

Full Length Research Paper

Oil Palm Frond for the Production of Bioethanol

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In order to investigate the possibility of using oil palm frond (OPF) as biomass resource for alternative energy production, the chemical composition of OPF was examined. OPF used in this study contained 50.7% holocellulose. It was enzymatically hydrolyzed by *Aspergillus niger* USM AI1 in a solid substrate fermentation (SSF) and ethanol was produced from the hydrolyzates in a second fermentation with the yeast, *Saccharomyces cerevisiae*. The yield of fermentable sugars was 200.2 mg/ g substrate which contained 69.2% of glucose. The fermentable sugar obtained from OPF could be used by *S. cerevisiae* to produce ethanol. After 36 h of fermentation, the ethanol was about 23.1 g/L with 6% (w/v) of the fermentable sugar used as the carbon source in the fermentation medium at 30°C. Thus, this finding indicates that OPF can be used as suitable lignocellulosic material for the production of fermentable sugars through *in-situ* enzymatic hydrolysis under the SSF condition for second generation bioethanol production.

Keywords: Oil palm frond, fermentable sugars, bioethanol, *Saccharomyces cerevisiae*

INTRODUCTION

Natural resources such as petroleum have been consumed at high rate over last decades. The heavy reliance on this fuel is bound to end, due to environmental impact and to the fact that they might eventually run out. Therefore, alternative resources like bioethanol are becoming more important to overcome those problems. Some bioprocesses have rendered possible routes for producing ethanol in large volumes using the low cost substrates (Gunasekaran and Raj, 1999). Late 1990, the concept of waste to wealth has been focused, especially the agrowaste which can be converted into value added products while reducing waste generation and enhancing eco-efficiency (Goh et al., 2010).

Malaysia is the world's second largest palm oil producer. In the year 2008, Malaysia has generated approximately 51 million tons of OPF, accounting for 53% of the total palm biomass (Goh et al., 2010; MPOB, 2009). Thus, OPF is a solid agrowaste which is

abundantly available on oil palm plantations (Goh et al., 2010). Currently, the disposal of the OPF is by direct decaying in the natural environment or by burning on site, with only a small amount being composted. These practices are creating environmental problems, and alternative ways to utilize and/or dispose OPF are needed (Tan et al., 2011). Hence, utilization of oil palm biomass for the production of environmental friendly biofuel has become an attractive approach instead of creating environmental pollution problems (Chew and Bhatia, 2008).

OPF consists primarily of lignocellulosic components, i.e. celluloses, hemicelluloses and lignin (Sjostrom, 1981). Only a few studies have focused on utilizing the lignocellulosic components of OPF. One approach is to hydrolyze the lignocellulosic materials into fermentable saccharides, which are then converted to fuels such as bioethanol, this is a promising alternative energy source for the limited crude oil (Sun and Cheng, 2002). However, the cost of ethanol production from lignocellulosic materials is relatively high based on current technologies, and the main challenges are caused by the low yield and high cost of the hydrolysis process (Sun and Cheng 2002). Therefore, recently

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considerable research efforts have been made by researcher to improve the hydrolysis of lignocellulosic materials.

Hydrolysis of cellulosic materials has been intensively explored since the 1970s (Wen et al., 2004). Degradation of OPF to monomeric sugars by cellulolytic enzymes is important, since the sugars can serve as raw materials in biotechnological production processes (Juhász et al., 2005) like ethanol (Lawford and Rousseau, 2003), lactic acid (El-Hawary et al., 2003) and hydrogen (Taguchi et al., 1996). Enzymatic hydrolysis has many advantages such as high yields of pure glucose, low environmental impact, and mild reaction conditions (Wen et al., 2004).

Over the past decades, interest in enzyme-based technologies has increased worldwide and recently research for optimization of physical and chemical parameters for the production of fermentable sugars has been of particular interest. Production of enzymes on-site instead of using commercial enzymes can improve the economy of the process. According to Mojovic et al. (2006), the main advantages of using enzymatic hydrolysis of biomass for fermentable production are lower energy consumption, environmental friendly process and lower content of non-glucosidic impurities, and yet much better serve as ethanol production medium.

Increases in energy consumption and dependence on crude oil to meet energy demands, have led to interest in alternative energy sources, especially those that use abundant, low cost agrowaste as the substrates in a fermentation. However, no report has been seen on hydrolyzing OPF which contain high holocellulose through the *in-situ* enzymatic hydrolysis by fungus. Therefore, the aim of the present work was to study OPF conversion of its lignocellulosic component into fermentable sugars. Furthermore, conversion of fermentable sugars from OPF to ethanol was attempted.

MATERIALS AND METHODS

Chemical Analysis of OPF

Processed OPF fiber strands were obtained from a local palm oil mill in Sungai Bakap, Penang, Malaysia. Proximate chemical analyses of the OPF in relation to ash and lignin were carried out according to standard methods (AOAC, 1997). Protein content was determined using the method of Makro-Kjeldahl (AOAC, 1997). Holocellulose which is the sum of hemicelluloses and cellulose in the biomass was analyzed using the method reported by Teramoto et al. (2009).

Substrate preparation, culture maintenance and inoculums preparation

OPF obtained was dried in oven at 50°C and milled with grinder machine (Rong Tsong Precision Technology Corporation, Taiwan) to 0.5 mm. It was then weighed and put into a conical flask and autoclaved at 121°C for 20 min for sterilisation purpose.

The fungus used, *A. niger* USMA11 was maintained on 3.9% (w/v) potato dextrose agar (Himedia, India) slants at 37°C for 5

days aerobically until sporulate, before keeping them at 4°C until further used. The inoculum was prepared by adding 5.0 ml of sterile distilled water to the agar slants and shaking vigorously. The spore suspension that obtained was adjusted to the desired concentration by using a haemocytometer slide chamber (Neubauer, Germany) and used as the inoculums.

Solid-substrate fermentation (SSF)

The 5 g of 0.5 mm sterilized OPF was inoculated with 1.0 ml of inoculums (1×10^7 spores/ml), 1.5% (w/w) of corn steep liquor (nitrogen source), 1.0% (w/w) carboxymethyl cellulose (inducer), initial pH of 7 and the moisture content was adjusted to 160% (v/w) with sterile distilled water. The contents were mixed thoroughly by using a sterile spatula and incubated at room temperature ($30 \pm 2^\circ\text{C}$). Sample as a whole flask was withdrawn every day for one week of cultivation. The experiment was carried-out in triplicate and the results obtained were reported as mean of the triplicate experiments.

A small quantity of OPF before fermented and after fermented with *A. niger* USMA11 were observed with field emission scanning electron microscopy (Leo Supra 50 VP, Germany). Samples were mounted on aluminum sample stubs and sputter coated with a thin layer of gold before observe under the scanning electron microscope (Lim et al., 2010).

Fermentable sugars extraction

The crude fermentable sugar from the fermented OPF was extracted by a simple contact method. The fermented OPF was suspended in 100 ml of distilled water and then incubated for 1 h at room temperature ($30 \pm 2^\circ\text{C}$) in a rotary shaker at 150 rpm. At the end of the incubation, the contents of the flask were filtered through Whatman No. 1 filter paper, and the filtrate was further centrifuged for 30 min at 5000 rpm, 4 °C (Sigma 4k15, Sartorius, Germany), and then used as the crude fermentable sugars for analysis. The crude fermentable sugar was concentrated by using rotary-evaporator (Eyela OSB 2100, China) and kept in the freezer as a fermentation medium for the production of bioethanol.

Ethanol fermentation

This fermentation was conducted in a 250 ml shake flask with 200 rpm at 30°C, initial pH medium of 5, 6% (w/v) concentration of fermentable sugars, and inoculated with 10% (v/v) inoculum of *S. cerevisiae* with the concentration of $\text{OD}_{540}=0.5$. The fermentation was carried out for 48 h. All experiment was conducted in triplicate.

Analyses

Fermentable sugar obtained was measured by the Nelson and Somogyi method (Breuil and Saddler, 1984). The fermentable sugar obtained was further characterized by a High Performance Liquid Chromatography (HPLC) (Waters Corporation, USA), pump equipped with an automatic injector, 10 µl injection capacity loop, a 4.6 mm x 250 mm (C-18) High-Performance Carbohydrate Analysis Cartridge Column (Waters, Ireland) and chromatography computing integrator with ELSD detector (Polymer laboratories, UK). The mobile phase used was 50% of acetonitrile and the flow rate was adjusted to 1.0 ml min⁻¹ at room temperature ($30 \pm 2^\circ\text{C}$).

The fungal biomass was measured by determining the amount of N-acetyl glucosamine released by the acid hydrolysis of the chitin, that present in the cell wall of the fungus (Sakurai et al., 1977). Ethanol production was estimated by Gas Chromatography (GC)

Table 1: Chemical composition of oil palm fronds strands

Component	Value (%)
Cellulose	31.5 ± 0.3
Hemicellulose	19.2 ± 0.1
Lignin	14.0 ± 0.5
Ash	12.3 ± 0.2
Protein	9.4 ± 0.1

Clarus 500 (Perkin Elmer, USA) with J & W GC column, USA (Length of 30.0 m, Inner diameter of 0.32 mm and Film of 0.1 µm). The temperature of the column and the injector were set at 200°C. The pressure of injector and column were adjusted to 5 kg/cm². Nitrogen gas was used as gas carrier in this analysis.

RESULTS AND DISCUSSION

Table 1 summarizes the results of chemical composition of oil palm frond strands. The OPF is herbaceous and contains 50.7% of holocellulose with 31.5% cellulose and 19.2% hemicelluloses in raw biomass. OPF strands are rich in holocellulose (50.7%) and contain higher of the level of this important compound found in other part of oil palm plant, like oil palm trunk, showed 41.9% (Choo, 2002). However, lignin content in OPF was present at a lower level (14.0%) than that normally found in hardwoods, such as aspen, about 18.1% (Law and Jiang, 2001) and eucalyptus, 22.0% (Alcaide et al., 1990). This difference may be due to the fact that oil palms are non-woody plants and that requirement for structural support differs from those of trees (WanRosli et al., 2007). The low lignin content of OPF makes enzyme hydrolysis of this material more efficient in the SSF. Nevertheless, OPF has relatively high ash content (12.3%), perhaps it is due to the presence of silica (Choo, 2002). The protein content of OPF was 2.6%, which is lower than that found in soybean, which had 9.4% protein (Siqueira et al., 2008). The protein content of a substrate in SSF plays the important roles for stimulating microbial growth.

Figure 1 shows the growth profile and total reducing sugars production of *A. niger* USMA11 on OPF which act as substrate in the SSF. The production of reducing sugars and the growth of the fungus was monitored every day for a week. The highest level of reducing sugars were obtained after four days cultivation time, a total of 200.2 mg / g substrate with a growth of 2.7 mg glucosamine / g substrate was observed. Mamma et al. (2008) obtained higher reducing sugars production by *A. niger* using orange peel as substrate in SSF, with 249.7 mg/ g substrate. Nevertheless, the decomposition of white fungus on the stems of bamboo only produced 38

mg of reducing sugar/ g substrate (Zhang et al., 2007). According to Mulimani et al. (2000) physical factors such as cultivation time is important in the adherence of the fungus to the solid substrate, which in turn affects the production of the hydrolytic enzymes and the fermentable sugar yield. This information is needed to minimize production costs and to optimize the cost-effectiveness of the overall production process (Yaoyu et al., 2003).

Fungus growth was found to increase exponentially after four days cultivation, and reached stationary phase followed by entering death phase (Figure 1). Reducing sugar production began to rise after one day of cultivation time and entered exponential stage after two days of cultivation. After four days of cultivation the production is reached a maximum level. However, reducing sugars is decreased gradually after reaching the maximum level. The same observation was reported by Ramachandran et al. (2004) for the production of reducing sugars by the fungus in SSF process.

HPLC evaluation showed fermentable sugars obtained during OPF *in-situ* enzymatic hydrolysis including xylose, fructose and glucose; however, the most common product was glucose, about 69.2%. Therefore, with 5 g of OPF inoculated with *A. niger* USMA11 then 0.69 g of glucose was obtained, or as 13.9% conversion. It is an important key because glucose is a preferred carbon source for the bioethanol production process. Sanchez and Cardona (2008) and Gupta et al., (2009) have reported that *S. cerevisiae* uses glucose to produce high level of ethanol.

Figure 2A shows the surface area of raw OPF at the initial day of fermentation which was covered with *A. niger* USMA11 spore. The web structure of the fronds showed the typical smooth surface. After 5 days of fermentation (Figure 2B), fungal hyphae became visible on the OPF. At this stage, the fungus began to produce enzymes that degrade the parenchyma cells containing cellulosic and hemicellulosic materials into fermentable sugars. It is noted that the surface structure of fermented OPF was significantly changed, giving rise rough texture. Moreover, some irregular conglomeration also happened on the surface of fronds compared to the raw fronds. These alterations were attributed by enzymatic hydrolysis process and were supported by fermentable sugars obtained from enzymatic hydrolysis of OPF.

Carlos and Ball (1994) reported that fungal hyphae can readily colonize solid biomass. Itoh et al. (2003) have reported that white rot fungi normally are used for the enzymatic hydrolysis of agrowaste and the production of fermentable sugars. *Neurospora crassa* can secrete high levels of cellulolytic and hemicellulolytic enzymes to hemicellulosic materials under SSF (Yazdi et al., 1990). Dogaris et al., (2008) reported *Neurospora crassa* DSM 1129 could assimilate the major fermentable sugars obtained under SSF during direct conversion into ethanol. Enzymatic hydrolysis of lignocellulosic biomass by enzymes such as endoglucanases, which randomly

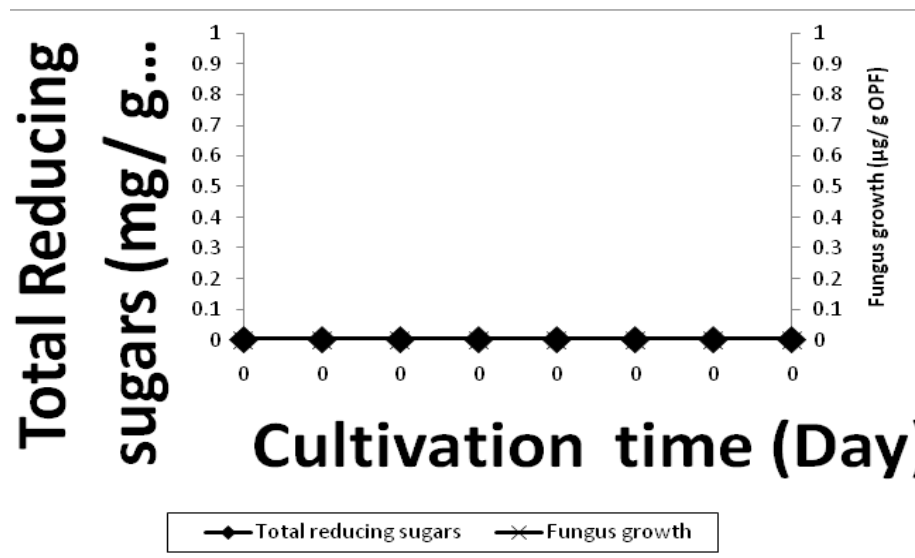


Figure 1: Growth profile and reducing sugars production by *A. niger* USM A11 on OPF. Note: The SSF process was carried out at room temperature, $30 \pm 2^\circ\text{C}$; 160% (v/w) moisture content; initial pH 7; inoculums size of 1×10^7 spore/ml; 0.5 mm of OPF, 1.5% (w/w) of corn steep liquor (nitrogen source), 1.0% (w/w) carboxymethyl cellulose (inducer)

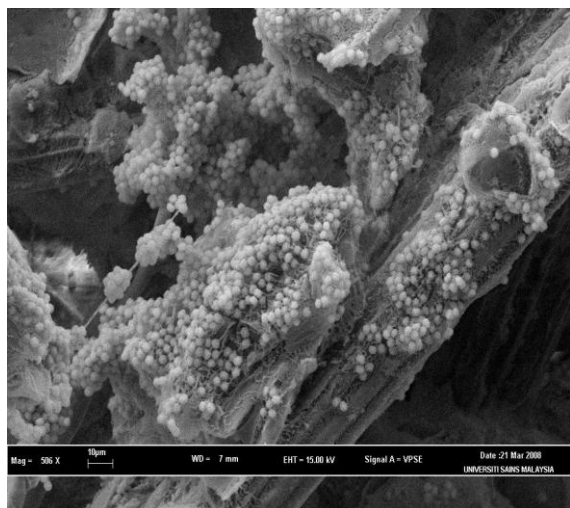


Figure 2 A : SEM micrographs of OPF fermented with *A. niger* USMA11. (A) Initial day of fermentation; (B) After 5 days of fermentation. (Note: The SSF process was carried out at room temperature, $30 \pm 2^\circ\text{C}$; 160%(v/w) moisture content; Initial pH 7; inoculums size of 1×10^7 spore/ml; 0.5 mm of OPF)

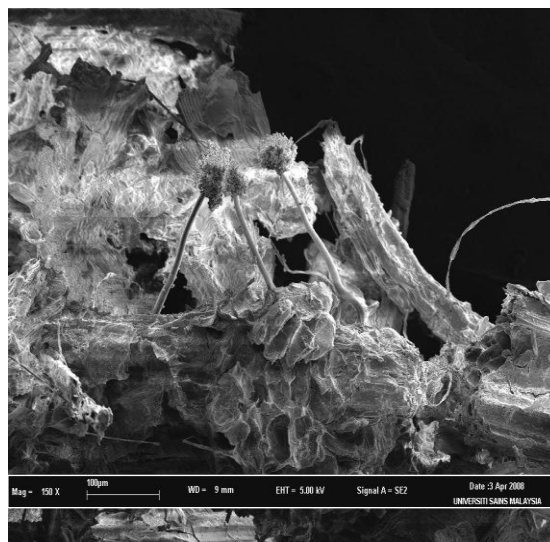


Figure 2. Cont. B

attack cellulose chains and release cello-oligosaccharides, exoglucanases; which cleave cellobiose units from the end of cellulose chains and β -glucosidase, which convert the resulting cellobiose to glucose (Bhat and Bhat, 1997). Figure 3 shows the time course of ethanol fermentation

using 6% (w/v) fermentable sugars obtained from the *in-situ* enzymatic hydrolysis of OPF as fermentation substrate. Theoretically 50% is a maximum percentage for converting reducing sugar to ethanol through fermentation (Siqueira et al., 2008). The results obtained showed OPF to be an effective substrate in providing glucose for ethanol fermentation. The highest ethanol concentration produced after 36 h of fermentation by *S. cerevisiae* reached approximately 23.1 g/L (45.5% of the

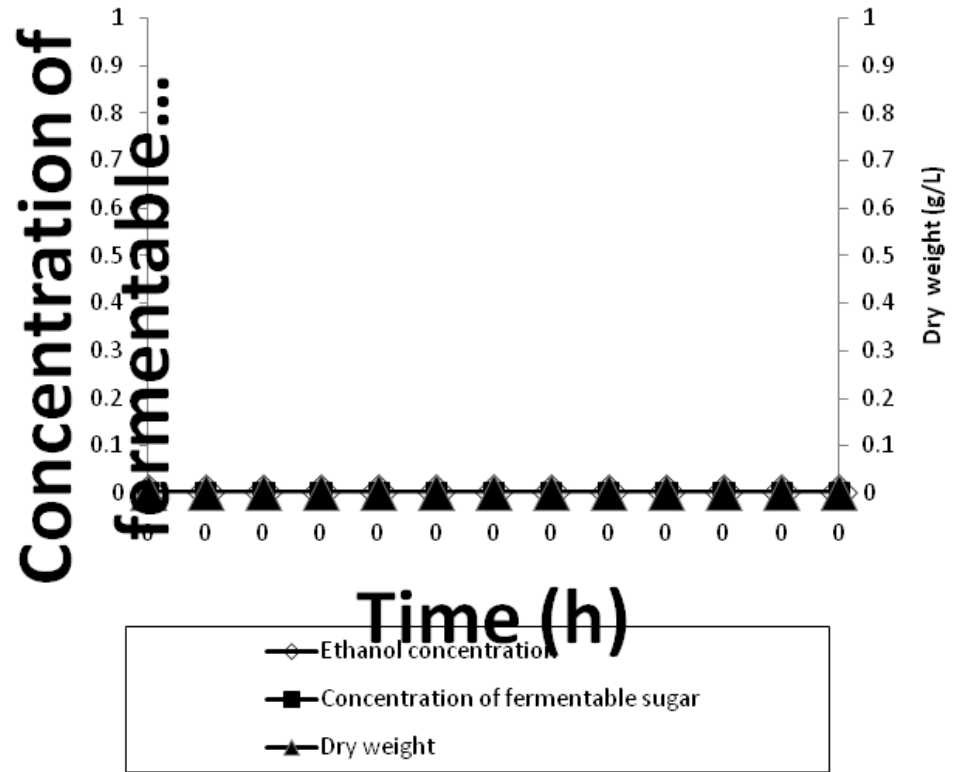


Figure 3: Time course of ethanol production by *S. cerevisiae* from fermentable sugar obtained from the OPF cultivated with *A. niger* USM A11. Note: The ethanol fermentation was carried out under the indicated yeast; 10% (v/v) inoculum size; 6% (w/v) concentration of reducing sugar; initial medium of pH 5; agitation rate of 200 rpm; temperature of fermentation at 30°C.

yield) with 84.7% of reducing sugars was consumed. The concentration of fermentable sugars declines as the ethanol fermentation time increases. Similar patterns of fermentable sugars utilization also was observed in the fermentation of bioethanol by *S. cerevisiae* (Matsushika *et al.*, 2009). Glucose conversion to ethanol by *S. cerevisiae* has been reported since the 19th century. Thus, the fermentable sugar obtained from this SSF is suitable as a carbon source for bioethanol production without adding other nutrient in the fermentation medium.

CONCLUSION

The present work shows that OPF has potential for development as an economical alternative energy source because of easy conversion to fermentable sugars. Hydrolysis of untreated OPF by the fungal enzymes was associated with the formation of monomeric carbohydrates. *S. cerevisiae* could assimilate most of the sugars released from OPF through the SSF system into ethanol. Thus, the fermentable sugars obtained can be used as medium for producing value added products such as bioethanol. As further study, the pretreatment of

OPF should be presented to enhance the production of fermentable sugars for practical application in industrial case like ethanol production.

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REFERENCES

- Alcaide LJ, Parra IS, Baldovin FL (1990). Characterization of Spanish agricultural residues with a view in obtaining cellulose pulp. *Tappi J.*, 73 (8): 173-177.
- AOAC (1997). Official Method of Analysis of AOAC International. (Cunniff, P. A. ed). 16th Ed. Virginia: AOAC Intl., pp. 25-116.
- Bhat MK, Bhat S (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv.*, 15: 583-620.
- Breuil C, Saddler N (1984). Comparison of the 3,5-dinitrosalicylic acid and Nelson-Somogyi methods of assaying for reducing sugars and determining cellulase activity. *Enzym. Microbial. Technol.*, 7: 327-332.
- Carlos T, Ball AS (1994). Is the solubilized product from the

- degradation of lignocelluloses by actinomycetes a precursor of humic substances? *J. Microbiol.*, 140: 3145-3152.
- Chew TL, Bhatia S (2008). Catalytic processes towards the production of biofuel in a palm oil and oil palm biomass-based biorefinery. *Bioresour. Technol.*, 99: 7911-7922.
- Choo KH (2002). *Development of bioprocess for converting the agrowaste to fermentable sugars and ethanol. Ph.D Thesis.* Universiti Sains Malaysia, Pulau Pinang, Pp. 63-76, 226-293.
- Dogaris I, Vakontios G, Kalogeris E, Mamma D, Kekos D (2008). Induction of cellulases and hemicellulases from *Neurospora crassa* under solid-state cultivation for bioconversion of sorghum bagasse into ethanol. *Ind. Crops Prod.*, 29: 404-411.
- El-Hawary FI, Mostafa YS, Laszlo E (2003). Cellulase production and conversion of rice straw to lactic acid by simultaneous saccharification and fermentation. *Acta Aliment Hung.*, 30: 281-295.
- Goh CS, Tan KT, Lee KT, Bhatia S (2010). Bio-ethanol from lignocelluloses: status, perspective and challenges in Malaysia. *Bioresour. Technol.*, 101: 4834-4841.
- Gunasekaran P, Raj KC (1999). Ethanol fermentation technology- *Zymomonas mobilis*. *Curr. Sci.*, 77: 56-68.
- Gupta R, Sharma KK, Kuhad RC (2009). Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. *Bioresour. Technol.*, 100: 1214-1220.
- Itoh H, Wada M, Honda Y, Kuwahara M, Watanabe T (2003). Bioorganosolve pretreatment for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. *J. Biotechnol.*, 103: 273-280.
- Juhasz T, Szengyel Z, Reczey K, Silika-Aho M, Viikari L (2005). Characterization of cellulase and hemicellulases produced by *Trichoderma reesei* on various carbon sources. *Proc. Biochem.*, 12: 1-7.
- Law KN, Jiang X (2001). Comparative papermaking properties of oil-palm empty fruit bunch. *Tappi J.*, 84(1): 13.
- Lawford HG, Rousseau JD (2003). Cellulosic fuel ethanol- alternative fermentation process designs with wild-type and recombinant *Zymomonas mobilis*. *Appl. Biochem. Biotechnol.*, 105: 457-469.
- Lee JW, Koo BW, Choi JW, Choi DH, Choi IG (2008). Evaluation of waste mushroom logs as a potential biomass resource for the production of bioethanol. *Bioresour. Technol.*, 99: 2736-2741.
- Lim SH, Darah I, Ibrahim CO (2010). Microscopic studies of oil palm frond during processing for saccharification. *Intl. J. Bioeng.*, 4 (2): 1-13
- Mamma D, Kourtoglou E, Christakopoulos P (2008). Fungal multienzyme production on industrial by-products of the citrus-processing industry. *Bioresour. Technol.*, 99: 2373-2383.
- Matsushika A, Inoue H, Murakami K, Takimura O, Sawayama S (2009). Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. *Bioresour. Technol.*, 100: 2392-2398.
- Mojovic L, Nikolic S, Rakin M, Vukasinovic M (2006). Production of bioethanol from corn meal hydrolazates. *Fuel*, 85: 1750-1755.
- MPOB (Malaysian Palm Oil Board) (2009). Monthly fresh fruit bunches received by mills: 2008 (Tonnes). <http://econ.mpob.gov.my/economy/annual/stat2008/Processing2.2pdf> . Accessed May 2010.
- Mulimani VH, Patil GN, Ramalingam (2000). α -Amylase production by solid-state fermentation: a new practical approach to biotechnology course. *Biochem. Educ.*, 28: 161-163.
- Pandey A, Soccol CR, Mitchell D (2000). New developments in solid-state fermentation: I-bioprocess and products. *Proc. Biochem.*, 35: 1153-1169.
- Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G, Pandey A (2004). Coconut oil cake – a potential raw material for the production of α -amylase. *Bioresour. Technol.*, 93: 169-174.
- Sakurai Y, Lee TH, Shiota H (1977). On the convenient method of the glucosamine estimation in koji. *Agric. Biol. Chem.*, 41: 619-624.
- Sanchez OJ, Cardona CA (2008). Trends in biotechnological production of fuel ethanol from different feedstock. *Bioresour. Technol.*, 99: 5270-5295.
- Siqueira PF, Karp SG, Carvalho JC, Sturm W, Rodriguez-Leon JA, Tholozan JL, Singhania RR, Pandey A, Soccol RC (2008). Production of bio-ethanol from soybean molasses by *Saccharomyces cerevisiae* at laboratory, pilot and industrial scales. *Bioresour. Technol.*, 99: 8156-8163.
- Sjostrom E (1981). Wood chemistry. In: *Fundamentals And Applications*. New York: Acad. Press Inc., pp. 223.
- Sun Y, Cheng J (2002). Hydrolysis of lignocellulosic material for ethanol production: a review. *Bioresour. Technol.*, 83: 1-11.
- Taguchi F, Yamaha K, Hasegawa K, Takisaito T, Hara K (1996). Continuous hydrogen production by *Cloridium* sp. strain no 2 from cellulase hydrolysate in an aqueous two-phase system. *J. Ferment. Bioeng.*, 82: 80-83.
- Tan HT, Lee KT, Rahman M (2011). Pretreatment of lignocellulosic palm biomass using a solvent-ionic liquid [BMIM]Cl for glucose recovery: An optimization study using response surface methodology. *Carbohydrate Polymers*. 83: 1862-1868.
- Teramoto Y, Lee S, Endo T (2009). Cost reduction and feedstock diversity for sulfuric acid-free ethanol cooking of lignocellulosic biomass as a pretreatment to enzymatic saccharification. *Bioresour. Technol.*, 100: 4783-4789.
- WanRosli WD, Zainuddin Z, Asro R (2007). Pulp from oil palm fronds by chemical processes. *Ind. Crops Prod.*, 25: 89-94.
- Wen Z, Liao W, Chen S (2004). Hydrolysis of animal manure lignocellulosics for reducing sugars production. *Bioresour. Technol.*, 91: 31-39.
- Yaoyu F, Zhimin H, Say Leong O, Jianguyong H, Zhigang Z, Wun-Jern Ng. (2003). Optimization of agitation, aeration, and temperature conditions for maximum β -mannanase production. *J. Enzym. Microb. Technol.*, 32: 282-289.
- Yazdi MT, Radford A, Keen JN, Woodward JR (1990). Cellulase production by *Neurospora crassa*: purification and characterization of cellulolytic enzymes. *Enzym. Microbiol. Technol.*, 12: 120-123.