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Full Length Research Paper

Factors affecting endoglucanase production by *Trichoderma reesei* RUT C-30 from solid state fermentation of oil palm empty fruit bunches using Plackett-Burman design

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A study was conducted to screen parameters affecting the production of endoglucanase by *Trichoderma reesei* RUT C-30 on solid state fermentation of oil palm empty fruit bunch using the Plackett-Burman design. Factors involved in the screening process were peptone concentration, urea concentration, ammonium sulfate concentration, calcium nitrate concentration, yeast extract concentration, tween 80 concentration, pH, incubation time, initial moisture content, inoculum size and substrate amount. Through analysis of variance (ANOVA), it was found that initial moisture content (p= 0.001), incubation time (p= 0.001), inoculum size (p= 0.032) and ammonium sulfate concentration (p= 0.023) have been recognized as significant factors affecting endoglucanase activity in solid state fermentation (SSF) of oil palm empty fruit bunch (OPEFB) by *T. reesei* RUT C-30. The model established from the ANOVA analysis have a significant value of $P_{model}>F=$ 0.0008 and R^2 of 0.9132. The pre-optimized media showed 2.6 fold increased of endoglucanase activity.

Key words: Cellulase, oil palm empty fruit bunches, solid state fermentation, *Trichoderma reesei* RUT C-30, Plackett-Burman design.

INTRODUCTION

Cellulases are enzymes that are widely used in many industrial processes such as in feed, pulp, detergent and textile industries (Gavrilescu and Chisti, 2005). Cellulases are a group of enzymes which hydrolyses cellulose into glucose. There are three main classes of cellulase namely endoglucanase (endo-1, 4-β-D-glucanases), exoglucanase (1, 4- β-D glucosidase) and β-glucosidase having structures and mechanical actions differ from one another. Endoglucanase functions in breaking the internal bonds within the fibre to disrupt the crystalline structure of cellulose and exposes the individual cellulose poly-saccharide chains. Meanwhile, exoglucanase produces tetrasaccharides or disaccharides that cleave the 2 or 4 units from the ends of the exposed cellulose chains produced by endoglucanase, respectively whereas, β-glucosidase hydrolyzes exoglucanase products into simple glucose.

Currently, majority of the commercial cellulases were produced from the submerged (liquid) fermentation (SmF) process using the fungi such as *Trichoderma*, *Penicillium*, *Aspergillus* and *Humicola* (Tolan and Foody, 1999). The production cost of cellulases commercially is

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expensive due to the use of pure grade cellulose as the fermentation substrate. Apart from this the system produced low cellulases yields in terms of per unit of purecellulose used (Chahal, 1985). It is imperative to note with the increasing global demand of cellulases enzyme, the use of cheaper fermentation substrate with economical process is desired.

Solid state fermentation (SSF) is a process which involves the growth of microorganisms on moist solid particles substrate (Mitchell et al., 2006). The micro-organisms get air supply from water droplets or film and gas phase within the spaces between the substrate particles. Meanwhile, food is acquired from nutrients dissolved in water droplets or film and also from the particle substrate. SSF has become an alternative process over submerged fermentation (SmF) in the production of some chemicals and enzymes from ligno-cellulosic substrate due to the use of simple cultivation equipments, lower capital investment, higher productivity per reactor volume, reduced bacterial contamination (Mitchell et al., 2006), low effluent generation as well as low requirements for aeration and agitation during enzyme production (Pandey, 2001).

Trichoderma reesei RUT C-30 strain is a mutant fungi strain which was developed having special characteristic of not being repressed by glucose accumulation during cellulose hydrolysis (Seidl et al., 2008). The strain was reported to produce more endoglucanase (CMCase) compared with exoglucanase (FPase) and β-glucosidase based on the binding affinity of many cellulases onto crystalline and/or amorphous region of cellulose during cellulose degradation (Ohmiya et al., 1997). It is among one of the industrial strains being used to produce high level of cellulases in SmF (Montenecourt and Eveleigh, 1977). The highest ever reported cellulases production by mutant *T. reesei* Rut-C30 was 290 IU/g of cellulose in SmF (Tangnu et al., 1981). Meanwhile, *T. reesei* RUT C-30 was reported to produce three to nine times higher CMCase than the one produced by mutant *Alternaria alternata* in SmF (Macris, 1984).

There are growing interests in research with respect to use lignocellulosic wastes as fermentation substrate. Lignocellulosic wastes can be used as a cheaper alternative substrate over pure cellulose in cellulase enzymes production by various types of microbes under solid state fermentation (SSF) (Pandey et al., 1999). Cellulase enzymes were reported to be produced from rice husk *Penicillium citrinum* (Kuhad and Singh, 1993), wheat bran by *T. reesei* (Smits et al., 1996), wheat straw by *Lentinus edodes* (Giovannozzisermanni et al., 1994), cassava waste by *Trichoderma harzianum* (Onilude, 1996) and from sago hampas by *Pleurotus sajor-caju* (Kumaran et al., 1997). Meanwhile, cellulase enzymes derived from SSF of lignocelluloses by mutant *T. reesei* RUT C-30 have been produced from sugar cane baggase (Mekala et al., 2008); wheat bran (Singhania et al., 2007; Sukumaran et al., 2008) and *Ocimum gratissimum* seed (Das et al., 2008).

In Malaysia, palm oil production is the major agri-cultural industry which has been estimated to generate about 6.93 million tonnes of dry oil palm empty fruit bunch (OPEFB) (Ng et al., 2011). Most of the generated OPEFB ends up in landfill or being burned. Only small amount of it was utilized as fertilizer (Lim, 2000), mulch (Hamdan et al., 1998) and to generate energy at the mill (Ma et al., 1993). OPEFB fiber generally contains 40 to 45% cellulose, 19 to 21% hemicellulose and 18 to 21% lignin (Astimar et al., 1997). Cellulase has been reported to be produced from OPEFB in liquid fermentation by *Chaetomium globosum* (Umikalsom et al., 1997) and by *T. harzianum* through SSF (Alam et al., 2009). However, cellulases production from SSF of OPEFB has not been much explored by mutant strain *T. reesei* RUT C-30.

In a fermentation process for cellulases production, there are various environmental and nutritional factors that may affect cellulases production under both SmF and SSF. Substrate concentration and pH have been reported to affect cellulase production from wheat straw by *Neurospora crassa* under SmF (Romero et al., 1999). Meanwhile, nitrogen from various sources were found to affect production of cellulase from treated oil palm empty fruit bunch fibre by *Chaetomium globosom* under SmF (Umikalsom et al., 1997). Incubation time has been reported to affect cellulase production by *Aspergillus niger* on various types of agricultural waste such as millet, guinea corn, rice husks and maize straw under SmF (Milala et al., 2005). Inoculum size has been reported to affect cellulase production by *Trichoderma* sp. on apple pomace under SSF (Sun et al., 2010) whereas, cellulase production by *Nectria catalinensis* has been found to be improved by the addition of surfactant tween-80 under SmF of microcrystalline cellulose (Pardo and Forchiassin, 1999).

Plackett-Burman design is a saturated fractional factorial design method that can be used to determine many factors affecting simultaneously the fermentation process without having to investigate all the possible combinations of the factors (Dürig and Fassihi, 1993). It has been used as a screening method to identify significant parameters in fermentation process in many biotechnology studies such as in lipase production by *Candida rugosa* (Rajendran et al., 2008), endoglucanase production from *Aspergillus terreus* (Youssef and Bereka, 2009), endo-polygalacturonase production by mutants of *A. niger* (Siva Kiran et al., 2010), glucoamylase production by a *Rhizopus microsporus* (Arnthong et al., 2010) and Ras farnesyl protein transferase inhibitor production from *Bacillus licheniformis* (Son et al., 1998).

In this study, physical and chemical parameters affecting endoglucanase production from oil palm empty fruit bunch (OPEFB) by *T. reesei* RUT C-30 under SSF were screened by using Plackett-Burman design. The information obtained from this study will be useful for further optimization process towards the development of

Table 1. Plackett Burman experimental design matrix with observed and predicted responses of different trials on endoglucanase production.

Standard order	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	CMCase(U/gds)	
												Observed	Predicted
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	1.607	1.600
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.219	0.404
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.15	0.000
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.097	0.000
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	1.946	1.637
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	0.149	0.406
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	1.826	2.042
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.804	0.789
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	1.233	1.252
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.978	0.752
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	0.007	0.347
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.582	0.406

cost-effective process for cellulases production.

MATERIALS AND METHODS

Preparation of inoculum

In this study, *T. reesei* RUT C-30 (ATCC 56765) was used as inoculum. It was grown on potato dextrose agar until the whole agar was fully covered with green spores on the fifth day of the incubation at 30°C. The spores were collected by pouring 5 ml of sterile mineral salt solution containing 5% (v/v) of mineral stock solution containing (g/l): KH_2PO_4 - 0.06, K_2HPO_4 - 0.04, $MgSO_4.7H_2O$ - 0.05, $CaCl_2.2H_2O$ - 0.0074, Ferric acid citrate - 0.0012, $ZnSO_4.7H_2O$ - 0.0006, $MnSO_4$ - 0.0005, $CuSO_4.5H_2O$ - 0.001 and Thiamine hydrochloride - 0.00001, into the sporulated plate. Then, a sterile hockey stick was used to dislodge spores into suspension mineral media. The spore suspension was then collected in a sterile 250 ml Erlenmeyer flask by filtering the suspension through a sterile Whatman No.1 filter paper on a funnel. The collected spore suspension was washed several times with sterile mineral salt solution by centrifugation at 6037 × *g* for 10 min for each washing. Serial dilutions of spores between 1×10^5 and 1×10^7 spores/ml were prepared by using sterile mineral salt solution. The numbers of spores within the washed suspension were counted under light microscope by using a neubauer haemocytometer counting chamber (GmBH, Germany). All procedures were carried out under aseptic condition.

Preparation of OPEFB

Shredded OPEFB was generously provided by Seri Ulu Langat Palm oil Mill Sdn. Bhd., Dengkil, Selangor, Malaysia. It was first washed with water 10 times to remove dust and oil from the OPEFB fibers before it was dried in 100°C oven for 24 h. Then, it was ground by a disc mill (Qingdao Dahua Double Circle FFC-23, China) and sieved through screens sizes between 215 and 425 µm (Retsch AS 300 control, Germany). The particles retained between these two screens were collected. The sieved OPEFB particles have a range size between 215 and 425 µm. Then, the sieved particles were again dried at 60°C overnight to remove remaining water. The particles were then cooled at room temperature before it was used as fermentation substrate.

Plackett-Burman design of parameters

The screening of factors which affect the activity of endoglucanase were monitored through Plackett-Burman design by using the Design-Expert version 6.0.8 software (State-Ease Inc., USA). A total number of 11 parameters were involved. The tested parameters were peptone concentration, urea concentration, ammonium sulfate concentration, calcium nitrate concentration, yeast extract concentration, tween 80 concentration, pH, incubation time, initial moisture content, inoculum size and substrate amount with each coded as X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, X_{10} and X_{11}, respectively. Plackett-Burman design having two-level design parameters for examining n (n= number of runs) parameters in $k = n+1$ (k= main effects) were used. The higher level value was coded as +1 and lower level was coded with -1 as shown in Table 1. Therefore, the design contained 12 runs with minimum value (-1) for each of the parameters on the last row of the design (Table 1). The nitrogen concentration, tween 80 concentration, pH level, duration of incubation time, level of initial moisture content, number of inoculums size and substrate amount that were used in this study were within the ranges used in Singhania et al. (2007) study. The effect of individual parameters on endoglucanase production was calculated based on the first order equation (Equation 1) as follows:

$$E=\beta_0+\sum\beta_i X_i \quad (1)$$

Where, E is the effect of parameter under study (endoglucanase activity); β_0 and β_i is the constant coefficients and X_i is the coded independent variables or factors. The significance of the fitted model and the significance effect of the individual parameters on endoglucanase production were determined through analysis of variance (ANOVA).

Preparation of culture flask for solid state fermentation (SSF)

An amount of sieved OPEFB particles (between 3 and 5 g as

Table 2. The levels of variables tested and their effect on endoglucanase production.

Code	Name	Low level (-1)	High level (+1)	Effect estimate	*P>F*
X_1	Peptone (g/l)	0.002	0.009	0.033	-[a]
X_2	Urea (g/l)	0.429	2.145	-0.084	-[a]
X_3	Yeast extract g/l)	0.002	0.008	-0.023	-[a]
X_4	$(NH_4)_2SO_4$ (g/l)	0.943	4.717	0.221	0.023
X_5	KNO_3 (g/l)	1.45	7.246	0.077	-[a]
X_6	Tween 80 (g/l)	0.1	0.5	-0.085	-[a]
X_7	Incubation time (day)	2	6	0.395	0.001
X_8	Substrate amount (g)	3	5	-0.13	-[a]
X_9	Inoculum size (spores/ml)	4	7	0.202	0.032
X_{10}	Initial moisture (%)	30	50	-0.424	0.001
X_{11}	pH	4	7	0.04	-[a]

[a] Terms not included in the model.

indicated by the experimental design in Table 2) were mixed with an appropriate amount (based on initial moisture level indicated by the experimental design in Table 2) of mineral salt solution in a 250 ml Erlenmeyer flask. The mineral salt solution contained appropriate amount of peptone concentration, urea concentration, ammonium sulfate concentration, calcium nitrate concentration, yeast extract concentration and Tween 80 concentration as indicated in Table 2 including 15% (v/v) of mineral stock solution. The initial pH of the prepared mineral salt solution was adjusted to a desired initial pH (as indicated in Table 2) as described by Mekala et al. (2008) by using 1 M potassium dihydrogen phosphate and 1 M potassium hydrogen phosphate. The well mixed substrate was then autoclaved at 121°C under 15 psi for 15 min.

Solid state fermentation and enzyme production

A 1 ml of diluted spore suspension with a desired inoculum size (between 1 x 10^5 and 1 x 10^7 spores/ml as indicated by the experimental design in Table 2) was inoculated into an Erlenmeyer flask containing sterilized fermentation substrate. Then, the Erlenmeyer flask was incubated at 30°C for duration of time as indicated by the experimental design (Table 2). At the end of each incubation period, enzyme was extracted with 0.05 M citrate buffer (pH 4.8) and was filtered with a Whatman No. 1 filter paper on a funnel connecting to a 250 ml Erlenmeyer flask. Filtrate was then centrifuged at 6037 × *g* for 10 min and the supernatant was then used for analysis.

Enzyme assay

The estimation of endocellulase activity within the collected supernatant was analyzed through carboxymethyl cellulase assay (CMCase), as described by Ghose (1987). One unit of cellulase activity was defined as the amount of enzyme required for liberating 1 mg of reducing sugar per milliliter per minute and was expressed as U/gds (Units per gram dry substrate).

RESULTS

Screening of significant parameters affecting endoglucanase production by Plackett-Burman design

T. reesei RUT C-30 produced 0.76 U/gds of CMCase activity from OPEFB moisturized with 45% (v/w) distilled water within 12 days (data not shown). Parameters of peptone concentration, urea concentration, ammonium sulfate concentration, calcium nitrate concentration, yeast extract concentration, Tween 80 concentration, pH, incubation time, initial moisture content, inoculum size and substrate amount were screened through Plackett-Burman design on their significant level on affecting endoglucanase activity by *T. reesei* RUT C-30 from OPEFB under SSF. The software has predicted that between 0 and 2.042 U/gds of CMCase activity was obtained (Table 1). However, experimental results indicate the values obtained were slightly lower than the predicted values which were between 0.007 and 1.946 U/gds of CMCase activity (Table 2).

Therefore, an increase of 2.6 fold of endoglucanase activity was obtained through screening experiment with Plackett-Burman design from endoglucanase activity produced on OPEFB moisturized with just 45% (v/w) of water.

ANOVA analysis, model establishment and effect estimate

The individual variant interaction with the model established as analyzed by ANOVA is shown in Table 1. The individual parameters interaction with the first order equation has established a model equation as follows (Equation 2):

$$E=0.8+0.22X_1+0.39X_2+0.2X_3-0.42X_4 \quad (2)$$

Coefficients estimated from the regression analysis from the table showed that four out of 11 parameters tested were significant in affecting endoglucanase activity with each having confidence level higher than 95%. The most significant factor affecting the enzyme production were initial moisture content (p= 0.001), incubation time (p=

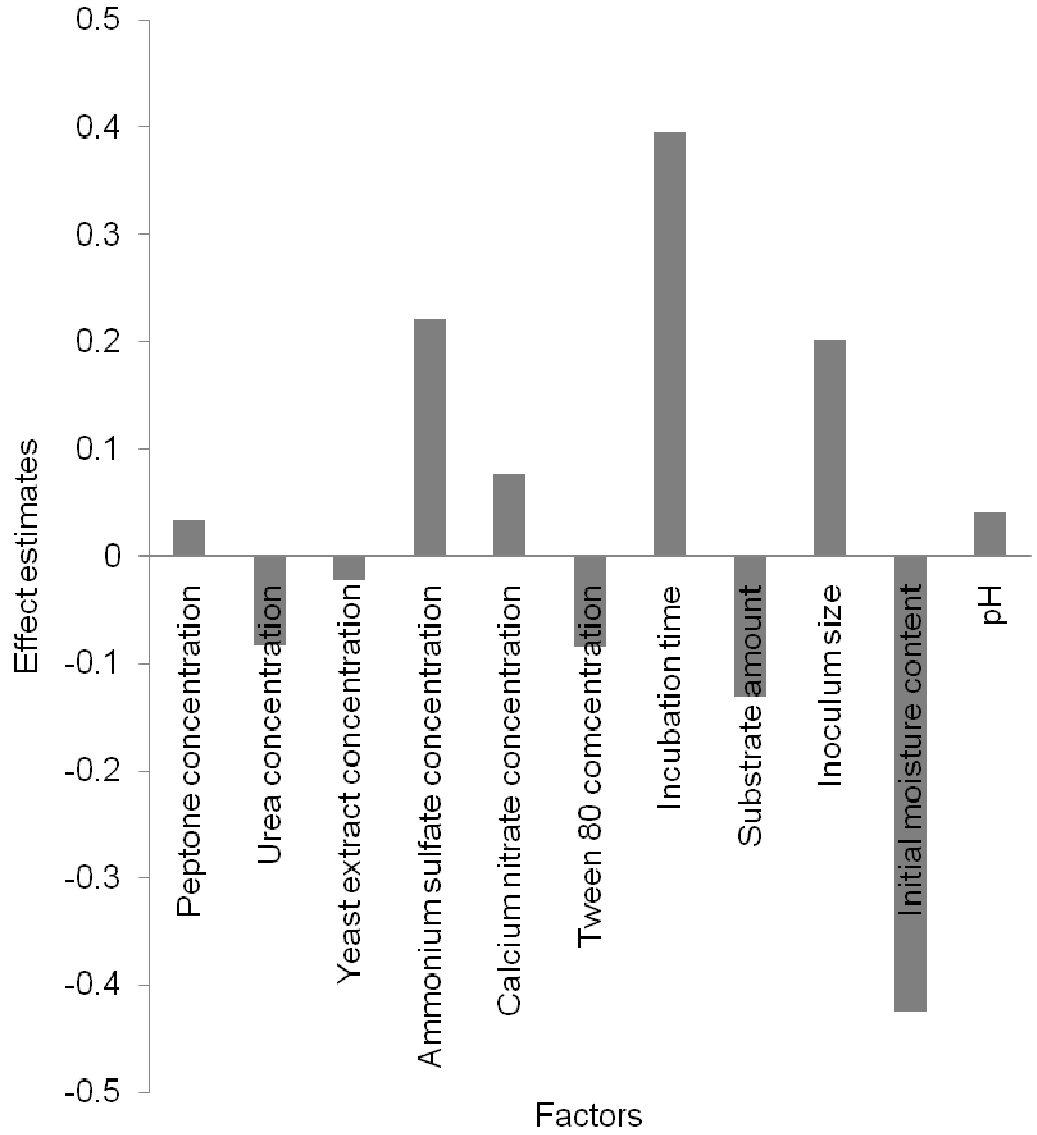

Figure 1. Effect of independent variables on endoglucanase production.

0.001), ammonium sulfate concentration (p= 0.023) and interaction of the system has a very low probability value ($P_{model}>F$= 0.0008) and *F*-value of 18.4 which indicated the reliability of the model equation in interpretation of the system interaction. The correlation measures for the estimation of the model regression equation are the multiple correlation coefficients *R* and R^2. The R^2 value of the model was 0.9132 which indicated that 91% of the variables content contributed positively to the respond and only 9% of the total variations were not explained by the endoglucanase activity. Meanwhile, the *R* value was 0.9556 which was closer to 1 indicating good correlation between the experimental and predicted value.

The effect estimate of the variance interaction with the model equation is stated in Table 2 and also illustrated in Figure 1. In sequence, from the most to the least parameters that affect positively on the endoglucanase activity in this study were incubation time, ammonium sulfate concentration, inoculum size, calcium nitrate concentration, pH and peptone concentration. Meanwhile, from the most to the least parameters that affect negatively on the endoglucanase activity were initial moisture, substrate amount, Tween 80 concentration, urea concentration and yeast extract concentration.

DISCUSSION

Parameters of initial moisture, ammonium concentration, incubation time and inoculum size have been recognized to affect endoglucanase activity from the crude sample derived from SSF of OPEFB by T. reesei RUTC-30 as

observed in this study.

The initial moisture content has been identified to be the most significant factors affecting endoglucanase activity in this study. This came in agreement with Singhania et al. (2007) and Mekala et al. (2008) findings in which they identified that the initial moisture content has affected cellulase activity in their study under SSF of wheat bran and sugar cane bagasse, respectively by T. reeseei RUT C-30. The levels of initial moisture content were believed to determine the level of water droplets present in between substrate particles in SSF. According to Sun et al. (2010), the water droplets may become the carrier for nutrients and air transfer between particles and microorganism in SSF in which, lower moisture level provided a lower degree of swelling. Whereas, higher water tension may decreases the particle porosity, changes particle structure, promotes development of stickiness, decreases diffusion, lowers oxygen transfer or increases formation of aerial hyphae. In other words, if the initial moisture level is too low or too high, lower level of product may be produced. Therefore, initial moisture content is a very important factor to be considered in the optimization process for endoglucanase production.

Incubation time has also been identified as the second factors in affecting endoglucanase activity in this study. In a study by Sun et al. (2010), enzyme activity from apple pomace by Trichoderma sp. was maximum at 120 h in SmF. Meanwhile, Ouyang et al. (2009) has reported that endocellulase production by T. reesei RUT C-30 reached a maximum concentration of 1.01 U/ml at 96 h in SmF. Therefore, it is believed that proper cultivation time allows maximum microorganism growth and product formation to a certain degree in a fermentation system.

Ammonium sulfate concentration was found to be the third factors in affecting endoglucanase activity tested in this study. Among the nitrogen compound tested, ammonium sulfate was observed to affect the endoglucanase activity the most and followed by calcium nitrate, urea, peptone and yeast extract. This was most probably due to the role played by ammonia as it was transported into the cell in the form of metabolite nitrate, nitrite, urea and amino acids (Mikeš et al., 1994) which triggered the synthesis of protein and cellulase (Spiridonov and Wilson, 1998). Apart from that some reports has supported that ammonium sulfate facilitated cellulase production in many fungus such as *Penicilium funiculosum, Myrothecium* sp., *Chaetomium cellulolyticum, T. reesei*, *A. niger, A. terreus* (Fadel, 2000) and *Rhizopus oryzae* (Fadel, 2000) and Rhizopus oryzae (Karmakar and Ray, 2010) in both submerged and solid state fermentation.

The initial concentration of spores inoculated at different concentrations into the SSF in this study has also influenced the endoglucanase activity. According to Jensen et al. (2002) fungal spores produced by SSF are typically more robust and have longer shelf-lives than those produced by liquid fermentation. The increase of the inoculum size results in the rapid culture growth and enzyme production due to fast degradation of the substrate fermentation (Sarao et al., 2010; Raimbault and Alazard, 1980). Therefore, with certain spores number cultivated into a SSF is believed to be able to improve the endoglucanase production.

Although, other parameters such as pH, Tween 80 concentration and substrate concentration has been identified to have less significant effect, however, they have played smaller roles in affecting endoglucanase activity in this study. The pH may contribute to cellulase production as the hydrogen ion affect enzyme production and control its stability (Kalra and Sandhu, 1986). Meanwhile, the hydrophorbicity character of surfactant Tween 80 is believed to have helped to minimize the absorption of cellulases by the lignin of the substrate particles (Chandra et al., 2007) which improve the detection of cellulases activity. According to Iqbal et al. (2010), low amount of substrate used in SSF system increased cellulase production and hydrolysis reaction on cellulose. This is because when a small amount of wet fermentation substrate was used in SSF system, better mass transfer of air and nutrients can be achieved due to the less compactness of the substrate used. This will then help to improve growth and production formation. Meanwhile, high amount of substrate may cause poor fungal growth and low cellulase production (Liu and Yang, 2007). The use of larger amount of substrate in SSF may increase the thickness of substrate bed. This will eventually limit the availability of air and nutrients within the static fermentation system for growth and product formation (Mitchell et al., 2006). Therefore, minimum amount of substrate will be considered to be used in future optimization process.

In this study, temperature was not included as one of the tested parameters affecting endoglucanase production. Instead, all cultures were incubated at 30°C based on optimum temperature suggested by Singhania et al. (2007) for cellulase production by *T.* reesei RUT C-30 from wheat bran. Singhania et al. (2007) also has claimed that different types of substrates does not ruled out as an interfering factor in controlling the optimal temperature of fungus for cellulase production. This claimed has been supported by Mekala et al. (2008) in which the same optimum temperature was preferred by *T.* reesei RUT C-30 to produce cellulase from sugar cane baggase.

Through this study, it has been suggested that lower initial moisture level, higher ammonium concentration, longer incubation time and a large inoculum number may further improve the endoglucanase production from OPEFB by *T.* reesei RUT C-30. It is also believed that there is a probability of interaction among these four identified significant factors in affecting endoglucanase production. Therefore, the interactions among these significant parameters will be investigated in the optimization process by using respond surface method

(RSM) in an effort to further improve endoglucanase production by *T.* reesei RUT C-30 on OPEFB under SSF in the near future.

Conclusions

The pre-optimization of endoglucanase production from SSF of OPEFB by *T. reesei* RUT C-30 has been successfully achieved through screening of significant parameters using Plackett-Burman design. Incubation time, ammonium sulfate, inoculum size and initial moisture content have been identified as significant factors affecting endoglucanase production in this study.

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Abbreviations

ANOVA, analysis of variance; **SSF,** solid state fermentation; **OPEFB,** oil palm empty fruit bunches; **SmF,** submerged fermentation; **CMCase,** carboxymethyl cellulose; **FPase,** Filter paperase; ***T. reesei,*** *Trichoderma reesei;* ***T. harzianum****, Trichoderma harzianum;* ***A. niger,*** *Aspergillus niger;* ***A. terreus****, Aspergillus terreus,* **sp.,**species; **β-glucosidase,** Beta-glucosidase; **U/gds,** Unit per gram dry substrate; **ATCC,** American Type Culture Collection; **M,** molarity; **v/v**, volume per volume; **v/w,** volume per weight, **g**, gram; **g/L**, gram per litre, **µm**, micrometer; ^{0}**C**, degrees Celsius, **min, minute; h,** hour; **ml,** milliliter; KH_2PO_4, potassium dihydrogen phosphate; K_2HPO_4**,** dipotassium hydrogen phosphate; $MgSO_4.7H_2O$,magnesium sulfate heptahydrate; $CaCl_2.2H_2O$**,** calcium chloride dehydrate; $ZnSO_4.7H_2O$**,** zinc sulfate heptahydrate; $MnSO_4$, manganese sulphate; $CuSO_4.5H_2O$**,** copper (II) sulfate pentahydrate; ***g,*** gravity; **Psi,** pressure

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