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Bioconversion of Oil Palm Frond by *Aspergillus niger* to Enhances It's Fermentable Sugar Production

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Abstract: The aim of this study was to develop an economical bioprocess to produce the fermentable sugars at laboratory scales Using Oil Palm Frond (OPF) as substrate in Solid State Fermentation (SSF). OPF waste generated by oil palm plantations is a major problem in terms of waste management. However, this lignocellulosic waste material is a cheap source of cellulose. We used OPF as substrate to produce fermentable sugars. The high content of cellulose in OPF promises the high fermentable sugars production in SSF. Saccharification of OPF waste by *A. niger* USMAI1 generates fermentable sugars and was evaluated through a solid state fermentation. Physical parameters, e.g., inoculum size, initial substrate moisture, initial pH, incubation temperature and the size of substrate were optimized to obtain the maximum fermentable sugars from oil palm fronds. Up to 77 mg of fermentable sugars per gram substrate was produced under the optimal physical parameter conditions. Lower productivity of fermentable sugars, 32 mg fermentable sugars per gram substrate was obtained under non optimized conditions. The results indicated that about 140.6% increase in fermentable sugar production after optimization of the physical parameters. Glucose was the major end component amongst the fermentable sugars obtained. This study indicated that under optimum physical parameter conditions, the OPF waste can be utilized to produce fermentable sugars which then convert into other products such as alcohol.

Key words: Oil palm frond, fermentable sugars, solid state fermentation, *Aspergillus niger*

INTRODUCTION

Elaeis guineensis, oil palm, originated in tropical West Africa. In Malaysia oil palm is planted commercially for the production of palm oil. Malaysia currently produces more than 80% of the world's crude palm oil. In year 2008, approximately 51 million tons of oil palm frond (OPF) was generated from oil palm plantation (Goh *et al*., 2010). OPF is a lignocellulolytic biomass that is an inexpensive raw material containing 40-60% cellulose, 20-40% hemicellulose and 15-30% lignin (Clarke, 1997). Recently, the enzymatic hydrolysis of lignocellulosic residues to fermentable sugars is considered as biotechnological process of high potential (Cara *et al*., 2007; Ibrahim *et al*., 2012a).

A variety of microorganisms, including bacteria, yeast and filamentous fungi, can produce xylanolytic enzymes (Coughlan and Hazlewood, 1993). The potential applications of xylanases with or without concomitant use of cellulose include the bioconversion of lignocelluloses to sugar, ethanol and other useful substances, clarification of juices and wine and nutritional value improvement of silage and green feed (Viikari *et al*., 1994). *Aspergillus niger*, a filamentous fungus, is one of the most commonly used organisms in the industrial production of enzymes. These enzymes play an important role in the degradation of lignocellulolytic material to monomers such as fermentable sugars.

Solid State Fermentation (SSF) is the growth of organisms on moist substrates in the absence of free-flowing water (Sun *et al*., 2009; Ibrahim *et al*., 2012b). The use of SSF for the production of enzymes and other products has plenty advantages over submerged fermentations (Lonsane and Ramesh, 1990). Some of the advantages are as follows: there will be no requirement for complex machinery or sophisticated control systems; only lower volume of liquid is required for product recovery, which is more cost-effective for downstream processing and subsequent waste treatment; usability of simple, cheap media for the fermentation; lower energy demand but a high product yield; lower risk of contamination due to the inability of most contamination to grow in the

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absence of free-flowing water (Pandey, 1992). A number of fungal species grows well on moist substrates in the absence of free-flowing water. However, many bacteria are unable to grow under these conditions. Thus, most studies involving SSF have been conducted by using fungi (Gessesse and Mamo, 1999).

The purpose of this communication is to report the use of OPF as a substrate for fermentable sugar production via SSF by a local isolate *Aspergillus niger* USM A11. In addition, the optimum physical parameter condition in SSF which to optimize the production of fermentable sugars was also studied in this study.

MATERIALS AND METHODS

Microorganism and inoculum preparation: *A. niger* USMAI1 was isolated earlier and kept at the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The culture was grown on Potato Dextrose Agar (PDA) (Oxoid, England) slants at 37°C until sporulation for 5 days and then maintained at 4°C on PDA slant. The 5 day old slants were used for inoculum preparation. The inoculum was prepared by adding 5 mL of sterile distilled water into the agar slant resulting in a spore suspension of 1×10^7 spores mL^{-1}.

Substrate preparation: OPF was air dried and milled to a 0.5 mm. Five grams of milled OPF was put into a 250 mL Erlenmeyer flask and autoclaved at 121°C for 20 min.

Solid-state fermentation (SSF): SSF was carried out with sterilized solid substrate inoculated with 1 mL of inoculum the spore suspension and the moisture content was adjusted to a desired level with sterile distilled water. The contents were mixed thoroughly by using sterile glass rod and then incubated at the desired temperature. Some of the OPF particle fermented with *Apergillus niger* USMAI1 were observed with a Scanning Electron Microscope (SEM).

Fermentable sugars extraction: The fermented substrate was added into 100 mL distilled water and mixed thoroughly by shaking for 1 hour at room temperature (30±2°C) in a rotary shaker at 150 rpm. The suspension was then filtered through filter paper (Whatman No. 1). The filtrate collected and centrifuged at 5000 rpm for 20 min at 5°C. The clear supernatant was used as the crude fermentable sugar for analysis.

Analysis of fermentable sugars: The fermentable sugars were measured according to the Nelson and Somogyi method (Breuil and Saddler, 1985). One milliliter of crude filtrate solution was added to one mL of cuprum reagent. The solution was placed in boiling water for 15 min. After cooling to room temperature (30±2°C) 1 mL of Nelson reagent was added. The solution was diluted with 10 mL of distilled water and A_{540} nm measured.

Biomass estimation: Fungal biomass was measured by determining the N-acetyl glucosamine released by the acid hydrolysis of the chitin that present in the cell wall of the fungus (Sakurai *et al*., 1977). Glucosamine released from the chitin by acid hydrolysis was mixed with 1 mL acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, 6 mL ethanol was added followed by the addition of 1 mL of Ehrlich reagent and incubated at 65°C for 10 min. After cooled to room temperature (30±2°C), OD_{530} was measured against the reagent blank.

Fermentable sugar analysis: The fermentable sugars obtained from the hydrolysis of OPF were analyzed by using a HPLC (Waters Corporation, USA) pump equipped with an automatic injector, 10 µL injection capacity loop, a 4.6×250 mm (C-18) High-Performance Carbohydrate Analysis Cartridge Column, a chromatography computing integrator with ELSD detector. The mobile phase used was acetonitrile and the flow rate was adjusted to 1.0 mL min^{-1} at room temperature (30±2°C).

Statistical analysis: Statistical comparisons were conducted using the Student's t-test and $p<0.05$ was considered significant. All statistical analyses were carried out with SPSS 12.0.1.

RESULTS

Figure 1 presents the production of fermentable sugars and fungal growth for 7 days of cultivation period. The production of fermentable sugars and fungal growth started to increase gradually and achieved maximum value on the 5 days cultivation with fermentable sugars of 32 mg g^{-1} substrate and fungal growth of 427 µg glucosamine g^{-1} substrate. However, further cultivation caused the level of fermentable sugars production did not increase.

When moisture content in the substrate was optimized at 120% (v/w), 49 mg g^{-1} of fermentable sugars were produced with the fungal growth of 387 µg glucosamine g^{-1} substrate (Fig. 2). However, when the moisture level was at 60% (v/w) or 80% (v/w) the amount of fermentable sugars obtained was less than 43.1 mg g^{-1}. The same condition occurred when the moisture level was more than 120% (v/w). The results showed that the moisture content in the substrate can significantly affect the fermentable sugars production ($p<0.05$) in SSF.

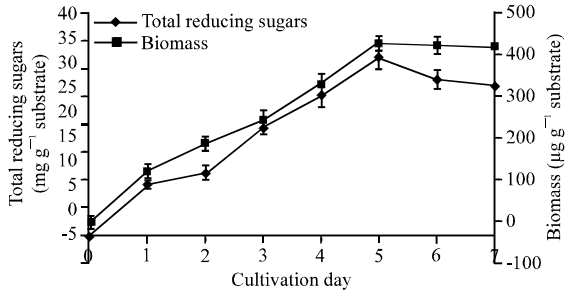

Fig. 1: Time course of fermentable sugars production by *A. niger* USM AI1 grown on OPF using solid state fermentation, The moisture content and incubation temperature were 80% (v/w) and 30°C, respectively, Particle size 0.75 mm, inoculums size was 1×10^6 spores mL^{-1}, Experiment were performed in triplicates (n = 3)

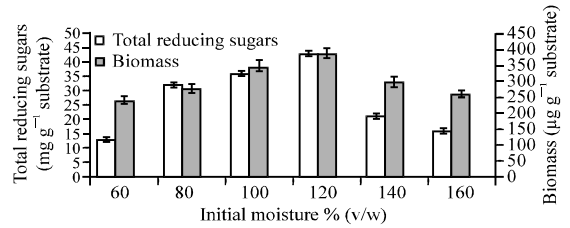

Fig. 2: Effect of moisture level on fermentable sugars production by *A. niger* USM AI1, The incubation temperature and particle size were 30°C and 0.75 mm, respectively, Inoculum size was 1×10^6 spores mL^{-1}, Experiment were performed in triplicates (n = 3)

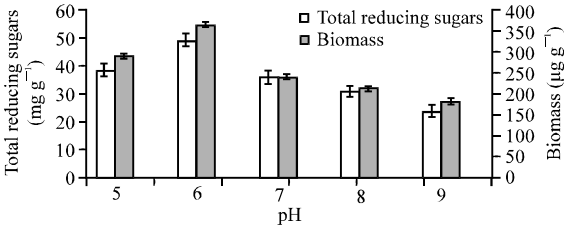

Fig. 3: Influence of initial pH of moistening solution on fermentable sugars production under SSF by *A. niger* USM AI1, Culture conditions were particle size 0.75 mm, initial moisture content, 120% (v/w), incubation temperature 30°C and inoculums size was 1×10^6 spores mL^{-1}, Experiment were performed in triplicates (n = 3)

Figure 3 showed the production of fermentable sugars was significant different at various initial pH in substrate ($p<0.05$). The optimum initial pH for fermentable

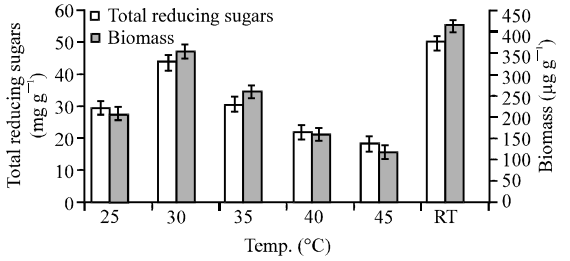

Fig. 4: Effect of incubation temperature on fermentable sugars production by *A. niger* USM AI1, Culture conditions were particle size 0.75 mm, initial moisture content 120% (v/w), pH of moistening solution was 6 and inoculum size was 1×10^6 spores mL^{-1}, Experiment were performed in triplicates (n = 3), RT: Room temperature, 30±2°C

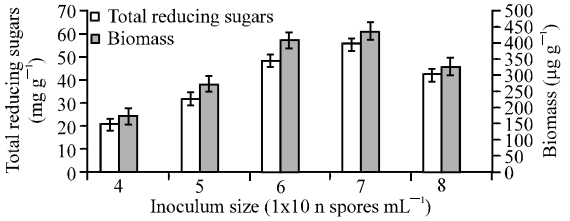

Fig. 5: Effect of inoculum sizes on fermentable sugars production by *A. niger* USM AI1 under SSF, Fermentation conditions include particle size 0.75 mm, initial moisture content, 120% (v/w), pH of moistening solution was 6 and incubation temperature 30±2°C (room temperature), Experiment were performed in triplicates (n = 3)

sugar production was at pH 6 and the maximum value of fermentable sugars and fungal growth obtained were 48.8 mg g^{-1} substrate and 363 glucosamine g^{-1} substrate, respectively. It was noted that fermentable sugar production was favoured by an acidic pH as an alkaline pH decreased fermentable sugars production of considerably.

The results of the present study (Fig. 4) reveal that incubation at room temperature, 30±2°C (50 mg g^{-1}) was the optimal for fermentable sugar production. The effect of temperature on growth of *A. niger* USMAI1 as well as on fermentable sugar production suggests that temperature has a similar effect on both growth and production of enzyme to hydrolyse OPF to fermentable sugars ($p<0.05$).

An inoculum of 1×10^7 spores mL^{-1} yielded the maximum (56 mg g^{-1} substrate, $p<0.05$) fermentable sugars production (Fig. 5). The fermentable sugar yield was reduced at lower and higher inoculum size. A very

Table 1: The optimum of physical parameter conditions for the production of reducing sugars (fermentable sugars) from OPF by *A. niger* USM AI1 using SSF system

Parameter	Optimum condition
Inoculum size	1×10^7 spores mL^{-1}
Initial moisture	120% (v/w)
pH	pH 6
Incubation temperature	30±2°C (room temperature)
Particle size of OPF	0.5 (mm)

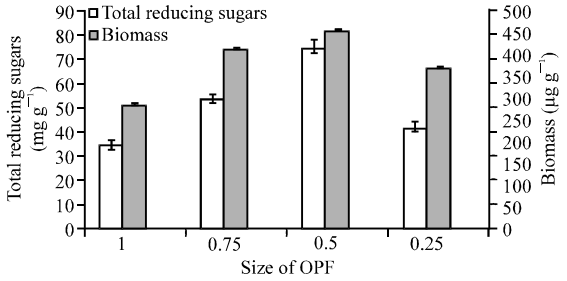

Fig. 6: Effect of particle size on fermentable sugars production by *A. niger* USM AI1, Fermentation conditions included inoculum size of 1×10^7, initial moisture content, 120% (v/w), pH of moistening solution was 6 and incubation temperature 30±2°C (room temperature), Experiment were performed in triplicates (n = 3)

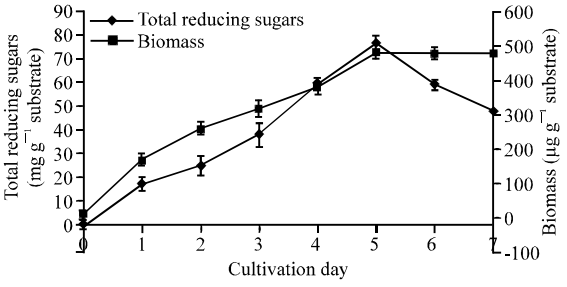

Fig. 7: The profile of growth and fermentable sugars production by *A. niger* USM AI1 after optimization of physical parameters on OPF, Experiment were performed in triplicates (n = 3)

low inoculum size was inadequate for fermentable sugar production, while an inoculum size above the optimum level reduced the yield, probably due to the competition for nutrients by the microorganisms in the SSF.

Different sizes of substrate have an impact on fermentable sugars production in SSF from OPF ($p<0.05$). We found that Fig. 6 shows the size of 0.5 mm substrate (OPF) gave the highest fermentable sugar production of 75 mg g^{-1} substrate with fungal growth of 453 mg glucosamine g^{-1} substrate.

Optimum culture condition was shown in Table 1. After 5 days of cultivation period, the maximum

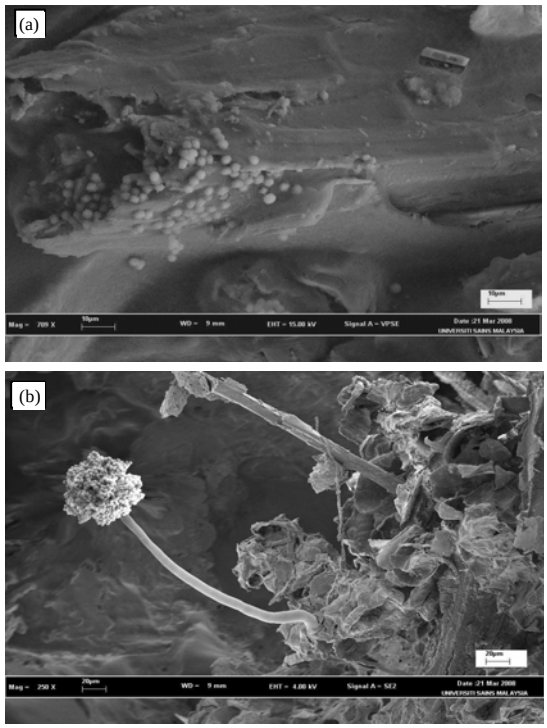

Fig. 8(a-b): Scanning electron micrographs (SEM) of OPF cultivated with *A. niger* USM AI1 with different cultivation days (a) Day 0 and (b) Day 3

production of fermentable sugars and fungal growth were 77 mg g^{-1} substrate and 478 µg glucosamine g^{-1} substrate, respectively (Fig. 7). There was about 140.6% increase in fermentable sugar production compared to before optimization of culture condition which only achieved 32 mg g^{-1} substrate.

SEM micrograph show that the use of the OPF for fermentable sugar production by *A. niger* USMAI1 resulted in distortion of the cell wall layer (Fig. 8). Figure 8(a) shows an initial day of OPF fermentation. The surface of OPF only had the spores of *A. niger* USMAI1. After 3 days of fermentation, the fungus was visibly growing on OPF (Fig. 8b).

HPLC analysis for the fermentable sugars obtained during OPF hydrolysis includes xylose, fructose and glucose (Fig. 9). However, the end product was primarily glucose. It was found that 5 gram of OPF that was inoculated with *A. niger* USMAI1 can yield 0.19 g of glucose, a 3.8% conversion.

DISCUSSION

Throughout the SSF process, the selection of an appropriate substrate is a key factor in the success of this

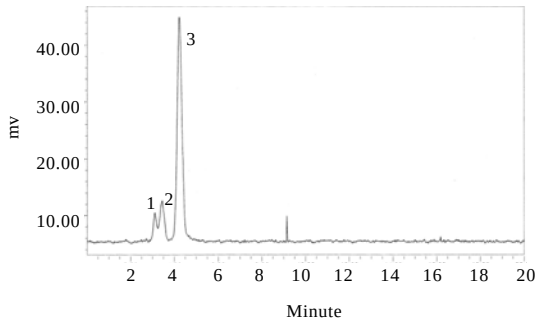

Fig. 9: HPLC chromatogram of fermentable sugars obtained from the OPF cultivated with *A. niger* USM AI1, 1: Xylose, 2: Fructose and 3: Glucose

process (Niladevi *et al*., 2007). According to Nasser Al-Shorgani *et al*. (2012), it has been common practice to use lignocellulosic agroindustrial residues to produce fermentable sugars. In Malaysia, OPF is a solid agrowaste which is abundantly available on oil palm plantation (Ibrahim *et al*., 2012b). Thus, in this study SSF was carried out for the production of fermentable sugars from OPF inoculated with local isolate *A. niger* USMAI1. Physical factors such as incubation temperature, moisture, initial pH, inuculum size and particle size of substrate have play an important role in the adherence of the fungus to the solid substrate, which in turn affects the production of enzyme and may affect the production of fermentable sugars (Mulimani and Patil Ramalingam, 2000). Therefore, in this study the physical parameters were optimized individually in order to increase fermentable sugar production.

The moisture content of the fermentation medium often determines the success of a SSF process (Lonsane *et al*., 1985; Rashid *et al*., 2011). The critical importance of moisture level in SSF media and its influence on the hydrolysis of substrate have been attributed to the interference of moisture with the physical properties of the solid particles. In SSF processes involving fungi and bacteria, higher moisture levels decrease porosity, change OPF particle structure, promote development of stickiness and reduce gas volume, gas exchange and gas diffusion. These conditions result in lowered oxygen transfer and enhance the formation of aerial mycelium (Zadrazil and Brunnert, 1981). However, lower moisture contents of the solid substrate reduce swelling and increases water tension (Battan *et al*., 2006). Thus, the microorganisms might not grow on the substrate due to lack of water content. Nevertheless, the optimum moisture content for fermentable sugars production under SSF is dependent upon the water binding properties of the substrate as well as the microorganism used.

Controlling the pH of a multi-phase growth medium is a difficult task, the majority of laboratory scale of SSF studies deal with the effect of the initial pH of the moist solid substrate on process outcome (Rajoka *et al*., 2005). The optimum initial pH in this study was pH 6. According to Gawande and Kamat (1999), fermentable sugar production by *A. niger* in SSF occurs at optimum pH of 5.5. At the optimum pH, the metabolic pathways of fungus might have been changed and caused the secretion of more lignocellulolytic enzyme which can enhance the degradation on lignocellulolytic material (Feng *et al*., 2003).

Temperature is important factor in SSF system. This is because the temperature of the fermenting mass increases due to respiration during fermentation (Pandey and Radhakrishnan, 1992). The impact of temperature not only is prominent in scale-up processes, but it is also important in all fermentation systems due to its impact on microbial growth and metabolite production. Within a certain temperature range, an appropriate decreasing the temperature would increase mRNA stability and extend the enzyme production period. However, the operating temperature cannot be too low since the biochemical reaction rate usually decreases with decreasing temperature. In this study, room temperature as the optimum cultivation temperature which is similar to the natural habitat of *A. niger* USM AI1. Feng *et al*. (2003) have concluded temperature is a significant physical parameter that turns effect in SSF.

The adherence and penetration of microorganisms as well as enzyme action on the substrate depend upon the physical properties of the substrate such as the accessible area, surface area, porosity, particle size, etc. Particle size plays a major role because all of the other physical properties of the substrate depend on it (Niladevi *et al*., 2007). The enzyme yield was lower for either larger or smaller particle sizes which was consistent with the hypothesis that lower particle size results in substrate agglomeration, enhanced channelling problems and decreased heat transfer, while larger particles reduce enzyme production due to limited surface area for microbial attack (Pandey *et al*., 2000).

The results of optimization study suggested that OPF has potential to be a substrate for the fermentable sugars production in SSF. Similar finding on the effect of physical parameter towards production of fermentable sugars using lignocellulosic biomass as substrate in SSF have been reported by Lim (2011).

The micrographs obtained confirm that enzymatically degradable lignocellulosic substrate produced the observed fermentable sugar. Trigo and Ball (1994) reported that fungal hyphae can readily colonize plant biomass. Our finding is also similar to that of Itoh *et al*.

(2003) who reported that fungi were commonly used for the enzymatic hydrolysis of agricultural wastes and to produce fermentable sugars.

The fermentable sugar obtained contain mainly of glucose is an important key for the bioethanol production process. Given all that has been reported, OPF is rich in glucose and it has recorded as high as 66% of the percentage of polysaccharide composition of OPF (Wanrosli *et al*., 2007). The high glucose content in the fermentable sugar obtained could ensure the degree of conversion to bioethanol. Previous studies have reported that the *Saccharomyces cerevisiae* is a yeast which can use glucose as a carbon source to produce a high concentration of ethanol (Sanchez and Cardona, 2008; Gupta *et al*., 2009; Hong *et al*., 2012).

CONCLUSION

From this study, we found that physical parameters could significantly affect the amount of fermentable sugars produced. This information is necessary as it would enable someone to minimize the production cost and optimize the cost-effectiveness for the overall production process. As further study, the pretreatment of OPF should be presented to enhance the production of fermentable sugars for practical application in industrial case.

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