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CELLULASE ENZYME PRODUCTION FROM LIGNOCELLULOSIC SUBSTRATES BY INDIVIDUAL AND CONSORTIUM OF NEW FUNGI ISOLATED FROM ASIAN ELEPHANT (*Elephas maximus*) DUNG

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Abstract- One of the major challenges of cellulosic bioethanol production is cellulase enzyme which is used in the hydrolysis of cellulose to sugars for fermentation to bioethanol. Commercial cellulase enzyme preparations are very expensive. Fungi are recognized for their ability to produce a large variety of extracellular enzymes. In this study, the potential of two new native fungi (*T. aureoviride* UPM 09 (JN811061) (UPMC 389) and *F. equiseti* strain UPM 09 (JN811063) (UPMC391) isolated from Asian elephant (*E. maximus*) dung to produce cellulase enzymes from rice husk (RH), rubber wood saw dust (RW) and oil palm empty fruit bunch (EFB) using solid state cultivation (SSC) and submerged cultivation (SMC) was investigated. From the results of the statistical analysis, there is no significant difference ($P>0.05$) in cellulase production between the two methods for all the three substrates. However, there is significant difference ($P<0.05$) between the two fungi in cellulase enzyme production both SSC and SMC among the three substrates used. The result, therefore, indicates that any of the two fungi can be used for cellulase production. In addition, the type of substrate is influential in cellulase production by the fungi. The two new fungal strains may serve as good candidates for cellulase production in the cellulosic bioethanol industry. However, consortium of the two fungi produced relatively slightly higher cellulase enzyme in each of the methods than each when used alone.

Keywords- lignocellulosic, bioethanol, cellulase, *T. aureoviride, F. equiseti,* consortium

Abbreviations- RH: rice husk, RW: rubber wood, EFB: empty fruit bunch, SSC: solid state cultivation, SMC: submerged cultivation, FPase: filter paper assay for saccharifying cellulase

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Introduction

One of the major setbacks in cellulosic bioethanol production is cellulase enzyme which is used in the hydrolysis of cellulose to sugars for fermentation to bioethanol. Commercial cellulase enzyme preparations are very expensive [1]. Cellulase enzymes are classified into three: endoglucanase (endo-1, 4-ß-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase (exo-1, 4-ß-D-glucanase, CBH, EC 3.2.1.91) and ß-glucosidase (1, 4-ß-D-glucosidase, BG, EC 3.2.1.21) [5,11]. Substantial cost reduction may be possible by exploring ways of cellulose conversion using microorganisms that produce cellulolytic enzymes. It is therefore imperative to look for microorganisms that have a high rate of cellulase production [9]. Fungi are recognized for their extreme facility in producing a large variety of extracellular enzymes. The principal organisms responsible for lignocellulose degradation are Basidiomycetes [8]. Therefore, for reducing the cost of enzyme, selection of a cheap and easily available substrate appears to be essential [2,16]. Lignocellulosic biomass is known to be an excellent carbon source for microbial enzyme production [3]. Lignocellulosic biomass such as rice husk (RH), rubber wood saw dust (RW) and oil palm empty fruit bunch (EFB) are very abundant, cheap and easily available especially in Malaysia where they are generated in large quantity annually [20]. Various agricultural substrates-byproducts and microbial cultures have been used successfully for cellulase production [22]. In this study, therefore, two new native fungi isolated from Asian elephant (*E. maximus*) dung were used individually and in consortium to produce cellulase enzymes from three substrates, namely,

rice husk (RH), rubber wood (RW) and oil palm empty fruit (EFB).

Materials and Methods

Preparation of Fungal Strains

The fungal strains used were isolated from Asian elephant (*Elephas maximus*) dung (sourced in the Malaysian forest reserve) in our laboratory at the Institute of Bioscience, Universiti Putra Malaysia. The sequences of the fungi were deposited in the database of Gen Bank NCBI, USA while the fungi were deposited at Universiti Putra Malaysia culture collection (UPMC) and were assigned accession numbers (in parenthesis). These are *Trichoderma aureoviride* UPM 09 (JN811063) (UPMC 389) and *Fusarium equiseti* strain UPM 09 (JN811061) and were selected and used for fungal pretreatment in this study. Prior to inoculation, the fungal strains were sub cultured on potato-dextrose agar (PDA) plates at 30ºC for 7 days and the fungal spores were harvested and stored at 4ºC before inoculation. The two fungi were earlier on screened for cellulolytic activity as described by Pointing [13].

Preparation of Substrates

The RH, RW and EFB used as substrates in this study were sourced locally in Malaysia. They were firstly dried and chopped into small pieces before grinding into smaller particles and sieving to 1.0 mm sieve.

Preparation of Minimal Salts Medium

For submerged cultivation, the minimal salts medium (100ml) was prepared by dissolving one liter of distilled water (g/l) in 250ml Erlenmeyer flask: Urea (0.3), KH_2PO_4 (2.0), $(NH_4)_2SO_4$ (1.4), $MgSO_4.7H_2O$ (0.3), Peptone (0.75), Yeast extract (0.25), $CaCl_2.2H_2O$ (0.002). Trace elements: $FeSO_4.7H_20$ (0.005), $MnSO_4.7H_2O$ (0.0016), $ZnSO_4.7H_2O0.0014$), $CoCl_2$ (0.002) were added. The medium was stored at 4ºC before use.

Preparation of Spores

Spore suspensions were prepared by washing the agar surface with distilled water. The spore counts were determined with a haemocytometer (Marienfeld®, Germany) and the final spore inoculums had a concentration of 5.6×10^9 for spores/ml for *Trichoderma aureoviride* UPM 09 and 9.6 x 10^9 *Fusarium equiseti* strain UPM 09.

Effect of Temperature on Growth Condition of the Fungi

The effect of temperature on the growth condition of the native fungal isolates was investigated based on optical density, dry weight, temperature (30, 40 and 50ºC) and FPase (cellulase activity of the fungi (FPase). One gram (1 g) each of 1mm of rubber wood saw dust, rice husk, oil palm empty fruit bunch in 250ml Erlenmeyer flasks separately was added to 100ml of minimal salts medium and autoclaved at 12lºC for 15 minutes before inoculating each of the fungi. The flasks were incubated in incubator shakers (120 rpm) at 30, 40 and 50ºC for 7 days. The optical density (OD), filter paper activity (FPase) and dry weight (DW) were measured every 24 hrs.

Enzyme Production

A modified method of Shi, et al. [17] was employed namely, Submerged Cultivation (Agitated) (SMC) and Solid State Cultivation (SSC) for enzyme production.

Submerged Cultivation (Agitated)

Seven grams each of 1 mm of the substrates was weighed and put in 250ml Erlenmeyer flask. Minimal salts medium (100ml) was added into each flask. The pH was recorded and the flasks were sterilized at 121°C for 15 minutes in an autoclave. The biomass substrate in each flask was each separately inoculated with the fungal spores individually and in consortium of the two fungi. Spore suspensions were prepared by washing the agar surface with 10ml of sodium acetate buffer (50 mM, pH 4.5). The final spore inocula had a concentration of 5.6×10^9 for spores/ml for *Trichoderma aureoviride* UPM 09 (JN811061) (UPMC 389) and 9.6 x 10^9 *Fusarium equiseti* strainUPM-09 (JN811063). The flasks were incubated at 30°C at 120 rpm shaking rate for 14 days. Samples were taken daily and centrifuged at 10,000 rpm for 5 min. The supernatants were recovered for enzyme assay in triplicate. The flasks were then stored at 4ºC until needed.

Solid State Cultivation (SSC)

This carried out in 250ml Erlenmeyer flasks, each of which contained rice husk (RH), rubber Wood (RW) and oil palm empty fruit bunch (EFB) 7 g 1.00 mm particle size. Distilled water (12ml) was added to obtain the appropriate substrate moisture content (75%). The flasks containing the substrates were sterilized at 121ºC for 20 minutes, then cooled and aseptically inoculated with 5.6×10^9 spores/ml of *T. aureoviride* UPM 09 (JN811061) (UPMC 389) and 9.6 x 10^9 spores/ml of *F. equiseti* strain UPM 09 (JN811063) individually and in consortium. Para film was wrapped around flasks to serve as a barrier against moisture loss and contamination. Small perforations were made to the film to avoid moisture condensation and allow ventilation of chambers. Flasks were maintained statically in an incubator at 30ºC for 14 days. A set of flasks of untreated sterilized substrates for each sample was used as control. Samples were taken daily and centrifuged at 10,000 rpm for 5 min. The supernatants were recovered for enzyme analysis at least in triplicate. After cultivation, the flasks were stored at 4ºC before use.

Cellulase Enzymes Assay

The following enzymes assays were conducted for each sample as supernatants from above as described by Ghose [4] and Rajoka and Malik [14].

Filter Paper Assay (FPase)

To a test tube was added 1.0ml 0.05 M Na-citrate, pH 4.8 and 1.0ml supernatant, diluted in citrate buffer. The mixture was allowed to temperate to 50°C and Whatman No.1 filter paper strip (1.0 x 6.0 cm (≈50 mg)) was added and mixed and incubated at 50°C for 60 minutes. Then 3.0ml DNS reagent was and mixed. The tube was transferred to a rack on the table and boiled the mixture for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzymes blanks, glucose standards and the spectro zero were boiled together. After boiling, the tube was transferred to a cold water bath. 20ml deionized or distilled water was added and mixed by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion. The colour formed was measured against

the spectro zero at 540nm. One unit of activity (U/ml) was defined as amount of enzyme required to liberate 1µ mol glucose equivalents per minute.

Carboxymethyl Cellulase Assay (CMCase)

To a test tube of volume at least 25ml was added 0.5ml supernatant, diluted in citrate buffer and temperate to 50°C. Then 0.5ml substrate solution of 2% Carboxymethyl cellulose CMC 7L2 (degree of substitution = 0.7) (Sigma Aldrich, Malaysia) in 0.05 M sodium citrate buffer, pH 4.8 was added and mixed well and incubated at 50ºC for 30 minutes. 3.0ml DNS was added and mixed well before transfer to a rack on the table. The mixture was boiled for about 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzymes blanks, glucose standards and the spectro zero were boiled together. After boiling, the tube was transferred immediately to a cold water bath. About 20ml deionized or distilled water was added and mixed well by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion. The color formed was measured against the spectro zero at 540nm. When necessary (i.e., then small dilutions are used), the colour formed in the enzyme blank is subtracted from that of the sample tube. The absorbance of the sample tube was translated (corrected if necessary by subtraction of the enzyme blank) into glucose production during the reaction using the glucose standard curve. One unit of activity was defined as amount of enzyme required to liberate 1µ mol glucose equivalents per minute.

ß-Glucosidase (BGL) Assay

To a test tube containing 0.2ml citrate buffer was added 0.2ml paranitrophenyl β-glucopyranoside and 0.2ml enzyme. The test tubes were incubated at 50°C for 15 minutes in water bath shaker at 150 rpm. After incubation, 3ml of 1 M Na_2CO_3 was added to stop reaction and colour development. A control was run along them. A blank was prepared by adding 0.6ml of water and 3ml Na_2CO_3 to adjust the zero absorbance at 410nm. One unit of β-glucosidase activity was defined as amount of enzyme required to liberate 1µ mol glucose equivalents in 1ml enzyme solution in 1 minute.

Statistical Analysis

Factorial ANOVA was used in the analysis of the cellulase enzymes produced by the fungi JMP, Version 9.0.3 SAS Institute Inc., Cary, NC, 1989-2010. P value less than 0.05 was considered significant.

Results and Discussion

Fungal Strains

The new native fungi isolated and used in this study were deposited in Universiti Putra Malaysia culture collection (UPMC) while the sequences of the fungi were deposited at the Gen Bank NCBI (USA) and were given the accession numbers JN and UPMC (shown in parenthesis), respectively. These are *Trichoderma aureoviride* UPM 09 (JN811061) (UPMC 389) and *Fusarium equiseti* strainUPM-09 (JN811063) (UPMC391).

Effect of temperature of Growth Condition of the Native Fungi and Enzyme Production (FPase)

The effect of temperature on growth condition was investigated based on optical density, dry weight and enzyme production (FPase). The optimum temperature for the growth of the two fungi was also determined. The results of the growth condition of *T. aureoviride* UPM 09 (designated P) and *F. equiseti* UPM 09 (designated F1) presented in [Fig-1] (optical density, OD 600nm), [Fig-3] (FPase, 540nm) and [Fig-4] dry weight (mg), the two fungi grew only at 30ºC with clear visible mycelia spread on the on the three biomass substrates. While at 40 and 50ºC no visible mycelia were observed growing on any of the substrates, only turbid growth was manifest after 7 days showing that growth of the two fungi is not favored at 40 and 50ºC. Relatively uniform growth was observed at 30ºC with log phase at 3 to 5 days for *T. aureoviride* (P).

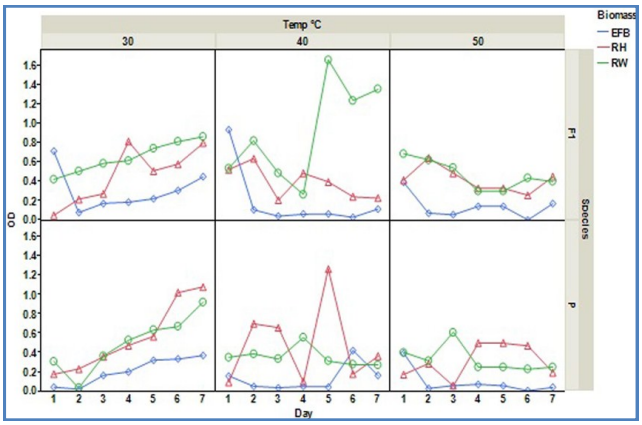

Fig. 1- Growth profile (OD, 600nm) of *T. aureoviride* UPM 09 (P) and *F. equiseti* UPM 09 on RH, RW and FFB at 30, 40 and 50ºC.

[Fig-2] shows the effect of temperature on the Filter paper activity (FPase) of the *T. aureoviride* strain UPM 09 (P) and *F. equiseti* strain UPM 09 at 30, 40 and 50ºC.At 30ºC, the former (P) had increased FPase production at day 5and 6 for EFB and RW and at day 4 for RH while the latter (F1) produced low yield which fluctuated for all the three substrates.

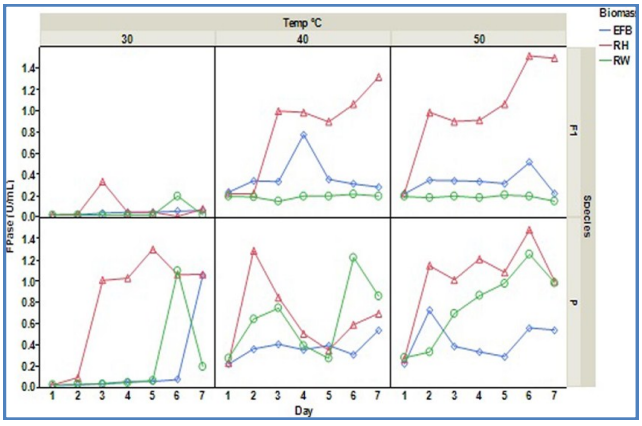

Fig. 2- FPase (U/mL) (Y-axis) of *T. aureoviride* strain UPM 09 (P) and *F. equiseti* strain UPM 09 on RH, RW and FFB at 30, 40 and 50ºC.

[Fig-3] shows the result of dry weight (mg) of mycelia of the two fungi increasing at 30ºC for all the three subsrates daily with peak at day 7. There was, however,unsteady growth at 40 and 50ºC. This result shows that the fungi grow best at 30Cº and, therefore, 30ºC is the optimum temperature for cellulase production.

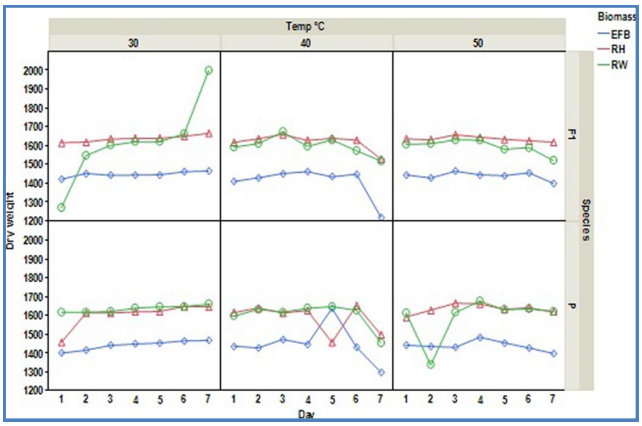

Fig. 3- Dry weight (mg) of *T. aureoviride* UPM 09 (P) and *F. equiseti* UPM 09 on RH, RW and FFB at 30, 40 and 50ºC.

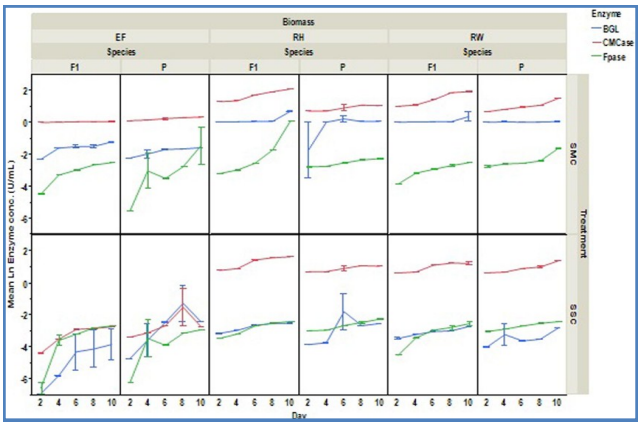

Fig. 4- Mean enzyme produced by *T. aureoviride* strain UPM 09 (P) and *F. equiseti* strain UPM 09 (F1) using RH, RW and EFB by SSC and SMC.

Cellulase Activities

A large number of microorganisms are capable of producing cellulases, but fungi are considered the most promising. Different strains belonging to *Trichoderma* sp. have been the most extensively studied Sohail, et al. [18]. Other studies revealed production of higher levels cellulases of by indigenous strains [6,7]. The present study confirms cellulase production by the two fungi from both SMC and SSC [Fig-4] and [Fig-5] and also shows a relative increase in cellulase production by the two fungi in the pretreated substrates (RH, RW and EFB) using the two pretreatments. From the results of the statistical analysis of the cellulase activity (FPase), carboxymethylcellulase (CMCase), ß-glucosidase (BGL), there is no significant difference (P>0.05) between *T. aureoviride* strain UPM 09 (P) and *F. equiseti* UPM 09 (F1) in the overall mean cellulase production in the two pretreatment methods (SSC and SMC) for all the three substrates. However, there is significant difference (P<0.05) between the two fungi in cellulase enzyme in each of the pretreatments SSC or SMC among the three biomass used. The result, therefore, indicates that any of the two methods can be used for cellulase production, but the importance of substrate on the cellulase production by each of the fungi is of great significance. As can be seen in [Fig-4], the production of CMCase (0.01 to 7.2 U/ml) in *F. equiseti* strain UPM 09 (F1) from RH and RW using SMC and SSC was higher than *T. aureoviride* strain UPM 09 (P) (0.0009 to 4.8 U/ml) while BGL and FPase produced by *F. equiseti* strain UPM 09 from RH and RW are higher than *T. aureoviride* strain UPM 09 (P) and continued to rise daily. However, consortium of both *T. aureoviride* strain UPM 09 and *F. equiseti* strain UPM 09 [Fig-5] produced relatively higher CMCase (0.2 to 1.8 U/ml for RH, 0.2 to 1.5 U/ml for RW, 0.2 to1.0 U/ml for EFB) and BGL (4 to 6 U/ml for RH, 2 to 4.5 U/ml for RW, 1 to1.8 U/ml for EFB) in SMC than SSC up to day 10.

According to Kovacs, et al. [10] BGL supplement is always sourced in order to attain high cellulose conversion because this is lacking in the most studied. *T. reesei.* However, considering the relatively high amount of BGL (1.0-2.0 U/mL) produced by the two fungi individually and by consortium from RH and RW in both SSC and SMC in addition to CMCase and FPase prduction, this finding could be considered as a step forward towards addressing the costly commercial cellulase enzymes in the bioethanol industry.

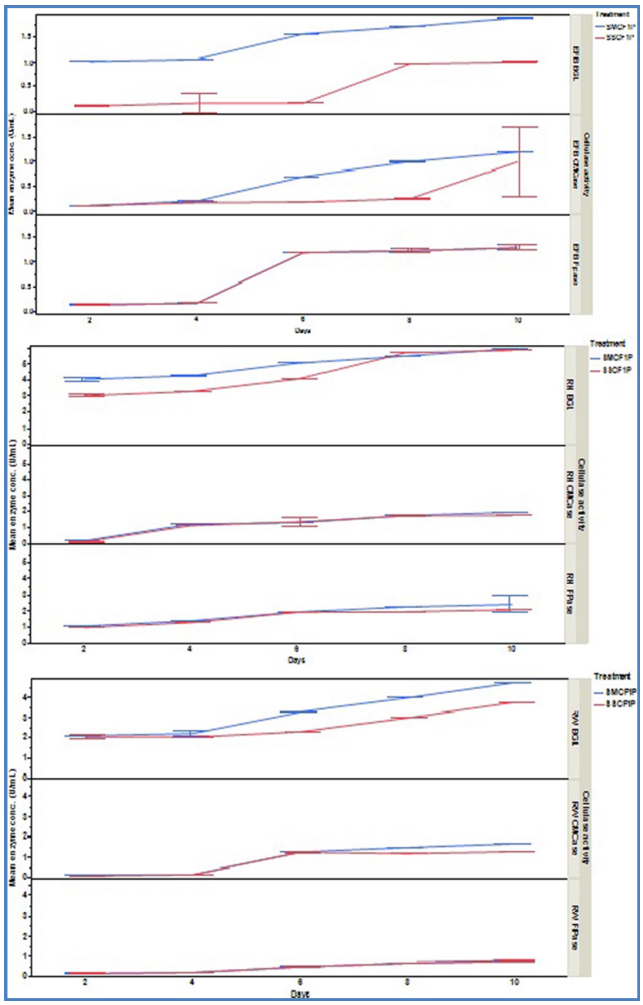

Fig. 5- Cellulase activities using consortium of *F. equiseti* strain UPM 09 (F1) and P (*T. aureoviride*) strain UPM 09 by SSC and SMC

Conclusion

The ability of newly isolated *T. aureoviride* strain UPM 09 (JN811063) and *F. equiseti* strain UPM 09 (JN811061) to produce cellulases from rice husk, rubber wood saw dust and oil palm fruit

bunch using SMC and SSC was investigated. The results suggested the potential use of *T. aureoviride* strain UPM 09 (JN811063) and *F. equiseti* strain UPM 09 (JN811061) for enzyme production individually and as consortium of the two fungi. The above properties suggested that the cellulases from these fungi might be used as key enzymes in the production of bioethanol from cellulosic biomass.

Acknowledgements

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