Research Article

Optimization of Endoglucanase Synthesis by *Trichoderma harzianum* via Taguchi Approach

Hwee Li Teo, Roswanira Abdul Wahab *

Universiti Teknologi Malaysia, Skudai 81310, Malaysia

ABSTRACT

The oil palm biomass produced by plantations and mills in Malaysia is the largest contributor to the nation's agro-waste, with the oil palm leaves (OPL) topping the list. Nevertheless, the surplus of OPL might have applicability as the substrate for cultivating lignocellulolytic bio-degraders. *Ipso facto*, this study employed raw OPL as the carbon source for cultivating *Trichoderma harzianum* under solid-state fermentation (SSF). Optimizing the SSF process using the Taguchi orthogonal design to produce endoglucanase (CMCase) successfully established the optimal fermentation conditions: 7.00×10^8 spore/g inoculum size, 50% moisture content, pH 12 Mandel's medium, with 3-day incubation at 40°C. The crude enzyme cocktail exhibited the corresponding maximum activity of 417.49 ± 6.61 U/g CMCase. The SSF process parameters significantly affected the enzymatic activities, namely, moisture content, inoculum size, and initial pH (p-value < 0.05). It can be construed that the high extracellular CMCase activity of the *T. harzianum* crude enzyme cocktail could be useful in accelerating the saccharification of cellulose for biofuel-and nanocellulose production.

Keywords: Cellulase, Endoglucanase, Oil palm leaves, Solid-state fermentation, Taguchi orthogonal design, Trichoderma harzianum

Introduction

Cellulase, the vital enzyme in saccharification of insoluble cellulosic polymer, proves its multifunctionality in various application fields covering textile, paper and pulp, food, and biofuel production industries [1-3]. Differences in the specificities or active sites shape the cellulases into three main groups, namely endoglucanase (EC 3.2.1.4), exoglucanase, or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). The random hydrolytic action of endoglucanase (CMCase) on the internal *o*-glycosidic bonds of the amorphous cellulose region results in the formation of polysaccharides and oligosaccharides [4, 5]. The formation of new ends on the poly- or oligo-saccharides released enables the subsequent binding to the exoglucanase [3, 4]. This shows the synergism between endos- and exo- glucanases in the cellulose depolymerization process.

Undeniably, commercialized cellulases and specific endoglucanases are commonly applied in both industrial and research fields, owing to the minimal reaction time. However, the expenses on endoglucanase production could further add to the manufacturing cost and even become the key constraint in the process [3]. Cellulase production can be achieved via solid-state fermentation (SSF), where the simultaneous saccharification and fermentation of solid substrate induces the secretion of enzymes into the environment with little or no water content by the fungi species. Significantly, the metabolic process of fungal growth, related to its activity in substrate degradation, depends on environmental factors. Therefore, researchers have been working on optimizing the fermentation process in synthesizing fungal cellulases [6-9]. The fermentation process can be manipulated by various parameters, including the fungi species, inoculum size, carbon source, temperature, and humidity.

In an enzyme-assisted reaction, fungi are usually opted for due to their outstanding ability to

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depolymerize the amorphous components of cellulose compared to the bacteria. Plus, fungi are known extracellular enzyme secretors where the cellulases are produced outside the fungi bodies [2]. In this context, *Trichoderma* species have been widely studied and applied in industrial cellulase synthesis [2, 10], owing to their good performance in cellulose degradation. Literally, *Trichoderma harzianum* is known as the prolific producer of cellulases and the secretion of well-balanced cellulolytic complexes [11].

Lignocellulolytic biomass waste is commonly used as the readily available substrate or carbon source for fungal fermentation activity. As established by the previous reports, various kinds of biomass waste such as sugarcane bagasse [12], wheat bran [12, 13], corn straw [7], groundnut shell [14], and oil palm biomass [8, 9, 15, 16] would result in enzyme production showing different enzymatic activities. The higher cellulose content in oil palm leaves (OPL) (40%) than other biomass such as trunks (34%) and empty fruit bunches (23%) [17] shows its potential to produce cellulolytic and xylanolytic enzymes. The feasibility of OPL in the fermentation process was demonstrated by Ezeilo et al. [8] in the cellulase synthesis by *Trichoderma asperellum*. However, the OPL employed in this study has a more accessible particle size, which could boost endoglucanase production in a shorter period.

Optimization of endoglucanase production is a precondition for realizing large-scale industrial production. The traditional optimization technique, such as the one-factor-at-a-time (OFAT) design, can be time-consuming with the mass consumption of experimental materials and chemicals. A previous study optimized endoglucanase production by OFAT for four process parameters, set at 6 – 10 levels, which carried a sum of 35 experimental runs [18]. Pertinently, the statistical approach, i.e., Response Surface Methodology (RSM), can be an alternative investigation tool in the mathematical analysis of multivariate systems, allowing better observation of the interactions between factors [19]. The design of the experiment (DOE) selected in this study was the Taguchi orthogonal array (OA) which investigates the correlations between a set of independent variables at a specific multi-level system [20]. It is advantageous to attain consistency and reliability at minimal experimental expenses with lesser experimental runs than the commonly-applied RSM

methods, viz. central composite design (CCD) and Box-Behnken design (BBD). Recognized as a simple statistical tool, Taguchi technique has been widely applied in multiple studies [21-23] for the enhancement on protocol design and process efficacy.

This study aimed to statistically optimize the process parameters of SSF by *T. harzianum* to favour endoglucanase production. The Taguchi L16 array design of the Design Expert v13.0 software was used to optimize five SSF parameters (inoculum size, moisture content, initial medium pH, incubation period, and temperature) to assess the enzymatic activity (U/g). The established optimal SSF process parameters were then used to maximize endoglucanase production for further application in nanocellulose synthesis. To the best knowledge, Taguchi OA-assisted endoglucanase production by *T. harzianum* has never been reported before.

Material and Methods
Substrate preparation

Oil palm leaves (OPL) employed in this study were collected from an oil palm plantation in Universiti Teknologi Malaysia (UTM), Johor, Malaysia. The petioles of the OPL were removed, and only the leaflets were subjected to washing, drying, and grinding for further use. The OPL leaflets were ground using a grinder (Wellmac RT-08, Taiwan) and sieved using an electronic sieving shaker (Endecotts Sieve Shaker Minor 200, UK). The non-treated OPL (63 – 106 μm) was sterilized at 121°C and 20 psi using an autoclave (HICLAVE HVE-50, Hirayama, Japan) before fermentation.

Inoculum preparation

The isolated strain of *T. harzianum* from the soil samples in the Mazandaran province, Iran [24], was used in this study. The fungal strain was cultured on potato dextrose agar (PDA) for 7 days at 30°C. Fungal spores of 7-day-old cultures were harvested using sterile Tween-80 (1% v/v) and centrifuged at 4000 rpm and 4°C for 20 min (Universal 32R Hettich, Germany). The pellet was mixed with sterile ultrapure water by a serial dilution, and the spore count was estimated using a haemocytometer under $40 \times$ magnification (Lieca Light Microscope, CME).

Table 1. Selected SSF process variables and their respective levels in Taguchi array

Factor	Symbol	Unit	Coded Level			
			1	2	3	4
Inoculum size	A	$\times 10^8$ spore/g	1	3	5	7
Moisture level	B	%	50	60	70	80
Initial pH	C	pH	3	6	9	12
Period	D	day	1	3	5	7
Temperature	E	°C	20	30	40	50

Solid-state fermentation (SSF)

The SSF was conducted in a 250 mL Erlenmeyer flask (HmBG) consisting of sterile OPL powder (10.0 g) and Mandel's medium (pH 3, 6, 9 and 12) to achieve a moisture content of 50 – 90%. Before fermentation, steam sterilization at 121°C and 20 psi were conducted for the substrate and Mandel's medium. Spore suspension with a concentration of $1 – 7 \times 108$ spore/g was inoculated into each SSF flask under an aseptic environment and incubated between 20 – 50°C for 1 – 7 days. After a specific fermentation period, the content was extracted using sodium acetate buffer (pH 5, 0.05M), followed by centrifugation at 4000 rpm and 4°C for 30 min. The cell-free supernatant was used as the crude enzyme and stored at 4°C until further use.

Taguchi orthogonal array design

The Design Expert, version 13 software (State Ease, Statistical Made Easy, MN, USA) was used in this study for the statistical analysis. The Taguchi method was selected to statistically optimize endoglucanase production by SSF of *T. harzianum* on raw OPL powder. The five-factor-four-level Taguchi L_{16} array in this study involved five selected process variables, i.e., (A) inoculum size, (B) moisture level, (C) initial pH, (D) incubation period, and (E) temperature, as presented in Table 1.

The objective function was given by the signal-to-noise ratio (S/N), where the signal denotes the mean and the noise denotes the standard variation. The analysis of variance (ANOVA) and regression analysis was performed by the Design Expert v13.0 software to investigate the fitness model and evaluate the model's statistical and each factor's significance.

Endoglucanase activity assay

The endoglucanase (CMCase) activity was estimated using carboxymethyl cellulose (1.0% w/v) (Sigma Chemical Co. St Louis, MO, USA) as the substrate, based on a protocol by Ghose [25]. The liberated reducing sugars in each assay were estimated by the 3, 5-dinitrosalicylic acid (DNS) method, measured at the absorbance of 540 nm. One unit of CMCase activity is expressed as the amount of enzyme needed for the liberation of 1 μmole of glucose per minute under the assay conditions.

Results and Discussion

Nutritional facts and the biochemical nature of the living environment directly impact the enhancement of metabolite production by the microorganism [3]. The Taguchi L_{16} OA was employed to identify the process parameters that most influenced the CMCase production via the SSF of *T. harzianum* on raw OPL powder.

The results (Table 2) suggest that the fermentation medium's initial moisture level was the highly significant factor (P-value < 0.0001) and was largest contributing factor (74.5%) in CMCase production by *T. harzianum*. The other factors, initial pH of the medium, inoculum size, incubation period, and temperature, contributed

Table 2. Results of ANOVA

Source	Sum of square	DF**	F-value	P-value	Percent (%)
Model	144000	5	14.15	0.0003*	
A	10703.5	1	5.26	0.0448*	7.43
B	107300	1	52.71	< 0.0001*	74.51
C	20856.4	1	10.25	0.0095*	14.48
D	5128.7	1	2.52	0.1435	3.56
E	10.9	1	0.0054	0.9431	0.01
Total	143999.5	10			

Remarks: *Indicate the source's significance with a $p < 0.05$ a cording to the ANOVA test.
**Degree of freedom.

14.48%, 7.43%, 3.56%, and 0.01%, respectively to the maximal CMCase production by the studied species. The other two factors with significant effects in this designed model were the initial pH (*P*-value = 0.0095) and inoculum size (*P*-value = 0.0448).

The regression analysis for this OA model were tabulated in Table 3. The acceptable R^2 value of 0.8761 shows that this OA model is a good-fitting model. The signal-to-noise ratio (S/N) is 12.0658, which is acceptable and indicates an adequate signal. Thus, this model is suitable for data navigation in the designed space. The mathematical equation can be used to represent this Taguchi model for the prediction on the CMCase production by *T. harzianum*, as expressed in Equation (1).

$$CMCase\ activity\ \left(\frac{U}{g}\right)$$
$$= 225.79 + 34.7A - 109.87B + 48.44C - 24.02D + 1.11E \dots\dots\dots\dots\dots (1)$$

Table 3. Regression analysis of Taguchi L$_{16}$ array

Parameter	Value
Mean (U/g)	225.79
Standard Deviation (U/g)	45.12
R^2	0.8761
Adjusted R^2	0.8142
Predicted R^2	0.7182
Coefficient of Variance (%)	19.98
S/N Ratio	12.0658

The response (actual and predicted CMCase activity) of each experimental run is presented in Table 4. Within the design space, the maximal CMCase activity (417. 49 ± 6.61 U/g) by *T. harzianum* is represented by Run 13, with the experimental conditions as follows: inoculum size of 7.00×10^8 spore/g, initial moisture content of 50%, pH 12 Mandel's medium, and 3-day incubation at 40°C. Notably, the SSF incubation period in this study was significantly shorter than in previous studies, i.e., 5 – 6 days [7, 26]. This is due to the smaller substrate particle size used. The finer particle size improves the fungi accessibility onto the substrate.

Synergistic effects can be observed by the factors, inoculum size, initial medium pH, and incubation temperature for CMCase production by *T. harzianum*, as indicated by the (+) sign in Equation 1 and the positive gradient as in the perturba-

Table 4. The Taguchi L$_{16}$ OA for the CMCase produced by *T. harzianum*

| Run	Parameters					CMCase activity (U/g)	
	A	B	C	D	E	Actual	Predicted
1	1	1	1	1	1	277.43 ± 3.02	275.43
2	1	2	2	2	2	233.17 ± 3.77	219.21
3	1	3	3	3	3	186.09 ± 3.72	162.98
4	1	4	4	4	4	105.70 ± 4.18	106.75
5	2	1	2	3	4	241.48 ± 6.86	301.05
6	2	2	1	4	3	177.33 ± 6.60	178.76
7	2	3	4	1	2	303.06 ± 7.91	249.70
8	2	4	3	2	1	77.94 ± 5.17	127.41
9	3	1	3	4	2	351.03 ± 7.87	338.98
10	3	2	4	3	1	321.68 ± 8.85	313.31
11	3	3	1	2	4	214.33 ± 8.64	161.41
12	3	4	2	1	3	62.53 ± 4.41	135.74
13	4	1	4	2	3	417.49 ± 6.61	427.17
14	4	2	3	1	4	346.37 ± 6.73	338.39
15	4	3	2	4	1	161.05 ± 7.11	182.60
16	4	4	1	3	2	136.01 ± 7.94	93.81
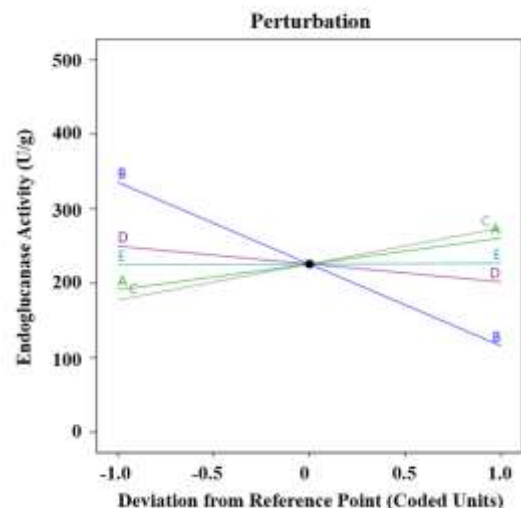
Figure 1. Perturbation graph of the design model

tion plot in Figure 1. Meanwhile, both initial moisture content and incubation period are antagonistic to the response, as seen in the (−) sign in Equation 1 and the declining line in Figure 1.

The interactions between two significant factors influencing the model's response can be demonstrated by contour and 3D-surface plots. The colour intensity from red (the highest) to blue (the lowest) represents the value of a response when two axes (factors) meet at a point. Notably, the inoculum size (A) antagonistically interacted with the initial moisture level (B). The maximal
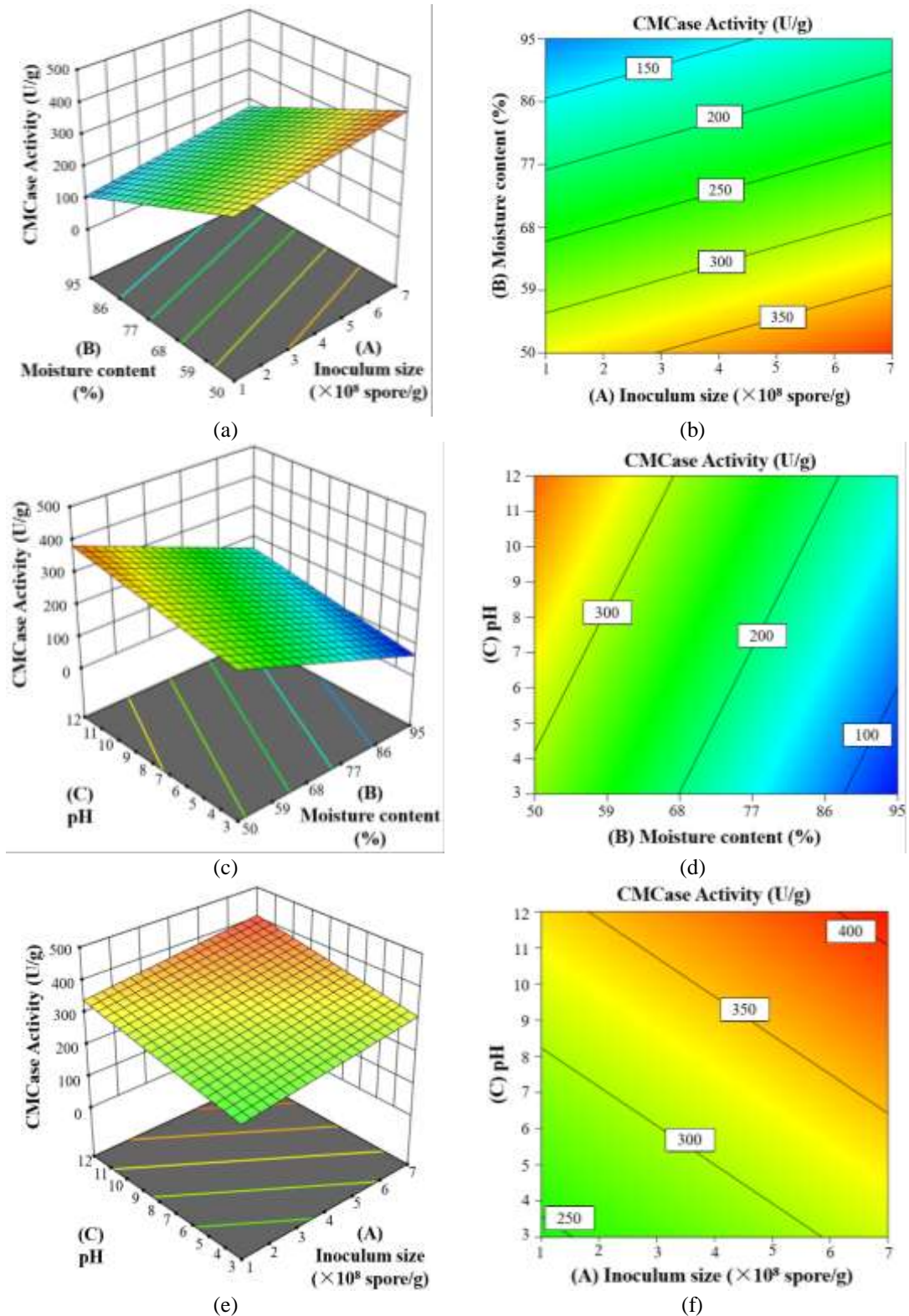
(a)

(b)

(c)

(d)

(e)

(f)

Figure 2. Contour and 3-D surface plot of interacting factors

CMCase activity is higher than the highest inoculum size (7.00×10^8 spore/g) and at minimal moisture level (50%) (Figure 2a – b). The optimal moisture level of 50% is in accordance with the

earlier research using *Trichoderma* species, where the recommended moisture content was in a range of 40 – 60% [18]. The same interactive trend is observed between the initial moisture level (B)

and the initial medium pH (C) where the CMCase production favours a fermentation condition with low moisture content but high pH medium (Figure 2c – d). Researchers reported that the optimum pH for endoglucanase production by *Trichoderma* spp. was at pH > 5.5. This value supports most of the industrial applications under an alkaline situation [26, 27]. Interestingly, mutual additive effect between the inoculum size (A) and the initial pH (C) was observed by the red colour, which represents the maximum pH and inoculum size (Figure 2e – f). The suitable inoculum size is vital in ensuring the ideal rate of metabolic activities of fungi in the fermentation process. This is because a too low spore concentration limits the mycelial production, hence slows down the enzyme secretion. Additionally, it was previously reported that the SSF of *Trichoderma* was optimum at 30 °C, which gave rise a maximum CMCase production at 160.67 U/mL [26].

Table 5. Validation of the model for CMCase production.

Factor	Set 1	Set 2
A ($\times 10^8$ spore/g)	3.00	7.00
B (%)	50	50
C (pH)	6	12
D (day)	5	5
E (°C)	50	30.34
Actual CMCase activity (%)	301.097 ± 9.18	409.304 ± 9.75
Predicted CMCase activity (%)	297.457	417.691
Deviation (%)	1.21	2.05

The mathematical calculation proposed the optimum SSF parameter conditions after analysing the ANOVA results. The experimental best conditions are identified: inoculum size of 6.98 × 10^8 spore/g, initial moisture content of 50.29%, pH 10.48 Mandel's medium, and 1.75-day incubation at 32.11°C. It is statistically predicted that the SSF by *T. harzianum* under the abovementioned conditions should produce the CMCase with the highest activity of 418.667 U/g. It was indicated that a 2.73-fold increase production of endoglucanase was observed compared to the produced CMCase, under an unoptimized conditions (153.467 ± 6.897 U/g). The reported SSF parameters using *Trichoderma* species were initial moisture content of 40 – 60% [18], pH > 5.5 Mandel's medium [27], and incubation temperature of 30 –

32 °C [7, 26], which agreed well with the with the current study.

The model is validated by running the simulated sets given by the model as a solution to the optimal CMCase production. Two sets of experimental conditions (Table 5) were selected based on the experiment's convenience. As seen in Table 5, the actual experimental CMCase produced by *T. harzianum* in both sets 1 and 2 had an activity of 301.097 ± 9.18 U/g and 409.304 ± 9.75 U/g, respectively. The deviations of the actual and predicted activities in both validation sets are within the acceptance range of 5.0%. Hence, it could be concluded that this Taguchi OA model is suitable for optimizing the SSF conditions to maximize cellulase production by *T. harzianum* with high CMCase activity.

Conclusion

This study demonstrated the feasibility of finer OPL powder (63 – 106 μm) as substrate to produce high level of CMCase in shorter incubation period of SSF by *T. harzianum*. The statistical optimization of five SSF parameters in favouring the CMCase production was successfully performed by the Taguchi L$_{16}$ OA method. It was ascertained that the optimal SSF conditions for the maximal production of CMCase by *T. harzianum* were using 7.00 × 10^8 spore/g inoculum size, a 50% initial moisture content, a pH 12 Mandel's medium, and 3 days incubation at 40°C. The most contributing and significant factor was the production medium's initial moisture content, with the significant concomitant contribution of inoculum size and initial medium pH in maximizing the CMCase production. The SSF optimized experimental parameters demonstrated an enhanced production of CMCase with an activity of 417.49 ± 6.61 U/g. It can be construed that this approach may prove promising for future applications in enzymatic saccharification of lignocellulosic residues for conversion into biofuels or nano-materials.

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