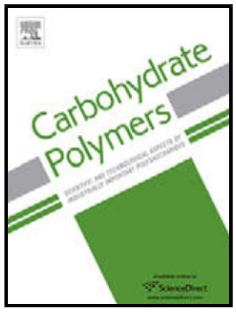

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Solid acid catalysts pretreatment and enzymatic hydrolysis of macroalgae cellulosic residue for the production of bioethanol

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Highlights

- Glucose yield of macroalgae cellulosic residue is greatly improved after solid acid pretreatment.
- 94.6% of the cellulose can be converted into fermentable sugars.
- The catalyst was separated and recycled eight times with slight deactivation.

ABSTRACT

The aim of this study is to investigate the technical feasibility of converting macroalgae cellulosic residue (MCR) into bioethanol. An attempt was made to present a novel, environmental friendly and economical pretreatment process that enhances enzymatic conversion of MCR to sugars using Dowex (TM) Dr-G8 as catalyst. The optimum yield of glucose reached 99.8% under the optimal condition for solid acid pretreatment (10% w/v biomass loading, 4% w/v catalyst loading, 30 min, 120^{o}C) followed by enzymatic

hydrolysis (45 FPU/g of cellulase, 52 CBU/g of β-glucosidase, 50^{o}C, pH 4.8, 30 h). The yield of sugar obtained was found more superior than conventional pretreatment process using H2SO4 and NaOH. Biomass loading for the subsequent simultaneous saccharification and fermentation (SSF) of the pretreated MCR was then optimized, giving an optimum bioethanol yield of 81.5%. The catalyst was separated and reused for six times, with only a slight drop in glucose yield.

Keywords: Solid acid catalyst; macroalgae cellulosic residue; pretreatment; cellulase; fermentation; bioethanol

1. Introduction

The world is currently facing the dilemma of high crude-oil price while simultaneously energy consumption keeps on increasing due to increasing world's population and rapid economic growth led by industrialization (Katinonkul et al., 2012). In addition, the emission of greenhouse gas (GHG) due to fossil fuels consumption has caused climate change. Considering these issues, it is now inevitable to shift toward

renewable sources of energy. Bioethanol production from biomass has gained considerable interest as a source of renewable transportation fuel. The production of first-generation bioethanol from food-based biomass is not sustainable because it competes with food sources (Nigam & Singh, 2011). Therefore, the focus on bioethanol has shifted to the development of second-generation bioethanol, by utilizing lignocellulosic biomass. Although second-generation bioethanol is attractive because it is made from non-edible feedstock, but the difficulty in separating lignin from lignocelluloses has impeded the commercialization potential of this renewable source (Karthika, Arun & Rekha, 2012). Now, macroalgae has emerge as the third-generation biomass that can be used in bioethanol production. Among the advantages of using macroalgae as feedstock is it does not need land and freshwater for their cultivation, it grows quickly, and is lignin free (Goh & Lee, 2010).

The red macroalgae, *Eucheuma cottonii*, is widely cultivated in Malaysia, and is used as biomass for the production of κ-carrageenan. The major polysaccharide constituents of red macroalgae are κ-carrageenan, which are the most commercially important polysaccharides for red macroalgae. κ-carrageenan can be easily obtain from red macroalgae through extraction or dissolving them into an aqueous solution (Chan et al., 2013). After extracting κ-carrageenan, a huge amount of residual materials are left behind, which is usually called macroalgae cellulosic residue (MCR). The residual biomass can be utilize for the production of bioethanol because it contains large amount of cellulose and with low lignin content. The utilization of MCR as an energy resource would pave the way for converting waste material to a product that has high commercial

value. In general, bioethanol production from biomass involves pretreatment, enzymatic hydrolysis, and fermentation. Thus, after extracting κ-carrageenan, the MCR must be pretreated prior to enzymatic hydrolysis for bioethanol production.

The advantage of pretreating cellulosic materials prior to hydrolysis have been well established in the literature (Ahmed et al., 2013b; Kootstra et al., 2009). However, the pretreatment process of macroalgae for the production of bioethanol is still at its infancy stage. The establishment of an efficient pretreatment method in order to facilitate the conversion of sugars during the enzymatic hydrolysis is the key step for bioethanol production (Monavari, Galbe & Zacchi, 2009; Schultz-Jensen et al., 2013). The goal of pretreatment process is to reduce the crystallinity of cellulose, increase the porosity of the cellulosic materials and thus enhance the enzymatic hydrolysis productivity (Cabiac et al., 2011). Different examples of bioethanol production and pretreatment methods for macroalgae have been described in the literature. One study reported that carbohydrates from *Laminaria japonica* can be effectively hydrolysed to simple sugars by dilute H_2SO_4 treatment (Ge, Wang & Mou, 2011). Another study reported that dilute acid pretreatment of *Saccharina japonica* can improve the efficiency of enzymatic hydrolysis followed by simultaneous saccharification and fermentation (SSF) (Lee et al., 2013). Although sulfuric acid are powerful agent for cellulose pre-treatment, however, it has its disadvantages. Sulfuric acid are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pretreatment process very expensive. In addition, it is also very difficult to recycle the catalyst and its disposal would require proper waste water treatment facilities. As an alternative to dilute acid pretreatment

99 method, there is the possibility of using heterogeneous solid acid catalyst which can
100 overcome the above drawbacks (Chareonlimkun et al., 2010). Recently, several solid
101 acids are reported in the literature as efficient catalytic systems for the hydrolysis of
102 cellulose, starch and other polysaccharides (Marzo, Gervasini & Carniti, 2012; Ormsby,
103 Kastner & Miller, 2012; Shen et al., 2013; Yamaguchi & Hara, 2010). However, to the
104 best of our knowledge, very little information is available on the use of solid acid catalyst
105 for the pretreatment of cellulosic biomass from macroalgae for the production of
106 bioethanol.

107 Thus, the objectives of this study are to, (1) optimize the pretreatment conditions
108 for macroalgae cellulosic residue (MCR) using solid acid catalyst, Dowex (TM) Dr-G8
109 base on the highest glucose yield that were obtained through enzymatic hydrolysis of the
110 pretreated MCR; (2) study the effect of enzyme loading on the enzymatic hydrolysis of
111 pretreated MCR; (3) study the effect of pretreated MCR loading on bioethanol production
112 through simultaneous saccharification and fermentation (SSF) by *Saccharomyces*
113 *cerevisiae*.

114 **2. Methods**

115 2.1. Raw materials and chemicals

116 *Eucheuma cottonii* were obtained from Futt Put Enterprise (north coast of Sabah,
117 Borneo). The macroalgae was first washed with tap water to remove impurities and dried
118 at room temperature for 24 h. Next 3% (w/v) of *E. cottonii* was boiled at 90^{o}C for 1 h
119 until the algae disintegrated. Subsequently, the κ-carrageenan extract was filtered (45 μm

mesh size). The extract residue was boiled for 30 min with 2 L of distilled water and filtered with pressure pump. The macroalgae cellulosic residue (MCR) was then dried at 50^{o}C, pulverized, screened through 80 mesh and used for subsequent experiments.

The enzymes used in the enzymatic hydrolysis were commercial cellulase (Celluclast 1.5L, Novozyme, Denmark) and β-glucosidase (Novozyme 188, Novozyme, Denmark), which were all purchased from Science Technics Sdn. Bhd. The activities of cellulase and β-glucosidase were reported by the manufacturer as 82.08 filter paper unit (FPU)/mL and 326.12 cellobiase unit (CBU)/mL, respectively. Strong acid cation-exchange resin Dowex (TM) Dr-G8, calcium hydroxide, standard D-glucose, hydroxyl-methyl furfurals, *Saccharomyces cerevisiae* (YSC2, type II), yeast extract, peptone, dextrose, and bicinchoninic acid (BCA) kit were purchased from Sigma-Aldrich (USA). Table 1 shows the properties of Dowex (TM) Dr-G8 catalyst. Acetic acid, sodium acetate, sodium hydroxide, sulfuric acid, hydrochloric acid and ethanol were purchased from Fisher Scientific (UK).

All the chemicals used in this study were analytical grade and all the experiments were performed in triplicates and the results are presented as mean ± standard deviation.

2.2. Yeast cultivation

For the inoculum preparation of *Saccharomyces cerevisiae* (YSC2, type II), dry yeast was dispersed in sterile YEPD medium (1% (w/v) yeast extract, 2% peptone, 2%

dextrose) at a concentration of 1.75% (w/v) in a 250 mL Erlenmeyer flask at pH 5.0. The preculture was incubated at 35^{o}C for 24 h in a shaking incubator (Benchmark Scientific Inc., New Jersey) at 120 rpm. Yeast cells were harvested by centrifugation (10, 000 × g, 15 min), suspended in sterilized water and used as inoculum in the SSF process.

2.3. Chemical compositional analysis of macroalgae cellulosic residue (MCR)

The cellulose, lignin and hemicellulosic fractions of pulverized macroalgae cellulosic residue (MCR) were determined according to a modified method that is based on the National Renewable Energy Laboratory (NREL, Golden, CO) analytical methods (Sluiter et al., 2008). Approximate 300 mg of MCR was initially subjected through a primary 72% sulfuric acid hydrolysis at 30^{o}C for 60 min. In the second step, the reaction mixture was diluted to 4% sulfuric acid and autoclaved at 121^{o}C for 1 h. The content of sugar was quantified with high performance liquid chromatography (HP-LC). The remaining acid-insoluble residue is considered as acid-insoluble lignin (AIL).

2.4. Pretreatment of MCR

2.4.1. Solid acid catalyst pretreatment

The macroalgae cellulosic residue (MCR) was pretreated using Dowex (TM) Dr-G8. The effects of the following parameters on the pretreatment of MCR were investigated: amount of MCR (8-14%, w/v), amount of Dowex (TM) Dr-G8 (0-6%, w/v), pretreatment time (15-60 min) and temperature (110-140^{o}C). The liquid amount was

fixed at 50 mL, and pre-determined amount of dried MCR were added at different S/L ratios. The mixture was then mixed with different amount of Dowex (TM) Dr-G8 and incubated at different temperature. The pretreatment was conducted in an autoclave reactor with gentle mixing at a speed of 320 rpm while the internal pressure of the autoclave reactor was kept constant at 10 bars. After a specific pretreatment time, the solid and liquid fractions were separated by filtration using filter paper. Subsequently, the residual substrates were washed and neutralized with distilled water and then dried at 50^{o}C until attain constant weight. The pretreated MCR was then used as the substrate for enzymatic hydrolysis.

2.4.2. Dilute acid and alkali pretreatment

The effectiveness of using Dowex (TM) Dr-G8 as catalyst for pretreatment was evaluated by comparing with conventional dilute acid and alkali pretreatment method. For dilute acid pretreatment method, 10% (w/v) MCR was treated with 1% (w/v) sulphuric acid at 120^{o}C for 30 min. After that, the residue solid material was separated and washed with excess distilled water until the washed water had a pH of 5.5. The sample was then dried in an oven at 50^{o}C until achieving constant weight. The solid fraction was used as the substrate in the subsequent enzymatic hydrolysis step.

For alkali pretreatment method, 10% (w/v) MCR was treated with 1% (w/v) NaOH at 120^{o}C for 30 min. After that, the pretreated substrate was filtered by using filter paper and washed with dilute acid solution (HCl) until pH 5.5 was attained. The sample

was then dried in an oven at 50°C until achieving constant weight. The solid fraction was used as the substrate in the subsequent enzymatic hydrolysis step.

2.5. Enzymatic hydrolysis

2.5.1. Enzymatic hydrolysis of pretreated macroalgae cellulosic residue (MCR)

To evaluate the effectiveness of MCR pretreatment at various conditions, the pretreated MCR was subjected to enzymatic hydrolysis with 2% (w/v) solid loading and 15 FPU/g of cellulase and 52 CBU/g of β-glucosidase in accordance with National Renewable Energy Laboratory (NREL)'s Chemical Analysis and Testing Standard Procedure no. 009 (NREL, 2004). For the enzymatic hydrolysis of pretreated MCR, a reaction mixture containing pretreated MCR, cellulase, β-glucosidase, and 50 mM sodium acetate buffer (pH 4.8) was incubated in a shaking incubator at 50°C and 120 rpm (Tan & Lee, 2014). Samples were taken periodically and in order to terminate the enzymatic activity, samples were boiled for 15 min immediately after each sampling. The residues were then separated from liquid by centrifugation (10, 000 × g, 5 min). The supernatants were filtered and preserved at -2°C and were analyzed for reducing sugar analysis to determine the percentage of hydrolysis by high-performance liquid chromatography (HP-LC).

2.5.2. Effect of cellulase concentration on enzymatic hydrolysis of pretreated MCR

Using the optimum pretreatment conditions obtained from the previous section (biomass loading= 10% (w/v), catalyst loading= 4% (w/v), pretreatment time= 30 min,

and temperature= 120^{o}C), pretreated MCR is subsequently used to investigate the effects of cellulase concentration on enzymatic hydrolysis of pretreated MCR. The cellulase concentrations studied are 5, 15, 30, 45 and 60 FPU/g while the β-glucosidase concentration were maintained constant at 52 CBU/g for all experiments. The solid loading and reaction time were fixed at 2% (w/v) and 72 h while other conditions were similar as reported in Section 2.5.1.

2.5.3. Determination of cellulase adsorption on cellulose

Cellulase adsorption during the enzymatic hydrolysis process was measured in a 250 mL Erlenmeyer flask with 2% (w/v) for MCR pretreated with different methods (Dowex (TM) Dr-G8, H_2SO_4 and NaOH). The dosage of cellulase for all the hydrolysis experiments is 45 FPU/g while other conditions were similar as reported in Section 2.5.1. Samples were withdrawn after 2 h of enzymatic hydrolysis and centrifuged at 10,000 × g for 5 min to remove insoluble materials. The supernatant obtained was analyzed for reducing sugar and protein content. The protein content of the supernatant (free cellulase) was determined using bicinchoninic acid (BCA) kit from Sigma Aldrich (Section 2.7.2). The amount of cellulase bound to the cellulose was calculated by subtracting the amount of free cellulase in the supernatant from the amount of cellulase initially added to the reaction medium (Pierre et al., 2011).

2.5.4. Recycling of catalyst

The possibility of re-using the Dowex (TM) Dr-G8 catalyst was determined by running pretreatment process at 120^{o}C for 30 min with solid loading of 4% (w/v) for

repeated cycles. After completing the first cycle of pretreatment, the solid acid catalyst mixed with the pretreated MCR was filtered. The resulting brown color solid residue was vigorously stirred in 250 mL of distilled water for 20 min and left standing for a while. Subsequently the catalyst will settle down to the bottom of the beaker while the tiny pretreated MCR will remain suspended in the solution. The solid acid catalyst was then easily recovered by decantation. The collected solid acid catalyst were dried at room temperature for 24 h and reused for another cycle of pretreatment. This was repeated for 6 cycles. For the enzymatic hydrolysis condition, pretreated MCR (2% w/v) was hydrolysed for 24 h in sodium acetate buffer (50 mM, pH 4.8) at 50^{o}C using a 250 mL Erlenmeyer flask. The enzyme loading used were 45 FPU/g of cellulase and 52 CBU/g of β-glucosidase.

2.6. Simultaneous saccharification and fermentation (SSF)

After establishing the optimal enzyme loading (Section 2.5.2), 4-10 % (w/v) concentration of pretreated MCR was used for simultaneous saccharification and fermentation (SSF) process to determine the suitable biomass concentration. *S. cerevisiae* was used for fermentation. Pretreated MCR was prehydrolyzed for 24 h in sodium acetate buffer (50 mM, pH 4.8) at 50^{o}C using a 250 mL Erlenmeyer flask. The enzyme loading used were 45 FPU/g of cellulase and 52 CBU/g of β-glucosidase. The temperature was subsequently reduced to 43^{o}C for further 8 h to allow for SSF after yeast inoculation. SSF experiments were performed in a shaking incubator operated at 120 rpm and was adjusted

242 to pH 5.0 by 5 M NaOH. The flasks were sealed with rubber stoppers equipped with
243 needles for CO_2 venting. 1.5 mL of sample was withdrawn at different interval time
244 during SSF and was centrifuged at 10, 000 × g for 15 min. The supernatant was stored in
245 -2^{o}C freezer and subjected for sugar and bioethanol analysis.

246

247 2.7. Analytical methods

248 2.7.1. Sugar, by-product and bioethanol analysis

249 Supernatants were analyzed for soluble sugar and by-product content using
250 Agilent series 1200 infinity high-performance liquid chromatography (HPLC). The
251 HPLC system was equipped with a 385-ELSD (evaporative light scattering detector) and
252 a Hi-Plex Ca column (300 × 7.7 mm). Distilled-deionised water was used as the mobile
253 phase with a flow rate of 0.6 mL/min and injection volume of 20 µL. Purified nitrogen
254 was used as carrier gas (70 psi) for the detector. The HPLC-ELSD's spray chamber
255 temperature was set at 40^{o}C whereas detector temperature at 80^{o}C. Prior to injection,
256 samples were diluted 50 times with deionized water and filtered with 0.20 µm syringe
257 filter (Nylon membrane, Fisher Scientific). The identities of the components were
258 authenticated by comparing their retention times with those of pure compounds (Sigma-
259 Aldrich, USA). The glucose content was calculated according to calibration curves
260 plotted with standard glucose. The sugar yield was calculated as:

261 $$\text{Yield}\,\% = \frac{\text{Concentration (g/L) of glucose at time of t}}{\text{Initial concentration (g/mL) of substrate}} \times 100\% \quad (1)$$

The bioethanol produced during the SSF process was quantified by gas chromatography (GC) using a 5890 Series II chromatography equipped with flame ionization detector (FID) (Hewlett Packard, Palo Alto, CA) . The column used was 2.0 m in length and 0.2 cm I.D, 80/120 mesh Carbopack B-DA/4% Carbowax 20M (Supelco, USA). The temperature of the injection unit and detector were 225^{o}C. The oven was heated to 100^{o}C for 2 min and the temperature was raised to 175^{o}C at a rate of 10^{o}C/min. Helium was used as the carried gas while 0.5% (v/v) 2-Pentanone was used as internal standard. Samples of 2 µL were injected. The bioethanol yield, expressed as percentage of the maximum theoretical yield that can produced from pretreated MCR, was calculated using the following equations (Keating et al., 2004):

$$\text{Biothanol yield } \% = \frac{[EtOH]_f - [EtOH]_o}{0.51 f [Biomass] \times 1.111} \times 100\% \quad (2)$$

where $[EtOH]_f$ is the bioethanol concentration at the end of the fermentation (g/L); and $[EtOH]_o$ is the bioethanol concentration at the beginning of the fermentation (g/L). The term "0.51 × *f* × [Biomass] × 1.111" corresponds to the theoretical bioethanol concentration, where [Biomass] is the dry biomass weight concentration at the beginning of the fermentation (g/L); *f* is the cellulose fraction of dry biomass (g/g); 0.51 is the conversion factor for glucose to bioethanol based on the stoichiometric biochemistry of yeast and 1.111 is the conversion factor for cellulose to equivalent glucose.

2.7.2. Determination of protein concentration

281 The solution obtained from Section 2.5.3 was diluted to 2 mL using 50 mM
282 sodium acetate buffer. After centrifuging the solution at 10, 000 × g for 2 min, 1 mL of
283 the diluted supernatant was transferred to a test tube, and 1 mL of the QuantiPro Working
284 Reagent (bicinchoninic acid reagent) was added. The test tube was vortex gently for
285 thorough mixing, left standing for 1 h in water at 60^{o}C and then cooled to room
286 temperature. The absorbance of the samples was measured at 562 nm using a
287 spectrophotometer (Agilent Technologies carry 60 UV-Vis). The protein concentration of
288 the samples was calculated from a bovine serum albumin (BSA) standard curve.

289 2.7.3. Scanning electron microscopy (SEM) analysis

290 The morphology and the physical structure of the untreated MCR and the MCR
291 treated with solid acid catalyst were observed by scanning electron microscopy (SEM)
292 using a Fei Quanta 450 FEG (Eindhoven, NL). The samples were dried at 50^{o}C to
293 constant weight and coated with Au/Pd film. All images were taken at a magnification of
294 10000× and observed using a voltage of 5 kV.

295

296 2.7.4. Brunauer-Emmett-Teller (BET)

297 The Brunauer-Emmett-Teller (BET) surface area, pore size and pore volume of
298 the untreated and treated MCR was determined using nitrogen adsorption/desorption
299 isotherms at -196^{o}C in a surface area analyzer (ASAP 2020, Micromeritics Co., USA).
300 Prior to analysis, the sample was degassed for 8 h at 120^{o}C under vacuum (5 mmHg) to

301 remove moisture and any contaminants. BET surface area was calculated from these
302 isotherms using the BET method. Total volume of pores was determined by single point
303 adsorption total pore volume of pores with P/P_o at 0.984. The average pore size was
304 calculated by Density Functional Theory (DFT) method (Seaton, Walton & quirke, 1989)
305 and software provided by Micromeritics Instrument Corporation.

306 2.7.5. Ammonia temperature programmed desorption (NH_3-TPD)

307 The acidity of the solid acid catalysts were measured by temperature
308 programmed desorption of ammonia (NH_3-TPD) using Autochem II 2920 chemisorption
309 analyser, (Micromeritics Instruments, USA) equipped with thermal conductivity detector
310 (TCD). 0.054 g catalyst was placed in an adsorption vessel and heated to 450^{o}C in He
311 flow for 1 h with a rate of 5^{o}C/min. Subsequently it was cooled to 100^{o}C in He flow and
312 15% NH_3 in He was passed through the sample for 1 h. NH_3 desorption was conducted
313 from 100 to 600^{o}C with heating rate of 5^{o}C/min under He flow.

314

315

316 **3. Results and discussion**

317 3.1. Macroalgae cellulosic residue (MCR) composition

318 Cellulose and acid insoluble lignin were analysed using the method of two-step
319 acid hydrolysis. The results showed that cellulose fraction comprised 68% and there was
320 no acid insoluble lignin detected in the macroalgae cellulosic residue biomass. The HP-

LC analysis of 72% sulfuric acid hydrolysate showed that glucose was the only main component of MCR. The high carbohydrate content of MCR makes it a very promising substrate for bioethanol production.

3.2. Pretreatment of macroalgae cellulosic residue (MCR)

3.2.1. Effect of biomass loading

The effect of macroalgae cellulosic residue (MCR) loading on the solid-acid pretreatment process was studied. Fig. 1 shows the enzymatic hydrolysis yield at 120^{o}C, 4% (w/v) Dowex (TM) Dr-G8 and 30 min pretreatment condition. Up to 24 h of enzymatic hydrolysis, there were no significant differences in glucose yield at all levels of biomass loading. However, after 24 h, it can be clearly seen that the glucose yield for biomass loadings with 8% (w/v) and 10% (w/v) are significantly higher than biomass loadings of 12% (w/v) and 14% (w/v). In fact for biomass loadings of 12% (w/v) and 14% (w/v), a slight drop in glucose yield was observed after 48 h. This indicated that the increase of biomass loading in the pretreatment process does not enhance the enzymatic hydrolysis of the pretreated MCR to some extent. When the biomass loading was increased higher than 10% (w/v), it became hard to keep the reaction system in homogeneous form because of insufficient liquid, which resulted to a slurry with high viscosity that is difficult to handle (Kim, Lee & Jeong, 2014). Besides that, when the biomass loading was too high, the MCR could not interact sufficiently with the solid acid catalyst, resulting to low glucose yield. Similar results were also reported by other

researchers in which the optimum *Gelidium amansii* content for H_2SO_4 acid pretreatment were 10% (Ra et al., 2013). Therefore, the biomass loading of 10% (w/v) was applied in further experiments.

3.2.2. Effect of catalyst loading

In this section, solid acid pretreatment experiments of macroalgae cellulosic residue (MCR) was carried out at 120^{o}C, pretreatment time of 30 min and by varying the catalyst loading from 0% to 6% (w/v). The pretreated MCR were then subjected to enzymatic hydrolysis and the content of glucose released was detected to evaluate the effectiveness of the pretreatment using Dowex (TM) Dr-G8. With regard to the environmental impact and operating cost, the lowest catalyst loading that can give the best hydrolysis performance would be desirable (Ho et al., 2013). Fig. 2 illustrates the effect of catalyst loading on pretreatment of MCR. It can be seen that the yield of glucose generally increases with time for all catalyst loading because longer hydrolysis time will allow more enzyme to depolymerize cellulose to glucose. Apart from that, the results also showed that the yield of glucose formation was significantly affected by the amount of catalyst loading. For example, when no catalyst was used in the pretreatment step, the highest glucose yield achieved at the 24 h of hydrolysis time was only 66%. However, when the catalyst loading was increased to 2% (w/v) and subsequent 4% (w/v), the glucose yield increased to 71.8% and 74.9%, respectively. Higher loading of Dowex (TM) Dr-G8 accelerates the pretreatment hydrolysis rate of MCR, which can be explained due to the increase in the total number of active catalytic sites available for the reaction

(Rinaldi, Palkovits & Schüth, 2008; Tan, Lam & Lee, 2013). Besides that, pretreatment could also help to reduce the crystallinity of cellulose by removing hydrogen linkages by solid acid hydrolysis that led to rapid and efficient enzymatic hydrolysis of MCR. In addition, no furfural or hydroxyl-methyl furfurals (HMF) were detected in the solid acid pretreated MCR hydrolysates for catalyst loading below 4% (w/v). On the other hand, there was a decreasing trend for glucose production when the loading of solid acid catalyst was increased above 4% (w/v) in which the yield dropped to 59.6%. This means that increasing the catalyst dosage beyond the optimal value resulted in a decrease of glucose yield. The main reason for this reduction would most probably be due to excessive acidity, which cause the degradation of the released sugars into unwanted side products such as furfurals and hydroxyl-methyl furfurals (Gupta, Khasa & Kuhad, 2011; Park et al., 2011). Thus a solid acid catalyst loading of 4% (w/v), which provided the highest glucose yield, was selected as the optimal catalyst loading for subsequent experiments in this study.

3.2.3. Effect of pretreatment time

The pretreatment time is another important factor that have significant effect on the productivity of glucose. The effect of pretreatment time (15-60 min) for MCR (10% w/v) pretreated with Dowex (TM) Dr-G8 (4% w/v) at 120^{o}C and subsequent enzymatic hydrolysis (50^{o}C, pH 4.8, 72 h) is presented in Fig. 3. At 48 h of hydrolysis time, the yield of glucose was found to increase from 97.6% to 99.8% when the pretreatment time

384 was increased from 15 min to 30 min. This is because longer pretreatment duration
385 allows sufficient time for the solid acid catalyst to degrade MCR biomass and thus more
386 cellulose site is available for enzymatic hydrolysis. However, further increase in
387 pretreatment time gave a negative effect on the glucose yield. The glucose yield decrease
388 to 82.8% (48 h hydrolysis time) when the pretreatment time was prolonged to 60 min.
389 This is because longer pretreatment time might degrade the cellulose and release soluble
390 glucose from MCR during the pretreatment process itself, which led to less amount of
391 cellulose that is available for enzymatic hydrolysis and hence a decrease in glucose yield.
392 This finding is supported by Harun and Danquah (2011), where they reported that
393 increasing pretreatment time up to 60 min does not increase the hydrolysis process of
394 microalgae but instead pose a risk in reducing the production of bioethanol. Besides that,
395 shorter duration for pretreatment process is actually favourable because it has a positive
396 impact on energy consumption. Therefore, 30 min of pretreatment time was chosen as the
397 optimum value for treating macroalgae cellulosic residue (MCR). (Harun & Danquah,
398 2011)

399 3.2.4. Effect of pretreatment temperature

400 The effect of pretreatment temperature on the enzymatic hydrolysis is presented
401 in Fig. 4. When the pretreatment temperature was increased from 110 to 120^{o}C, the
402 glucose yield of pretreated macroalgae cellulosic residue (MCR) improved significantly.
403 This shows that MCR pretreated at higher temperature was more acquiescent to
404 enzymatic hydrolysis. After 48 h of enzymatic hydrolysis, the glucose yield attained was

405 99.8% after being treated at 120°C, which increased over 7.2% in comparison with that at
406 110°C. This phenomenon could be explained as high pretreatment temperatures facilitate
407 cellulose fibers to dissolve faster and making more cellulose accessible to enzyme attack.
408 In other words the adsorption rate of enzyme onto the macroalgae cellulosic residue
409 surface increased at higher pretreatment temperature which leads to higher hydrolysis
410 rate. However, when the pretreatment temperature was increased more than 120°C, the
411 glucose yield decreased significantly. For example, at 140°C pretreatment temperature,
412 the glucose yield obtain was only 66.5% even at the longest hydrolysis time. This is again
413 possibly due to partial cellulose degradation during pretreatments under harsh conditions.
414 Harun and Danquah (2011) also reported that acid pretreatment temperature above 140°C
415 gave a significant decrease in the amount of reducing sugars from microalgae biomass.
416 Therefore, 120°C is the optimum pretreatment temperature and it is worthwhile to note
417 that this optimum temperature is relatively milder compared to that of terrestrial biomass
418 (Jung et al., 2013; Tucker et al., 2003). Up to this point, the optimum pretreatment
419 condition for macroalgae cellulosic residue (MCR) is 4% (w/v) Dowex (TM)-Dr G8,
420 120°C, and 30 min. At this condition, no degradation of cellulose was detected.

421 3.3. Enzymatic hydrolysis

422 3.3.1. Effect of enzyme loading

423 For evaluation on the effect of enzyme loading on MCR pretreated with Dowex
424 (TM) Dr-G8, samples were subjected to enzymatic hydrolysis with 5-60 FPU/g of
425 cellulase supplemented with β-glucosidase. The reason for adding β-glucosidase is to

hydrolyze cellobiose which is an inhibitor to cellulase activity. MCR pretreated at 120°C for 30 min with 4% (w/v) Dowex (TM) Dr-G8 were used as starting materials for this part of study and the results are shown in Fig. 5. When the hydrolysis time is below 36 h, increasing the cellulase loading from 5 to 30 FPU/g seems to have a significant increase in the glucose yield. However further increasing the cellulase loading beyond 30 FPU/g has little effect on the glucose yield. This finding can be easily justified as follow. High cellulase loading will increase the availability of enzymes and therefore more cellulase is adsorbed onto the substrate surface for reaction to occurs (Ran et al., 2012). However, increasing the cellulase loading beyond a certain value does not have significant effect on the glucose yield because the limiting factor has shifted from cellulase loading to β-glucosidase which was fixed at 52 CBU/g for all experiments. To explain further, initially cellulase will hydrolyze cellulose to cellobiose and subsequently β-glucosidase will hydrolyze cellobiose to glucose. Thus, initially, when cellulase loading was increased, more cellobiose will be obtained and being converted to glucose by β-glucosidase. This will go on until there is too much cellobiose that now the limiting factor is the amount of β-glucosidase that was fixed at 52 CBU/g. In other words, the amount of β-glucosidase is not sufficient for effective hydrolysis of cellobiose into glucose at higher cellulase loading causing an accumulation of cellobiose. Cellobiose is a dimer of glucose that has a strong inhibition toward endo- and exoglucanases (from cellulase) that can significantly slows down the entire enzymatic hydrolysis process (Ruangmee & Sangwichien, 2013; Teugjas & Väljamäe, 2013). Besides that, high cellulase loading could also reduce the absorption efficiency of the enzyme on cellulose due to high viscosity that could also

448 contribute to a lower glucose yield (Singh et al., 2009; Zheng et al., 2009). This was
449 supported by the findings shown in Fig. 5 whereby at hydrolysis time of 30 h, glucose
450 yield decreased by 14.4% when the cellulase loading was increased from 45 to 60 FPU/g.

451 In short, either too low or too high enzyme loading is not appropriate for
452 enzymatic hydrolysis. However, minimizing enzyme consumption is an important way to
453 reduce cost. Thus, the optimal loading of cellulase was determine as 45 FPU/g which will
454 be used for subsequent experiments. The optimum enzyme dosage was found lower as
455 compared to acid or alkaline hydrolysis and does not pose any corrosion problem
456 (Borines, de Leon & Cuello, 2013).

457 3.3.2. Effect of different pretreatment methods

458 In order to further understand the digestibility of the macroalgae cellulosic residue
459 (MCR) pretreated with Dowex (TM) Dr-G8, MCR was also pretreated with two other
460 methods (H_2SO_4 and NaOH) and the content of protein in the supernatant and glucose
461 yield were determined, as summarized in Table 2. It is well established that the
462 adsorption of cellulase on substrate is a prerequisite for enzymatic hydrolysis of cellulose
463 to occur. Among the different pretreated substrates evaluated in this study that were
464 subjected for enzymatic hydrolysis, the MCR pretreated with Dowex (TM) Dr-G8 were
465 observed to be more vulnerable to enzymatic hydrolysis and gave the highest glucose
466 yield (99.8%). Therefore, it could be speculated that for the same cellulase loading, MCR
467 pretreated with Dowex (TM) Dr-G8 would adsorb significantly more cellulase than MCR
468 pretreated with H_2SO_4 or NaOH. This is supported by the fact that only 19.6% protein

(cellulase) remained in the supernatant of the MCR pretreated with Dowex (TM) Dr-G8, which was significantly lower than that in the supernatant of the MCR pretreated with H_2SO_4 (22.3%) or NaOH (25.3%), respectively. This result proved the speculation that MCR pretreated with Dowex (TM) Dr-G8 was able to adsorb more cellulase at the beginning of the enzymatic hydrolysis than the MCR pretreated with H_2SO_4 or NaOH. This is probably because Dowex (TM) Dr-G8 act as a swelling agent, which enhances the surface area of the substrate and make the substrate more amenable for enzymatic action and better digestibility. This comparison shows that Dowex (TM) Dr-G8 is a far more superior catalyst than NaOH and slightly better than H_2SO_4. The role of Dowex (TM) Dr-G8 resins is merely an acidifier of the aqueous slurries, having a similar effect as an aqueous solution of H_2SO_4 and therefore it is expected that the mechanism of cellulose depolymerisation using Dowex (TM) Dr-G8 is similar to the depolymerisation mechanism with H_2SO_4 (Watanabe, 2010). In contrast, the MCR pretreated with NaOH when subjected to enzymatic hydrolysis resulted to a sugar yield of only 80.2%. As compared to MCR pretreated with Dowex (TM) Dr-G8, the lower enzymatic hydrolysis efficiency in MCR pretreated with NaOH might be due to lower digestibility. Furthermore, the advantage of using alkali pretreatment is that it can efficiently remove lignin from the lignocellulosic biomass, which was not applicable in this case as MCR does not contain lignin (Dagnino et al., 2013; Sambusiti et al., 2013). Besides that, Dowex (TM) Dr-G8 is more environmental friendly because it can be easily separated from the biomass after the pretreatment process and can be reuse. Additional experiments

were conducted to test the possibility of recycling Dowex (TM) Dr-G8 cation-exchange resin and will be presented in the section 3.3.3.

A mass balance of the process (from pretreatment to enzymatic hydrolysis) was summarized as shown in Fig. 6. The remaining solid fraction was separated from the hydrolysate prior to enzymatic hydrolysis. On the basis of 100 g of MCR, about 80-81 g of pretreated MCR can be recovered after pretreatment. Subsequently, about 0.77, 1.08 and 0.05 g glucose can be recovered in the prehydrolysate from solid acid, dilute acid, and sodium hydroxide pretreatment, respectively. The pretreated MCR was subjected to enzymatic hydrolysis with a buffer solution that comprising of 45 FRP/g of cellulose, 52 CBU/g of β-glucosidase and incubated at 50^{o}C for up to 30 h. Finally, the mass balance demonstrates that about 80.6, 80.0, and 64.9 g glucose can be recovered from enzymatic hydrolysis of solid acid, dilute acid, and sodium hydroxide pretreated MCR for the conditions selected, respectively.

3.3.3. Reuse of catalyst

In order to make the entire process more attractive and industrially viable, the catalyst must have a long life-usage and consistently give high activity. Catalyst recyclability study were performed with 4% (w/v) of Dowex (TM) Dr-G8 at 120^{o}C and 30 min pretreatment time, since these conditions resulted in the highest glucose yield during enzymatic hydrolysis. As shown in Fig. 7, the glucose yield maintained at around 94% till the fifth run, with only slight decrease during the sixth run. The results showed that the catalyst was not significantly deactivated even after repeated use. This means that

511 the functional groups of the solid acid catalyst remained intact even after repeated use
512 and the resin can be used repeatedly by simply washing with distilled water. Therefore, it
513 can be concluded that the catalyst is very stable even after being used repeatedly for six
514 times.

515

516 3.4. Simultaneous saccharification and fermentation (SSF)

517 In the previous section, the effect of pretreatment using Dowex (TM) Dr-G8 was
518 only evaluated up to the stage of glucose yield. Thus, in this section, the use of SSF
519 process for the production of bioethanol from MCR pretreated with Dowex (TM) Dr-G8
520 will be presented. For any bioethanol from biomass production process, it is important to
521 produce high concentration of bioethanol due to the energy intensive nature of distillation
522 and dehydration process (Ahmed et al., 2013a). In order to achieve a high final
523 bioethanol concentration, a high substrate loading, and hence a high cellulose content, is
524 crucial for the SSF process to be economically viable. The effect of solid loading
525 (pretreated MCR) on bioethanol production using the SSF process with prehydrolysis
526 was carried out at 43^{o}C and the results are shown in Fig. 8. It is important to differentiate
527 between conventional SSF and SSF process with prehydrolysis. SSF with prehydrolysis
528 has the potential to significantly improve the bioethanol yield because this will facilitate
529 more effective stirring at the beginning of the process (Hoyer, Galbe & Zacchi, 2013).
530 Fig. 8 indicates that the glucose accumulated during prehydrolysis was rapidly consumed
531 after the inoculation of *S. cerevisiae*. In general, the glucose concentration accumulated

532 during prehydrolysis increased with the higher solid loading, 37.8, 65.3, 85.5, and 110.6
533 g/L corresponding to solid loading of 4%, 6%, 8% and 10% (w/v), respectively. The
534 glucose concentration was found to decrease as the SSF process progressed which was
535 accompanied by a rapid increase in the fermentation product. The glucose was totally
536 consumed within 3 h after inoculation and then remained at a low level for all solid
537 loading, indicating that fermentation by the yeast was much faster that enzymatic
538 hydrolysis. Therefore, the rate-limiting step in SSF process for bioethanol production is
539 the enzymatic hydrolysis of the pretreated macroalgae cellulosic residue (MCR) into
540 glucose, rather than the fermentation of the reducing sugar to bioethanol by yeast.
541 Although the bioethanol concentration was found to generally increase with higher solid
542 loadings, but the percentage of theoretical yield was found to generally drop with higher
543 solid loadings. At the highest loading of 10% (w/v), the highest theoretical yield achieved
544 was only 31.1%. In fact, it was found that the lag phase (results not shown) for the
545 highest solid loading of 10% (w/v) reached almost 4 h. This lag phase is due to the
546 adaptation of the yeast to fermentation conditions and its duration is related with the solid
547 loading. The low theoretical yield at high solid loading could be due to insufficient
548 enzyme and furthermore the high initial sugar concentration at higher solid loading could
549 inhibit the enzyme leading to low cellulose hydrolysis. In addition, high solid loading can
550 also cause mixing problems due to higher viscosity of the mixture that lead to mixing
551 problem at the beginning of the SSF process. A similar result was also reported for
552 pretreatment of macroalgae by Lee et al. (2013). The substrate loading could not be
553 increased beyond the maximum of 6% due to high hygroscopic properties of macroalgae.

554 Thus for this study, SSF process loaded with 4% (w/v) pretreated MCR and 45 FPU/g of
555 cellulase and 52 CBU/g of β-glucosidase was optimal for efficient production of
556 bioethanol. Interestingly, the bioethanol yield from pretreated MCR was found
557 comparable with the previously reported bioethanol yields from various macroalgae
558 materials such as brown algae by Lee and Lee (2012). In their study, a bioethanol
559 concentration of only 2.7 g/L was attained after 7 days of fermentation by using *S.*
560 *cerevisiae* (KCCM50550). In another paper by Kim et al. (2013) using *S. cerevisiae* as
561 the fermentation agent, a bioethanol yield of only 37.1% was attained after 24 h of SSF.
562 This shows that the highest bioethanol concentration (14.1 g/L) obtained from MCR
563 pretreated with Dowex (TM) Dr-G8 and followed by SSF process is much higher than
564 that reported by several other studies and can be obtained at a much shorter time. In
565 addition, this work utilized an environmental friendly solid acid pretreatment process
566 with mild temperature (120^{o}C) and without the need of hazardous chemicals. (Lee,
567 Li,(Lee, Ryu & Oh, 2013) (Lee & Lee, 2012) (Kim et al., 2013)

568

569

570 3.5. Scanning electron microscopy (SEM)

571 Scanning electron microscopy (SEM) analysis revealed the morphological
572 features and surface characteristic of MCR pretreated with Dowex (TM) Dr-G8
573 compared with the untreated MCR. For the untreated MCR, there were highly ordered
574 fibrous structures of cellulose and the surface was continuous, even and smooth (Fig. 9A).

After the solid acid pretreatment, the structure of pretreated MCR seems to have loosened and resulting in exposure of internal structure and fibers (Fig. 9B). SEM image of the pretreated MCR indicated that the pretreatment process resulted to the removal of external fibers, which in turn increase surface area so that cellulose becomes more accessible to enzymes. Increase in surface area and pore volume in pretreated solid residues was reported to increase glucose yield during enzymatic hydrolysis of solid residue (Ge, Wang & Mou, 2011).

3.6 Porosity and surface analysis

In general, the substrate surface area is a primary indicator of cellulose accessibility. In this work, the Brunauer-Emmett-Teller (BET) surface area, pore volume, and pore size of MCR before and after pretreatment were determined using nitrogen adsorption. The values of surface area, pore volume, and pore size of the samples before and after pretreatment were calculated and the results are listed in Table 3. Compared with the untreated sample, the value of the surface area after the solid acid pretreatment increased from 7.1728 to 9.3145 m^2/g using the BET method. The cumulative pore volume increased from 0.002618 to 0.007180 cm^3/g, and the average pore size enlarged from 11.2436 to 40.0427 Å after pretreatment. All of these values indicated that the pretreatment using solid acid catalyst leads to a significant increase in the porosity of the MCR, thereby resulting in the significant enhancement of enzyme accessibility toward cellulose. (Guo & Catchmark, 2012).

596

597 3.7 NH_3-TPD measurement

598 Temperature-programmed desorption of ammonia (NH_3-TPD) was used to
599 characterize the acidic properties of the fresh Dowex (TM) Dr-G8 as shown in Fig. 10.
600 The strength of the acid sites can be determined by the temperature at which the adsorbed
601 NH_3 desorbs. The acid sites could be defined as weak, medium, strong and very strong at
602 desorption temperatures of 150-250, 250-350, 350-500, and >500^{o}C, respectively
603 (Azzouz et al., 2006). Fig. 10 shows that NH_3 desorption profile of fresh Dowex (TM)
604 Dr-G8 have two peaks at 200-350^{o}C and 350-450^{o}C, respectively, indicating that there
605 are two types of acid sites with different intensity. It was found that the peak maximums
606 of fresh Dowex (TM) Dr-G8 appeared at the temperature of 300^{o}C, implying that the
607 acidic center of this catalyst was related to strong acid sites of $–SO_3H$ groups. Generally,
608 a higher the activity in cellulose hydrolysis can be anticipated from a higher acid amount
609 and a stronger acid strength.

610

611 **4. Conclusion**

612 This study successfully demonstrated a novel pretreatment method coupled with
613 SSF process for the conversion of macroalgae cellulosic residue (MCR) to bioethanol.
614 The conversion of MCR to bioethanol would simultaneously provide a more sustainable
615 waste management system. The catalyst was found capable of being used repeatedly with

616 minimal drop in activity making it a green process. Thus, this study have pave the way
617 for utilizing macroalgae cellulosic residue by converting it to third-generation bioethanol.

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623

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Figure captions

Fig. 1. Effect of biomass loading on enzymatic hydrolysis of MCR. Pretreatment conditions: pretreatment time: 30 min, reaction temperature: 120^{o}C, catalyst loading: 4%

769 w/v. Enzymatic hydrolysis conditions: 2% w/v pretreated MCR, 15 FPU/g of cellulase,
770 52 CBU/g of β-glucosidase, pH 4.8, 50^{o}C.

771 Fig. 2. Effects of catalyst loadings on the pretreatment of MCR in the presence of a
772 heterogeneous catalyst Dowex (TM) Dr-G8. Pretreatment reaction conditions: biomass
773 loading: 10% w/v, pretreatment time: 30 min, reaction temperature: 120^{o}C. The
774 enzymatic hydrolysis conditions were the same as that in Figure 1.

775 Fig. 3. Effect of the pretreatment time on enzymatic hydrolysis of MCR. Pretreatment
776 conditions: biomass loading: 10% w/v, reaction temperature: 120^{o}C, catalyst loading: 4%
777 w/v. The enzymatic hydrolysis conditions were the same as that in Figure 1.

778 Fig. 4. Effect of the pretreatment temperature on enzymatic hydrolysis of MCR.
779 Pretreatment conditions: biomass loading: 10% w/v, pretreatment time: 30 min, catalyst
780 loading: 4% w/v. The enzymatic hydrolysis conditions were the same as that in Figure 1.

781 Fig. 5. Effect of different enzyme loadings on hydrolysis of pretreated MCR. Enzymatic
782 hydrolysis conditions: 50^{o}C, 72 h, 120 rpm at 2% w/v of pretreated MCR in 50 mM
783 sodium acetate buffer at pH 4.8.

784 Fig. 6. Overall mass balance for (A) solid acid, (B) dilute acid, and (C) sodium hydroxide
785 pretreatment.

786 Fig. 7. Performance of the recycled catalyst at repeated runs. Pretreatment conditions:
787 catalyst loading: 4% w/v, s/l ratio: 4% w/v, pretreatment time: 30 min, reaction
788 temperature: 120^{o}C.

789 Fig. 8. Profile of bioethanol production and glucose consumption for 8 h SSF of
790 pretreated MCR suspended in deionized water at different solid loadings. (A) 4% w/v, (B)
791 6% w/v, (C) 8% w/v and (D) 10% w/v. Conditions: enzyme loading of 45 FPU/g of
792 cellulase and 52 CBU/g of β-glucosidase, 50^{o}C for prehydrolysis, and 43^{o}C for SSF.

793 Fig. 9. Scanning electron micrographs of (A) untreated, (B) solid acid pretreated MCR
794 under optimized conditions.

795 Fig. 10. Temperature programmed desorption (TPD) profiles of NH_3-TPD for fresh
796 Dowex (TM) Dr-G8 (B).

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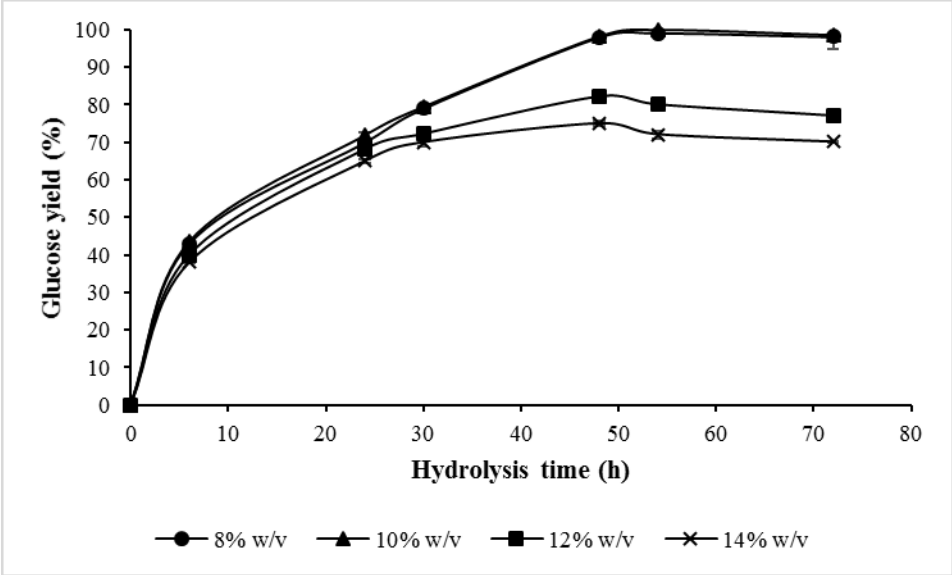
804 Figure 1



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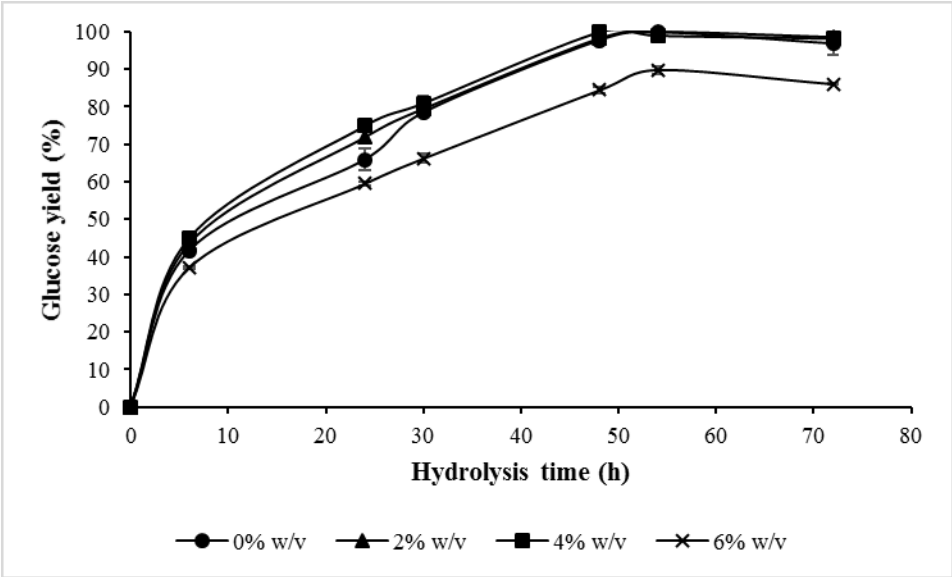
815 Figure 2



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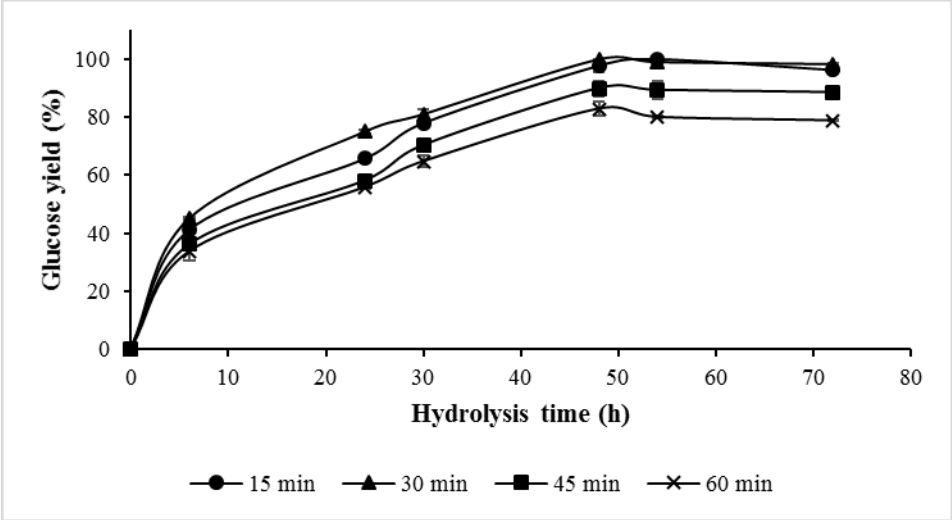
826 Figure 3



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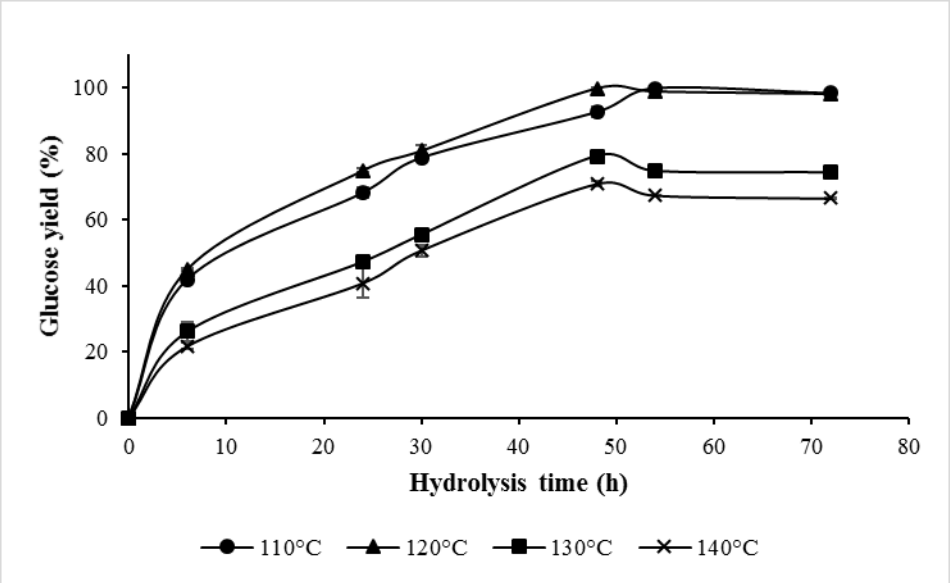
837 Figure 4



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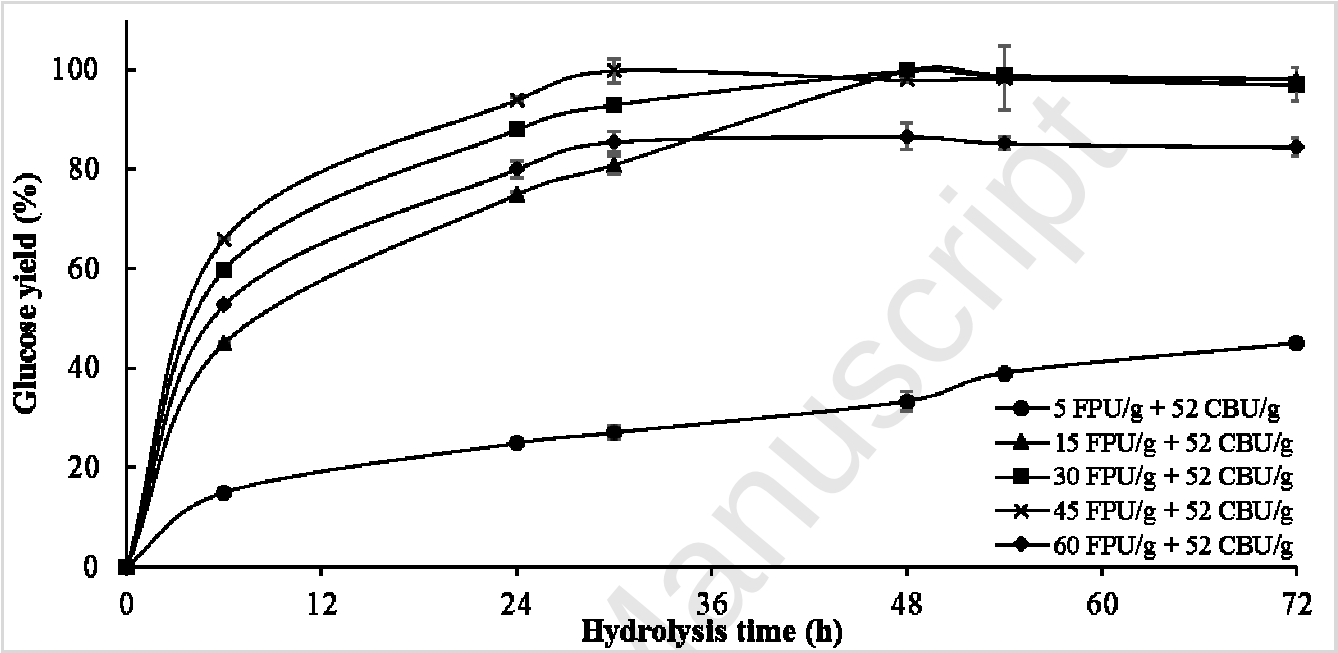
848 Figure 5



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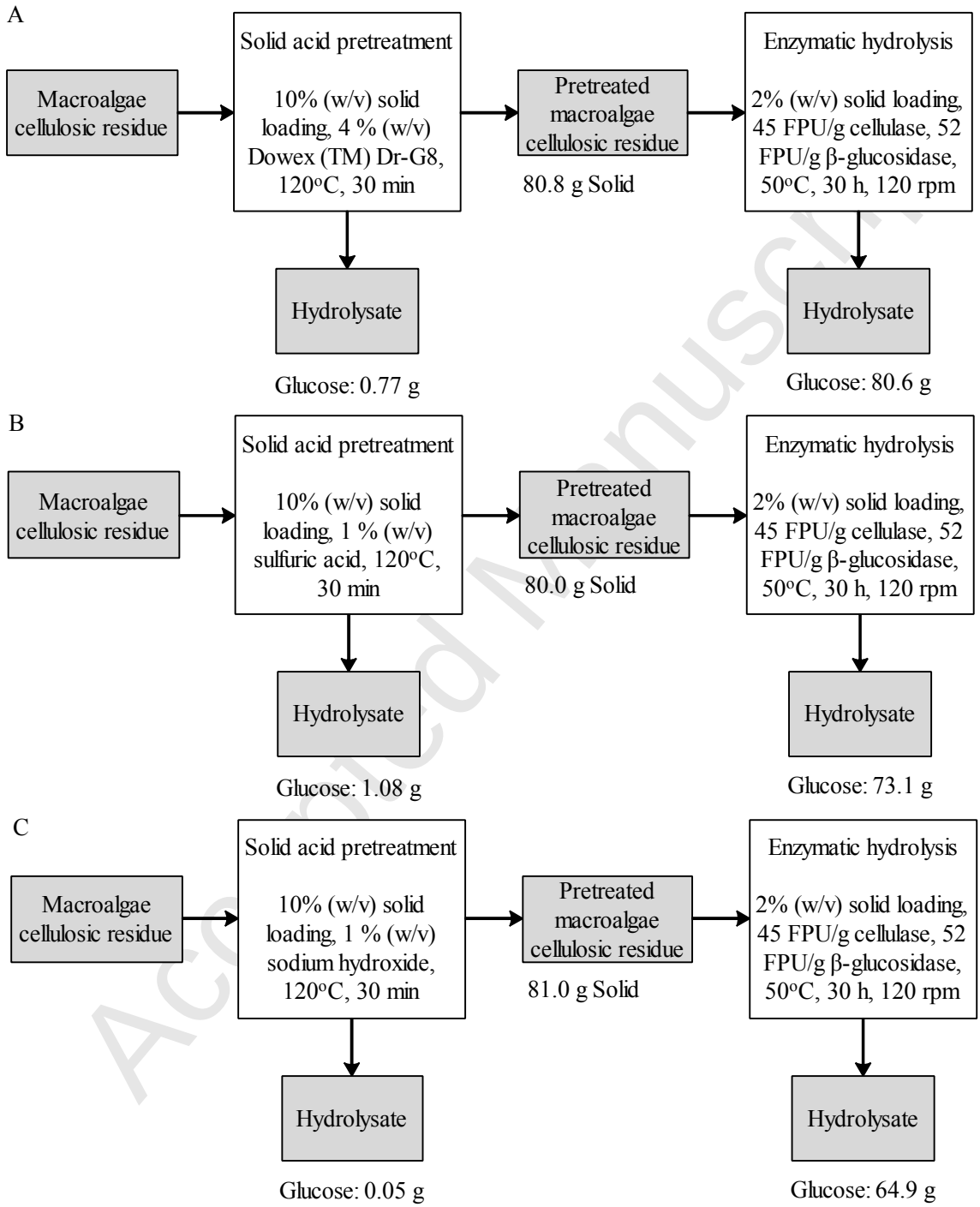
858 Figure 6



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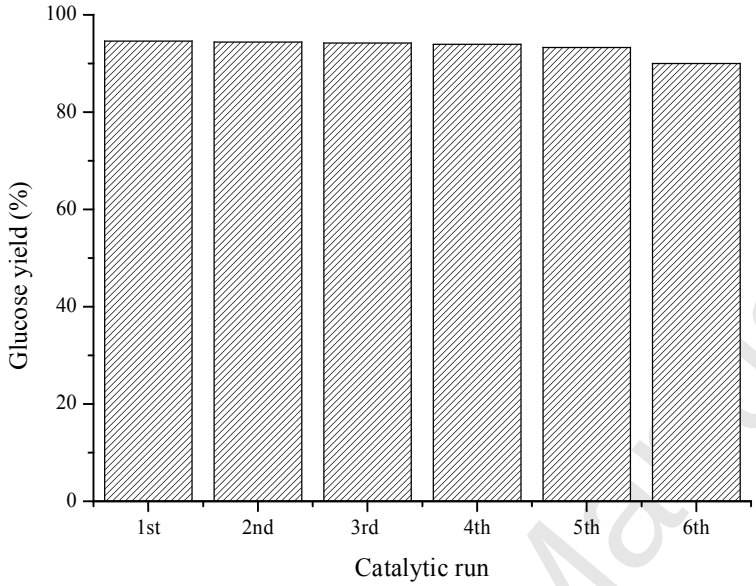

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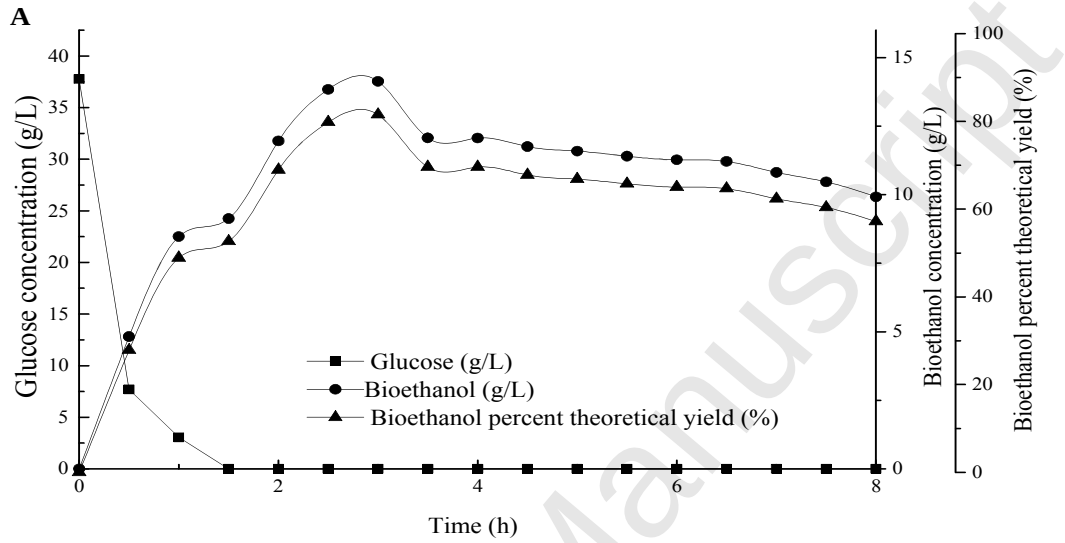
871 Figure 8



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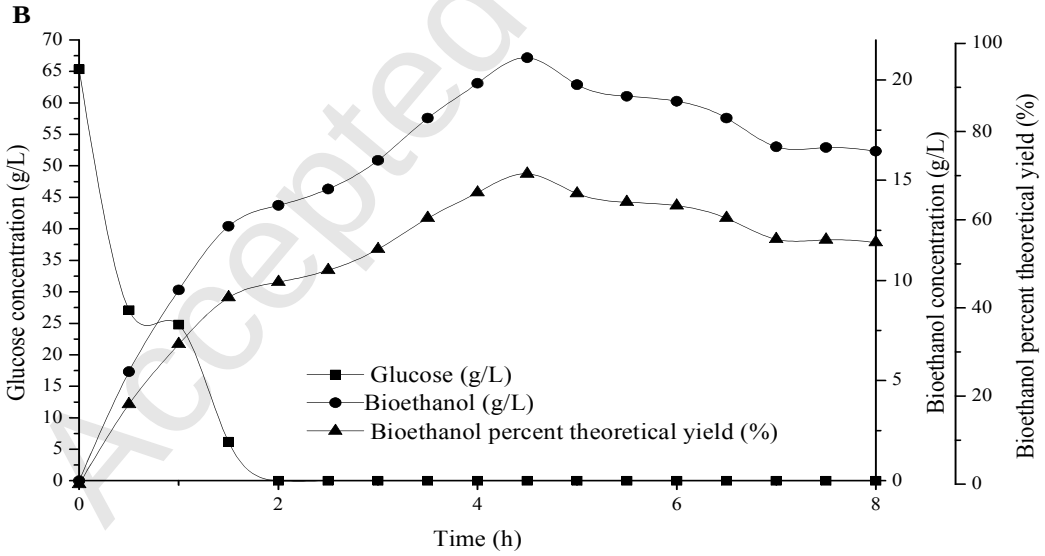

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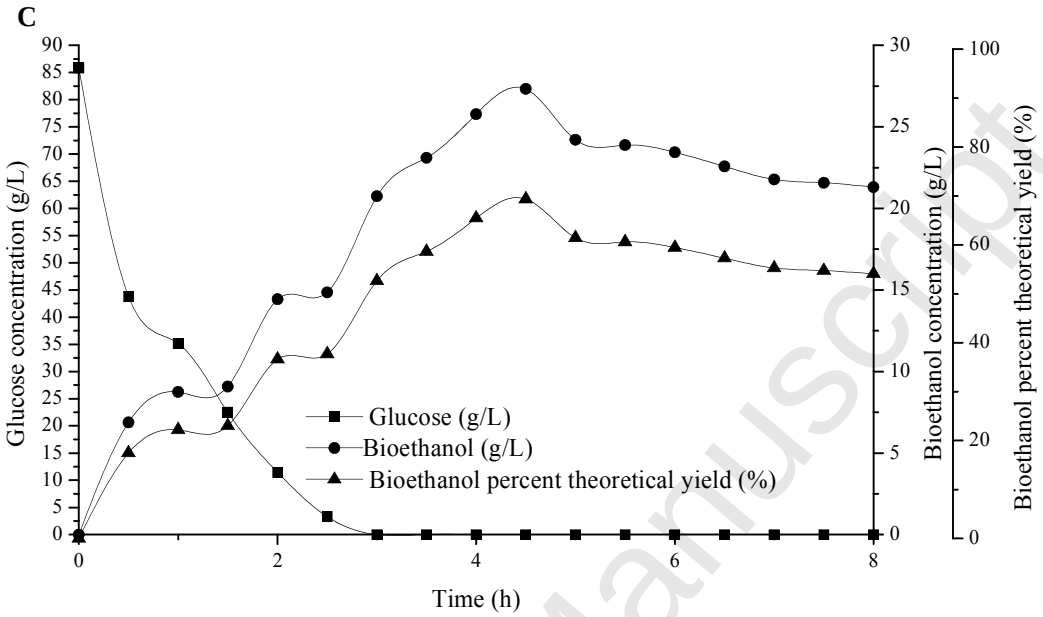

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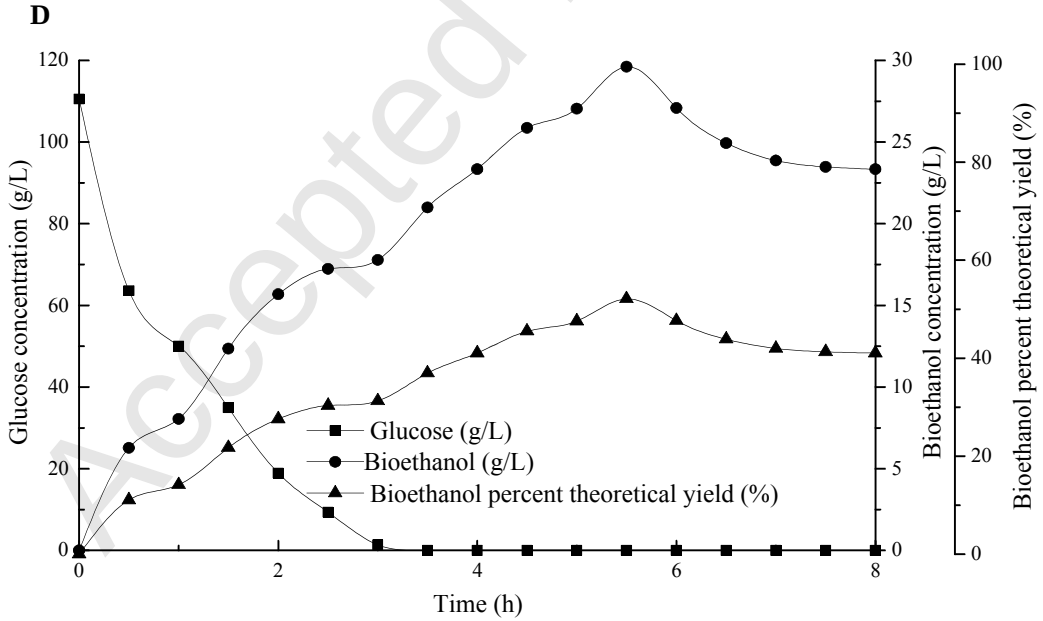

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878 Figure 9

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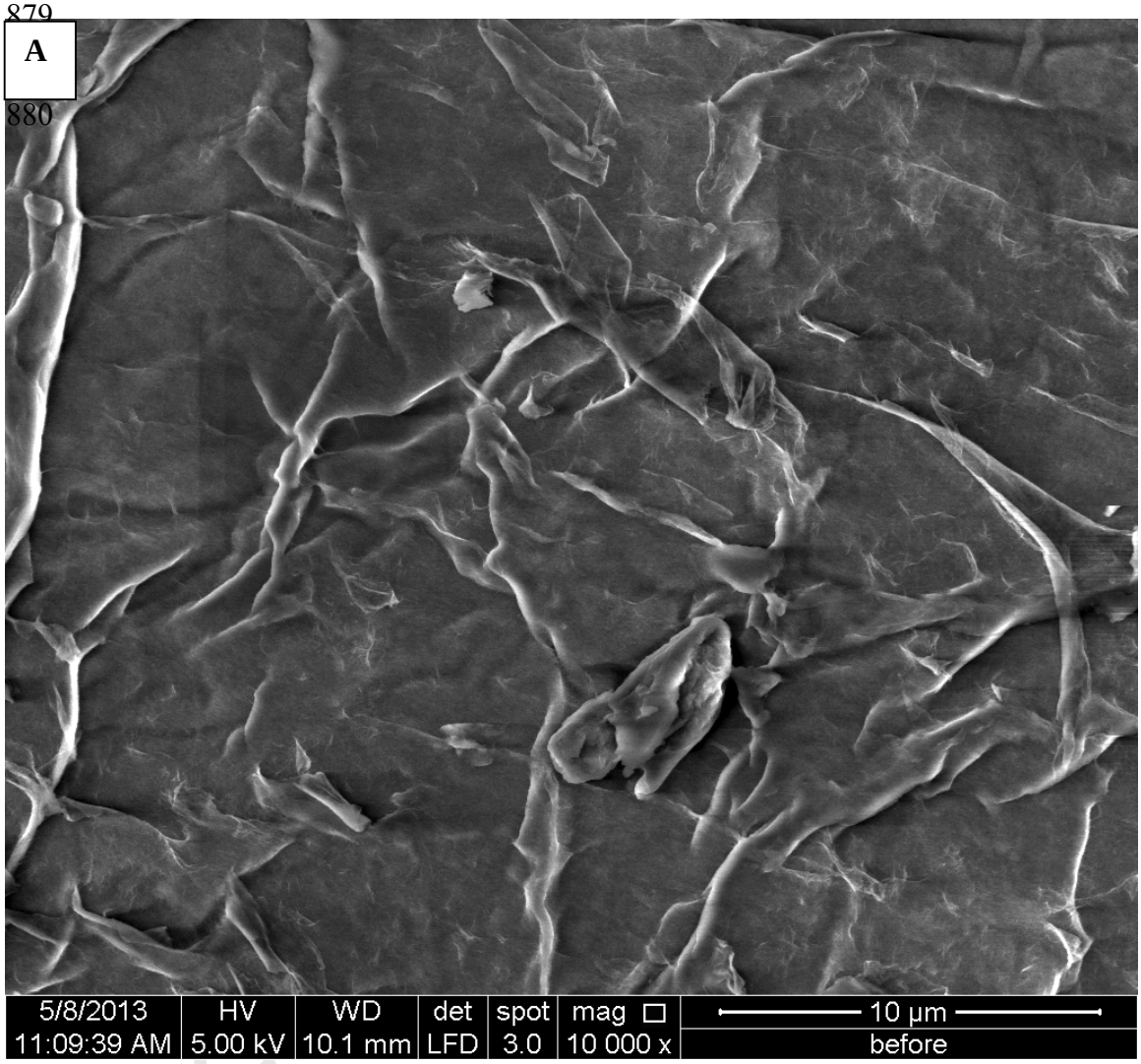

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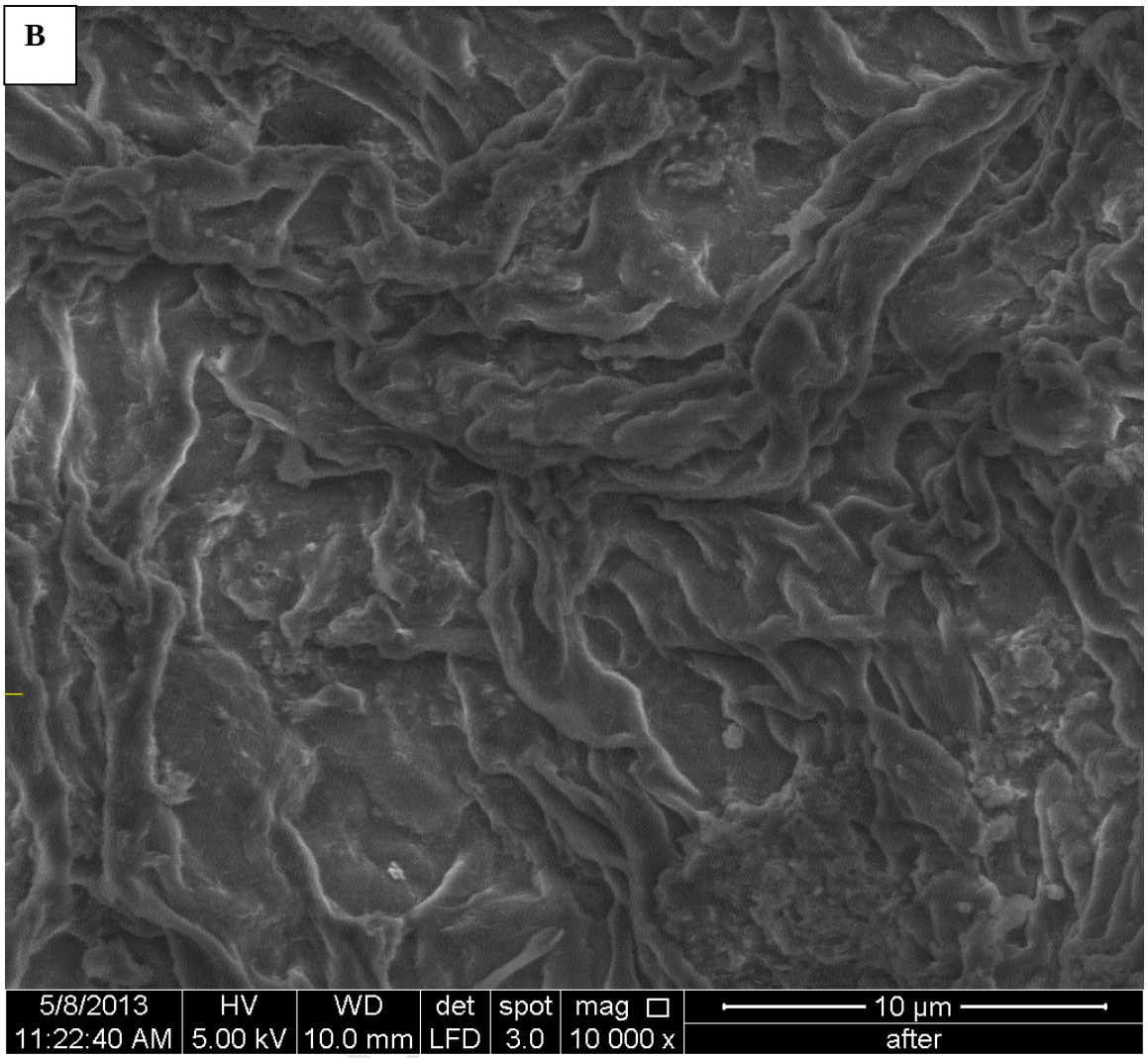

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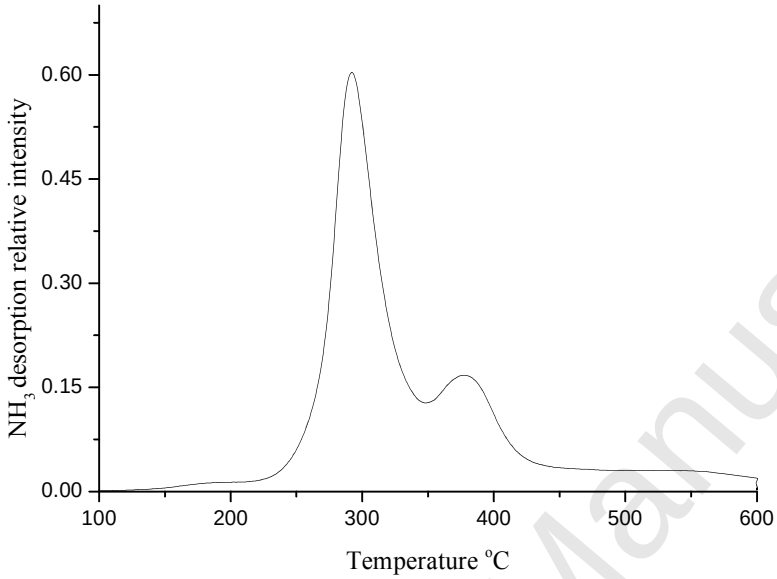
900 Figure 10



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910 List of table

911 Table 1: Properties of Dowex (TM) Dr-G8 catalyst.

912 Table 2: Comparison with other pretreatment methods of macroalgae cellulosic residue.

913 Table 3: Physical properties of macroalgae cellulosic residue before and after
914 pretreatment with Dowex (TM) Dr-G8 catalyst.

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927 Table 1

Property	Dowex (TM) Dr-G8
Shape	Bead
Particle size (µm)	300-1,200
Capacity (meq/gm)	4.5
Particle density (g/mL)	1.22
Functional group	Sulfonic acid

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930 Table 2

Substrates	T (^{o}C)	Time (min)	Protein content in supernatant (%) at 2 h	Glucose yield (%) at 30 h of enzymatic hydrolysis
4% (w/v) Dowex (TM) Dr-G8	120	30	19.6%	99.8%
1 % (w/v) H_2SO_4	120	30	22.3%	90.5%
1 % (w/v) NaOH	120	30	25.3%	80.2%

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940 Table 3

Sample	**BET surface area (m^2/g)**	**Pore volume (cm^3/g)[a]**	**Pore size[b] (Å)**
Untreated MCR	7.1728	0.002618	11.2436
Treated MCR	9.3145	0.007180	40.0427

941 [a] Single point adsorption total pore volume of pores less than less than 1244.577 Å width
942 at P/Po = 0.984.
943 [b] Adsorption average pore width (4 V/A by BET).
944