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Bioproteins Production from Palm Oil Agro-Industrial Wastes by *Aspergillus terreus* UniMAP AA-1

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ABSTRACT

Presently, the animal feed industry is suffering from inadequate and expensive conventional protein ingredients due to the increasing demand for food and feed products. This has led to the search for unconventional protein sources to fulfil market needs. In this study, the potential of selected palm oil wastes, namely palm pressed fibre (PPF) and palm oil decanter cake for bioprotein production, was investigated. Fermentation process was carried out aerobically in conical flasks with the working mass of 20 g each at 32°C for seven days. The performance of these palm oil wastes as substrates in solid state bioconversion of *Aspergillus terreus* UniMAP AA-1 strain were evaluated. A substrate with higher protein yield was chosen for the subsequent parameter screening using 2-level factorial design. Results showed that the protein content in PPF and palm oil decanter cake was increased up to 401 mg/L and 493 mg/L, respectively post-fermentation. Among the parameters studied, substrate concentration and inoculum size were found to significant affect bioprotein production. The highest protein content of 1683 mg/L was successfully produced from palm oil decanter cake at temperature of 35°C with 50% substrate concentration and 15% of inoculum size, suggesting its potential as an alternative protein source. Thus, this study provides preliminary data for future process optimisation of bioprotein production using the statistical approach.

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INTRODUCTION

Animal feeds with high and balanced nutritional composition determine the productivity of livestock. Presently, good quality of feeds require higher costs due to some limitations in the raw materials of feed and competition with human nutrition (Villa-Boas *et al.*, 2002). Therefore, in order to reduce the cost of animal feeds, the industry uses cheap and locally available feed ingredients such as agro-industrial by-products and waste. These agro-industrial by-products, better known as lignocellulosic wastes, are high in fibre and carbon but lacking in nutrients such as protein and vitamins (Villa-Boas *et al.*, 2002). Hence, the addition of micro-ingredients is essential to improve the nutritional content of the low-cost raw material and other advanced processes, making the final prices of feeds even higher.

One of the most promising approaches to solving this problem is by utilising microorganisms, mainly fungi, to convert agro-industrial waste to produce valuable products (Villa-Boas *et al.*, 2002; Jamal *et al.*, 2007, 2008). These wastes can be regarded as new sources for bioprotein production, which have high nutritional value with higher digestibility, do not compete with food for human consumption and is economically feasible and locally available (Jamal *et al.*, 2009; Gad *et al.*, 2010).

Bioproteins, also known as microbial protein or single cell protein (SCP), are proteins that are obtained by biosynthesis during growth and multiplication of microbial biomass. Bioproteins can be produced using varieties of microorganisms such as fungi, bacteria and algae (Anupama & Ravindra, 2000). In addition, protein produced from microbial sources is cheaper and high in nutritional value (Asad *et al.*, 2000), has a potential use as food additive, is fat binding and can be used as an alternative to costly conventional animal feed (Gad *et al.*, 2010). In addition, bioproteins produced from microbial cells could improve the digestibility of lignocellulosic materials (Villa-Boas *et al.*, 2002).

In this study, agro-industrial wastes from palm oil industries, palm oil decanter cake and palm-pressed fibre (PPF) were chosen as potential substrates for bioproteins production. The utilisation of these substrates is mainly due to their availability and can be obtained at a cheaper cost. As stated before, agricultural wastes are known to be low in protein content and high in fibre. Therefore, in order for it to be high in protein content and to improve digestibility, these wastes are fermented together with a selected fungal strain, *Aspergillus terreus* UniMAP AA-1 (Ahmad Anas & Arbain, 2012). The influence of biotechnological parameters, temperature, substrate concentration and inoculum size upon biosynthesis yield of proteins was also investigated as a basis for process optimisation in the future.

MATERIALS AND METHODS

Sample and Substrates Preparation

Palm oil decanter cake and PPF were obtained from Norstar Palm Oil Mill Sdn. Bhd situated in Kuala Ketil, Kedah,

Malaysia. The substrates were washed and dried in an oven at 60°C for 24 hours. The dried substrate was ground (RT Precision Tech.) and sieved (Retsch) to obtain 500 mm mesh size and was pretreated using 1% NaOH at 90°C for 1 hour as described by Hamisan *et al.* (2009) with some modifications. Then, the pre-treated substrates were washed until the pH was neutral (pH 7) and dried in an oven (Sartorius) at 60°C for 24 hours. The substrates were kept in separate air-tight containers until further use.

Microorganism and Fungal Inoculum Preparation

The *Aspergillus terreus* UniMAP AA-1 strain was obtained from the School of Bioprocess Engineering culture collection (School of Bioprocess Engineering, UniMAP, Perlis, Malaysia) (Ahmad Anas & Arbain, 2012) and was grown on potato dextrose agar (PDA) at 32°C. Inocula were prepared by washing the growing culture with 25 mL of sterile distilled water. The spore suspensions were rubbed and adjusted to final concentration of 10^7 spores per ml. The suspension inocula were kept in a chiller at 4°C for further use.

Fermentative Media Preparation

Evaluation of the potential of palm oil decanter cake and palm-pressed fibre (PPF) as fermentative substrates was carried out in order to determine the best and potential substrate that can be used to produce the highest bioproteins by applying the solid-state fermentation approach. Each substrate was evaluated using a constant amount of substrate concentration, inoculum size and media composition and process condition. The 70% of moisture content was maintained for every 6 g substrate in a conical flask. The 70% was equivalent to 14 mL of solution, which divided into 0.4 mL of inocula suspension and 13.6 mL of growth media solution that contained 0.2% of KH_2PO_4, 0.5% of NH_4NO_3 and 0.1% each NaCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$. The solution was autoclaved prior to usage.

Solid-State Fermentation

Solid-state fermentation was carried out in a 250 mL flask with the working mass of 20 g consisting of 30% substrate, 2% of inocula and 68% of growth media (0.2% of KH_2PO_4, 0.5% of NH_4NO_3 and 0.1% each of NaCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$. Samples were prepared in duplicate and incubated at 32°C for seven days. For the testing of biotechnological process parameters' influence upon yield of protein production, the amount of substrate used was in a range of 30 to 50% (w/w), while 5 to 15% (v/w) of inoculum size was inoculated into the fermentation medium. The temperature of the fermentation process was varied between 30 and 35ºC. These values were tabulated according to data indicated in Table 1 samples were incubated for six days.

Total Protein Determination

The biomass was withdrawn daily for analysis. The samples were dried for 24 hours at 60°C. Dried samples were

TABLE 1
2-Level factorial design for screening of process parameters with actual values and observed results

Run	Temperature (℃)	Substrate (% w/w)	Inoculum size (% v/w)	Protein content (mg/L)
1	30	30	5	582
2	30	30	5	565
3	35	30	5	678
4	35	30	5	715
5	30	50	5	1287
6	30	50	5	939
7	35	50	5	1153
8	35	50	5	1061
9	30	30	15	548
10	30	30	15	611
11	35	30	15	939
12	35	30	15	592
13	30	50	15	1189
14	30	50	15	1481
15	35	50	15	1507
16	35	50	15	1683
17	32.5	40	10	1287
18	32.5	40	10	1238

macerated in a pestle and 50 mL of 1N NaOH was added and incubated at 4^{o}C, overnight. The mixtures were then centrifuged at 8000 rpm for 20 minutes. The supernatant obtained was kept in the refrigerator for further analysis. The protein content in the supernatant was analysed using Lowry method (Lowry *et al.*, 1951).

Screening of Process Parameters

Two-level factorial design was carried out for screening of process parameters using the statistical software package Design Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, version 7.1.5) and the statistical analysis of experiment data. The screening process was done in duplicate involving three parameters, temperature, substrate concentration and inoculum size as shown in Table 1. All the parameters values were varied at two levels, which were -1 and +1. Value -1 indicates low level and +1 indicates high level.

RESULTS AND DISCUSSION

The fermentation was performed at constant media composition and process conditions in order to study the potential of palm oil wastes as substrates of the highest bioprotein concentration in fungal biomass. The fermentation was carried out for seven days and protein concentration was analysed daily. The production profiles for each substrate are illustrated in Fig.1.

From Fig.1, it can be observed that the proteins produced by each substrate have the same trend but differ in concentration. Protein production by PPF was gradually increased from 159 mg/L after the first day of fermentation to the maximum concentration of 401 mg/L after five days of fermentation. The protein concentration gradually decreased after six days of fermentation and on day seven, the protein concentration dropped to 199 mg/L.

In comparison, the protein concentration of fermented palm oil decanter cake also gradually increased over the fermentation time. The maximum protein concentration was obtained on the sixth day of fermentation while the lowest protein concentration was observed during the first day of fermentation, which was 146 mg/L. Unlike PPF, the protein content of the decanter cake started to decrease at day 7 and the protein content was 337 mg/L.

Fig.1 depicts that the protein produced by decanter cake was higher than the protein concentration of PPF. It is known that the growth of fungi mainly depends on the carbon, nitrogen and inorganic sources as their nutritional sources, and the main nutrients are carbon sources such as cellulose and hemicelluloses (Amal Nafissa *et al.*, 2008). Oil palm agro-industrial wastes such as PPF and palm oil decanter cake contain high amounts of lignocellulosic materials, which can act as a carbon source for the growth of fungi. According to Mahlia *et al.* (2000), PPF contains 47.2% carbon and 1.4% nitrogen while decanter cake contains 33% carbon and 3.6% nitrogen (Parveen *et al.*, 2010). Since both substrates consist of a sufficient amount of carbon and nitrogen, this factor contributes significantly to their capability in bioprotein production.

Fig.2 describes the comparison of bioprotein production for each substrate.

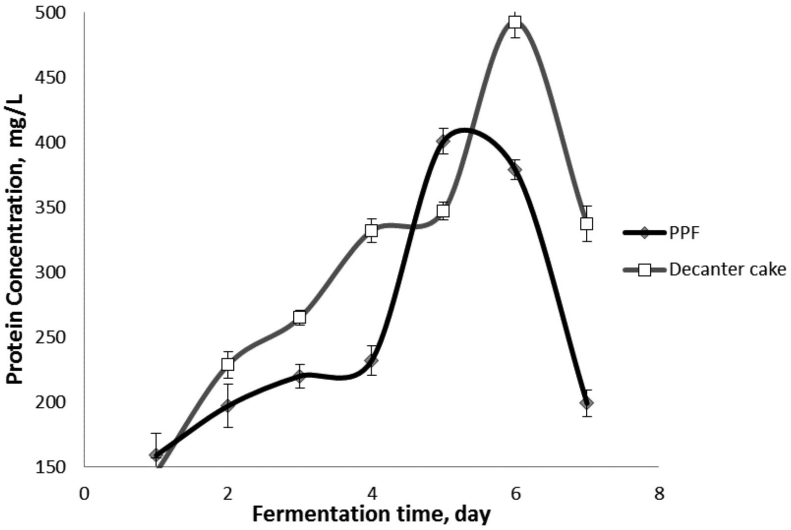

Fig 1: Profile of bioprotein production using different substrates.

The protein content of solid state cultivation on PPF was increased by 2.5 fold while the protein content of palm oil decanter cake was increased over three folds. In addition, the result also showed that the protein increment in palm oil decanter cake was higher than PPF by 85%.

Literature data described incorporations of different type of substrates and microorganisms where protein enrichments were successfully demonstrated. Among these microorganisms, fungi are the most commonly used for bioprotein production as they have the ability to secrete large amount of protein into the growth medium (Jahwarhar *et al.*, 2011). A number of studies were conducted using *Aspergillus niger* with various substrates including coconut dregs, orange waste, empty fruit bunch and palm kernel cake (Marini *et al.*, 2008; Hafiza *et al.*, 2011, 2012, Khadijah Hanim *et al.*, 2012; Alemu, 2013). In addition, *Aspergillus terreus* was employed in combination with rice, *Eichhornia* (water hyacinth) and banana peel (Shahzad & Rajoka, 2011; Jaganmohan *et al.*, 2013), while palm kernel cake was utilised with newly isolated *Rhizopus orizae* ME01 (Mohd Firdaus *et al.*, 2013).

In order to compare bioprotein production, Jaganmohan *et al.* (2013)

TABLE 2
Statistical analysis of 2-level factorial design for each variable

Process parameter	Main effect	F-value	p-value	Confidence level (%)
Temperature	140.75	4.40	0.0599	94.01
Substrate Concentration	633.75	89.17	0.0001	99.99
Inoculum Size	196.25	8.55	0.0138	98.62

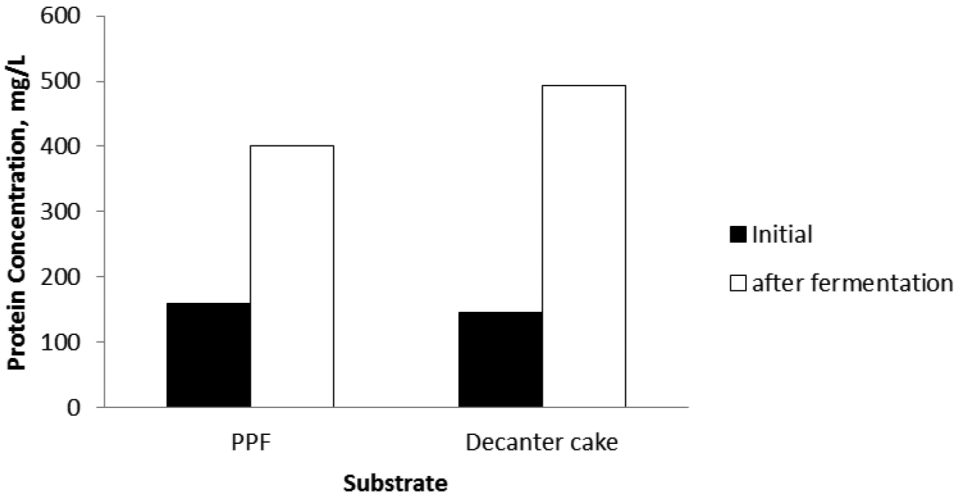

Fig 2: Comparison of bioprotein produced for each substrate.

performed solid-state fermentation using *Aspergillus terreus* with different raw materials namely rice bran, wheat bran, banana peel and combinations of these agricultural wastes for five days at room temperature. *Eichhornia* and banana peel mixture produced the highest mycelial protein, surpassing other substrate combinations with 6 mg/g of yield following process parameters and media optimisation. Conversely, Hafiza *et al.* (2012) studied the application of *Aspergillus niger* for bioprotein production from coconut dregs at 32°C for seven days and achieved a huge protein production of 76.6 mg/g. However, in another study using similar fungi with empty fruit bunches as substrate, the protein produced was significantly low (Khadijah Hanim *et al.*, 2012).

In comparison, protein produced in the present study was slightly lower than that reported earlier (Hafiza *et al.*, 2012, Jaganmohan *et al.*, 2013). The possible reason could have been the difference in nutritional content of the substrates used. As compared to PPF and decanter cake, banana peel contains several important nutrients such as potassium, calcium, sodium, iron, numerous essential amino acids, starch, hemicelluloses and important simple sugars. Moreover, the degradative reactions caused by endogenous enzyme might have increased its sugar content (Saheed *et al.*, 2012). A proximate analysis of coconut dregs revealed that it contained 56.5% (w/v) of carbohydrates, 3.5% (w/v) protein, 24.1% (w/v) of crude fibre and 515 Kcal/100g of energy (Hafiza *et al.*, 2012). These nutritional values obviously are more than those available in the substrate used, thus contributing to a higher fungal growth and subsequently, bioprotein production. However, the low protein yield can be maximised by screening the parameters that influence the yield followed by optimising them using statistical approach.

Since the protein content in palm oil decanter cake increased dramatically after fermentation, the protein content in this substrate has potential to be improved. Thus, decanter cake has shown a better performance as a substrate for bioprotein production and was selected to undergo a screening process prior to optimisation.

Screening of Process Parameters Affecting Bioprotein Production

The screening of process parameters was done according to 2-level factorial design to evaluate the significant process parameters for bioprotein production (Table 1). The highest protein concentration obtained was 1683 mg/L while the lowest protein concentration was 548 mg/L. Analysis of variance (ANOVA) results are presented in Table 2. The R-squared for this experiment was 0.9085. The significant parameters were substrate concentration and inoculum size since the parameters showed a confidence level of above 95% and p-value less than 0.0500 (Table 2). The main effect for each variable was estimated and graphically presented in Fig.3, which revealed that substrate concentration has the most positive effects on the fungal bioproteins production followed by inoculum size. This positive

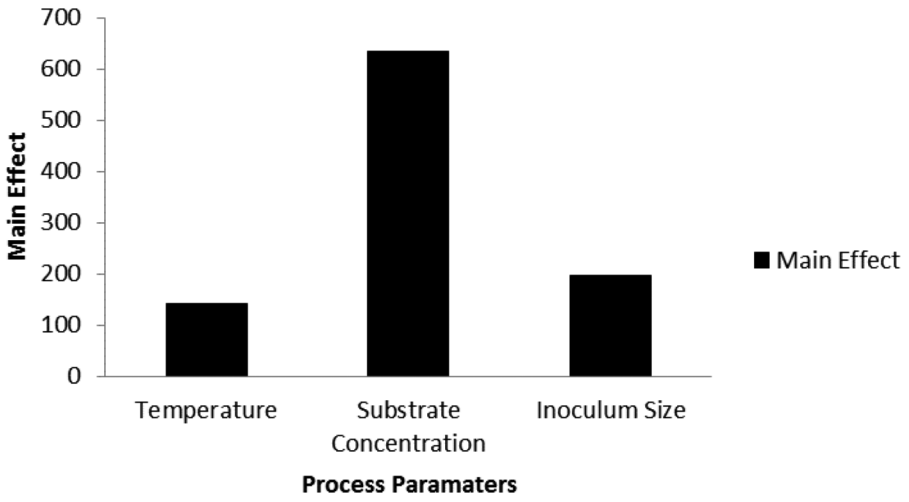

Fig 3: Main effects of process condition on bioprotein production.

effect means that these parameters will increase the fungal bioproteins production by increasing their concentrations and nutritional quality.

The substrate concentration shows the highest positive effect on bioprotein production. Substrate concentration plays a crucial role in solid-state fermentation. Mostly, the substrate concentration used for solid state concentration is around 50-55%, which is equivalent to moisture content of 45-50% (Iluyemi *et al.*, 2006). Variation of moisture content influences microbial growth. Lower moisture content causes reduction in solubility of substrates, low degree of swelling and high water tension (Kheng & Omar, 2005). Increasing moisture content can lead to the reduction of substrate porosity, thus limiting oxygen transfer into the substrat,e which in turn results in decrement of fungi growth and product formation (Abdeshahian *et al.*, 2010). In a previous study by Abdeshahian *et al.* (2010), the cultivation of *A. niger* strain under solid state fermentation produced higher levels of mananase at substrate concentration of 40% to 60% and the production began to reduce when lower levels of substrate concentration was applied (20-30%). This substrate concentration contributed to the highest confidence level of 99.99%, which proved that it is important for optimisation of bioprotein production.

Fig.3 shows that besides the substrate concentration, inoculum size also provided a significant effect on bioprotein production. In a previous study, it was demonstrated that higher inoculum size of *P. chrysosporium* produced more bioproteins (Gad *et al.*, 2010). Higher inoculum size should produce more bioproteins since the term bioproteins itself refers to the total protein extracted from microbial biomass such as fungal, yeasts and bacteria (Handan *et al.*, 2002), provided sufficient substrate concentration is available.

In this study, fermentations were carried out at temperature range of 30 to 35°C. The cultivation temperature was found to be an insignificant factor for bioprotein production due to their confidence level of less than 95%. The temperature for fungal growth varies between species; the most optimum is between 25ºC and 30ºC (Pietikainen *et al.*, 2005). In a study by Jaganmohan *et al.* (2013), it was reported that protein yield and biomass turnover of *A. terreus* increased at 25ºC to 35ºC and decreased gradually to 45°C. Ravinder *et al.* (2003), also studied the effect of temperature on protein yield in *A. oryzae* mutants. They discovered that protein production increased when the temperature was between 20 and 35ºC and decreased rapidly when approaching 45°C. For other species such as *Candida utilis*, *Chetomnium* sp. and *A. niger*, maximum protein production was at 32ºC (Li *et al.*, 2009; Yalemtesfa *et al.*, 2010) Therefore, the selection of temperature range is an important factor when observing fungal growth as to compensate for the diversity between species. In this case, a greater range is expected to provide better profiles, thus significantly justifying the influence of temperature in bioprotein production.

CONCLUSION

This study aimed to explore the potential of palm oil wastes to be used as a substrate for bioprotein production. Based on the experimental results, the solid state bioconversion of palm-pressed fibre (PPF) and palm oil decanter cake with a selected *Aspergillus terreus* UniMAP AA-1 strain successfully increased the protein content of the fermented final by-product from 159 mg/L to 401 mg/L for PPF and from 146 mg/L to 493 mg/L for palm oil decanter cake. Based on this protein production profile, oil palm decanter cake has higher potential thus, should be selected to undergo screening processes. Substrate concentration and inoculum size were identified by 2-level factorial design as important parameters for improving the production of bioproteins by solid-state fermentation on oil palm decanter cake. Protein production dramatically increased to 1683 mg/L following screening at temperature 35°C with substrate concentration of 50% (w/w) and inoculum size of 15% (v/w). This study provides preliminary data for future studies in optimisation of media composition and process condition using the statistical approach.

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