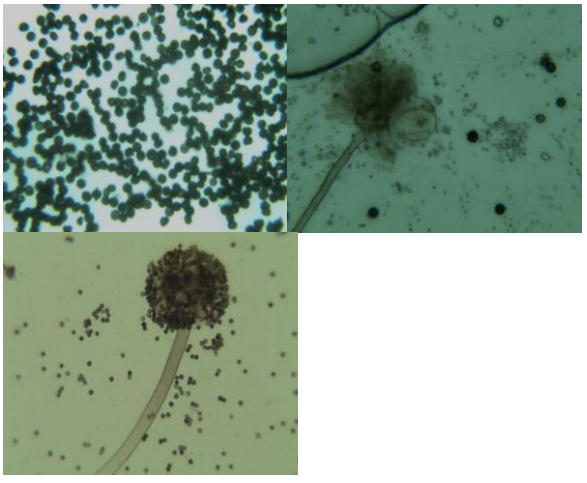
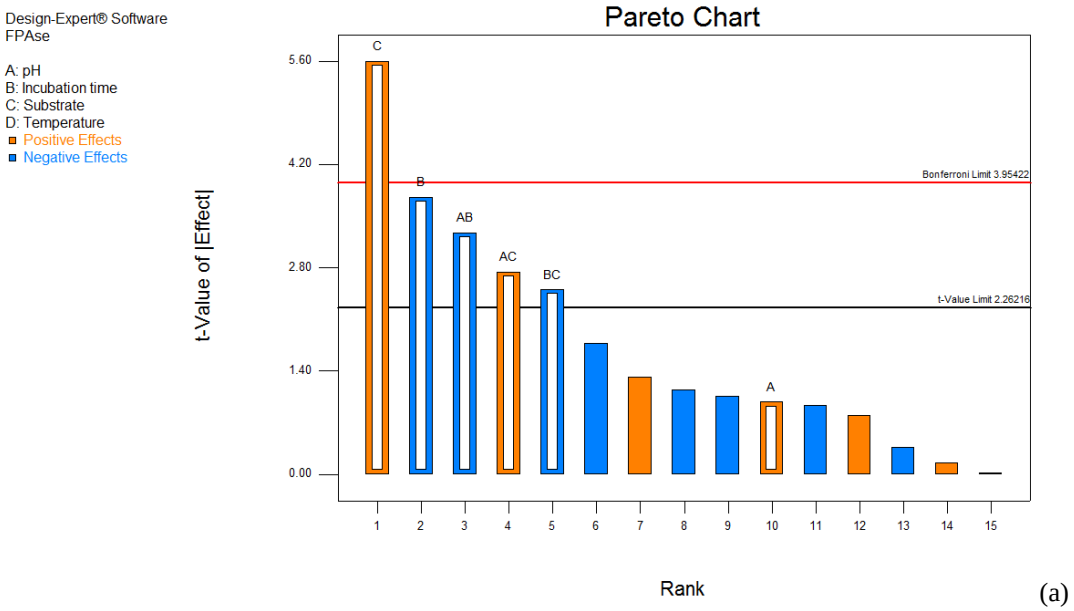


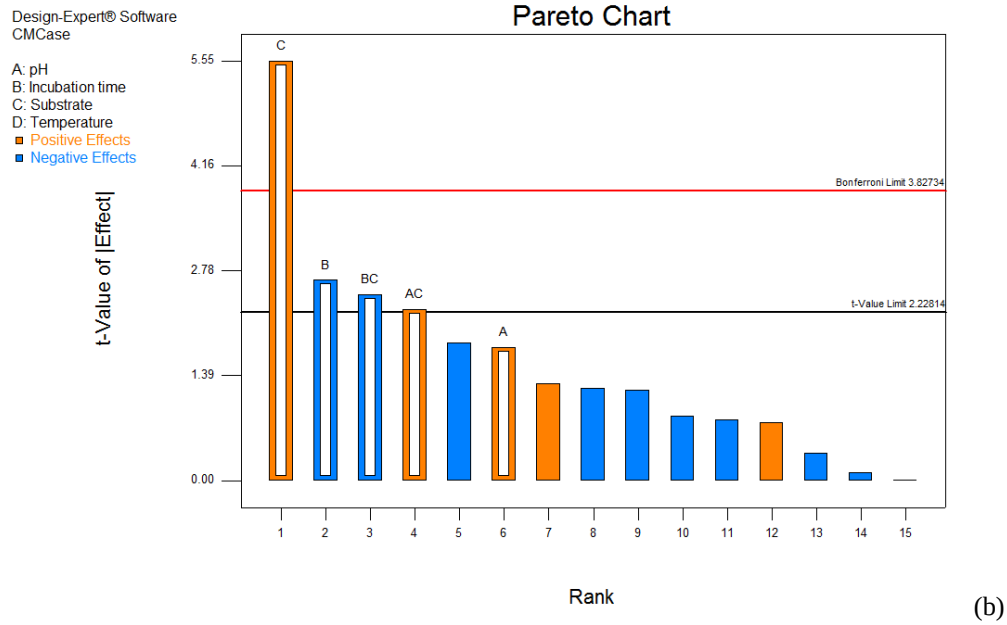
Figure 3: Optical microscopic images of the isolated-cocoa pod fungi (expected as *Rhizopus sp*.)

As shown in the Pareto chart, it can be seen that the amount of substrate had a significant influence at 10% and the other parameters did not statistically influence enzymatic activity. Therefore, the parameter values were fixed at the level where the maximum activity of each enzyme was obtained and this condition will be validated by repeating the experiments.

ISSN Number: 2289-3946



(a)



(b)

Figure 4: Pareto charts of the effects of the four parameters studied: amount of substrate, pH, incubation time, and temperature of the selected isolate (a) FPAse activity and (b) CMCase

4. Conclusions

Funga strain *Rhizopus sp*. was successfully screened for cellulose enzyme production using cocoa pod husk (CPH) as substrate. In the screened of culture conditions, amount of was statistically significant ($p<0.10$) for both response variables; CMCase and FPAse activities.

Acknowledgement

This project was funded by IIUM Endownment Fund B.

References

[1] Abo-State, M. A. M., Hammad, A. I., Swelim, M., & Gannam, R.B. (2010), Enhanced production of cellulase(s) by *Aspergillus* spp. isolated from agriculture wastes by solid state

ISSN Number: 2289-3946

fermentation, American-Eurasian J. Agric. & Environ. Sci., 8(4):402-410

[2] Bhat, M. K. (2000), Research Review Paper: Cellulases and related enzymes in biotechnology, Biotechnol. Adv., 18:355-383

[3] Chandel, K., Jandaik, S., Kumari, V., Sarswati, S., Sharma, A., Kumar, D. & Kumar, N. (2013), Isolation, purification and Screening of cellulolytic fungi from mushroom compost for production of enzyme (cellulase), International Journal of Current Research, 5(1):222-229

[4] Ghose, T. K., (1987), Measurement of cellulase activities, Pure and Appl. Chem., 59:257-268

[5] Milala, M. A., Shugaba, A., Gidado, A., Ene, A. C., & Wafar, J. A. (2005), Studies on the use of agricultural wastes for cellulase enzyme production by *Aspergillus* niger. Res. J. Agric. Biol. Sci, 1(4):325-328

[6] Ojumu, T. V., Solomon, B. O., Betiku, E., Layokun, S. K., & Amigun, B. (2003), Cellulase Production by *Aspergillus* flavus Linn Isolate NSPR 101 fermented in sawdust, bagasee and corncob. African Journal of Biotechnology, 2(6):150-152

[7] Onsori, H., Zamani, R. M., Motallebi, M., & Zarghami, N. (2005), Identification of over producer strain of endo-β-1,4-glucanase in *Aspergillus* species: characterization of crude carboxymethyl cellulase, African Journal of Biotechnology, 4(1):26-30

[8] Qurat-ul-Ain, Baig, S., & Saleem, M. (2012), Production and characterization of cellulases of *Aspergillus* niger by using rice husk and saw dust as substrates, Pak. J. Bot., 44:377-382

[9] Solomon, B. O., Amigun, B., Betiku, E., Ojumu, T. V., & Layokun, S. K. (1999), Optimization of Cellulase Production by *Aspergillus* flavus Linn Isolate NSPR 101 Grown on Bagasse, JNSChE, 16:61-68

ISSN Number: 2289-3946