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Enzymatic Saccharification of Dilute Alkaline Pre-treated Microalgal (*Tetraselmis suecica*) Biomass for Biobutanol Production

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***Abstract*—**Enzymatic saccharification of biomass for reducing sugar production is one of the crucial processes in biofuel production through biochemical conversion. In this study, enzymatic saccharification of dilute potassium hydroxide (KOH) pre-treated *Tetraselmis suecica* biomass was carried out by using cellulase enzyme obtained from *Trichoderma longibrachiatum*. Initially, the pre-treatment conditions were optimised by changing alkali reagent concentration, retention time for reaction, and temperature. The *T. suecica* biomass after pre-treatment was also characterized using Fourier Transform Infrared Spectra and Scanning Electron Microscope. These analyses revealed that the functional group such as acetyl and hydroxyl groups, structure and surface of *T. suecica* biomass were changed through pre-treatment, which is favourable for enzymatic saccharification process. Comparison of enzymatic saccharification of untreated and pre-treated microalgal biomass indicated that higher level of reducing sugar can be obtained from pre-treated *T. suecica*. Enzymatic saccharification of pre-treated *T. suecica* biomass was optimised by changing temperature, pH, and enzyme concentration to solid ratio ([E]/[S]). Highest conversion of carbohydrate into reducing sugar of 95% amounted to reducing sugar yield of 20 (wt%) from pre-treated *T. suecica* was obtained from saccharification, at temperature: 40°C, pH: 4.5 and [E]/[S] of 0.1 after 72 h of incubation. Hydrolysate obtained from enzymatic saccharification of pretreated *T. suecica* biomass was further fermented into biobutanol using *Clostridium saccharoperbutyliticum* as biocatalyst. The results from this study demonstrate a positive prospect of application of dilute alkaline pre-treatment to enhance enzymatic saccharification and biobutanol production from microalgal biomass.

***Keywords*—**Microalgal biomass, enzymatic saccharification, biobutanol, fermentation.

I. Introduction

INCREASED demand of gasoline price and decreasing petroleum reserves have led the exploration of potential alternatives for fuel in the future [1]. Biobutanol, which has high energy content, is safe for blending with gasoline, and can be used in conventional engine without modification, is advantageous over bioethanol [2]. Generally, biobutanol can be produced from different materials such as woody biomass and starch feedstock [3], [4]. However, several issues such as food versus fuel, and presence of recalcitrant component in the biomass have made biobutanol production from these feedstocks unviable [1]. This has led to exploring new biobutanol feedstock that are sustainable and are of low cost.

Renewable biomass feedstock such as algal biomass is believed to have the potential to become biobutanol feedstock [5]. Microalgae, which has high biomass productivity, is able to use CO_2 as a carbon source, and has high carbohydrate content. Hence, it is a great candidate to replace woody and starch materials for biobutanol production feedstock [6].

In order to produce biobutanol from microalgal biomass, it has to go through a series of processes, namely; biomass production, pre-treatment, enzymatic saccharification and anaerobic fermentation [7]. Enzymatic saccharification is one of the crucial steps involving the release of reducing sugar from biomass as a carbon platform prior to the fermentation process. Rigid microalgal cell wall needs to be hydrolysed to break the wall and release polysaccharide such as starch, carbohydrate, and other nutrients prior to enzymatic saccharification [8].

Thus, the objective of this study was to determine the suitable conditions including pH, temperature, and enzyme concentration on the enzymatic saccharification of alkaline pre-treated *Tetraselmis suecica* biomass. The potential to produce biobutanol from microalgal hydrolysate was also investigated in this study.

II. Materials and Methods

A. Microalgal Cultivation Conditions

Microalgal species, *Tetraselmis suecica* was used in this study. Modified algae growth medium (MLA medium) with 0.49 g/L magnesium sulfate ($MgSO_4.7H_2O$), 1.7 g/L sodium nitrate ($NaNO_3$), 0.14 g/L di-potassium phosphate (K_2HPO_4) 0.03 g/L calcium chloride ($CaCl_2.2H_2O$) was used as the seed culture and biomass production medium. The medium was initially sterilized using a 0.22 µm Millipore filter. Microalgal seeds were cultivated in 1 L Schott bottle containing 700 mL of the modified MLA. The bottle was incubated in an illuminated incubator chamber with 0.3 L/min compressed air under light with a photon intensity of 450 µmol/m^2s. The cultivation temperature was 30.0±0.2°C. The microalgae cultures were harvested during the late logarithmic growth phase. Each harvested sample was centrifuged at 4500 rpm for 15 min. The resulting pellet was rinsed twice with distilled

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water and subsequently dried at 60°C for 24 h. The dried biomass obtained was used for further study.

B. Chemical Composition

The lipid, carbohydrate and protein contents of microalgal biomass were determined using soxhlet extraction [9], phenol-sulfuric acid method [10], and Lowry method analyses [11] respectively.

C. Pre-treatment of Microalgal Biomass

A total of 1.0 g dried microalgae biomass samples was measured and soaked in 100 mL of 2 %(w/v) of potassium hydroxide (KOH) in 250 mL Scott bottle. The mixture was placed in an oven and incubated at 120°C for 2 h. After the incubation period, the sample was removed and cooled at room temperature. The sample was then centrifuged at 3000 rpm for 10 min. The supernatant was separated and subjected to reducing sugar analysis. Surface structure and functional group on *T. suecica* biomass after pre-treatment was characterised using FTIR and SEM analyses.

D. Enzymatic Saccharification

Preliminary enzymatic saccharification of both raw and pre-treated microalgal biomass were soaked in 10 mM acetate buffer (pH 5.5) and mixed with cellulase from *Trichoderma longibrachiatum* at 45°C and rate of agitation of 150 rpm in an orbital shaker (Thermoline Scientific) for 96 h.

A further study to determine the suitable enzymatic saccharification conditions was carried out using one-variable at a time design (OVAT) method. Alkaline pre-treated *T. suecica* biomass was used in this study. Enzymatic saccharification of pre-treated *T. suecica* biomass was carried out by incubating the sample at a temperature ranging between 30-60°C, pH ranging between 3.5-7.5 and an enzyme concentration to solid ratio ([E]/[S]) of 0.02 to 1 for 72 h. Five different enzyme concentrations varied from 5 mg to 25 mg with initial amount of 250 mg microalgal biomass was used to evaluate this study. The samples were withdrawn at regular intervals for analysis and heated at 100°C to deactivate the enzymaes. The samples were then centrifuged at 3500 rpm for 5 minutes and the supernatant was used for reducing sugar analysis as described in section F.

E. Acetone-Butanol-Ethanol Fermentation

All fermentation experiments were conducted using 100 mL serum bottle with a working volume of 70 mL medium. The hydrolysate obtained from enzymatic saccharification process was used as a fermentation medium. The pH value of the medium was adjusted to 6.0±0.2 using 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH) followed by sterilization by autoclaving it at 121°C for 10 minutes. After sterilization, an anaerobic condition was attained by passing nitrogen gas through the medium for about 2 to 5 min prior to inoculation with 10% active *Clostridium saccharoperbutylicticum* N-14. The sample was then incubated in an incubator oven at 35°C for 96 hours. The fermentation sample was withdrawn at regular intervals for analytical monitoring. All experiments were conducted in triplicates and the average value was reported.

F. Chemical Analysis

Biomass concentration was determined using a dry cell weight (DCW) by measuring the optical density at 680 nm using a spectrophotometer (Hach, DR-5000). The total reducing sugar was determined using 3, 5 dinitrosalysilic acid method and the reducing sugar was determined using high-pressure liquid chromatography (HPLC) with a RHM-monosaccharide column (Phenomenex, REZEX^{TM}).

The acetone, butanol, ethanol and butyric acid produced during fermentation were determined using gas chromatography (Shimadzu-1200), equipped with a capillary column (HP-FFAP).

G. Statistical Analysis

All samples were analysed in triplicates. T-test was used to determine the statistical difference between the control and the experimental parameters. Statistical analysis was performed using Minitab 14.3 software.

III. Results and Discussion

A. Microalgal Species and Chemical Composition

Table I shows the chemical composition of *T. suecica* biomass. The major component of its biomass was protein (58.32±3.08), followed by carbohydrate (27.41±2.08), and lipid (14.25±0.08). The significant amount of carbohydrate content in *T. suecica* biomass endows the species with a high potential to be used as a fermentation feedstock.

TABLE I
CHEMICAL COMPOSITION OF *T. SUECICA* BIOMASS

Chemical compound	*T. suecica* biomass (%)
Carbohydrate	27.41±2.08
Protein	58.32±3.08
Lipid	14.25±0.08

B. Enzymatic Saccharification of Raw and Dilute Alkaline Pre-treated T. suecica Biomass

Alkaline pre-treatment of microalgal biomass was carried out using 2% KOH at 120° C for 2 h. Preliminary enzymatic saccharification for both untreated and pre-treated *T. suecica* were evaluated using the degradation enzyme produced from *Trichoderma longibrachiatum*. Fig. 1 shows the reducing sugar concentrations obtained from untreated and pre-treated *T. suecica* biomass. As expected, pre-treated microalgal biomass produced higher reducing sugar compared to untreated microalgal biomass.

Similar observation has been reported on the effect of alkaline pre-treatment on enzymatic saccharification of poplar wood, sorghum straw, corncobs, and rice hull [12]-[14]. A study on the effect of alkaline treatment on poplar wood indicated that saccharification of untreated poplar wood yielded lower reducing sugar compared to pre-treated sample [15].

Higher reducing sugar concentrations obtained from pre-treated biomass is mainly contributed by the physical structure

of the biomass. According to Kim et al. [16], the change in biomass features due to the pre-treatment process has improved enzymatic saccharification of alkaline treated corn stover. Similar observations have also been reported on enzymatic saccharification of pre-treated hybrid poplar and wood chips [17]. Alkaline pre-treatment was reported to remove lignin and acetyl groups in the biomass that makes it easily saccharified in the enzymatic saccharification process.

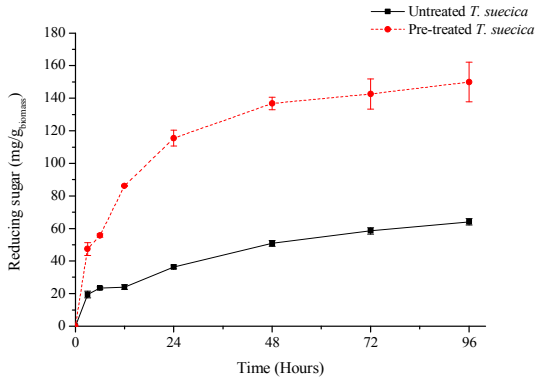

Fig. 1 Reducing sugar produced from enzymatic saccharification of untreated and alkaline pre-treated *T. suecica* biomass

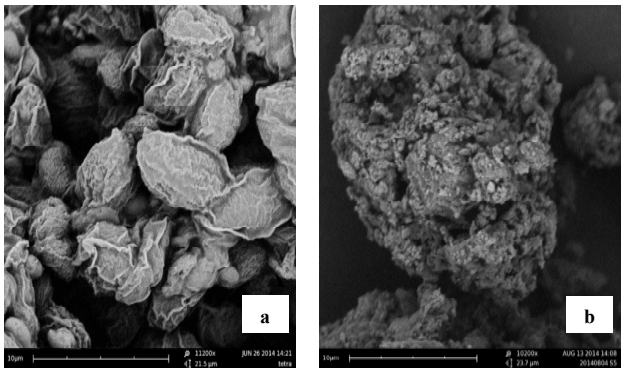

Fig. 2 SEM of *T. suecica* biomass (a) before alkaline pre-treatment, (b) after alkaline pre-treatement

C. Scanning Electron Microscopy (SEM)

SEM analysis was conducted to determine the surface structure changes and surface characteristic of microalgal biomass. SEM images of microalgal biomass before and after alkaline pre-treatment are shown in Fig. 2. Comparisons of SEM images showed significant changes in the biomass structure after alkaline treatment process. Untreated *T. suecica* biomass seemed to have actual cell structure form, while uneven distribution and rough surface were observed in pre-treated *T. suecica* biomass. These results indicate that dilute alkaline pre-treatment disrupted and changed the surface structure of microalgal biomass. The cracks and uneven structure resulted in higher surface area facilitating more rapid accessibility for the degradation enzyme to attack the inner structure of the biomass during enzymatic saccharification. These results agreed with previous studies on the effect of alkaline pre-treatment on the structural changes in biomass [15], [18], [19]. The fragment of biomass structure was disrupted and separated from its initial structure, and exposing it for further reaction. This would provide better enzyme assessment to attack the inner linkage, hence accelerating the degradation process.

D. Fourier Transform Infrared (FTIR) Spectroscopy

During alkaline pre-treatment, the cleavage of hydrolysable linkage such as α- and β- aryl ether in lignin and glycosidic bonds in carbohydrates constitutes the primary reaction that leads to the dissolution of lignin and carbohydrate [18]. In order to determine the effect of alkaline treatment on biomass sample, FTIR analysis has been widely used to characterize the functional group on biomass surface after pre-treatment process [15]. Fig. 3 shows the comparison of FTIR spectra for *T. suecica* biomass before and after pre-treatment. The spectrum shows obvious effects near 3400 to 3200 cm^{-1}, 1720 to 1600 cm^{-1}, 1245 cm^{-1}, and 1098 to 900 cm^{-1} bands. The peak near 3400 to 3200 cm^{-1} was representative of the hydroxyl (OH) group in the samples [20], [21]. Significant reduction in intensity was observed in this region, which indicates that hydrogen bond in cellulose was disrupted during the pre-treatment process. Reduction of 1720 to 1600 cm^{-1} band indicates that condensation or splitting of lignin aliphatic side chains occurred during the pre-treatment process. Kumar et al. [22] suggested that a complete disappearance of this band after alkaline treatment illustrated lignin removal during the pre-treatment process.

FTIR analysis also indicate that dilute alkaline pre-treatment had an obvious effect on band 1245 cm^{-1}, which was associated with the acetyl group in the biomass [23]. Obvious reduction of this band in *T. suecica* biomass after pre-treatment process strongly indicates cleavage or alteration of acetyl group on the biomass surface. The peak 1098 and 900 cm^{-1} represented crystalline and amorphous cellulose in the biomass [24]. This study also shows increased absorbance band at 1000 cm^{-1}, but reduced absorbance at 900 cm^{-1} (Fig. 3). This indicates that most of the cellulose in the sample was not disrupted by alkaline catalyst reaction.

Overall, this study found that most acetyl groups and microalgal biomass structure were disrupted during the pre-treatment process, thus confirming the effectiveness of dilute alkaline pre-treatment for selective removal of acetyl group and delignification. The alkaline pre-treatment process, therefore, provides greater surface area for enzymatic saccharification of microalgal biomass.

E. Carbohydrate Content after Alkaline Pre-Treatment

Further enzymatic saccharification was carried out to determine the suitable conditions to produce higher reducing sugar from pre-treated *T. suecica* biomass. Note that reducing sugar from microalgal biomass can be derived from its total carbohydrate content. Table II shows the carbohydrate content before and after alkaline pre-treatment. A small reduction of carbohydrate content was observed after pre-treatment, which indicates that a small amount of carbohydrate in the biomass

was dissolved in alkaline liquor during the pre-treatment process.

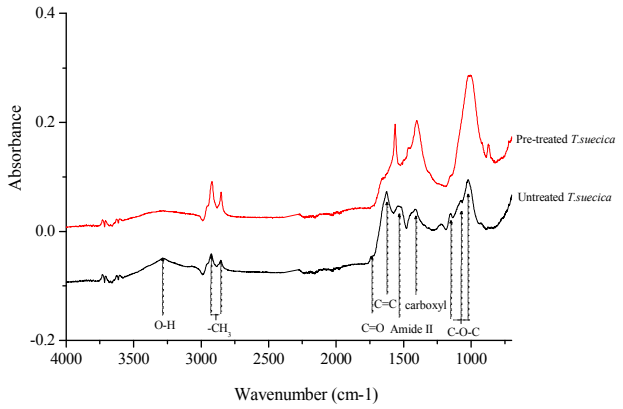

Fig. 3 FTIR spectra for raw and alkaline pre-treated microalgae biomass

TABLE II
T. SUECICA CARBOHYDRATE CONTENT BEFORE AND AFTER ALKALINE PRE-TREATMENT

Chemical compound	Before pre-treatment	After pre-treatment
Carbohydrate	27.41±2.08	19.26±1.21

F. Effect of Temperature

Fig. 4 shows the effect of temperature on reducing sugar produced from pre-treated *T. suecica* biomass. The maximum reducing sugar concentration was 133.53±5.51 mg/$g_{biomass}$ obtained at 40°C. Further increase in saccharification temperature to 60°C reduced the reducing sugar production. Further statistical analysis showed that there was a significant difference (p=<0.05) between tested temperatures, indicating that saccharification temperature played a significant role in reducing sugar production from *T. suecica* biomass. Similar observation has been reported on the saccharification of microalgae *Chlorococcum* sp. [8]. The highest reducing sugar production obtained at this temperature could be due to cellulase enzyme activity used in this study. Generally, cellulase enzyme produced from *T. longibrachiatum* has been reported to have an optimum activity within 30-45°C [25]. Low reducing sugar concentration produced at higher temperature may be attributed by the inactivation of cellulase enzyme during the process [26].

G. Effect of pH

The effect of pH on enzymatic saccharification of pre-treated *T. suecica* was also investigated and results are presented in Fig. 5. The suitable pH for enzymatic saccharification of *T. suecica* was 4.5 with a reducing sugar concentration of 169.55±3.25 mg/$g_{biomass}$. The results of the current study indicate that enzymatic saccharification at lower or higher than pH 4.5 produced lower reducing sugar. A suitable pH is required to maintain the three dimensional active site structure of the enzyme [27]. Saccharification in strongly acidic and alkaline conditions will retard enzyme activity, which will then change the pH and affect the electrostatic binding of enzyme resulting in unfolded or denatured enzyme structure [8]. Consequently, the cellulase enzyme becomes inactive indirectly.

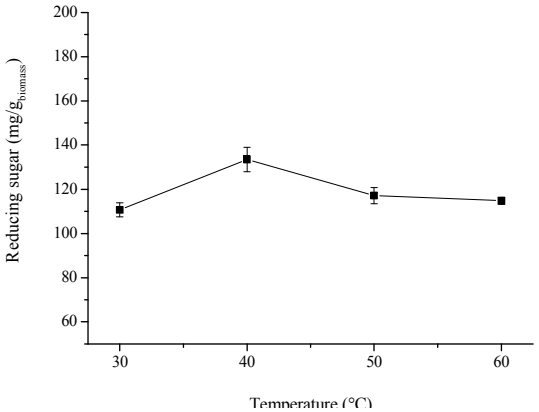

Fig. 4 The effect of temperature on saccharification of pre-treated *T. suecica* biomass Results represent the average value of 3 replicates

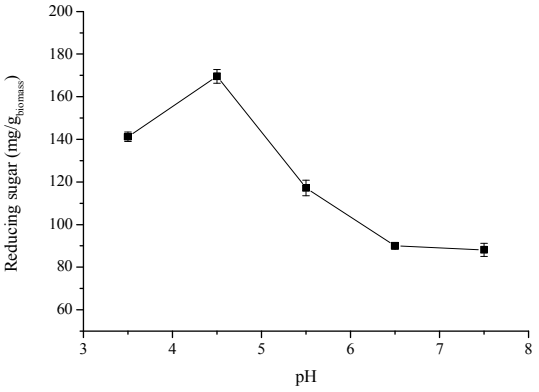

Fig. 5 The effect of pH on saccharification of pre-treated *T. suecica* biomass Results represent the average value of 3 replicates

H. Effect of Enzyme Concentration

The effect of enzyme concentration to substrate ratio in the range of 0.02 to 0.1 was studied and the results are presented in Fig. 6. The highest reducing sugar concentration was 190.49±10.41 mg/$g_{biomass}$ obtained when the saccharifiation was carried out with E/S of 0.1. The rate of saccharification was increased with increase of enzyme concentration. Eventhough high reducing sugar was obtained using ratio of 0.1, however, further statistical analysis indicated that there was no significant effect (p=0.678) on enzyme to solid ratio on final reducing sugar production. This finding indicates that low enzyme concentration can be applied to saccharify pre-treated *T. suecica* biomass. Slow decrease of reducing sugar production at higher enzyme loading would be due to the saturation of the substrate surface with enzyme and reduced enzyme activity during saccharification [28]. Thus, this study shows that low enzyme concentration to produce maximum reducing sugar from microalgal biomass offers the advantage of reducing the production of fermentation inhibition, making the process economically effective.

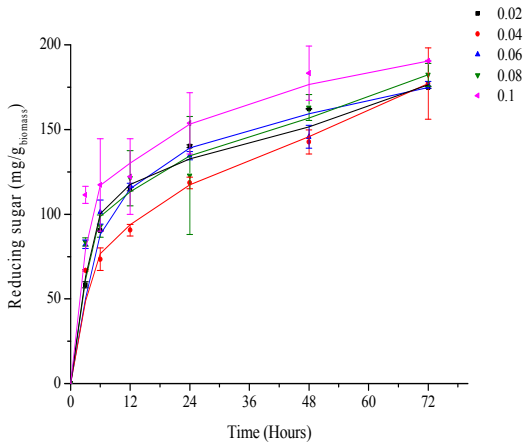

Fig. 6 The effect of enzyme to solid ratio ([E]/[S]) of pre-treated *T.suecica* biomass Results represent the average value of 3 replicates

I. Biobutanol Production from Enzymatic Hydrolysate of T. suecica

The potential to produce biobutanol from enzymatic saccharification of pre-treated *T. suecica* was evaluated in this study and the result is shown in Fig. 7. The acetone-butanol-ethanol (ABE) fermentation result shows that the total solvent production was 0.14 g/L, which included 0.07 g/L of biobutanol. On the other hand, the productivity of biobutanol production yield was 0.07 $g/g_{reducing\ sugar}$ after 72 h of incubation. These results indicate that biobutanol can be produced using the hydrolysate obtained from the saccharification of pre-treated *T. suecica* biomass.

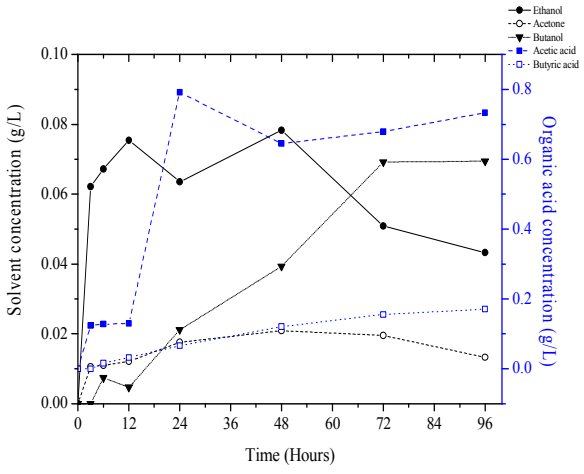

Fig. 7 ABE fermentation of hydrolysate enzymatic saccharification of alkaline pre-treated *T. suecica* biomass

IV. Conclusion

A combination of dilute alkaline pre-treatment, and enzymatic saccharification resulted in the conversion of carbohydrate into reducing sugar of 95% amounted to reducing sugar yield of 20 (wt%) from pre-treated *T. suecica* biomass. Suitable enzymatic saccharification is crucial in converting the total carbohydrate content in microalgal biomass into reducing sugar. Based on this study, enzymatic saccharification parameters such as temperature, pH, and enzyme concentration showed a significant effect on reducing sugar production from *T. suecica* biomass. In this study, the maximum reducing sugar from *T. suecica* biomass obtained from saccharification was 190.49±10.41 $mg/g_{biomass}$, at 40°C, pH 4.5 and [E]/[S] of 0.1 after 72 h of incubation. The reducing sugar obtained from the saccharification process also proves its ability to become a feedstock for biobutanol production.

Acknowledgement

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References

[1] Naik, S.N., Goud, V.V., Rout, P.K., Dalai, A.K. (2010) Production of first and second generation biofuels: A comprehensive review. Renew Sust Energ Rev 14: 578-597.
[2] Dürre, P. (2007) Biobutanol: An attractive biofuel. Biotechnol J 2: 525-1534.
[3] Jang, Y.S., Malaviya, A., Cho, C., Lee, J., Lee, S.Y. (2012) Butanol production from renewable biomass by *Clostridia*. Bioresource Technol 123: 653-663.
[4] Qureshi, N., Ezeji, T.C. (2008) Butanol, 'a superior biofuel' production from agricultural residues (renewable biomass): recent progress in technology. Biofuels, Bioprod Bior 2: 319-330.
[5] Ellis, J.T., Hengge, N.N., Sims, R.C., Miller, C.D. (2012) Acetone, butanol, and ethanol production from wastewater algae. Bioresource Technol 111: 491-495.
[6] Harun, R., Singh, M., Forde, G.M., Danquah, M.K. (2010) Bioprocess engineering of microalgae to produce a variety of consumer products. Renew Sust Energ Rev 14: 1037-1047.
[7] Balat, M. (2011) Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energ Convers Manage 52: 858-875.
[8] Harun, R., Danquah, M.K. (2011) Enzymatic hydrolysis of microalgal biomass for bioethanol production. Chem Eng J, 168: 1079-1084.
[9] Zeng, X., Danquah, M.K., Halim, R., Yang, S., Chen, X.D., Lu, Y. (2013) Comparative physicochemical analysis of suspended and immobilized cultivation of *Chlorella* sp. J Chem Technol Biotechnol 88: 247-254.
[10] Nielsen, S.S. (2010) Phenol-Sulfuric Acid Method for Total Carbohydrates, Food Analysis Laboratory Manual, Springer US2010, pp. 47-53.
[11] González López, C.V., García, M.D.C.C., Fernández, F.G.A., Bustos, C.S., Chisti, Y., Sevilla, J.M.F. (2010) Protein measurements of microalgal and cyanobacterial biomass. Bioresource Technol 101: 7587-7591.
[12] McIntosh, S., Vancov, T. (2010) Enhanced enzyme saccharification of Sorghum bicolor straw using dilute alkali pretreatment. Bioresource Technol. 101: 6718-6727.
[13] Saha, B.C., Cotta, M.A. (2008) Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. Biomass Bioenerg 32: 971-977.
[14] Baadhe, R.R., Potumarthi, R., Mekala, N.K. (2014) Influence of dilute acid and alkali pretreatment on reducing sugar production from corncobs by crude enzymatic method: A comparative study. Bioresource Technol 162: 213-217.
[15] Rawat, R., Kumbhar, B.K., Tewari, L. (2013) Optimization of alkali pretreatment for bioconversion of poplar (*Populus deltoides*) biomass into fermentable sugars using response surface methodology. Ind Crop Prod 44: 220-226.
[16] Kim, S., Holtzapple, M.T. (2005) Lime pretreatment and enzymatic hydrolysis of corn stover. Bioresource Technol 96: 1994-2006.
[17] Zhu, L., O'Dwyer, J.P., Chang, V.S., Granda, C.B., Holtzapple, M.T. (2008) Structural features affecting biomass enzymatic digestibility, Bioresource Technol 99: 3817-3828.

[18] Chen, Y., Stevens, M.A., Zhu, Y., Holmes, J., Xu, H. (2013) Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. Biotechnol Biofuels 6: 1-10.
[19] Lima, M.A., Lavorente, G.B., Silva, H.K.D., Bragatto, J., Rezende, C.A., Bernardinelli, O.D., deAzevedo, E.R., Gomez, L.D., McQueen-Mason, S.J., Labate, C.A., Polikarpov, I. (2013) Effects of pretreatment on morphology, chemical composition and enzymatic digestibility of eucalyptus bark: a potentially valuable source of fermentable sugars for biofuel production – part 1. Biotechnol Biofuels 6: 1-17.
[20] Grierson, S., Strezov, V., Shah, P. (2011) Properties of oil and char derived from slow pyrolysis of *Tetraselmis chui*. Bioresource Technol 102: 8232-8240.
[21] Siengchum, T., Isenberg, M., Chuang, S.S.C. (2013) Fast pyrolysis of coconut biomass – An FTIR study. Fuel 105: 559-565.
[22] Kumar, L., Chandra, R., Saddler, J. (2011) Influence of steam pretreatment severity on post-treatments used to enhance the enzymatic hydrolysis of pretreated softwoods at low enzyme loadings. Biotechnol Bioeng 108: 2300-2311.
[23] Sun, X.F., Xu, F., Sun, R.C., Fowler, P., Baird, M.S. (2005) Characteristics of degraded cellulose obtained from steam-exploded wheat straw. Carbohyd Res 340: 97-106.
[24] Laureano-Perez, L., Teymouri, F., Alizadeh, H., Dale, B. (2005) Understanding factors that limit enzymatic hydrolysis of biomass. Appl Biochem Biotechnol, 124: 1081-1099.
[25] Gautam, S.P., Bundela, P.S., Pandey, A.K., Khan, J., Awasthi, M.K., Sarsaiya, S. (2011) Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. Biotechnol Res Intern 1-8.
[26] Andreaus, J., Azevedo, H., Cavaco-Paulo, A. (1999) Effects of temperature on the cellulose binding ability of cellulase enzymes. J Mol Catal B: Enzym 7: 233-239.
[27] Hamzah, F., Idris, A., Shuan, T.K. (2011) Preliminary study on enzymatic hydrolysis of treated oil palm (Elaeis) empty fruit bunches fibre (EFB) by using combination of cellulase and β 1-4 glucosidase. Biomass Bioenerg 35: 1055-1059.
[28] Xu, Z., Wang, Q., Jiang, Z., Yang, X.X., Ji, Y. (2007) Enzymatic hydrolysis of pretreated soybean straw. Biomass Bioenerg 31: 162-167.