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Abstract. Bioprotein is an alternative source that can be used to substitute the conventional protein source. In order to produce bioprotein, agricultural wastes can be utilized by microorganisms as substrates. Therefore, in this study, the utilization of oil palm fronds (OPF) as a substrate for the production of bioprotein by *Aspergillus terreus* strain UniMAP AA-1 through solid-state fermentation was investigated. The objectives of this study are to evaluate the production of bioprotein developed from OPF as a substrate and to optimize the physical parameters affecting bioprotein production. Solid state fermentation was carried out in conical flasks with 20 g of working volume at 30°C for 7 days. The fermentation time which produced the highest bioprotein was recorded at day 5. After that, the effects of temperature, substrate concentration and inoculum size were screened through 2-Level factorial design. Substrate concentration and temperature for the fermentation process were further optimized using Response Surface Methodology through Central Composite Design. As a result, maximum bioprotein produced was 0.7348 mg/mL at the temperature of 34.69°C, 58.06% w/v of substrate concentration and 5% v/v inoculum size. The data obtained in this study is potentially applicable in the scale-up production of bioprotein from OPF by *A. terreus* strain UniMAP AA-1 in the future.

1. Introduction

Oil palm plantations constitute a major agricultural industry in Malaysia. The plantations generated huge amounts of wastes such as shells, fibers, empty fruit bunches (EFB), oil palm fronds (OPF), trunks and palm oil mill effluent (POME). These wastes are rich in lignocelluloses which can be used for generating energy or can be turned into other value-added products [1]. Among the wastes generated, oil palm fronds (OPF) are the most abundant, which comprised 70% of the wastes generated from oil palm plantation [2]. Thus, OPF can be converted into value added products such as bioprotein for animals' utilization.

Protein is a major nutritional source for the growth of humans and animals. Increasing in the population growth has led to the deficiency of protein in human food and animal feed. Because of this, animal feed industries are having an impact due to inadequate supply and high cost of conventional protein sources [3]. Therefore, alternative ways are necessary to increase the production of protein and replace the conventional sources for animal feeding. Bioprotein is a formulation of alternative protein

sources to supplement the conventional protein source [4]. Bioprotein is a microbial biomass. The protein is extracted from the cultured cell and can be produced using a number of different microorganisms including bacteria, fungus and algae [5]. Protein obtained from microorganisms is cheaper compared to other protein sources and it also has a good nutritive value. Besides having a good quality of bioprotein, it can also be produced in large quantities. This is due to the rapid growth rate of microorganisms which results in high production of bioproteins [6].

The usage of fungi in bioprotein production is desirable due to their capability to propagate on agricultural wastes within a short period and ability to produce high protein content in their biomass [7]. The selection of potential microorganisms is important to produce the maximum quantity of bioprotein. Therefore, this study is attempted to produce bioprotein from *A. terreus* UniMAP AA-1 using oil palm fronds as a substrate. Physical parameters such as temperatures, substrate concentrations and inoculum size were screened using 2-factorial design and parameters that affect bioprotein production were further optimized using Response Surface Methodology (RSM) through Central Composite Design (CCD) approach.

2. Methodology

2.1. Collection and preparation of substrate

Oil palm fronds (OPF) was collected from Institute of Sustainable Agrotechnology (INSAT), UniMAP, Sungai Chuchuh, Padang Besar, Perlis. The substrate was washed and dried in an oven at 60°C for 24 hours. Then, the dried substrate was milled with grinding machine and sieved to obtain 0.5 mm of particle size [4]. The substrate was undergone pretreatment process by soaking in 1M NaOH and incubated at 90°C for 1 hour [8] in water bath. The substrate was filtered and washed with distilled water until pH 7 was recorded [4]. After that, the substrates were dried in an oven at 60°C for 24 hours and were kept in air-tight container until further use.

2.2. Preparation of fungal inoculum

A. terreus strain UniMAP AA-1 was obtained from the Faculty of Chemical Engineering Technology culture collection, Universiti Malaysia Perlis. The microorganisms were grown on Malt Extract Agar (MEA) plate at 30°C for 7 days. The inoculum was prepared by washing the growing culture with 25 mL sterile distilled water. The spore suspension was rubbed with hockey stick and adjusted to a final concentration of 10^7 spores/mL using haemocytometer [3]. The microorganism was sub-cultured every 2 weeks for further use.

2.3. Preparation of growth media

The growth media was prepared by mixing 0.2% w/v of KH_2PO_4, 0.5% w/v of NH_4NO_3, 0.1% w/v of NaCl, 0.1% w/v of $MgSO_4.7H_2O$, 0.1% w/v of $FeSO_4.7H_2O$, 0.1% w/v of $CuSO_4.7H_2O$ and 0.1% w/v of $ZnSO_4.7H_2O$ [4] in a beaker. The pH of the media was adjusted to pH 5.5. Then, the mixture was stirred using a magnetic stirrer on the hot plate without applying heat. Then, the growth media solution was autoclaved at 121°C for 15 minutes.

2.4. Solid state fermentation

Solid state fermentation process was carried out in 250 mL conical flask with 20 g of working volume. For the determination of growth curve *A. terreus* UniMAP AA-1, the ratio used was 70:30 which is 70% for the moisture content and 30% for the substrate [4]. In the laminar flow, 12 mL of sterile growth media was added into 6 g of substrate in the flask. Then, 2 mL of inoculum was pipetted into the flask. After that, the flask was incubated at 30°C in the incubator for 7 days [9]. For the screening process, the percentage of moisture content and substrate concentration depended on the 2-Level factorial design.

2.5. Total protein determination

The fermentation product was analysed daily. The samples were dried for 24 hours at 60°C in the oven [4]. Then, the dried samples were added with 50 mL of phosphate buffer and incubated in an incubator shaker at 4°C and 150 rpm for 3 hours. After that, the samples were filtered through Whattman No.1 filter paper. The sample solutions were collected and centrifuged at 8000 rpm for 20 minutes [3]. The supernatant was taken and underwent further analysis to determine total protein content using Lowry method.

2.6. Screening of physical parameters

The screening process was carried out using 2-Level Factorial Design from Design Expert Software Version 7.1.5. There were three parameters involved for the screening process in bioprotein production which are temperatures, substrate concentrations and inoculums size. All parameters were prepared in two levels which are +1 (high level) and -1 (low level).

2.7. Optimization of significant physical parameters in bioprotein production

From the screening of process parameters, the main effect from each variable was evaluated. The optimization process was carried out by using Response Surface Methodology (RSM) through Central Composite Design (CCD). By using CCD, the optimum levels of significant parameters were determined.

3. Results and discussion

3.1. Growth profile determination

The solid-state fermentation was carried out for seven days at constant temperature, inoculum size and substrate concentration. The temperature was maintained at 30°C with 30% w/v of substrate concentration and 10% v/v of inoculum size. The experiment was conducted in triplicates. Based on the growth curve, the fermentation time which produced the highest bioprotein was obtained. The reducing sugar and bioprotein production were analysed from day one until day seven. Figure 1 showed the profile of bioprotein production and sugar consumption of *A. terreus* UniMAP AA-1 on OPF as substrate. The highest glucose concentration produced is on day 4 which was 0.7789 mg/mL while the highest protein concentration produced is on day 5 which was 0.0851 mg/mL. Glucose concentration was higher due to the hydrolysis of cellulose to sugar by *A. terreus* UniMAP AA-1 during the fermentation process. These sugars can then be consumed by the fungi for their growth.

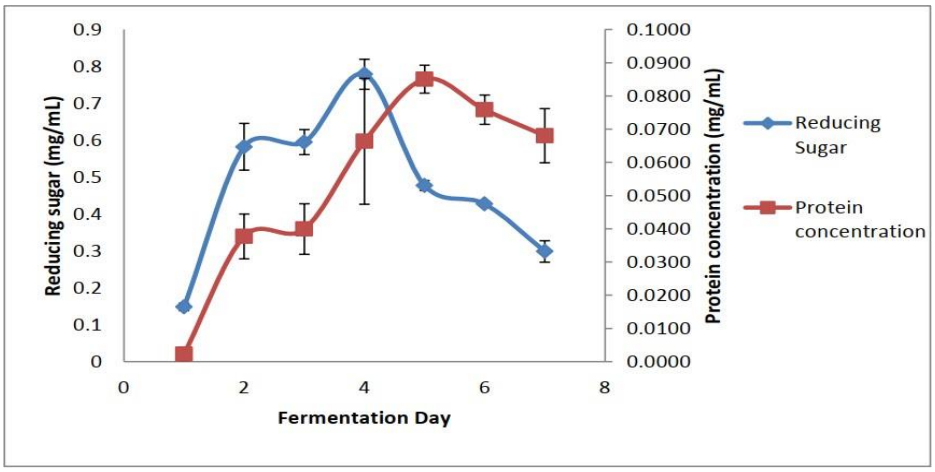

Figure 1: Profile on bioprotein production and reducing sugar consumption.

3.2. Screening of physical parameters

Parameters studied were temperatures, substrate concentrations and inoculum sizes. These parameters were evaluated by 2-Level factorial design. The main effect and interaction for each of parameters can be studied through pareto chart as shown in Figure 2. Pareto chart measured and showed graphically significant and insignificant parameters that affected bioprotein production. It consists of two reference lines which are t-value limit (black line) and Bonferonni limit (red line). Any effects above the Bonferonni limit indicated significant variables while any effect below the t-value limit indicated insignificant variables.

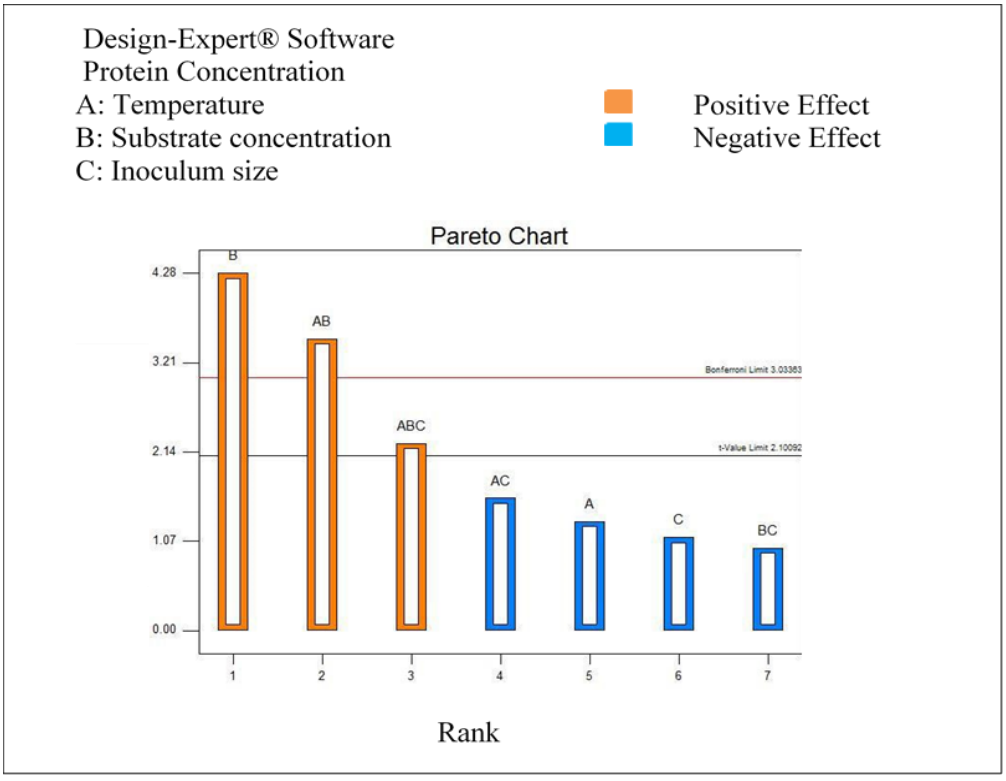

Figure 2: Pareto chart of process parameter on bioprotein production.

From Figure 2, variables B, interaction AB and interaction ABC showed significant variables while variables A, variables C, interaction AC and interaction BC showed insignificant variables since the effects were below t-value limit. Although the interaction ABC indicated significant variables, the effect was lower and closer to the t-value limit. Since variables B and interaction AB showed the higher effect, these variables were further analysed.

Based on the three parameters, substrate concentration has the most positive effect while inoculum size has the most negative effect followed by the temperature. This positive effect indicated that this parameter will increase the bioprotein production by increasing its concentration. Meanwhile, the negative effect means the parameter will increase the bioprotein production by decreasing the temperature and concentrations. Although temperature shows negative effect, the interaction between temperature and substrate concentration indicated positive effect. This means the temperature and substrate concentration needs to be higher in order to obtain higher bioprotein production.

3.3. Optimization of significant physical parameters in bioprotein production

In this study, optimization of bioprotein production was done using Response Surface Methodology (RSM) through Central Composite Design (CCD). CCD was used to optimize the variables and to obtain maximum bioprotein production. The experiment was conducted using rotatable design with 5 centre

points, 1 replicate of factorial points and 1 replicate of axial point. From the screening process, there were two parameters involved which are temperature and substrate concentration. Based on the design, 13 experimental runs were carried out. The fermentation was done for 5 days. The working volume of fermentation process was conducted at 20 g with varied substrate concentration and temperature while inoculum size was maintained at 5% v/v of concentration for each run. Equation 1 showed, the polynomial regression equation was developed relating to the production of bioprotein as shown as below:

$$Y = 0.75–0.021*A+0.031*B – 1.750E\text{-}003*A*B – 0.17 *A^2 – 0.077*B^2 \quad (1)$$

Table 1 showed the results obtained from Analysis of Variance (ANOVA). ANOVA demonstrated the significance of the model. Based on Table 1, it was showed that the model is significant with *p*-value 0.0054. Thus, it was showed that only 0.54% of model could not be significant due to the noise. Besides that, the significant variables that affect bioprotein production were studied. In this case, quadratic temperature (A^2) and quadratic substrate concentration (B^2) were the significant model with *p*-value of 0.0004 and 0.0262 which has ($p < 0.05$). Although other variables were not significant since *p*-value more than 0.05, the variables were included in the model as the A^2 and B^2 were significant. This is because the model was constructed based on the hierarchy. Meanwhile, the lack of fit was not significant which showed there were not much error occurred. Thus, it showed that the model fit with the data well [10]. The R-squared of this model was 0.8686 which explained 86.86% of the variables were correlated with one another and contributed to the production of bioprotein.

Table 1: Analysis of Variance (ANOVA) data for quadratic model of bioprotein production

Source	Sum of Squares	df	Mean Square	F value	p-value Prob>F	
Model	0.24	5	0.048	9.25	0.0054	Significant
A- Temperature	3.597E-003	1	3.597E-003	0.69	0.4339	
B- Substrate Concentration	7.849E-003	1	7.849E-003	1.50	0.2597	
AB	1.225E-005	1	1.225E-005	2.347E-003	0.9627	
A^2	0.21	1	0.21	40.07	0.0004	
B^2	0.041	1	0.041	7.89	0.0262	
Residual	0.037	7	5.220E-003			
Lack of Fit	9.926E-003	3	3.309E-003	0.50	0.7037	Not significant
Pure Error	0.027	4	6.653E-003			
Cor total	0.28	12				

From the regression model, the graph model graphically generated to obtain the optimum value for bioprotein production. It shows the interaction effect between temperature and substrate concentration when the inoculum size was set at a low level which is 5% v/v. From the analysis, the model was quadratic with elliptical response surface. It was observed the optimum temperature was near 35°C and the optimum value for substrate concentration was near 55% w/v. Based on Figure 3, as the substrate concentration and temperature increased, the production of bioprotein was also increased. However, as

the substrate concentration more than 55% w/v and temperature more than 35°C, the production of bioprotein started to decline.

The higher temperature caused the higher rate of reaction of microorganisms which enhanced the activation energy of enzyme to degrade the cellulosic component into sugar [11]. Thus, the bioprotein production was increased as the rapid growth of microorganism. However, every microorganism has their own optimum temperature for their growth. At higher temperature beyond its optimum level will inhibit their growth. From Manpreet et al. most of the fungi groups grow between 20°C - 40°C. Based on the result obtained, the optimum temperature obtained was near 35°C [12]. This was supported by Jaganmohan et al. where the optimum temperature for *A. terreus* was at 35°C by using Eichornia and banana peel as a substrate to produce bioprotein [13]. The optimum temperature for bioprotein production by *A. terreus* was also reported by Rahman et al. In the paper, the most optimum temperature for bioprotein production using palm decanter cake as substrate were temperature at 35ºC, substrate concentrations of 50 % and 15% inoculum size producing 1683 mg/L of protein [4].

The fungi mycelium penetrates into the particles of the substrates to grow. Therefore, in the presence of high substrate concentration, more bioprotein produced as the fungi grows rapidly on the substrate. However, higher substrate concentration than the optimum level necessary for microbial growth caused the lower activity of microorganisms since the enzyme to degrade the substrate became limiting factor and saturated [14].

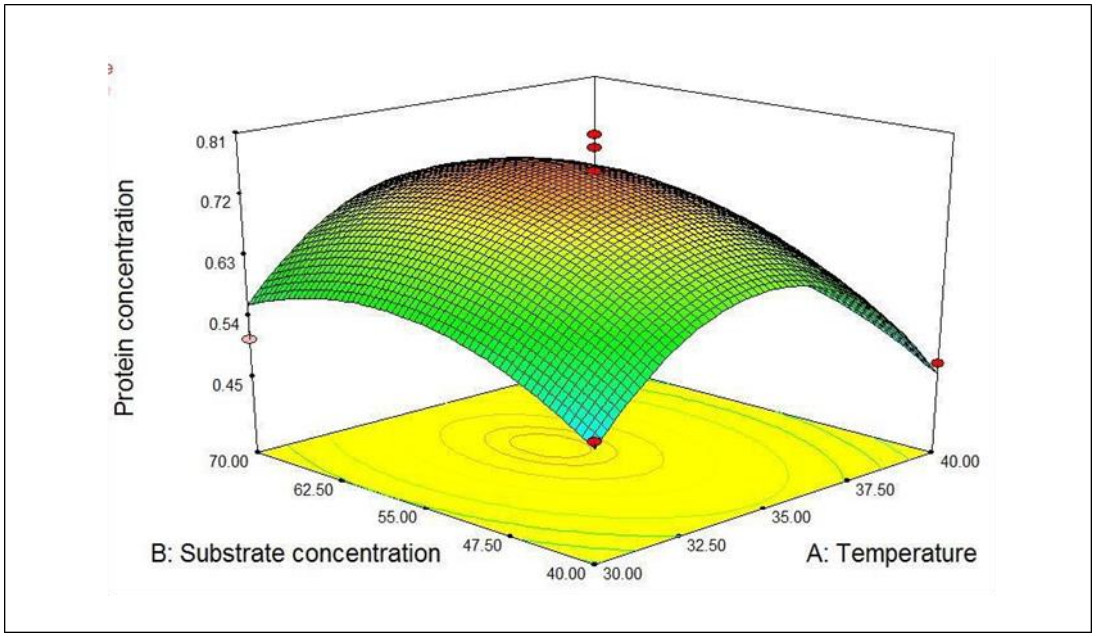

Figure 3: 3D response surface of optimization bioprotein production

3.4. Verification of model

In this study, the optimized value for bioprotein production was obtained at temperature of 34.69°C with 58.06% w/v of substrate concentration. The validation run was performed in triplicate. The inoculum size was maintained at low level which is 5% v/v. The parameter was set in a range while the bioprotein production was set at maximum level. Table 2 showed the result from optimization of bioprotein production. Based on the Table 2, the actual value w within the range of predicted value with a mean value of 0.7348 mg/mL. Meanwhile, the standard deviation was 0.035. The small standard deviation between predicted value and the actual value are in a good agreement [12]. The small error occurred probably due to the unfixed temperature used during conducted the experiment. Temperature gives

effect on the fermentation process. Since the temperature was not consistent, it will affect the product formation.

Table 2: Validation of optimum conditions for bioprotein production with predicted and actual value

Run	Temperature (°C)	Substrate concentration (% w/v)	Protein concentration (mg/mL)		Percentage error (%)	Standard deviation
			Predicted value	Actual value		
1	34.69	58.06	0.7578	0.7712	1.77	0.035
2	34.69	58.06	0.7578	0.7016	7.42	
3	34.69	58.06	0.7578	0.7317	3.44	

4. Conclusion

In this study, bioprotein was produced from oil palm fronds (OPF) by *A. terreus* UniMAP AA-1. Temperatures and substrate concentrations were the two physical parameters that significantly affects the production of bioprotein. Optimization using CCD approach recorded that the optimized value for temperature is 34.69^{o}C, substrate concentration of 58.06% and 5% inoculum size would produce the highest bioprotein of 0.7348 mg/mL. These optimization data can be potentially used for up-scale production of bioprotein from oil palm fronds in the future.

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Acknowledgements

This work was supported by Universiti Malaysia Perlis (UniMAP) under Short-Term Grant (STG) (Grant No. 9001-00308).