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### PRODUCTION OF CELLULASE AND PROTEASE FROM EMPTY FRUIT BUNCH AND PALM OIL MILL SLUDGE VIA SOLID-STATE FERMENTATION

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#### Abstract

Lignocellulosic biomass from the palm oil industry is one of the most abundant bio-renewable sources in Malaysia. However, the oil palm biomass especially empty fruit bunch (EFB) and palm oil mill sludge (POMS) are not being handled properly which will trigger environmental pollution. Therefore, solid-state fermentation (SSF) can be a good medium to valorize biomass into valuable products. Since SSF product was trapped in a solid-state, therefore it is crucial to know the appropriate extraction ratio to harvest a particular product. Thus, this research aimed to study the effect of the extraction ratio on the production of cellulase and protease enzymes via SSF using lignocellulosic biomass. The substrate was prepared by mixing EFB and POMS according to 1:2 (w/w) weight ratio. The samples were harvested every 3 days interval of the fermentation process until the substrate profile was constant. In the first stage of the study, the physical content of fermented mixtures for each interval was characterized according to pH, conductivity, temperature, moisture content, volatile solid and bulk density. The crude enzymes were then prepared by extracting the fermented mixtures with buffers for 45 min using 1:2, 1:3, 1:5, 1:7 and 1:10 (w/v) ratios. Upon observation, the optimal conditions for cellulase and protease productions were found in alkaline conditions (pH 8–9) with 74–76% of moisture content at the temperature of 31 °C–39 °C. In the present study, the extraction ratio of 1:2 (w/v) was found to be the highest yield of cellulase ( $5187.35 \pm 131.46$  FPU/g DW), while the ratio 1:7 (w/v) of extraction was found to be the highest amount of protease ( $102.4 \pm 10.66$  U/g). Both enzymes produced the highest yield on the 9<sup>th</sup> day of SSF. Therefore, the EFB and POMS have the potential as low-cost sources for cellulase and protease production.

#### INTRODUCTION

In the current situation, many agriculture industries produce large amounts of solid waste and liquid waste that may cause several environmental issues and turn out to be one of the main concerns in recent years. About 85.5% of agro-industrial waste in Malaysia came from the palm oil industry [1]. A million tonnes of palm oil fruit will produce 43% to 45% of mill residues such as empty fruit bunch (EFB), palm kernel shell (PKS), oil palm trunks (OPT), oil palm fronds (OPF), palm oil mill sludge (POMS), palm oil mill effluent

(POME) and mesocarp fruit fibre (MFF) [2]. Most of the agriculture industry wastes are disposed of by unplanned landfilling, burning and dumping [3]. As an alternative, solid-state fermentation (SSF) can be used to reduce the amount of solid waste generated from the palm oil industry, as it has proven to be a good process for producing valuable products [4]. In addition, the waste can be used as a source of indigenous mixed culture and substrate for the SSF process, especially in hydrolytic enzyme production such as cellulase and protease.

Cellulases are a complex group of enzymes including bacteria, actinomycetes, and fungi which are secreted by a wide variety of microbes [5; 6]. They hydrolyze  $\beta$ -1, 4 linkages in cellulose chains and synergistic interactions between cellulolytic microbes in the natural environment provide a beneficial role in the hydrolysis of lignocellulosic polymer materials. Lignocelluloses are the most munificent renewable carbon source with a production rate of hundreds of billion tons of biomass per annum [7]. As the cellulase is widely used in industries such as fermentation, animal feed, agriculture, textiles, commercial food processing pulp and paper industry, the global demand for cellulase is assumed to increase at a significant rate [8]. Cellulase is even used in pharmaceuticals for various purposes such as digestive enzyme products that are so vital for healthy cells [9]. It has also been utilized in the fermentation of biofuels by hydrolyzing the lignocellulosic materials that can be considered as an abundant resource for renewable and low-cost energy sources [10]. Also, making minor changes to the substrate using a nitrogen source at 30 °C, pH 4–5 with 70% initial moisture content and applying alkaline pre-treatment can generate the highest amount of cellulase [11].

Proteases are known as proteolytic enzymes, peptidases or proteinases that can be found in all creatures such as animals, plants and microorganisms provide an important role for normal growth and development [12]. The proteases can be classified into serine, cysteine, aspartic and metalloprotease. The classification of protease enzymes depends on the chemical groups that function in catalysis. Moreover, the protease enzymes can maximize the rate of the biochemical reactions and minimize retention time to achieve equilibrium. They have their biotechnological criteria that give advantageous effects in industrial enzymes [13]. All the criteria that proteases have can improve their development into some industrial processes such as biomedical, food [14], pharmaceutical, chemical, silk [15]

and leather. The high demands of these applications require a large number of setup costs for purification and isolation of biotechnological outcomes [16]. Some industrial technologies are often accomplished in utmost conditions and utilise the cheapest raw materials in enzymes production [17]. Therefore, the use of inexpensive substrates for cellulase and protease production is more preferable.

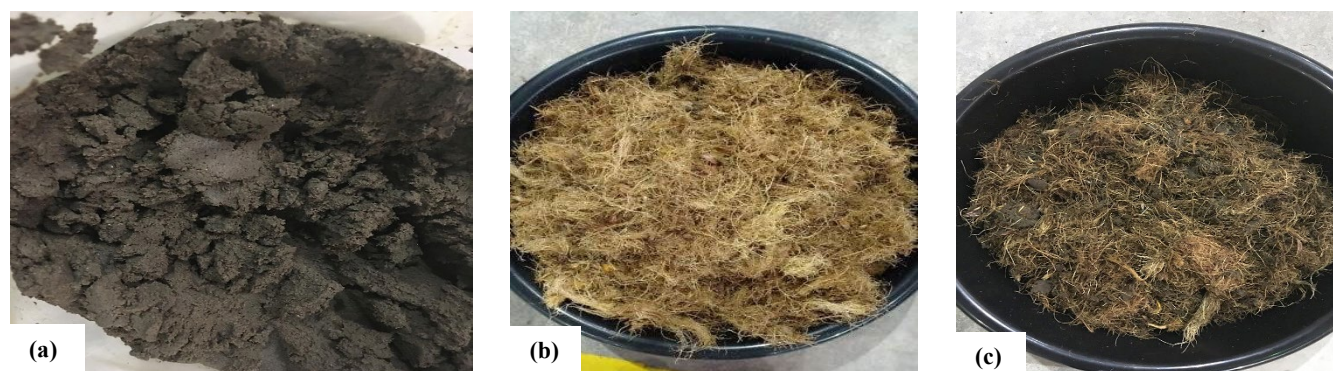
## MATERIALS AND METHODS

### Materials

The main agro-industrial by-products used for this research were isolated from all parts of empty fruit bunches (EFB) and palm oil mill sludge (POMS) collected from palm oil mill in Lepar Hilir, Gambang, Kuantan. Meanwhile, ethanol ( $C_2H_5OH$ ), sodium hydroxide ( $NaOH$ ), hydrochloric acid ( $HCl$ ), acetylacetone ( $CH_3COCH_2COCH_3$ ), 3,5-Dinitrosalicylic acid ( $C_7H_4N_2O_7$ ), p-dimethylaminobenzaldehyde ( $C_9H_{11}NO$ ), glucose and sucrose were purchased from Sigma-Aldrich. Tris(hydroxymethyl)aminomethane ( $NH_2C(CH_2OH)_3$ ), sodium citrate dihydrate, citric acid, anhydrous sodium carbonate, tyrosine, potassium sodium tartrate, trichloroacetic acid and casein from bovine milk sodium were purchased from Gardner Global Enterprise, Kuantan. Folin-Ciocalteu phenol reagent was purchased from Merck Malaysia, Ltd.

### SSF Substrate Preparation

As shown in Figure 1, the shredded empty fruit bunches (EFB) and fresh palm oil mill sludge (POMS) were used in this study. Both samples were homogeneously mixed using a weight ratio of 1:2 (w/w) to obtain a mixture as shown in Figure 1 (c).



**Figure 1.** SSF substrate preparation for particular hydrolytic enzymes productions; (a) POMS; (b) shredded EFB; (c) mixture

## Solid-State Fermentation

The solid-state fermentation was then conducted in two 5 L airtight bioreactors equipped with temperature probes, oxygen sensors and water trap. Samples of 50 g were collected at every time interval (0, 2, 7, 9, 13, 15 and 17 days until all mixtures are completely consumed) after manual homogenisation of the entire mass in all bioreactors. The oxygen was regulated using airflow manipulation in the gas exhaust gas to maintain the system in favourable aerobic conditions ( $\pm 21\%$  of oxygen in the air). The fermentation was slightly modified from [18].

## Characterization of Physical Content

Dry matter (DM) and organic matter contents (OM), total suspended solids (TSS), bulk density, moisture content, conductivity and pH in fermented solid samples were determined according to standard methods [19]. Total solid content (TS) which is corresponding to dry matter (DM) was estimated by drying the samples in the oven. The mass of the samples also has been taken before and after drying. In the meantime, the TS and moisture content (%) were expressed using Equation 1 and Equation 2 [4].

$$TS (\%) = \frac{M_w - M_d}{M_w - M_0} \times 100 \quad (1)$$

$$MC (\%) = 100 - TS (\%) \quad (2)$$

Where,  $M_w$  refers to the wet mass of samples (g),  $M_d$  is a dry mass of samples (g) and  $M_0$  indicates the mass of empty container (g). Bulk density (BD) was estimated on a wet basis dividing the sample weight by the sample volume and expressed as Equation 3 [4].

$$BD_w (\text{kg} \cdot \text{L}^{-1}) = \frac{M_s}{V_s} \quad (3)$$

Where,  $M_s$  is the mass of the sample (kg) and  $V_s$  is the volume of the sample (L). Volatile solid content (VS) which is corresponding to organic matter (OM) was obtained by sample ignition in a furnace. It was expressed as Equation 4 [4].

$$VS (\%) = \frac{M_d - M_a}{M_d - M_0} \quad (4)$$

Where,  $M_d$  is a dry mass of samples (g),  $M_a$  is a mass of ashes of the samples (g) and  $M_0$  refers to the mass of an empty container (g). Also, the pH was measured using an electrometric pH meter, where the pH probe was directly dipped in the extracted solution. The electrical conductivity

(EC) was also determined using an electrical conductometer [4].

## Enzyme Extraction

The samples were extracted with 50 mM HCl-Tris-buffer (pH 8.1) for protease analysis and citrate buffer (pH 4.8) for cellulase analysis at five different extraction ratios, which are 1:2, 1:3, 1:5, 1:7 and 1:10 (w/v) of the substrate to buffers, respectively for 45 min at room temperature. Then the mixtures were separated by centrifugation at 10 000 rpm for 10 min at 4 °C. The supernatant was then collected and filtered through a 0.45  $\mu\text{m}$  filter and used as crude enzyme extracts for further use [18]. All extractions were done in triplicate.

## Cellulase Assay

The Whatmann No. 1 filter paper was cut into 1  $\times$  6 cm strips (50 mg) before putting them into crude enzyme extract from SSF. The 0.5 mL of the crude enzyme were mixed into 1 mL citrate buffer in the test tube. The reaction mixture was then incubated for 1 h at 50 °C. After incubation, the reaction was terminated by adding 3 mL of 3,5-Dinitrosalicylic acid (DNS) reagent and the reaction mixture was boiled in the water bath for 5 min. After boiling, distilled water was added to make up until 15 mL of volume and optical density was taken at 540 nm against the blank. One unit of alkaline cellulase activity was defined as the liberation of 1  $\mu\text{g}$  of glucose per minute under the assay conditions. All activity tests were performed in triplicate. The standard curve was established using glucose as a standard [20].

## Protease Assay

Briefly, 1 mL of the crude enzyme extract was added into 4 mL Tris-buffer (pH 8.1). Then, 5 mL of a 2% (w/v) casein solution was added into the mixture and incubated at 50 °C with 100 rpm for 1 h. The reaction was terminated by adding 5 mL of 15% (w/v) TCA. The samples were centrifuged at 10,000 rpm for 10 min at 4 °C. An aliquot of 0.5 mL of the supernatant was added to the alkaline reagent and incubated for 15 min at room temperature prior to the addition of 0.5 mL of 25% (v/v) Folin-Ciocalteu phenol reagent. The resulting solution was incubated at room temperature in the dark for 1 h. The absorbance was measured at 700 nm using a tyrosine standard. One unit of alkaline protease activity was defined as the liberation of 1  $\mu\text{g}$  of tyrosine per minute under the assay conditions. All activity tests were performed in triplicate. The calibration curve was established by using tyrosine standard solution [18].

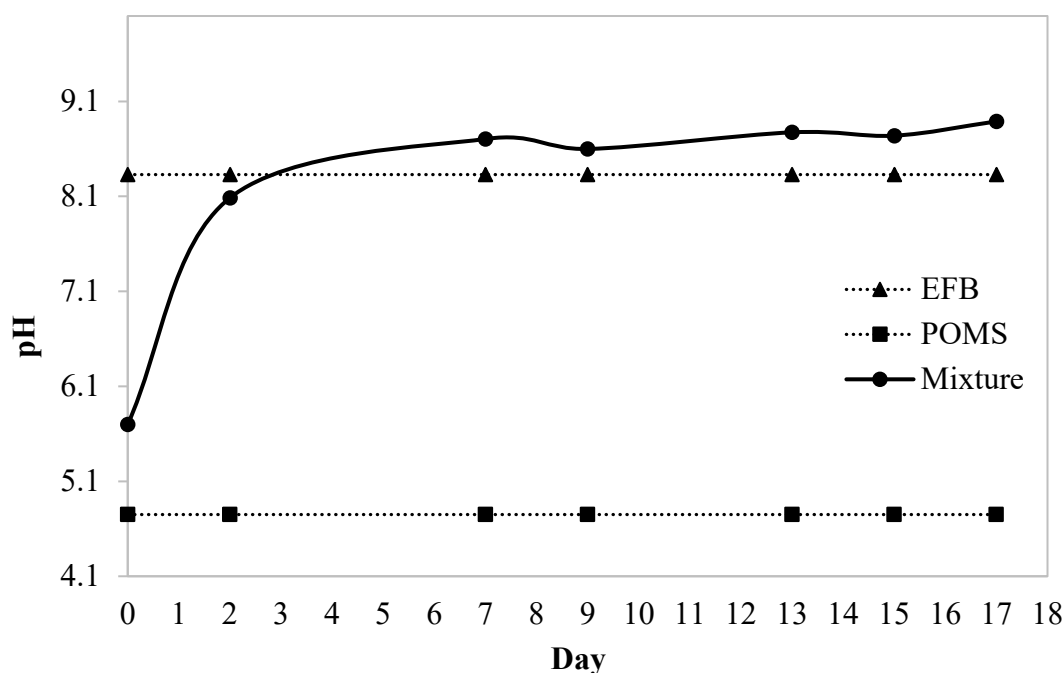
## RESULTS AND DISCUSSION

### Physical Content Characterization

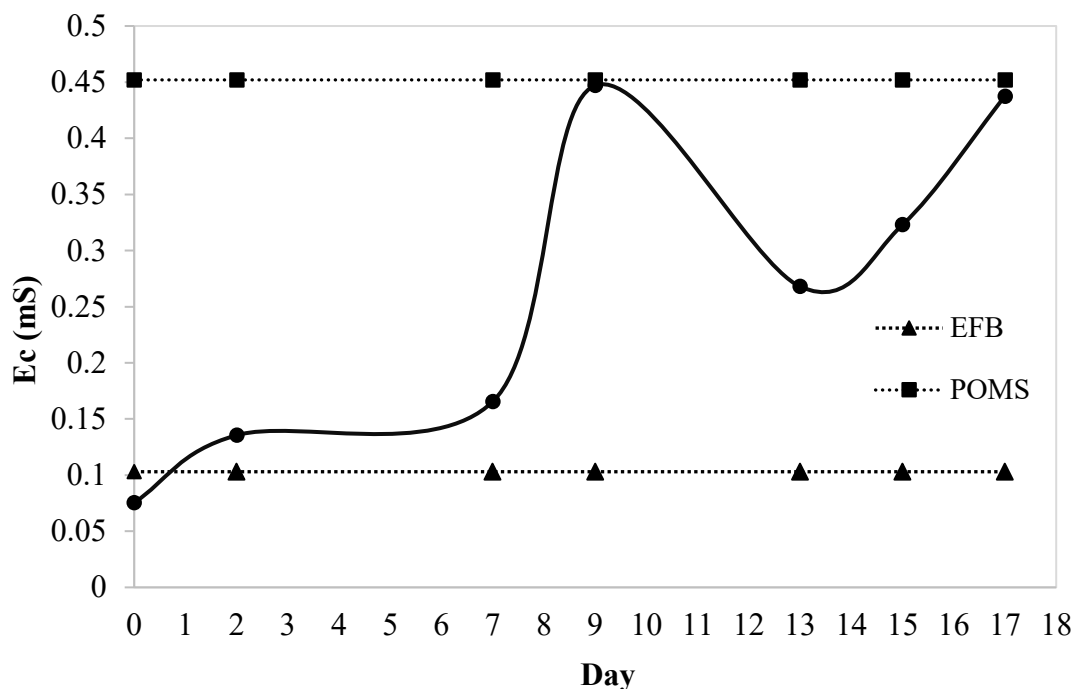
The sampling analysis of empty fruit bunch (EFB), palm oil mill sludge (POMS) and the mixture in the initial day were conducted to determine the total solid content (%TS), moisture content (%MC), volatile solid content (%VS), bulk density and conductivity. The total solid content (%TS) is expressed as a weight ratio gained before and after the drying process. Table 1 shows the quantity of mass in the raw material is less than 45%, which is applied to describe the dry matter and prevent the water content. Upon observation, POMS contains the highest amount of moisture content (84%), resulting in the samples degrading in the lowest time. The present finding of POMS is comparable to the previous study by [21] that recorded 86% of moisture content. Besides, all samples show a low yield of bulk density (0.2–0.25 g/mL), which can allow high movement of air into the inter-particle solid substrates and maintain the moisture levels to prevent the fermented beds from drying [22]. The

volatile solid content (%VS) is an approximation of the quantity of organic matter present. From the observation, the amount of organic matter present in the samples (80–91%) is adequate to be transformed into available nutrients and significantly correlated to all enzyme activities studied [23].

From Figure 2, the pH of EFB is an alkaline condition (pH 8.33), while POMS is acidic (pH 4.75). The mixture in the initial day shows that the pH is acidic (pH 5.70) that was affected by the weight ratio of EFB and POMS (1:2 w/w). However, the pH of mixtures during the fermentation process has increased from the second day onwards, resulting in the mixtures being in alkaline condition. The cellulase and protease production are highly suitable in alkaline conditions, thus generating high amounts of microorganisms during SSF [24, 25]. Regarding Figure 3, the mixtures were capable to produce maximum conductivity on the 9<sup>th</sup> day of SSF because the increasing of conductivity will increase the nutrient of the sample where the microbes excrete salt that is occurred as a nutrient. Thus, both pH and conductivity are included towards the good generation of the protease and cellulase enzymes.



**Figure 2.** pH versus time for every interval of fermentation that conducted in two 5 L two airtight bioreactors at minimum rate of 0.1 L/min using 1:2 (w/w) weight ratio of EFB to POMS



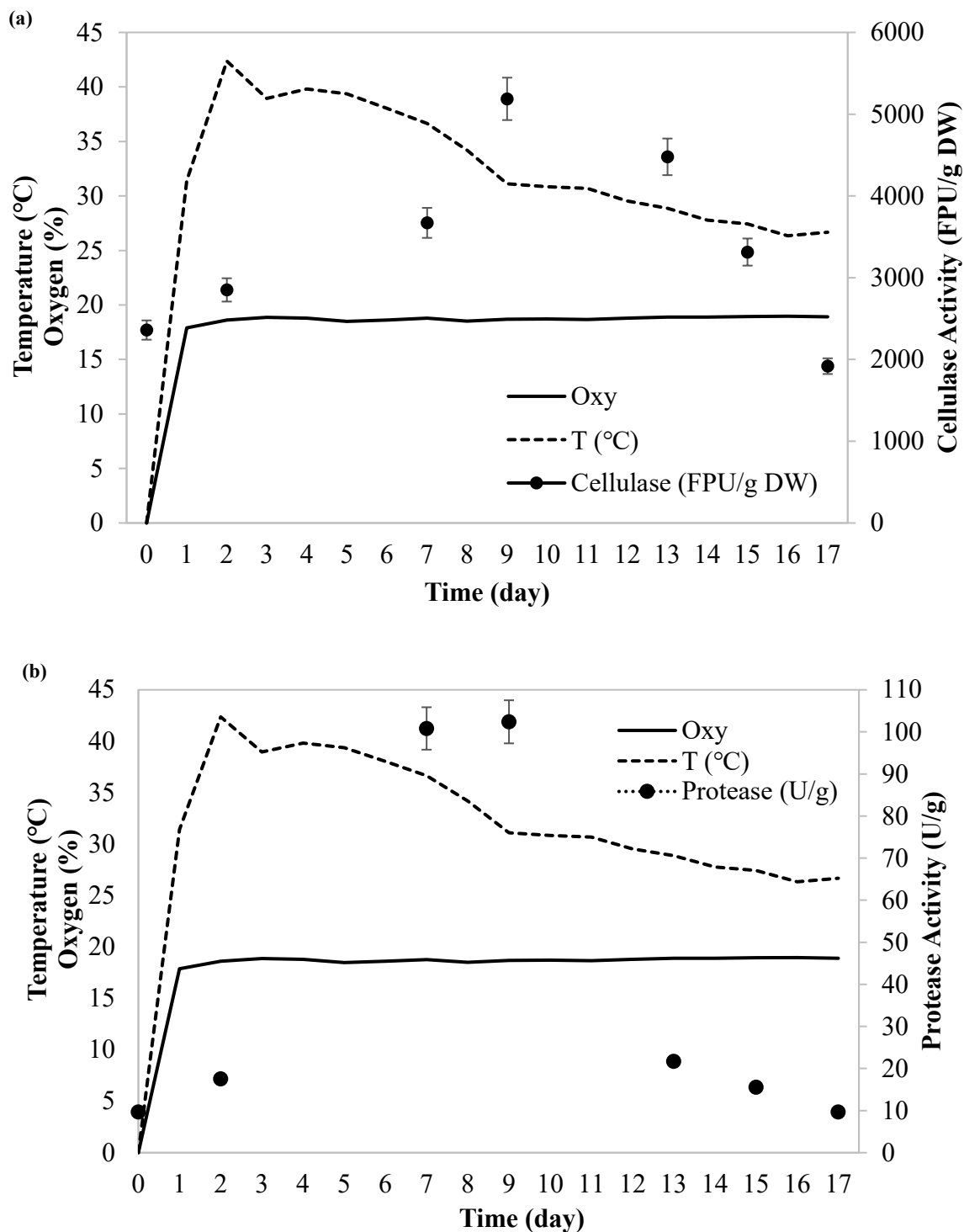
**Figure 3.** Conductivity versus time for every interval of fermentation that conducted in two 5 L airtight bioreactors at minimum rate of 0.1 L/min using 1:2 (w/w) weight ratio of EFB to POMS

### Solid-State Fermentation Profile

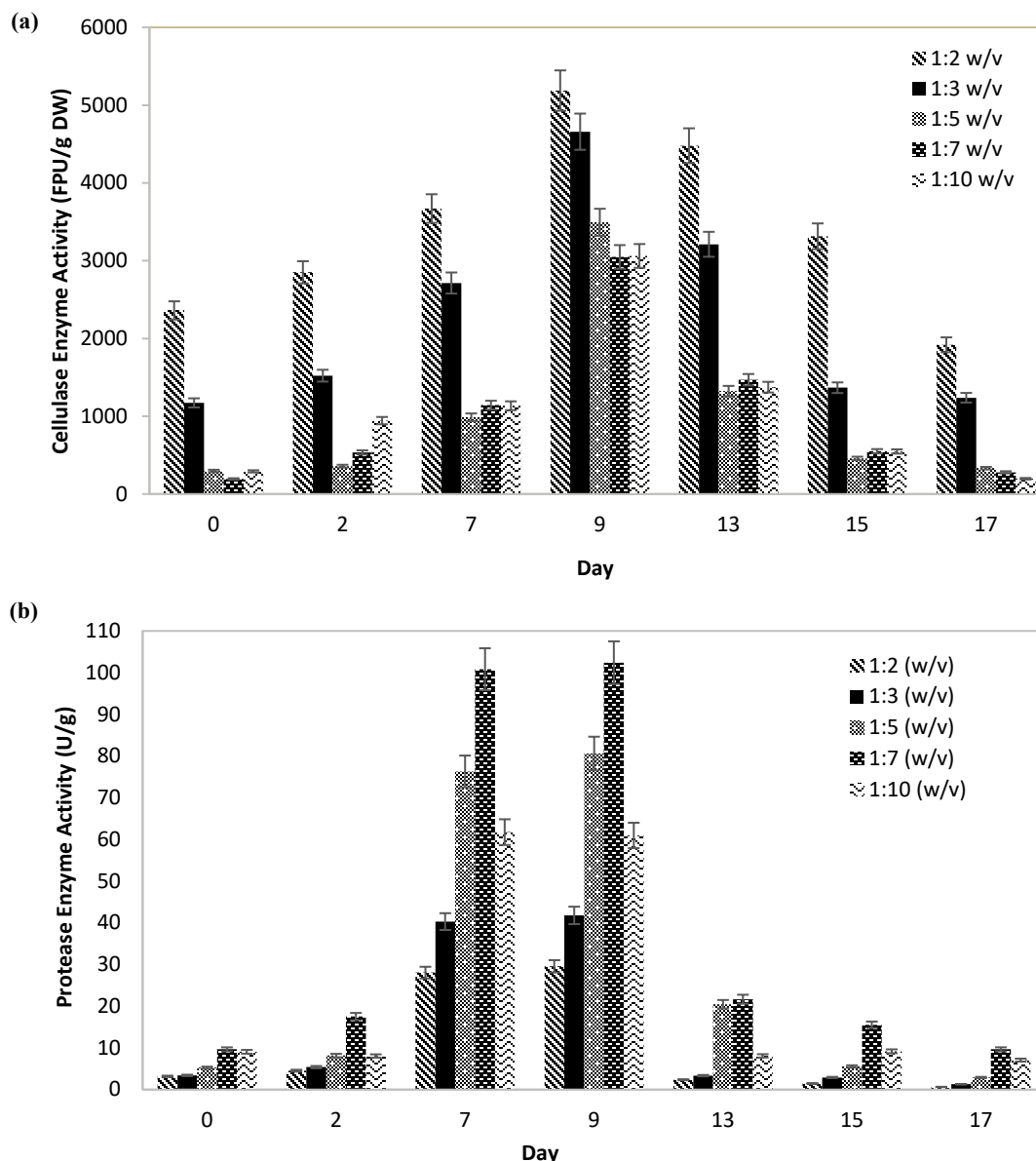
Based on the recent study, solid-state fermentation was applied when the samples supplied the oxygen and produced good aeration. The SSF can be defined as the cultivation of microorganisms on moist solid supports [26]. To validate the processes performances of the enzymes, these were assessed through the statistical comparison of temperature and oxygen profile for both particular hydrolytic enzymes that were plotted in Figure 4 (a) and (b). The fermentation revealed that the temperature was highest on the second day of SSF and decreased towards the end of the fermentation. The oxygen content, it was occurred in mesophilic conditions ( $< 45^{\circ}\text{C}$ ), resulting the aerobic respiration to have happened during the fermentation process. The result shows the production of protease and cellulase enzymes are activated at  $30^{\circ}\text{C}$  to  $39^{\circ}\text{C}$  showing the optimum temperature for highest enzyme activity (9<sup>th</sup> day) at  $32 \pm 0.9^{\circ}\text{C}$ . This result is in agreement with the theory that optimum growth temperature in the range of  $30^{\circ}\text{C}$  to  $39^{\circ}\text{C}$  [27]. Referring to the previous study [28], the highest yield of the enzyme from cow hair and digested sludge were observed on the 14th day of fermentation. Therefore, differences in results between the recent study and previous study can be due to the different inoculum, as in this work fresh sludge was applied.

### Effect of Extraction Ratio on Hydrolytic Enzymes

Factors influencing enzyme extraction from the solid matrix at the point of maximum cellulase activity during SSF were evaluated for the mixture of EFB and POMS under study. There are five different extraction ratios (1:2, 1:3, 1:5, 1:7, and 1:10 (w/v) of solid samples and citrate buffer) that were applied to evaluate the cellulase activity. All the data was done in triplicate to obtain consistency and the findings were then plotted in Figure 5 (a). Among the extraction ratios tested, ratio 1:2 (w/v) was found to be the highest production of cellulase enzymes ( $5187.35 \pm 131.46$  FPU/g DW), while 1:10 (w/v) ratio produced the lowest yield of cellulase ( $3060.93 \pm 18.39$  FPU/g DW). The solubility of cellulase from the mixture of EFB and POMS samples seems to be high and related to the equilibrium between the concentration of cellulase in the solid matrix and the solvent. In the literature, a direct relation between enzyme activity and weight ratio (w/v) is recorded. Most of the studies performed on this issue confirmed a direct relationship between weight ratio (w/v) and activity [29]. A similar finding of cellulase production was also observed in the previous study using banana peels via solid-state fermentation ( $5.56$  FPU/g DW) [11]. The findings revealed that the EFB is capable of producing more cellulase than banana peel in SSF. Hence, the EFB is capable of providing nutrients for cell growth and cellulase synthesis.



**Figure 4.** Solid-state fermentation profiles and hydrolytic enzymes production that conducted in two 5 L airtight bioreactors under optimal aerobic condition ( $\pm 21\%$  oxygen in air) at minimum rate of 0.1 L/min for 17 days; **(a)** cellulase production using 1:2 (w/v) ratio of substrate to citrate buffer; **(b)** protease production using 1:7 (w/v) ratio of substrate to Tris-buffer



**Figure 5.** Effect of extraction ratio on hydrolytic enzymes production using 1:2, 1:3, 1:5, 1:7 and 1:10 (w/v) of extraction ratio for 17 days; (a) cellulase enzyme; (b) protease enzyme

Based on Figure 5 (b), ratio 1:7 (w/v) was found to be the highest protease generation ( $102.4 \pm 10.66$  U/g), while ratio 1:2 (w/v) produced the lowest production of protease enzyme ( $29.56 \pm 0.09$  U/g). From the recent study, ratio 1:5 (w/v) to 1:7 (w/v) of mixtures and buffer proved that the solid wastes were capable to generate high amounts of protease enzymes via SSF. A similar finding of protease production was also observed using soy residue and hair waste via solid-state fermentation ( $319 \pm 34$  U/mg and  $459 \pm 34$  U/mg, respectively) [18]. The enhancement of protease production due to the growing phase by the bacteria is linked with nutrient elevation in the media [30]. Commonly, the protease production will be raised gradually with the fermentation time may due to high lipid content that

influenced by nutritional factors which are acting as inducers [31] and the microbes are completely used the simple sugar that enhances the production of enzymes [32]. Also, [33] stated that the higher ratio of extraction may produce lower enzyme concentration. Upon observation, all ratios revealed that the maximum production of cellulase and protease was generated on the 9<sup>th</sup> day.

## CONCLUSION

The agro-industrial wastes such as empty fruit bunch (EFB) and palm oil mill sludge (POMS) were utilised as low-cost substrates to generate hydrolytic enzymes such as cellulase and protease via solid-state fermentation (SSF). As SSF can valorize organic solid wastes into valuable products, it helps to reduce environmental pollution and concurrently contributes to solid waste management. Based on the observations, the optimum production of cellulase and protease enzymes were found in alkaline conditions (pH 8  $\pm$  0.5) at 39  $\pm$  1 °C of temperature. Also, the highest excretion of salt during SSF was found at 0.40  $\pm$  0.05 mS, resulting in a good generation of hydrolytic enzymes. Among the extraction ratios tested, ratio 1:2 (w/v) revealed the highest performance of cellulase enzymes (5187.35  $\pm$  131.46 FPU/g DW), while the highest yield of protease was found 102.4  $\pm$  10.66 U/g at ratio 1:7 (w/v). The differences in the performance of the extraction ratio may be affected by the characteristics of the enzyme produced. Therefore, the cellulase and protease enzymes were successfully produced from EFB and POMS as low-cost sources by using SSF.

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## CONFLICT OF INTEREST

There is no conflict of interest regarding the publication of this manuscript.

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