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Cellulase Production by *Pycnoporus sanguineus* on Oil Palm Residues through Pretreatment and Optimization Study

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Abstract: The ever expanding trend of the palm oil industries in Malaysia brings about environmental concern with various parties calling for global practice of sustainable palm oil production. In as much as researches in processing technologies are ongoing, utilization of palm oil industries' residues as a substrate for cellulases production has received little attention. This study addressed on the effect of pressed pericarp fibers sterilization on cellulase production by *Pycnoporus sanguineus* grown in shake flask culture using a statistical approach. Optimum condition was obtained in 70% (v/v) palm oil mill effluent supplemented with 6 g L^{-1} sterilized palm pressed fibers at pH 6.77 and 350 rpm with CMCase, FPase and β-glucosidase activities and net changes of biomass and suspended solid at 50.11, 29.01, 5.58 IU mL^{-1} and 2.49 g L^{-1}, respectively. Under such conditions, the predicted maximum growth and cellulolytic enzyme production were in good agreement with the experimental data with 0.016-0.358% error.

Key words: *Pycnoporus sanguineus*, cellulase production, oil palm residues, response surface methodology

INTRODUCTION

The Malaysian palm oil industry has grown aggressively over the last few decades and became the world's largest producer and exporter of palm oil. With the most ideal climate conditions for palm oil cultivation, it is not surprising that the crop's full potential has been realized and exploited (Ibarahim *et al*., 1999; Yusoff, 2006). In fact, Malaysia was the world's largest producer and exporter of palm oil with 13.4 million tons (49%) of world production and 12.2 million tons (58%) of total world exports, respectively (Ahmad *et al*., 2005).

With such high production of oil palm, various types of by-products and wastes are produced from the palm oil processing such as liquid effluent (e.g., POME), gaseous emission (e.g., smoke and dust) and solid waste materials (e.g., empty fruit bunches, potash ash, palm kernel, fiber and shell) (Ibarahim *et al*., 1999; Yusoff, 2006; Wu *et al*., 2007). Ramli *et al*. (2002) reported that palm pressed fibers (PPF) were clean, non-toxic, non-carcinogenic, non-hazardous biodegradable material and also free from pesticides and soft parenchyma cells. While raw palm oil mill effluent (POME) was a colloidal suspension containing 95-96% water, 0.6-0.7% oil and 4-5% total solids including 2-4% suspended solids. It is estimated that for 1 ton of crude palm oil produced, 5-7.5 tons of water are required and more than 50% of the water will end up as POME (Ahmad *et al*., 2005; Wu *et al*., 2007). Usually, the raw POME is a thick brownish liquid discharged at 80-90°C. It is acidic (pH 4-5) and non-toxic since no chemicals is added during oil extraction (Ahmad *et al*., 2003; Okwute and Isu, 2007). The characteristics of palm oil mill effluent varied widely (Choorit and Wisarnwan, 2007) from day to day and from plant to plant, depending on the raw material (fresh fruit brunches) and the efficiency of the machines involved during the extraction process as well as the operational control. So, the treatment efficiency of POME using different samples may not the same due to the variations in characteristics.

Previously, it was reported that POME and PPF could be used as a substrate for cellulases production (Mashitah *et al*., 2002). And the demand for cellulases is growing more rapidly than ever before and this demand acts as the main driving force for research on cellulose degrading enzymes. Currently, they are used in the textile industry for cotton softening and denim finishing; in detergent market for colour care, cleaning and antideposition; in the food industry for mashing; in pulp and paper industry for deinking, drainage improvement and fibre modification (Kirk *et al*., 2002; Cherry and Fidantsef, 2003).

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White-rot fungi, also known as wood degrading fungi, were reported to have unique ability to degrade all components of the lignocellulosic material by a range of non-specific extracellular enzyme (Park *et al*., 2006). In order to improve the degrading process, effective pretreatment methods need to be carried out based on some requirements (Kang *et al*., 2004; Ji *et al*., 2008): (a) production of reactive cellulosic fiber for enzymatic attack, (b) avoiding destruction of hemicelluloses and cellulose, (c) avoiding formation of possible inhibitors for hydrolysis enzymes and fermenting microorganisms, (d) minimizing the energy demand (Keller *et al*., 2003), (e) reducing the cost of size reduction for feedstock, (f) reducing the cost for material construction of pretreatment reactors (Chum *et al*., 1985), (g) producing less residues and (h) consumption of little chemical and using a cheap chemical (Taherzadeh and Karimi, 2007). As an example, high pressure steaming process would increase the accessible surface area and pores size; while decreased the cellulase crystallinity and also degree of polymerization (Taherzadeh and Karimi, 2007). However, pretreatment of oil palm residues, such as PPF for subsequent use as a substrate of cellulase production has received little attention. In fact, its degradation using white rot fungi is less reported. Hence, the present study aims to investigate the effectiveness of sterilized PPF to be used as a substrate with POME for the production of cellulases by *Pycnoporus sanguineus* (*Pyc. sanguineus*). Optimization of fermentation parameters using response surface methodology coupled with central composite design were also highlighted.

MATERIALS AND METHODS

Fungus strain: The fungal strain, *Pyc. sanguineus* was obtained from Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. This stock culture was grown on agar slants comprised of 20 g L^{-1} glucose, 10 g L^{-1} yeast extract and 10 g L^{-1} malt extract agar. The pH of the mixture was adjusted to 7.0±0.2 prior autoclaving at 121°C for 15 min. The culture was grown at 30°C and maintained on agar slants prior to the fermentation process.

Preparation of mycelia suspension: Mycelia suspension was prepared by suspending mycelia discs from 5 days *Pyc. sanguineus* culture plate in sampling bottle containing sterilized distilled water and a few drops of Tween 80. The disks of 0.5 cm diameter was punched on the mycelia mats of the agar plate using sterilized cork borer. A total of 10 discs for every 100 mL of sterile distilled water were vortexed for 5 min so that the mycelia suspensions became homogenous.

Table 1: Proximate composition of POME and PPF

	Range (% dry weight basic)	
Components	POME	PPF
pH	4.0-5.0	-
Cellulose	0.25-8.0	1.70-13.97
Lignin	2.90-7.89	24.90-25.60
Crude protein	11.11-16.66	6.00-6.70
Crude fiber	14.44-16.66	35.00-40.00
Moisture content	8.0-12.0	5.00-5.25
Ash	18.88-22.22	10.20-10.40
Nitrogen free extract	28.88-53.33	35.40-41.79
Suspended solid*	25.0-29.0	-

*In g L^{-1} of palm oil mill effluent

Fermentation condition: Ten milliter (10% v/v) of the mycelia suspension was added to 90 mL medium containing different POME concentration in 250 mL Erlenmeyer flasks. POME was supplied by United Oil Palm Industrial Sdn. Bhd., Nibong Tebal, Malaysia. The proximate analysis of POME was determined in order to utilize it as a carbon source as well as trace elements source prior to the fermentation process (Table 1). Before transferring the mycelia into the culture media, the POME-based-media need to be autoclaved at 121°C for 15 min. The culture was incubated at 30±1°C, pH 7 in an incubator shaker at 150 rpm for 7 days. The culture broth was then harvested and centrifuged at 4000 rpm for 20 min. The supernatant obtained is hereafter referred to as crude enzyme extract and was used for cellulolytic activities analysis, reducing sugar estimation and total sugar estimation. The residues were used to determine the net changes of biomass and suspended solid. Three replicates were run simultaneously.

Effects of different POME concentration on growth and cellulases production: The effects of different POME concentration on cellulase production by *Pyc. sanguineus* was studied in the range of 30% (v/v) to 90% (v/v). The POME-based-media (90 mL) was adjusted to pH 7.0±0.2 using 1 M NaOH and 1 M HCl and incubated with 10 mL cell suspension for 7 days at 30±1°C and 150 rpm. Samples were harvested and analyzed for cellulolytic and laccase activities, growth (net changes of biomass and suspended solids), sugar concentration and cellulose conversion.

Effects of PPF pretreatment on cellulase production by *Pycnoporus sanguineus*: PPF used in this experiment was supplied by United Oil Palm Industrial Sdn. Bhd., Nibong Tebal, Malaysia. The chemical composition of PPF used as substrate was examined and result shown in Table 1. In this study, ground PPF were passed through 250 μm mesh size and kept until used. Then, the effect of PPF

pretreatment on cellulase production were carried out in two series: series 1- the media comprised of POME supplemented with 5 g L^{-1} untreated-PPF and series 2- the media was POME supplemented with 5 g L^{-1} sterilized-PPF. The PPF was previously sterilized at 121°C for 15 min prior using it in the fermentation process. The concentration of POME used in this study was based on the optimum concentration obtained in the previous section. Working on the premise that cellulase is a substrate induced enzymes, POME supplemented with sterilized-PPF of varying concentration (4-8 g L^{-1}) were carried out in an attempt to increase cellulase production by *Pyc. sanguineus*.

Experimental design using response surface methodology (RSM): RSM is a collection of mathematical and statistical techniques that are useful for the modelling and analysis of problem in which a response of interest is influenced by several variables and the objective is to optimize this response (Rodrigues *et al*., 2006; Zinatizadeh *et al*., 2007). The RSM used in this present study was a central composite face-centered design involving two different factors, pH and agitation. The growth and cellulase production by *Pyc. sanguineus* on POME-based-medium was assessed based on the face-centered experimental plan as shown in Table 2. The results were analyzed using Analysis of Variance (ANOVA) by Design Expert 6.0.6 software. Three-dimensional plots and their respective contour plots were obtained based on the effect of the levels of the two factors. From these three-dimensional plots, the simultaneous interactions of the two factors on the responses were studied. The optimum region was also identified based on the main parameters in the overlay plot. The experiment was repeated for 5 times and each result obtained was compared with the predicted values in order to determine the validity of the model.

Analytical method: The cellulolytic activities and laccase productivity were analyzed using the method as described by Ghose (1987) and Alves-Garcia *et al*. (2006). The reducing sugar was estimated based on DNS method (Ghose, 1987) whereas the total sugar estimation was done according to R-P method (Taylor, 1995). Besides, cellulose content was determined using a slight modification of Updegraff method (Abdulrazzak *et al*., 2006).

Table 2: Variables and levels used for a central composite design

Independent variable	Symbol		Level		
	Actual	Coded	-1	0	+1
pH	x1	A	5	7	9
Agitation (rpm)	x2	B	150	250	350

RESULTS AND DISCUSSIONS

Effects of different POME concentration on growth and cellulases production by *Pycnoporus sanguineus* Fig. 1 shows the effect of different POME concentration on growth and cellulolytic activities by *Pyc. sanguineus* in shake flask culture. Higher the POME concentration higher was the cellulase (CMCase, FPase and β-glucosidase) and laccase activities. Beyond 70% (v/v) POME, a reverse trend was observed. This could be due to the substrate inhibition at higher substrate concentration, thus resulting in decreased enzyme activity. Due to that, the 70% (v/v) of POME was used for further studies.

The results also show that the β-glucosidase activity was the lowest among all the cellulolytic enzymes tested. According to Juhasz *et al*. (2005), endoglucanases (CMCase) randomly attacked cellulose chains and released cello-oligosacchrides; exoglucanases (FPase) cleave cellobiose units from the end of cellulose chains and β-glucosidase converts the resulting cellobiose to glucose. In fact, the β-glucosidase activity was found to be low. This is in agreement with the result of the present study in which the enzyme activity was only 6.36 IU mL^{-1}. For the laccase activity, it was found to increase gradually during fermentation and the maximum activity (8.0 IU mL^{-1}) was found to be in 70% (v/v) POME in shake flask culture. According to Vikineswary *et al*. (2006) and Munusamy *et al*. (2008), *Pyc. sanguineus* have also been reported to produce laccase as the sole lignolytic enzyme. Being a white rot fungus, *Pyc. sanguineus* are known to have evolved complex enzymatic machinery to degrade lignin, the non-hydrolysable part of wood, to any extent (Garzillo *et al*., 1998). Due to that fact, reducing sugar was produced for thr first 50 h and then reduced gradually till then end of the fermentation period. Thus showing that inhibition might have occurred under such condition.

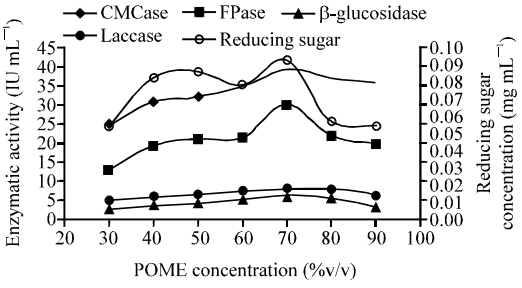

Fig. 1: Cellulolytic enzyme and reducing sugar production by *Pyc. sanguineus* in various POME concentrations after incubation for 7 days

Table 3: Effect of sterilization on growth, cellulolytic and laccase activities, sugar and cellulose conversion by *Pyc. sanguineus* in 70% (v/v) POME based media supplemented with PPF after 7 days of fermentation period

Substrate	70% (v/v) POME	70% (v/v) POME+untreated PPF	70% (v/v) POME+sterilized PPF
Net changes of biomass and suspended solids (g L^{-1})	4.49±0.81	9.62±0.82	15.72±0.71
Cellulase Activities (IU mL^{-1})			
Fpase	39.09±0.83	40.15±0.88	42.14±0.73
CMCase	30.08±0.90	31.13±0.94	36.41±0.83
β-glucosidase	6.36±0.93	6.478±0.94	7.25±0.81
Laccase Enzyme Activity (IU mL^{-1})	12.86±0.12	13.33±0.16	15.58±0.11
Sugar (mg mL^{-1})			
Total sugar	0.24±0.65	0.27±0.63	0.29±0.61
Glucose	0.09±0.62	0.11±0.83	0.11±0.21
Cellulose conversion (%)	23.80±0.31	40.00±0.37	49.00±0.35

Values are as Mean±SD

Table 4: Effect of different PPF weight on growth, cellulolytic and laccase activities, sugar and cellulose conversion by *Pyc. sanguineus* in 70% (v/v) POME after 7 days of fermentation period

	Weight of sterilized-PPF (g)				
Analysis	4	5	6	7	8
Net changes of biomass and suspended solids (g L^{-1})	15.51±0.83	15.72±0.71	17.54±0.74	16.88±0.86	16.52±0.81
Cellulase activities (IU mL^{-1})					
(a) FPase	39.54±1.13	42.14±0.73	51.60±0.84	50.22±0.86	49.46±0.81
(b) CMCase	31.72±1.65	36.41±0.83	40.99±0.91	40.40±1.03	39.82±0.92
(c) β-glucosidase	7.07±0.88	7.25±0.81	8.02±0.30	5.75±0.36	5.63±0.37
Laccase Activity (IU mL^{-1})	12.30±0.45	15.58±0.11	17.48±0.10	17.23±0.17	17.10±0.15
Sugar (mg mL^{-1})					
(a) Total sugar	0.22±0.78	0.29±0.61	0.31±0.32	0.27±0.39	0.14±0.89
(b) Glucose	0.09±0.98	0.11±0.21	0.12±0.03	0.10±0.65	0.09±0.64
Cellulose conversion (%)	44.00±0.82	49.00±0.35	55.00±0.08	52.00±0.12	50.00±0.45

Values are as Mean±SD

Effects of PPF pretreatment on growth, cellulase and laccase production by *Pycnoporus sanguineus*: The enzymatic conversion of cellulosic materials requires some form of pretreatment to improve cellulose accessibility and digestibility. It was apparent that the most effective pretreatment was one which allowed the substrate to be hydrolyzed partially and completely, using a minimum amount of enzyme. These include physical, chemical and biological processes, either singly or in combination (Chum *et al*., 1985; Zaheh and Karimi, 2008; Ji *et al*., 2008; Taherzadeh and Karimi, 2007). As in the present study, PPF was pretreated using sterilization process at 121°C for 15 min prior to fermentation. This is to increase the pore size and surface area and to reduce cellulose crystalline (Zaheh and Karimi, 2008).

As can be seen in Table 3, highest cellulolytic and laccase activities, net changes of biomass and suspended solids, sugar and cellulose conversion were obtained for POME-sterilized PPF series. In fact, medium with POME-sterilized PPF showed that CMCase and β-glucosidase activity increased significantly by 16.96 and 11.90%, respectively compared to POME-untreated PPF series. Higher the weight of PPF, higher was the cellulolytic activities (Table 4). The maximum Fpase, CMCase and β-glucosidase activities were at 40.99, 51.59 and 8.02 IU mL^{-1} using 6 g L^{-1} PPF, respectively. This is in agreement with the theory that pretreatment made the lignocelluloses available to the enzymatic attacked (Taherzadeh and Karimi, 2007; Zaheh and Karimi, 2008) in which it would broke the cellulose-lignin complex and hence increased the tendency for cellulase to attack on cellulose (Umikalsom *et al*., 1997). Besides, Keller *et al*. (2003) reported that the lignin network covering the holocellulose is broken down by successive fungal pretreatment and sterilization pretreatment, which together maximize the subsequent enzymatic production.

In this study, the highest laccase activity during Submerged Liquid Culture (SLC) was also resulted in POME supplemented with 6 g L^{-1} sterilized-PPF series. Thus showing that the laccase enzyme secreted by the white-rot fungus, *Pyc. sanguineus* might have degrade the cellulose and lignin as well as reducing sugar into the media (Table 3, 4). According to Madhavi and Lele (2006), the white rot fungus were considered to be the most promising group of microorganisms that degrade lignin because they produced extracellular polyphenol oxidases particularly lignin peroxidases, manganese peroxidases and laccases which are highly effective in degrading lignin. And Vikineswary *et al*. (2006) for their studies also stated that, the tested strain *Pyc. sanguineus* produced laccase during solid state fermentation of sago hampas and oil palm frond parenchyma tissue at 7.60 U g^{-1} substrate and 7.52 U g^{-1}, respectively. This study was then proved with the highest cellulose conversion (55%) was also achieved using POME

supplemented with 6 g L^{-1} sterilized PPF after 168 h of the fermentation process.

However, these enzymes activities and sugar content decreased when the PPF concentration exceed 6 g L^{-1}. Thus, indicating that PPF concentration of more than 6 g L^{-1} inhibit growth and cellulase production. This can be explained by the fact that at higher PPF concentration, the resistance of mass transfer and mixing increased, thus reducing the cellulase activity. The extent of inhibition also depend on the ratio of total enzyme to total substrate concentration (Taherzadeh and Karimi, 2007; Deshpande *et al*., 2008). In fact, the enzyme became saturated as the substrate concentration increased as it is bound to the active site of every enzyme molecules. This meant that under such condition, adding more substrate to the POME-based media did not increase the reaction rate further as there is no free enzyme binding site to which it can be bounded.

Optimization of cellulase production by *Pycnoporus sanguineus* using response surface methodology: A central composite design was used to develop a correlation between the pH and agitation speed in order to improve the cellulase production by *Pyc. sanguineus*. The complete design matrixes together with the values of corresponding responses were obtained from the experimental works. By using multiple regression analysis, the polynomial equation with the coefficients of the full regression model equation and their statistical significance were determined and evaluated using Design-Expert 6.0.6 software. The final models for each response in terms of coded values are:

$$\text{CMCase} = 47.96-5.34A+1.48B-6.49A^2+0.39B^2+2.25AB \quad (1)$$

$$\beta\text{-glucosidase} = 5.64-0.67A+0.31B-1.37A^2-0.93B^2+0.31AB \quad (2)$$

$$\text{Biomass} = 2.39-0.11A+0.27B-0.69A^2-0.19B^2-0.082AB \quad (3)$$

where, A is pH and B is the agitation speed.

Based on the above equation, the coefficients with one factor represented the effect of that particular factor, while the coefficients with two factors and those with second-order terms represented the interaction between the two factors and quadratic effect, respectively. The positive sign in front of the terms indicated synergistic effect, while the negative sign indicated antagonistic effect (Wu *et al*., 2002; Joshi *et al*., 2008). The present model and data analysis allowed us not only to define the optimal media composition for lignocellulolytic enzyme production, but it also showed the combined effects among the two factors studied. Figure 2-4 represented the

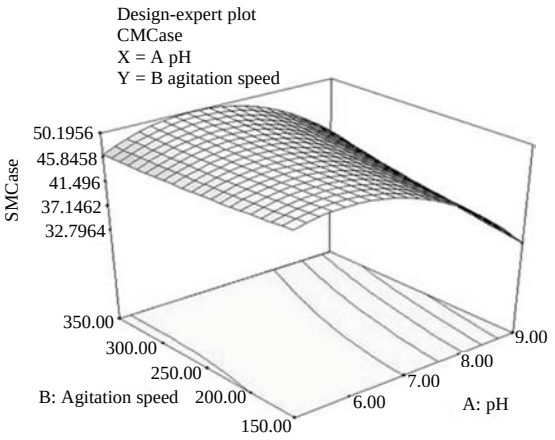

Fig. 2: 3D plot of CMCase production based on the pH and agitation rate

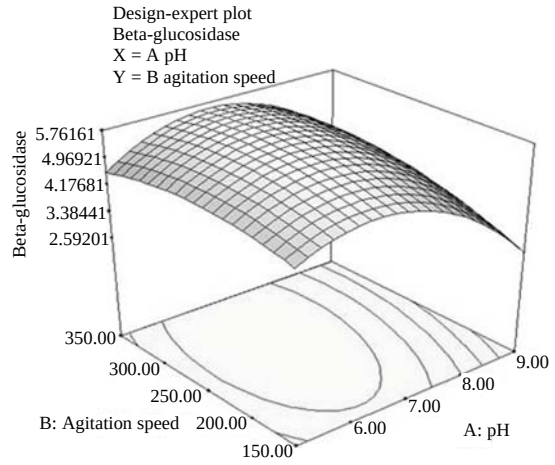

Fig. 3: 3D plot of β-glucosidase production based on the pH and agitation rate

3D plot of each individual response based on the variation of pH and agitation rate within the studied parameters range.

The pH of the growth medium played an important role by inducing morphological changes in the organism and in enzyme secretion. The pH changed was observed during growth of the organism which affected product stability in the medium (Asgher *et al*., 2007; Manivannan and Kathiresan, 2007). As in the present study, the secretion of cellulolytic enzymes and values of other parameters increased with increasing the pH medium from 5.0 to 7.0. However, the experimental design employed was not much affected by pH variations in the range of 6.0 to 7.0. Khan and Husaini (2006) reported that there appeared great stability in the enzyme within pH ranges of

6.0 to 7.0 for optimal α-amylase activity and cellulase activity. The sudden decrease in enzymatic hydrolysis after pH 7.0 could be due to the unfavorable reactions within high alkaline condition that resulted in a loss of nutritive value of protein and formation of potentially toxic substances such as lysinoalanine (Liu *et al*., 2008).

Besides pH, agitation intensity is another notable variable that influenced the mixing and oxygen transfer rates in many fungal fermentations and thus influencing mycelia morphology and product formation (Wang *et al*., 2003). Agitation also served to replenish the interparticle spaces with fresh air, which this could not be achieved in static culture since only upper most substrate was in contact with air while others were not. Such condition can lead to reduction of oxygen in interparticles space at limiting levels and the carbon dioxide can rise to inhibitory levels. It has been reported that a higher agitation speed is sometimes detrimental to mycelia growth and thus may decrease the enzyme production (Khan and Husaini, 2006). By observing the 3D surface plot of this study, the speed of agitation showed slightly positive effect on the enzyme production. The small increment of enzyme activities as increasing the agitation speed from 150 rpm to 350 rpm showed that aeration is an important factor for the growth of aerobic strains. In fact, greater aeration and agitation could result in excessively breakdown of the enzyme, thus decreasing the production of extracellular protease or cellulases (Rahman *et al*., 2005).

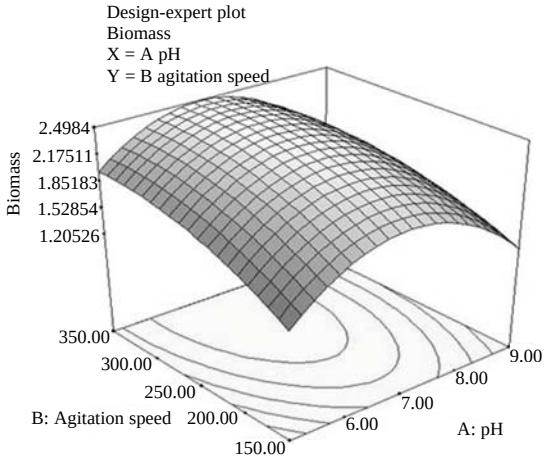

Fig. 4: 3D plot of net changes of biomass and suspended solids obtained based on the pH and agitation rate

Optimum range of parameter: Design-Expert plot illustrates the interaction between agitation speed and solution pH corresponding to different responses. The optimal condition to obtain maximal cellulases production were initial pH 6.77 and agitation speed of 350 rpm (Fig. 5). In this study, the desirability value for optimum media fermentation condition was 0.971, which is evidence for the application of this model.

Verification experiments: In order to confirm the model adequacy, five sets of experiments were repeated randomly at optimum condition to obtain a maximum

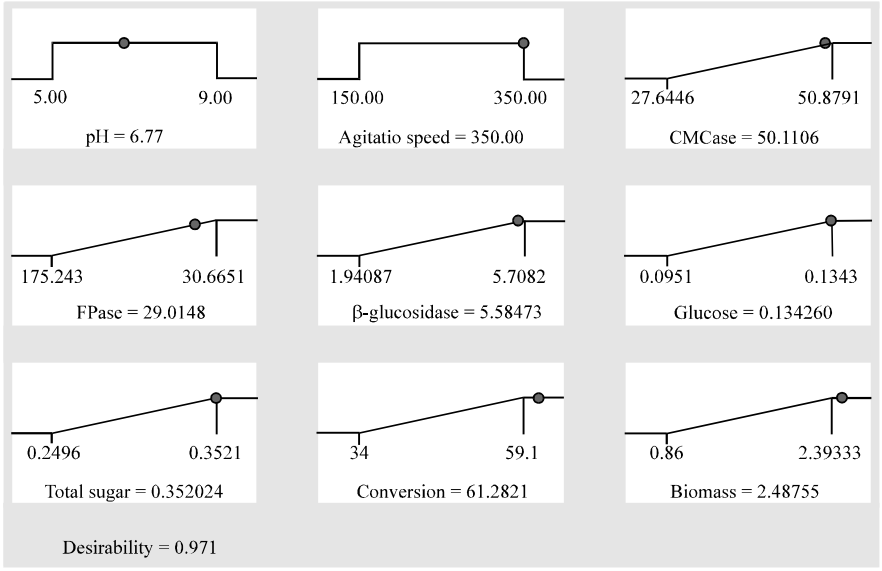

Fig. 5: Optimal condition and the desirability for growth and cellulase production by *Pyc. sanguineus* after 7 days cultivation with 6 g L^{-1} sterilized-PPF mixed with 70% (v/v) POME

Table 5: Validation of the data and models constructed

Results	Experimental result (average)	Predicted result (average)	Error (%)
CMCase (IU mL^{-1})	50.1621	50.1106	0.102
FPase (IU mL^{-1})	28.9990	29.0148	-0.056
β-glucosidase (IU mL^{-1})	5.5826	5.5847	-0.038
Laccase (IU mL^{-1})	6.6273	6.6423	-0.227
Glucose (mg mL^{-1})	0.1345	0.1343	0.174
Total sugar (mg mL^{-1})	0.3529	0.3521	0.222
Cellulose conversion (%)	61.5000	61.2800	0.358
Biomass (g L^{-1})	2.4880	2.4876	0.016

secretion of enzyme experimentally. As shown in Table 5, the percentage error difference between the experimental and predicted value were in the range of 0.016-0.358%. Since the differences between actual and predicted response were always less than 1% thus providing its validity.

CONCLUSION

POME was found to support growth and cellulases production by *Pyc. sanguineus* in shake flask culture. However, PPF has some drawbacks due to the presence of lignin, the crystallinity of cellulose and its low specific surface area. Thus, pretreatment of PPF by sterilization at 121°C for 15 min enhanced cellulases production or enzymatic hydrolysis. A statistical tool, RSM was used to optimize cellulases production with pH and agitation speed as the coded variables. Results showed that the highest CMCase (50.11 IU mL^{-1}) exoglucanase (29.01 IU mL^{-1}), β-glucosidase (5.58 IU mL^{-1}), laccase (6.6273 IU mL^{-1}) and the net changes of biomass and suspended solids (2.49 g L^{-1}) were detected at pH 6.76 and agitation speed 350 rpm after 7 days of the fermentation process. The experimental data fitted well with the model predicted values within 0.016-0.358% error.

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