Bioethanol Fermentation from Acid/Base-Treated Water Hyacinth Biomass using Fermentation Yeasts *Saccharomyces cerevisiae* YRK 017 and *Candida Shehatae* ATCC 22984

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Abstract- Water hyacinth is considered as one of most abundant lignocellulosic feedstock in Thailand. It has been selected as a substrate for producing ethanol at an economically feasible manner. This study focuses on the bioethanol production from enzyme-treated hydrolysate of acid/base pretreatment water hyacinth. Two different pretreatment methods of water hyacinth were used including either 2 %(v/v) sulfuric acid or 2 %(w/v) sodium hydroxide at 121°C for 15 min. After acid/base pretreatments, the biomass residues in order to release glucose molecules were treated with a commercial cellulase enzyme complex (ACCELLERASE® 1500) at a concentration of 0.30 mL enzyme/g biomass residue. Two yeast species consisting of *Saccharomyces cerevisiae* YRK 017 and *Candida shehatae* ATCC 22984 were separately used as inoculum in fermentation process of the hydrolysate from enzymatic treatments. The results showed that ethanol yield obtained from acid pretreatment was slightly higher than the yield from base pretreatment. In acid pretreatment, *S. cerevisiae* YRK 017 could produce bioethanol in a yield of 3.82 ± 0.10 g/L, whereas *C. shehatae* ATCC 22984 could produce yielding 2.85 ± 0.04 g/L.

Keywords- water hyacinth; acid/base pretreatment; bioethanol; *Saccharomyces cerevisiae*; *Candida shehatae*.

1. Introduction

A fast-growing plant water hyacinth *Eichhornia crassipes* is listed as an alien species in Thailand. They widely distribute over many water-canal regions around the country as an aquatic weed. Due to a huge number of this weed biomass, many approaches attempt to utilize cellulose-rich parts as a starting material for bioenergy production such as bioethanol [1], biogas [2, 3, 4], biodiesel [5], and liquid fuel [6]. Nowadays, cellulosic ethanol from cellulose-rich

materials is one of the most impressive energy sources since its ability in reducing carbon dioxide emission from the atmosphere [7, 8, 9]. Generally, the bioethanol production from lignocellulose comprises three steps including hydrolysis (to break down carbohydrate polymers to their monomers), fermentation (to metabolize those monomeric sugars to form ethanol molecules) and distillation (to separate ethanol molecules out from other contaminants). The hydrolysates can contain a various types of monosaccharide such as pentose and hexose sugars. However, there are obstacles from some contaminants derived from a raw material preparation including phenolic compounds from lignin degradation. These substances may play a role as a growth inhibitor on the microorganisms in subsequent fermentation [10]. Lignocellulosic materials from agricultural waste such as wheat straw, sugarcane bagasse, corn stover, coffee processing waste, empty palm bunch, banana stalks and other plants including *Japropha curcas* have been identified as a source for low-cost ethanol production [11, 12, 13].

During ethanol fermentation process, some microbes can metabolize monosaccharides presenting in the hydrolysates to form ethanol as reported by many studies. Nigam [10] investigated that the bioethanol production from acid-hydrolyzed water hyacinth using yeast *Pichia stipitis* NRRL y-7124. The results showed that the ethanol molecules could be produced even the yield was in low proportion. It was due to the fact that the sugar in samples will be converted partially to acetic acid and as a result in decreasing the ethanol yield. However, an industrial yeast *Saccharomyces cerevisiae* is one of the best choice to be known as bioethanol producer, even the cells can only utilize hexose sugars during fermentation process [14]. In contrast, among the xylose fermentative yeasts, *Candida shehatae* is a promising strain for bioethanol fermentation because cells can utilize both xylose and glucose sugars. However, even a high yield of bioethanol could be obtained from *C. shehatae*, the cells also showed low ethanol tolerance [15] A. Stavrinides *et al*. [16] studied on selection of a hyper-cellulolytic fungus for cellulase gene selection, the construction of a cellulolytic ethanogen through the expression of the fungal cellulolysis gene in *Saccharomyces* spp.

Recently, our previous report showed that water hyacinth could be used as a starting material for bioethanol production. Acid/base treatment could allow a release of reducing sugars including hexose and pentose sugars at about 50% from a total cellulose content. The study also found that acid/base-treated biomass remained an interesting lignocellulose content. Thus, this study aims to utilize acid/base treated water hyacinth as a starting material in bioethanol production. The objectives of this study are (1) to hydrolyze the remaining lignocellulose with a commercial cellulase enzyme complex (ACCELLERASE® 1500), (2) to produce ethanol using enzyme-treated hydrolysate from acid/base pretreated biomass and (3) to compare ethanol yields produced from *Saccharomyces cerevisiae* YRK 017 and *Candida shehatae* ATCC 22984 yeasts.

2. Materials and Methods

2.1 Microorganisms and maintenance

The yeast strain was isolated from wine rice's starter (Look-Pang-Lao) in Thailand. The organism was later identified as *Saccharomyces cerevisiae* YRK 017 in our laboratory. *S. cerevisiae* YRK 017 was maintained on YEPD medium (yeast extract 10.0 g/L, peptone 20.0 g/L, glucose 20.0 g/L, and agar 20.0 g/L) with pH 5.0.

Candida shehatae ATCC 22984 was purchased from the Culture Collection Center of Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The *C. shehatae* was maintained on SXA slants (neopeptone 10.0 g/L, xylose 20.0 g/L, and agar 25.0 g/L) with pH 6.5.

2.2 Inoculum preparation of S. cerevisiae YRK 017 and C. shehatae ATCC 22984

Both *S. cerevisiae* YRK 017 and *Candida shehatae* ATCC 22984 were grown in according medium broth. The culture density was adjusted to the cell density of about 10^6-10^7 CFU/mL (OD600 ≈ 0.5) [17] prior to inoculate into the experiment batch.

2.3 Preparation of enzyme-treated hydrolysate

Water hyacinth was randomly collected from Pasricharoen canal, Sumutsakorn Province, Thailand. It was continuously dried at 105°C for 5-6 h. in hot-air oven. The dried plant material was then cut into 2-2.5 cm. in size and further pulverized to obtain 3 mm. in size powder by milling process using an electrical blender. A 100 ml of 2.0 % (w/v) sulfuric acid or 2.0% (w/v) sodium hydroxide were separately added into 10 g of water hyacinth powder. The mixtures were autoclaved at 121°C for 15 min and further discarded a supernatant. The acid/base treated biomass was harvested and then washed with excess amount of water until filtrate pH reached 7.0 [18]. The biomass powder was dried out at 60°C for 48 h. and further used in enzymatic treatment. One gram of dried acid/base treated biomass was added in a round-bottle flask containing 100 ml of 50 mM acetate buffer, pH 5.0. A 300 µl of a commercial cellulase enzyme complex (ACCELLERASE® 1500) was added into a mixture. These

flasks were incubated at 50 °C for 48 h without shaking prior to centrifuged at 18,000 x g for 15 min. The supernatants were collected and further quantified for the reducing sugar concentration by DNS method [19] and analyzed for sugar compositions (glucose, xylose and arabinose) by HPLC system.

2.4 Ethanol fermentation

The enzyme-treated hydrolysate was supplemented with 1.0% (w/v) peptone and adjusted the pH to 6.0. Ninety milliliter of the mixture was poured into 250 mL Erlenmeyer flask and autoclaved at 121℃ for 15 min. Ten milliliter of either *S. cerevisiae* YRK 017 or *C. shehatae* ATCC 22984 cultures was separately inoculated into a 90 mL hydrolysate. The culture flask was then kept in a rotatory shaker with a speed of 150 rpm at 30 ºC. Ten milliliters of culture was withdrawn at time intervals and centrifuged at 18000×g for 10 min at 4 ℃. The supernatant was used to analyze for the remaining reducing sugar concentration by DNS method along with the produced ethanol concentration by gas chromatography.

The value of productivity from hydrolyzed water hyacinth was calculated by the following equation:

$$\text{Productivity (g/L/h)} = \frac{\text{maximum ethanol concentration} - \text{initial ethanol concentration}}{\text{fermentation time (h)}}$$

2.5 Analytical methods

2.5.1 Reducing sugars

The reducing sugars were determined using DNS method [16]

2.5.2 Sugar composition

For sugar composition determination, ten microliter of enzyme-treated supernatant was analyzed for sugar composition by HPLC system (Shimadzu, UV 1601) using 300 mm x 7.8 mm BP-800 Ca^{2+} column (Benson polymeric™) equipped with a refractive index detector (RI). The running parameters were used with the column temperature of 80 °C, detector temperature of 40 °C. The eluent was filtered water with flow rate of 0.4 ml/min.

2.5.3 Ethanol concentration

For ethanol determination, one microliter of supernatant was quantified for ethanol concentration by gas chromatograph (Shimadzu, GC-2014 (using 2B –Wax column (30 mm x 0.25 mm) with a flame ionization detector (FID). The running parameters were previously described elsewhere [20] with the column temperature of 150 °C, injection temperature of 175 °C and detector temperature of 250 °C. The carrier gas was N_2 with flow rate of 40 ml/min.

2.6 Statistical analysis

Data was reported as mean ± standard deviation from triplicate determination. Analysis of variance (ANOVA) accompanied with DMRT test (SPSS for Windows) were conducted to identify the significant difference between sample ($p < 0.05$).

3. Results and discussion

3.1 Sugar composition in enzymatic hydrolysate

Even water hyacinth is considered as fast-growing weed, the structure of this weed also contain lignocellulose as a major component which could be used in several biotechnology applications. Our previous study showed that acid or base pretreatment under the autoclave condition could hydrolyze β-1,4-glycosidic bond of those core structure releasing reducing sugars as products that could be further used in any aspects [13]. After acid or base pretreatment, our previous results also revealed that some cellulose still remained in acid-treated biomass, whereas cellulose and hemicellulose still remained in base-treated biomass. In this study, the experiment extended the utilization of cellulose-remaining biomass by enzymatic treatment. The biomass was treated with a commercial cellulase enzyme complex (ACCELLERASE® 1500) as described in section 2.3. The hydrolysate from acid or base pretreatment was later determined for reducing sugar content by DNS method. The result showed that the hydrolysates from acid-pretreated biomass produced a slightly higher reducing sugar concentration than those from base pretreatment. This may be due to the fact that acid-treated biomass contained only cellulose, thus the cellulase enzyme could easily hydrolyze its substrate without obstacle, whereas base-treated biomass contained both cellulose and hemicellulose (0.86 ± 0.11%), thus the cellulase might be obstructed by hemicellulose structure. Moreover, lignocellulose biomass treated by base could only be delignified by breaking the cross-linking ester bonds between lignin and xylan, thus increase the porosity of biomass [21].

After cellulase treatment, the sugar compositions in hydrolysate were checked by HPLC (Fig.1). As expected, it was clear that the hydrolysates contained only the respecting retention time of glucose. This evidence was inferred to our previous finding that hemicellulose was not found in biomass

after acid pretreatment. Then, this led to the loss of some hexose and pentose sugar content on water hyacinth biomass.

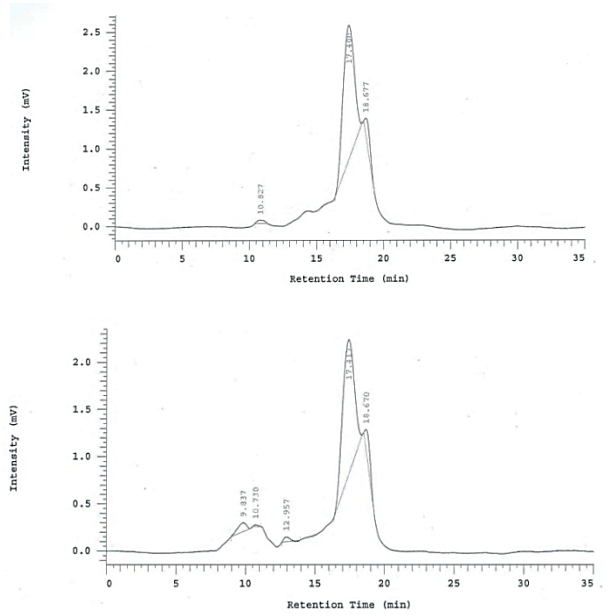

Figure 1 HPLC chromatogram of enzyme-treated hydrolysate from acid-pretreatment water hyacinth (upper) and base-treatment water hyacinth (lower).

Glucose in hydrolysate could be utilized by yeasts through their metabolisms to change sugar via glycolysis pathway prior to obtain pyruvate molecules. Under anaerobic condition, pyruvate molecules would be further metabolized through fermentation process releasing one carbon dioxide gas in order to form two-carbon alcohol, ethanol.

3.2 Ethanol production from acid-treated biomass hydrolysate

The fermentation of hydrolysate from acid-pretreated water hyacinth biomass by two yeasts, *S .cerevisiae* YRK 017 and *C. shehatae* ATCC 22984, was done at initial pH 6.0 with temperature of 30°C. The results showed in Figure 2 that *S. cerevisiae* YRK 017 rapidly utilized reducing sugars within 12 h. and remained constant concentration of about 1.2 ± 0.04 g/L to the end of experiment. As a result, ethanol production was sharply increased during the first 12 h. reaching the maximum yield of 3.65 ± 0.21 g/L. The production rate was constant after 12 h. at the average yield of 3.53 ± 0.15 g/L throughout the fermentation period with no significant increasing (Fig. 2-upper) Whereas *C. shehatae* ATCC 22984 could uptake the reducing sugar much slower than *S. cerevisiae* YRK 017. The reducing sugar was gradually decreased within 60 h. prior to remain constant afterward at the average concentration of 3.50 ± 0.03 g/L. However, ethanol production from *C. shehatae* ATCC 22984 was rapidly increased within first 12 h. yielding 2.65 ± 0.15 g/L (Fig. 2-lower) as observed the same pattern in *S. cerevisiae* YRK 017.

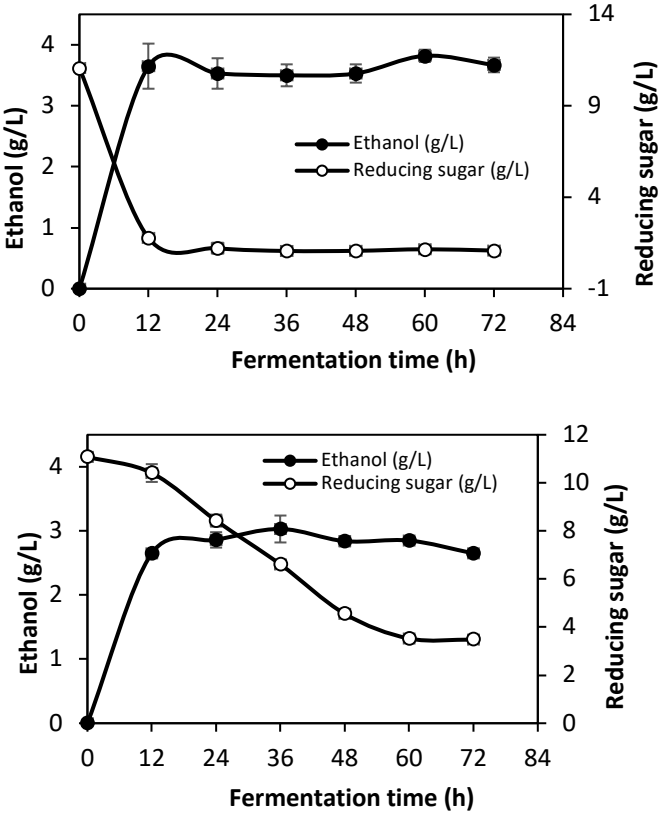

Figure 2 Concentration profiles of total reducing sugars and ethanol concentration during fermentation of acid-pretreated water hyacinth by *S. cerevisiae* YRK 017 (upper) and *C. shehatae* ATCC 22984 (lower).

Even a starting sugar concentration was not equal in both systems, *S. cerevisiae* YRK 017 still showed higher capacity to utilize most of sugar in a system and produced ethanol in higher concentration. However, our results showed a comparable yield to other reports. Isarankura-Na-Ayudhaya., *et al* [22] successfully fermented sugar in acid pretreated water hyacinth for bioethanol using *Candida shehatae*. The maximum ethanol yield of 0.19 g/g dried water hyacinth with the productivity of 0.008 $gL^{-1}h^{-1}$ was achieved. Our result revealed that the acid pretreated water hyacinth hydrolysate could be used in fermentation process by *S. cerevisiae* YRK 017 and *C. shehatae* ATCC 22984, resulted in high productivity (0.304 ± 0.002, 0.221 ± 0.080 $gL^{-1}h^{-1}$, respectively). Our results were comparable to other studies. Recently, M. Iram., *et al* [23] reported results of saccharified sugarcane bagasse fermentation using *S. cerevisiae* and *P. stipites* in mono- and/or co-culture. The maximum ethanol yield (Yp/s) of 0.49 g/g was observed in the use of

monoculture of *S. cerevisiae* after 96 h. of fermentation time. A.F. Shah., *et al* [24] also reported that the maximum yield of 0.49 g/g was obtained from the *S. cerevisiae* thermotolerant mutant when using starch industry waste as a source of sugar.

3.3 Ethanol production from base-treated biomass hydrolysate

Not only acid that could hydrolyze lignocellulose structure, base could also function in the same manner in some cases. However, hemicellulose composition (0.86 ± 0.11%) still remained in base-treated water hyacinth. A commercial cellulase enzyme complex (ACCELLERASE® 1500) was used to hydrolyse base-treated water hyacinth biomass to obtain reducing sugar solution. The enzymatic hydrolysate was used in the same aspect as in acid treated biomass hydrolysate. The results also showed a similar pattern as obtained from acid–pretreatment experiment .A starting sugar concentration was slightly lower than the concentration from acid-treatment experiment. This is due to the fact that base pretreatment on water hyacinth biomass was remained in hemicellulose which obstructed the enzymatic catalysis of lignocellulose, making the hydrolysate contained in lower sugar concentration when compared to acid pretreatment experiment.

The ethanol production results showed in Figure 3 that the overall pattern was almost similar to the pattern obtained from acid pretreatment experiment. *S. cerevisiae* YRK 017 could produce the ethanol with the maximum yield of 3.40 ± 0.18 g/L (productivity = 0.284 ± 0.032 $gL^{-1}h^{-1}$) within 12 h. and the production yield remained constant afterward with no significant difference. As far as *S. cerevisiae* YRK 017 showed a higher production yield, *C. shehatae* ATCC 22984 could produce ethanol yield in lower rate, yielding of 2.91 ± 0.02 g/L (productivity = 0.121 ± 0.052 $gL^{-1}h^{-1}$) within 24 h. With these production rates, some recent other studies reported the comparable results. A. Gul., *et al* [25] reported that Kallar grass (KG) treated by 0.625 M NaOH followed by steam treatment at 121 °C for 1 h was utilized as a substrate for ethanol production using *Kluyveromyces marxianus*. The result demonstrated that 10% pretreated KG, 0.6 mg/mL enzyme concentration, 40 °C temperature and 48 h of simultaneous saccharification and fermentation time were the optimized variables. The results showed ethanol production and the substrate conversion efficiency (SCE) as 40g/L and 83.74%, respectively. L.K. Bhullar, *et al*. [26] reported the study on bioethanol production using empty fruit bunch (EFB) of palm oil to and corn stover as the feedstock in NREL's model. The result showed that the bioethanol yield from EFB is higher than the yield from corm stover, as well as the production cost is lower.

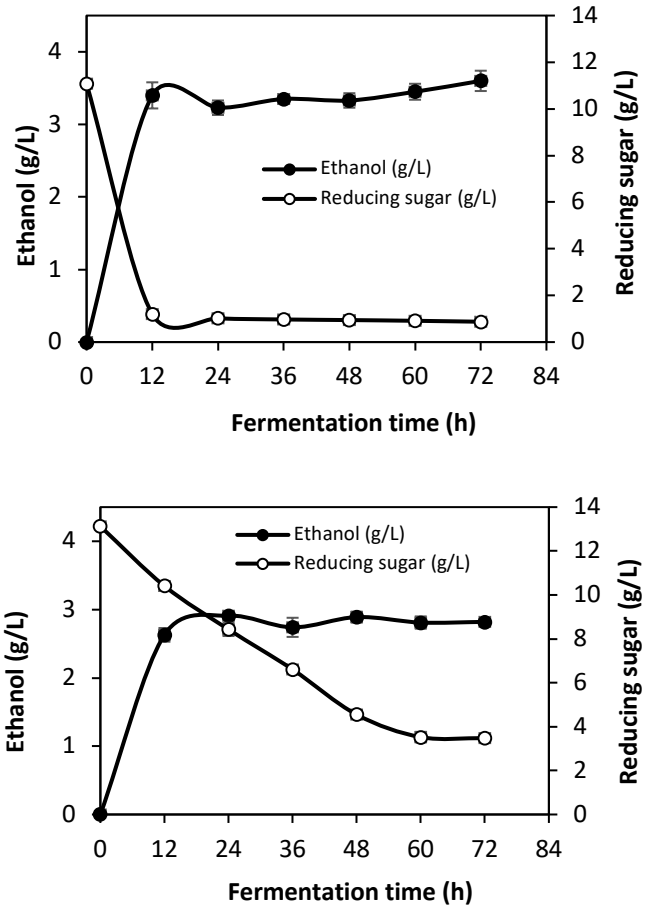

Figure 3 Concentration profiles of total reducing sugars and ethanol concentration during fermentation of base-pretreated water hyacinth by *S. cerevisiae* YRK017 (upper) and *C. shehatae* ATCC 22984 (lower).

In the overall, the highest ethanol concentrations from the acid–pretreated water hyacinth were higher than those from the base–pretreatment. The ethanol yield from yeast *S. cerevisiae* YRK 017 was slightly higher concentrations from both acid/base pretreatments than the yield from yeast *C. shehatae* ATCC 22984. As a result, the productivity from the acid–pretreated water hyacinth by *S. cerevisiae* YRK 017 and *C. shehatae* ATCC 22984 were 0.304 ± 0.002 and 0.221 ± 0.080 g/L/h, respectively, and 0.284 ± 0.032 and 0.121 ± 0.052 g/L/h. from alkali-pretreated of water hyacinth, respectively.

4. Conclusions

Water hyacinth is shown to be a good source of material for bioethanol production. The biomass after acid/base pretreatment still remained a valuable lignocellulose content to be further used in a release monosaccharide by

enzymatic reaction. The hydrolysate from acid-pretreatment water hyacinth yields higher concentration of ethanol than that from base-pretreatment. Furthermore, *S. cerevisiae* YRK 017 produced a higher yield of ethanol from both acid/base pretreatments than that in *C. shehatae* ATCC 22984.

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References

1. U. S. Aswathy, "Bio-ethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy", Bioresource Technology, vol. 101, pp. 925 – 930, 2010. (Article)

2. O. J. Reátegui, H. L. Cárdenas, D. G. Peña, V. J. Castro, R. F. Roque, N. F. Mejía, M. M. Ponce, and R. S. Mestas, "Biogas production in batch in anaerobic conditions using cattle manure enriched with waste from slaughterhouse", 6th International Conference on Renewable Energy Research and Applications (ICRERA), San Diego, CA, 2017, pp. 819-822, 5-8 December 2017. (Conference Paper)

3 Y. Ulusoy, A. H. Ulukardesler, R. Arslan, and Y. Tekin, "Energy and emission benefits of chicken manure biogas production — A case study", 6th International Conference on Renewable Energy Research and Applications (ICRERA), San Diego, CA, 2017, pp. 648-652, 5-8 December 2017. (Conference Paper)

4. Y. Ulusoy and A. H. Ulukardesler, "Biogas production potential of olive-mill wastes in Turkey", 6th International Conference on Renewable Energy Research and Applications (ICRERA), San Diego, CA, 2017, pp. 664-668. 5-8 December 2017. (Conference Paper)

5. S. Dewang, Suriani, S. Hadriani, Diana, E. S. Lestari and Bannu, "Viscosity and calorie measurements of biodiesel production from *Callophyllum Inophyllum* L using catalyst and time variations for stirring in tansesterfication process," *6th International Conference on Renewable Energy Research and Applications (ICRERA)*, San Diego, CA, 2017, pp. 734-738, 5-8 December 2017. (Conference Paper)

6. S. Hosokai, K. Matsuoka, K. Kuramoto, and Y. Suzuki, "Estimation of thermodynamic properties of liquid fuel from biomass pyrolysis", 3rd International Conference on Renewable Energy Research and Application (ICRERA), Milwaukee, WI, 2014, pp. 728-731, 19-22 October 2014. (Conference Paper)

7. J.F. Agwa-Ejon, C. Mbohwa, "The potential for bio-ethanol fuel from molasses in the Southern African sugar industry", PICMET 14 Conference: Portland International Center for Management Engineering and Technology; Infrastructure and Service Integration, Kanazawa, 2014, pp. 3589-3597, 27-31 July 2014 (Conference paper)

8. B. Eshton, and J.H.Y. Katina, "Carbon footprints of production and use of liquid biofuels in Tanzania", Renewable and Sustainable Energy Reviews, vol. 42, pp. 672-680, 2015. (Article)

9. R. Ndong, M. Montrejaud-Vignoles, O. Saint Giron, B. Gabrielle, R. Pirot, M Domergue, and C. Sablayrolles, "Life cycle assessment of biofuels from *Jatropha curcas* in West Africa A field study, GCB" Bioenergy, vol 1, pp. 197-210, 2015. (Article)

10. J. N. Nigam, "Bioconversion of water hyacinth (*Eichhornia crassipes)* hemicelluloses acid hydrolysate to motor fuel ethanol by xylose –fermenting yeast", Journal of Biotechnology, vol. 97, pp. 107 - 116, 2002. (Article)

11. Ayele K, Mesfin R, Araya, A, "Potential of bioethanol production and optimization test from agricultural waste: The case of wet coffee processing waste (pulp)", International journal of renewable energy research, vol 2, NO.3, pp. 446-450, 2012. (Article)

12. S. Echaroj and N. Pannucharoenwong, "Bioethanol production through enzymatic saccharification and fermentation of mechanically milled empty palm bunch", 5th International Conference on Engineering Technologies & Applied Sciences, Bangkok, Thailand, pp. 1-4, 22-23 November. 2018. (Conference paper)

13. Wubiao Zhu I, Kun Zhang, Suqing Zhao I, Wei Tan I, Baohua Huang I, and Yonglian Li I, "A study into preparation of bio-ethanol by degradation of banana stalks with enzyme", 2010 Asia-Pacific Power and Energy Engineering Conference, Chengdu, 2010, pp. 1-4, 28-31 March 2010. (Conference paper)

14. A. Kumar, L. K. Singh and S. Ghosh, "Bioconversion of lignocellulosic fraction of water –hyacinth *(Eichhornia crassipes)* hemicellulose acid hydrolysate

to ethanol by *Pichia stipites*", Bioresource Technology, vol. 100, pp. 3293 -3297, 2009. (Article)

15. A. Manivannan, P. H. Jayarani and R. T. Narendhirakannan, "Enhanced acid hydrolysis for bioethanol production from water hyacinth *(Eichhornia crassipes)* using fermentation yeast *Candida shehatae* NRRLY –981*",* Journal of Science and Industrial Research, vol. 71, pp. 51 – 56, 2012. (Article)

16. A. Stavrinides, A.I. Al-Shamma'a, and D.A. Phipps, "Investigation into the production of a cellulytic *Saccharomyces*-identification of a high cellulytic *Trichoderma* spp. For gene selection", Second International conference on developments in eSystem engineering, Abu Dhabi, pp. 409-412. 14-16 December 2009. (Conference paper)

17. S.K. Yadav, S. Naseeruddin, Sai, G. Prashanthi, L. Sateesh and L. Venkateswar Rao, "Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of *Saccharomyces cerevisiae* and *Pichia stipites*", Bioresource Technology, 193:103-109, 2011. (Article)

18. A. Singh and N. R. Bishnoi ."Comparative study of various pretreatment techniques for ethanol production from water hyacinth", Ind Crop Prod, vol. 44, pp. 283-289, 2013. (Article)

19. G. L. Miller, "Use of dinitrosalicylic acid reagent for the determination of reducing sugars", Analytical Chemistry, vol. 31, pp. 426- 428, 1959. (Article)

20. R. Gupta, K. K. Sharma and R. C. Kuhad, "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", Bioresource Technology, vol. 100, pp. 1214 –1220, 2009. (Article)

21. H. Tarkow and C.W. Feist, "A mechanism for improving the digestibility of lignocellulosic materials with dilute akali and liquid ammonia in cellulases and their applications", American Chemical Society, vol 95, 197-218, 1969. (Article)

22. C. Isarankuma-Na-Ayudhaya, T. Tantimongcolwat, T. Kongpanpee, P. Prabkate, and V. Prachayasittikul, "Appropriate technology for the bioconversion of water hyacinth *(Eichhornia crassipes)* to liquid ethanol: future prospects for community strengthening and sustainable development", EXCLI Journal*,* vol. 6, pp. 167 –176, 2007. (Article)

23. M. Iram, U. Asghar, M. Irfan, Z. Huma, S. Jamil, M. Nadeem and Q. Syed, "Production of bioethanol from sugarcane bagasse using yeast strains: A kinetic study", *Energy resource, Part A: Recovery,* Utilization, and Environmental Effects, vol. 40(3), pp. 364-372, 2018. (Article)

24. A. F. Shah, S. Aziz, H. R. Memon and M. I, "Ethanol production kinetics by a thermo-tolerant mutant of Saccharomyces cerevisiae from starch industry waste, Pakistan Journal of Analysis Environmental Chemistry, vol. 11, pp. 16 – 21, 2010. (Article)

25. A. Gul, M. Irfan, M. Nadeem, Q. Syed and I. ul. Haq, "Kallar Grass (*Leptochloa fusca L. Kunth*) as a feedstock for ethanol fermentation with the aid of response surface methodology", Environmental Progress & Sustainable Energy, Vol 37, pp. 569-575, 2018. (Article)

26. L.K. Bhullar, Z.A. Putra, M.R. Bilad, N.A. Hadi, and M.D. Hakim, "Process simulation of bio-ethanol production from empty fruit bunch via acid hydrolysis pretreatment", 5th IET International Conference on Clean Energy and Technology (CEAT 2018), Kuala Lumpur, Malaysia, 7 pp., 5-6 September 2018. (Conference paper)