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# Evaluation of Palm Oil Mill Effluent Treatment with Concomitant Phenolics Production by *Aspergillus niger* IBS-103ZA

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**Abstract:** Previous results show that fermentation of palm oil mill effluent (POME) with *Aspergillus niger* IBS-103ZA has successfully increased the phenolic content (from 856±2.22 to 941±3.72 GAE mg/l) and also antioxidant activity of the extract (with $IC_{50}$ value of 0.45 mg/ml compared to unfermented extract with $IC_{50}$ value of 1.13 mg/ml based on DPPH radical scavenging assay). In this study, the potential use of *Aspergillus niger* IBS-103ZA to treat the effluent for removal of chemical oxygen demand (COD) and heavy metals were evaluated. The percentage of COD removal was low. Only 24.44% of COD was removed after 120 hours of fermentation period. The biomass of *Aspergillus niger* IBS-103ZA absorbed $Pb^{2+}$ ions from POME more rapidly than $Zn^{2+}$ ions. Within 24 hours of fermentation period, the percentage removal of $Pb^{2+}$ ions was 76.08%. The $Pb^{2+}$ ions were totally removed after 72 hours of fermentation period. The removal of $Zn^{2+}$ ions from POME was not as efficient as $Pb^{2+}$ ions. Only 24.79% of $Zn^{2+}$ ions were removed after 120 hours of fermentation period. No Cd was detected in POME during the study. The highest phenolics production (949.56±3.82 GAE mg/l) and total protein (76.58±0.43 g/kg-dried biomass) were obtained at 72 and 48 hours of fermentation respectively. Thus, POME treatment via fermentation with *Aspergillus niger* IBS-103ZA not only increased the amount of its value added product but also reduced the pollution load in the effluent.

**Key words:** Palm oil mill effluent (POME); Aspergillus niger; Phenolics; Fermentation; Chemical oxygen demand (COD); Heavy metals.

## INTRODUCTION

Malaysia is the world's second leading palm oil producing country and it is estimated that approximately 0.65 tonnes of palm oil mill effluent (POME) is produced for every ton of fresh fruit bunches (FFB). It has been identified as one of the major sources of aquatic pollution in Malaysia (Yacob *et al.,* 2005). POME is highly polluting due to its organic nature and its discharge to a relatively small river can be devastating to its echo-system and its use for various purposes. It consists of water soluble components of palm fruits as well as suspended materials like palm fibre and oil. Although POME is biodegradable but it cannot be discharged without first being treated because it is acidic and has a very high biochemical oxygen demand (BOD), chemical oxygen demand (COD) and heavy metals. Realizing the adverse effect of this effluent to the environment, measures to counter pollution from POME have been deployed. Instead of using conventional treatment methods, the research on the possibility of reusing POME as an environmentally sustainable bioresource has been explored in order to find approachable solutions for managing POME along with value added phenolics production.

## MATERIAL AND METHODS

### *Preparation of Raw Material:*

Palm oil mill effluent (POME) was collected from East Oil Mill, Sime Darby Plantation Sdn. Bhd, Cary Island, Selangor, Malaysia and was stored at 4.0 °C in the laboratory cold room for further use. The POME sample having 4.0% (w/v) of total suspended solid (TSS) was prepared on the basis of material balances (Alam *et al.,* 2008).

### *Fungal Strain and Preparation of Inoculum:*

The culture of *Aspergillus niger* strain namely IBS-103ZA (IMI 385267) was obtained from laboratory stock, which was isolated from sewage treatment plant (STP) sludge (Bari *et al.,* 2009; Fakhru'l-Razi *et al.,* 2002). The

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cultures were maintained at 4 °C on potato-dextrose agar (PDA), with regular subculturing at interval of 30 days. Inoculum preparation (spore suspension) was done according to the suggested method (Fakhru'l-Razi *et al.,* 2002). Each strain of the *Aspergillus niger* was first cultured on four PDA plates for 7 days in an incubator at 32 °C. About 100 ml of sterilized water was used for suspension inoculum. The spores in the culture plates were harvested from, with sterilized water followed by filtration using filter paper to remove the mycelia from spore suspension. The spore count of the suspension was determined by hemocytometer, which showed 1 x $10^6$ spores/ml.

***Fermentation Conditions:***

In this experiment, fermentation conditions were fixed at optimum values for phenolic production. In previous study, highest phenolic content was obtained at 5.39% (w/v) sucrose, 2.22% (w/v) $MnSO_4$, 0.3% (w/v) $MgSO_4$ and inoculum size of 1% (v/v) (Jamal *et al.,* 2011). The experiment was performed at an agitation rate of 150 rpm for 120 hours. The initial pH of the fermentation media and temperature were maintained at pH 5 and 35° C respectively.

***Determination of Total Phenolic Content:***

The total phenolic content was determined using Folin-Ciocalteau assay based on the suggested method (Waterman and Mole, 1994) with slight modification. Gallic acid was used as a standard. In 15 ml test tube, 2.37 ml of distilled water, 0.03 ml of sample extract or blank and 0.15 ml of Folin-Ciocalteau reagent were added and vortexed. After 1 min, 0.45 ml of 20% saturated sodium carbonate ($Na_2CO_3$) was added, and then the mixture was vortexed and allowed to stand at 40 °C for 30 min. The absorbance was read at 750 nm and converted into total phenolic content using gallic acid standard curve. Standards of difference concentration (from 0 to 1 mg/ml of gallic acid) were prepared to make the standard curve. The total phenolic content was expressed as mg of gallic acid equivalent per liter (GAE mg/l).Distilled water was used as a blank for the background subtraction. All measurements were measured in triplicates.

***Determination of Total Sugar:***

The analysis of total sugar was carried out to determine the remaining sugar content after the fermentation using phenol sulfuric acid method (Dubois *et al.,* 1956). For each 15 ml test tube, 1 ml of 5% phenol reagent was added and followed 5 ml of concentrated $H_2SO_4$ (98%). The solution was mixed immediately and allowed to cool before the absorbance was read at 490 nm. The total sugar was determined against a standard curve of sucrose. Standards of difference concentration (from 0 to 0.2 mg/ml of sugar) were prepared to make the standard curve. The results were corrected for dilution. All analyses were run in triplicate. For the blank, distilled water was used for the background subtraction. All measurements were measured in triplicates.

***Determination of Total Protein:***

The total protein content in fermented substrate was determined by Folin-Phenol reagent method (Lowry *et al.,* 1951) and the fungal growth was recorded during the fermentation. The following reagents were prepared: Reagent 1, 1N NaOH; Reagent 2, 3% (w/v) of $NaCO_3$ in 0.1N NaOH; Reagent 3, 2% (w/v) of $CuSO_4.5H_2O$; Reagent 4, 2% (w/v) of sodium tartrate; and Reagent 5 which was prepared by mixing 96 ml of Reagent 2, 2 ml of Reagent 3 and 2 ml of Reagent 4 that was prepared fresh every day. Ten milligram of homogenized dried fungal biomass sample was taken into a test tube. About 5 ml of 1N NaOH was added in the test tube and allowed to stand at room temperature for 24 hours by closing the test tube with cap. The extract (0.5 ml) was pipetted into a test tube and 5 ml of Reagent 5 was added. The mixture was kept for 10 minutes. Then, 0.5 ml of 1N Folin reagent was added into the mixture and allowed to stand for 20 minutes. The absorbance was read at 660 nm and the total protein was determined against a standard curve of bovine serum albumin.

***Determination of COD Removal:***

The analysis of COD was carried out according to the reactor digestion method (Jirka and Carter, 1975). Two ml of diluted sample was added to the COD digestion reagent vial (for COD 0-1500 ppm range) and the mixture was mixed gently. The vial was placed in the preheated COD reactor at 150 °C for 2 hours. The vial was cooled to room temperature and the COD (in mg/l) was determined by the colorimetric method. The absorbance was read at 620 nm (for COD 0-1500 ppm range using Hach Program). For the blank, deionized water was used for the background subtraction. All analyses were run in triplicates.

***Determination of Heavy Metals Removal:***

The concentrations of Cd, Pb and Zn in the fermentation effluent were determined using Flame Atomic Absorption Spectroscopy (FAAS). The spectrometer, model Perkin Elmer AAnalyst 400, was equipped with hallow cathode lamps (HCL) specific for each element and deuterium lamp for continuous background correction. The instrument was also equipped with AS 90 Plus autosampler and FIAS 100 flow injection system

controlled by WinLab32AA system software. Calibration with stock standard solutions of 1000 mg/l of Cd, Pb and Zn was used to prepare a series of composite standards, the concentrations of which in 0.2% $HNO_3$ ranged between 0.02-0.5 mg/l for cadmium and zinc, and 0.2-8 mg/l for lead. To analyze concentrations over the calibration range, sample solutions were diluted using the appropriate acid diluent. All calibration curves for each heavy metal had a correlation of $R^2 \geq 0.999$.

## RESULTS AND DISCUSSION

### *Effect of Fermentation Period on Phenolics Production and COD Removal:*

Biological conversion of agro-industrial wastes has been successfully converted into many value-added products (Vattem and Shetty, 2002). The biological conversion of soluble and insoluble organic compounds like sugars, lipids, protein and phenolic compounds that were present in POME can be associated with the decrease in COD (Figure 1). In this study, the initial COD value was 107.60±0.00 g/l and it was reduced to 81.30±1.27 g/l after 120 hours of fermentation period. The percentage of COD removal was only 24.44% as compared to 61.28%, which was reported (Jamal *et al.,* 2005) during production of citric acid from a cheaper carbon source. The low percentage of COD removal by *Aspergillus niger* IBS-103ZA strain could be explained in the same way as reported by other researchers (Friedrich *et al.,* 1983). The COD reduction was more pronounced in lower solid content as compared to higher solid content of waste. High solid concentration may inhibit the growth of fungus.

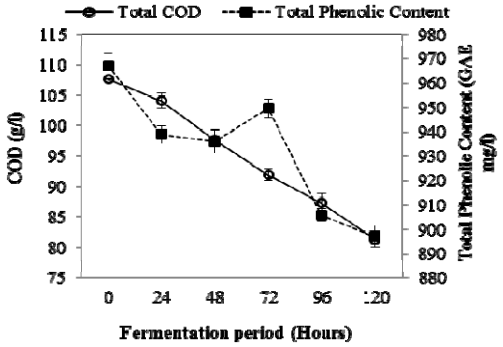


**Fig. 1:** Effect of fermentation period on production of phenolics and removal of COD. Data are expressed as means ± SD ($n$ = 3).

In this study, the ability of *Aspergillus niger* IBS-103ZA to enhance levels of free phenolics from POME at optimum fermentation conditions was also investigated. Fig. 1 shows the changes in the total phenolic content at different fermentation periods by the selected strain. The initial total phenolic content was found to be 967.09±5.03 GAE mg/l, which was contributed by the presence of free phenolics in the POME as, reported by other researchers (Wattanapenpaiboon and Wahlqvist, 2002). After 48 hours of fermentation period, the total phenolic content dropped to 935.93±4.65 GAE mg/l and then increased to 949.56±3.82 GAE mg/l at 72 hours of fermentation period. An increase of about 13.63 GAE mg/l of total phenolic content at the end of this period could be explained by the liberation of simple phenolic compounds after acid and enzymatic hydrolysis of polymerized phenolic compounds (Bouzid *et al.,* 2005).

The total phenolic content continued to decrease after 72 hours of fermentation period until the end of the fungal growth. At 120 hours of fermentation period, the total phenolic content obtained was 896.98±4.33 GAE mg/l. A decrease in total phenolic content during fermentation period was possibly due to the degradation of some phenolic compounds by the strain. *Aspergillus niger* has been reported by many researchers to have the ability to utilize many phenolic compounds as substrates for their growth (Shyamala *et al.,* 2004). According to another data (Seng, 1988), the decrease in the content of phenolics might also be contributed by the absorption of the polyphenols on the fungal mycelium. This absorption occurs, may be due to the hydrogen bond between phenolic compounds and protein or due to the chitin of the mycelial wall, which has a strong coagulant effect.

### *Determination of Total Protein Content and Total Sugar Consumption during Fermentation:*

The growth of *Aspergillus niger* IBS-103ZA during fermentation period was monitored by measurement of changes in the protein content and sucrose concentration level as shown in Fig 2. According to (Seng, 1988), protein measurement is a simple indirect method for the estimation of fungal growth. From Figure 2, the protein content of 59.36±0.53 g/kg-dried biomass at the beginning of the fermentation was actually contributed by the initial total protein that was present in the POME. Several researchers had reported that POME itself contains high concentrations of protein and other nutrients that make it suitable as fermentation media (Raghavarao *et al.,* 2003).

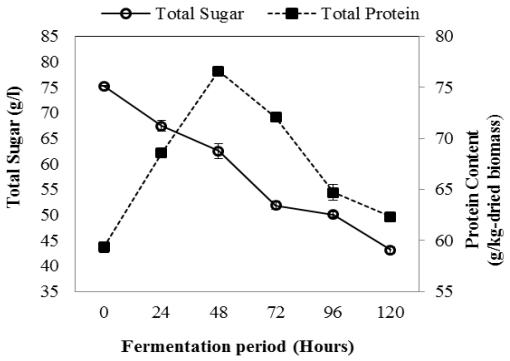


**Fig. 2:** Effect of fermentation period on total protein and total sugar consumption. Data are expressed as means ± SD ($n$ = 3).

After 24 hours of fermentation period, the protein content increased by about 15.49% to 68.56±0.11 g/kg-dried biomass. The highest protein content of 76.58±0.43 g/kg-dried biomass was observed after 48 hours of fermentation period, where there was an increase of about 17.22 g/kg-dried biomass. Increase in the protein level could result from slight protein synthesis by proliferation of the fungal biomass and a synthesis of enzyme protein or from rearrangement of the composition following the degradation of other constituents (Habib *et al.,* 1997). The protein content started to decrease after 72 hours of fermentation period. A decrease of about 18.58% to the final protein content of 62.35±0.11 g/kg-dried biomass was observed after 120 hours of fermentation period. A decrease in the protein level indicates the beginning of mycelium or cell autolysis (Raimbault, 1998).

Instead of protein content, substrate utilization is another important parameter for growth assessment. In this study, about 5.39% (w/v) or 75.29±0.15 g/l of sucrose was supplemented into the fermentation media to enhance the growth of *Aspergillus niger* IBS-103ZA. About 12.78 g/l of sucrose was consumed after 48 hours of fermentation period. The consumption of sucrose increased drastically to 10.64 g/l for the next 24 hours of fermentation period. Fast increment in sucrose uptake was probably due to rapid increase in the concentration of fungal biomass and enzyme production (Zhang *et al.,* 2008). The reason of lower rate of sucrose uptake at the beginning of 48 hours of fermentation period might be due to the starting of germination of fungal spores. For the last 48 hours of fermentation period, it was observed that the sucrose consumption decreased to 8.72 g/l. The less sucrose consumption at the last stage of the fermentation period could be explained with no further increase in fungal biomass and enzyme production (Kiel *et al.,* 1980). Throughout the fermentation period, about 42.69% of sucrose was consumed by *Aspergillus niger* IBS-103ZA.

***Analysis of Heavy Metals Removal and pH Profile:***

Among microorganisms, fungi are known for their superior ability to produce a large variety of extracellular proteins, organic acids, enzymes and other metabolites, and their waste biomass may be used as effective biosorbents for removal, reduction, and detoxification of heavy metals ions from industrial effluents (Favela-Torres *et al.,* 1997). Various fungal species under the genus *Aspergillus*, *Penicillium* and *Rhizopus* have been reported to be effective in biosorption of heavy metals from polluted effluents (Christian *et al.,* 2005).

In this study, the biosorption capacity of *Aspergillus niger* IBS-103ZA was evaluated for removal of Pb, Cd and Zn from POME for safe discharge of effluent. A continuous decrease in the concentration of Pb and Zn was observed throughout the fermentation period (Fig. 3). Meanwhile, no Cd was detected in POME. *Aspergillus niger* IBS-103ZA biomass absorbed $Pb^{2+}$ ions from POME more rapidly than $Zn^{2+}$ ions. Within 24 hours of fermentation period, the percentage removal of $Pb^{2+}$ ions was 76.08%. The $Pb^{2+}$ ions were totally removed after 72 hours of fermentation period. The removal of $Zn^{2+}$ ions from POME by *Aspergillus niger* IBS-103ZA was not as efficient as $Pb^{2+}$ ions. Only 24.79% of $Zn^{2+}$ ions were removed after 120 hours of fermentation period. The low percentage removal of $Zn^{2+}$ ions by *Aspergillus niger* biomass from industrial effluents was also reported by other researchers (Tsekova *et al.,* 2010).

The efficiency of the biosorption of heavy metal ions from aqueous solution depends on several factors such as properties of absorbent, pH, concentration of absorbate and the presence of co-ions in solution (Zhang *et al.,* 1998). The highest percentage removal of $Pb^{2+}$ ions was might be due to more electrostatic interaction with the biomass cell surface as compared to $Zn^{2+}$ ions. The binding of heavy metal ions to the cell surface due to electrostatic interaction leading to formation of complexes between metal cations and different functional binding groups found in carbohydrates, lipids, proteins and others biopolymers of microbial cell envelop (Gallil *et al.,* 2003).

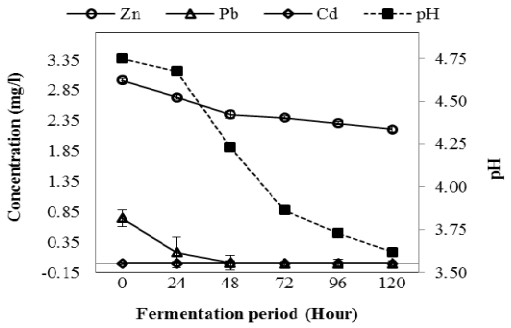


**Fig. 3:** Effect of fermentation period on total protein and total sugar consumption. Data are expressed as means ± SD ($n$ = 3).

The pH of the aqueous solution is also one of the main factors in determining the efficiency of the biosorption of heavy metal ions. Fig. 3 shows the effect of pH on biosorption of $Pb^{2+}$ and $Zn^{2+}$ ions. The highest total removal of $Pb^{2+}$and $Zn^{2+}$ ions (0.74 and 0.56 mg/l respectively) from POME took place in the pH range of 4.75 to 4.23. At pH below 4, the biosorption of $Zn^{2+}$ ions was found to be relatively slow. In total, only 0.19 mg/l of $Zn^{2+}$ ions were removed from POME in the pH range of 3.87 to 3.62. The low biosorption capacity of $Zn^{2+}$ ions at pH values below 4 could be explained in the same way as reported by (Huang, 1991). The competition between hydrogen ions and metal ions for the available biosorption sites attributes to the low biosorption of metal ions at low pH. At lower pH, due to protonation of the binding sites resulting from high concentration of protons, negative charge density on the sites reduced, which resulted in the reduction or inhibition of the binding of metal ions (Mullen *et al.,* 1992). With an increase in pH, the negative charge density on the surface increased due to deprotonation at the metal binding sites, which in turn increased the biosorption capacity of the biomass.

In this study, it was found that the *Aspergillus niger* IBS-103ZA biomass could be used as an economic and eco-friendly option to treat effluent especially for heavy metals removal. The IBS-103ZA fungal biomass removed $Pb^{2+}$ ions efficiently as compared to $Zn^{2+}$ ions from POME. Within 72 hours of fermentation period, $Pb^{2+}$ ions were totally removed but only 0.62 mg/l of $Zn^{2+}$ions was removed from initial concentration of 3.02±0.03 mg/l. The permissible discharge limits for Pb and Zn concentrations according to Environmental Quality (Sewage and Industrial Effluents) Regulation 1979 were 0.1 and 2.0 mg/l for Standard A respectively, and 0.5 and 2.0 mg/l for Standard B respectively (Department of Environment., Environmental Quality Report 2003). In this case, after 72 hours of fermentation, the concentration of remaining $Zn^{2+}$ ions in the fermentation effluent was still higher (2.40±0.02 mg/l) as compared to the standard permitted by Department of Environmental of Malaysia.

***Conclusion:***

In this study, *Aspergillus niger* IBS-103ZA was able to reduce the COD value to 24.44%. The low percentage of removal was might be due to high concentration of solid content in POME used. In the case of heavy metals removal, *Aspergillus niger* IBS-103ZA biomass absorbed $Pb^{2+}$ ions more effective as compared to $Zn^{2+}$ ions. Within 72 hours of fermentation period, $Pb^{2+}$ ions were totally removed. Only 24.79% of $Zn^{2+}$ ions were removed after 120 hours of fermentation period. The highest phenolics production (949.56±3.82 GAE mg/l) and total protein (76.58±0.43 g/kg-dried biomass) were obtained at 72 and 48 hours of fermentation hours respectively. Thus, treating POME via fermentation with *Aspergillus niger* IBS-103ZA not only increased its value added product but also reduces the pollution load in the effluent.

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