Project Code :
(for RCMO use only)

UNIVERSITI SAINS MALAYSIA

RU GRANT FINAL REPORT FORM

Please email a softcopy of this report to rcmo@usm.my

A	**PROJECT DETAILS**
i	**Title of Research: Production of Third Generation Bioethanol via Hydrolysis of Macroalgae (Seaweed) Using Heterogeneous Catalyst**
ii	**Account Number: 814187**
iii	**Name of Research Leader: Prof. Dr. Lee Keat Teong**
iv	**Name of Co-Researcher:** **1. Prof. Dr. Abdul Rahman Mohamed** **2. Prof. Dr. Ahmad Zuhairi Abdullah**
v	**Duration of this research:** **a) Start Date : 1 Feb 2013** **b) Completion Date : 31 Jan 2016** **c) Duration : 3 years (3 years and 9 months including extention period)** **d) Revised Date** *(if any)* **: 31 Oct 2016**
B	**ABSTRACT OF RESEARCH**
	(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English. This abstract will be included in the Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large) The efficacy of red macroalgae *Eucheuma cottonii* (EC) as feedstock for third-generation bioethanol production was evaluated. Dowex (TM) Dr-G8 was explored as a potential solid catalyst to hydrolyzed carbohydrates from EC or macroalgae extract (ME) and pretreatment of macroalgae cellulosic residue (MCR), to fermentable sugars prior to fermentation process. The highest total sugars were produced at 98.7 g/L when 16% of the ME was treated under the optimum conditions of solid acid hydrolysis (8% (w/v) Dowex (TM) Dr-G8, 120 °C, 1 h) and 2% pretreated MCR (P-MCR) treated by enzymatic hydrolysis (pH 4.8, 50 °C, 30 h). A two-stream process resulted in 11.6 g/L of bioethanol from the fermentation of ME hydrolysates and 11.7 g/L from prehydrolysis and simultaneous saccharification and fermentation of P-MCR. The fixed price of bioethanol obtained from the EC is competitive with that obtained from other feedstocks.

RECEIVED 14 APR 2017 Pej. Penyelidikan RCMO

C	BUDGET & EXPENDITURE

i

Total Approved Budget : **RM 247,500.00**

Yearly Budget Distributed

Year 1 : **RM 54,500.00**
Year 2 : **RM 99,000.00**
Year 3 : **RM 94,000.00**

Total Expenditure : **RM 246,918.97**

Balance : **RM 581.03**

Percentage of Amount Spent (%) : **99.8 %**

 # Please attach final account statement (eStatement) to indicate the project expenditure

ii **Equipment Purchased Under Vot 35000**

No.	Name of Equipment	Amount (RM)	Location	Status
NIL	NIL	NIL	NIL	NIL

 # Please attach the Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori) – Appendix 1

D	RESEARCH ACHIEVEMENTS

i **Project Objectives** *(as stated/approved in the project proposal)*

No.	Project Objectives	Achievement
1	To synthesize suitable heterogeneous catalyst that can be used for hydrolysis of macroalgae.	Achieved
2	To study the effects of hydrolysis/saccharification process variables on the yield of sugars (glucose or galactose) including optimization process – reaction temperature, reaction time, concentration of material and amount of catalyst.	Achieved
3	To develop a continuous hydrolysis system.	Achieved
4	To study the effectiveness of converting the hydrolysis products to bioethanol via fermentation process in a batch reactor.	Achieved
5	To determine the feasibility of continuous bioethanol production using the simultaneous saccharification and fermentation (SSF) process.	Achieved
6	To develop kinetic models for both the batch and continuous system.	Achieved

ii **Research Output**

a) **Publications in ISI Web of Science/Scopus**

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
1.	Inn Shi Tan, Keat Teong Lee, Man Kee Lam, "Hydrolysis of Macroalgae Using Heterogeneous Catalyst for Bioethanol Production", Carbohydrate Polymers, Elsevier, Vol. 94, 2013, pp 561–566.	Published
2.	Inn Shi Tan, Keat Teong Lee, "Enzymatic hydrolysis and fermentation of seaweed solid wastes for bioethanol production: An optimization study", Energy, Elsevier, Vol. 78, 2014, pp 53–62.	Published
3.	Inn Shi Tan, Keat Teong Lee, "Immobilization of b-glucosidase from Aspergillus niger on k-carrageenan hybrid matrix and its application on the production of reducing sugar from macroalgae cellulosic residue", Bioresource Technology, Elsevier, Vol. 184, 2015, pp 386–394.	Published
4.	Inn Shi Tan, Keat Teong Lee, "Solid acid catalysts pretreatment and enzymatic hydrolysis of macroalgae cellulosic residue for the production of bioethanol", Carbohydrate Polymers, Elsevier, Vol. 124, 2015, pp 311–321.	Published
5.	Inn Shi Tan, Keat Teong Lee, "Comparison of different process strategies for bioethanol production from *Eucheuma cottonii*: An economic study", Bioresource Technology, Elsevier, Vol. 199, 2016, pp 336–346.	Published
6.		

b) **Publications in Other Journals**

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
NIL	**NIL**	**NIL**

c) **Other Publications**
(book,chapters in book,monograph,magazine,etc.)

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
NIL	**NIL**	**NIL**

d) **Conference Proceeding**

No.	Conference (conference name,date,place)	Title of Abstract/Article	Level (International/National)
NIL	NIL	NIL	NIL

 # Please attach a full copy of the publication/proceeding listed above

iii **Other Research Ouput/Impact From This Project**
(patent, products, awards, copyright, external grant, networking, etc.)

Not Applicable

E HUMAN CAPITAL DEVELOPMENT

a) **Graduated Human Capital**

Student	Nationality (No.)		Name
	National	International	
PhD	1	-	1. **Tan Inn Shi** 2.
MSc	-	-	1. 2.
Undergraduate	-	-	1. 2.

b) **On-going Human Capital**

Student	Nationality (No.)		Name
	National	International	
PhD	-	-	1. 2.
MSc	1	-	1. **Teh Yong Yi** 2.
Undergraduate	-	-	1. 2.

c) **Others Human Capital**

Student	Nationality (No.)		Name
	National	International	
Post Doctoral Fellow	-	-	1. 2.
Research Officer	-	-	1. 2.
Research Assistant	-	-	1. 2.
Others (.....)	-	-	1. 2.

F COMPREHENSIVE TECHNICAL REPORT

Applicants are required to prepare a comprehensive technical report explaining the project. The following format should be used (this report must be attached separately):

- Introduction
- Objectives
- Methods
- Results
- Discussion
- Conclusion and Suggestion
- Acknowledgements
- References

G PROBLEMS/CONSTRAINTS/CHALLENGES IF ANY

(Please provide issues arising from the project and how they were resolved)

NIL

H RECOMMENDATION

(Please provide recommendations that can be used to improve the delivery of information, grant management, guidelines and policy, etc.)

NIL

Project Leader's Signature:

.....

Name : PROF. DR LEE KEAT TEONG

Pengarah
Pejabat Pengurusan & Kreativiti Penyelidikan
Universiti Sains Malaysia
11800 USM, Pulau Pinang.

Date : 6/4/17

I	COMMENTS, IF ANY/ENDORSEMENT BY PTJ'S RESEARCH COMMITTEE

Good achievement in terms of publication and training of students. Grant allocation is also well spent.

PROFESSOR DR AHMAD ZUHAIRI ABDULLAH
Deputy Dean (Research, Postgraduate & Networking),
School of Chemical Engineering,
Universiti Sains Malaysia, Engineering Campus,
Nibong Tebal, Penang, Malaysia.

Signature and Stamp of Chairperson of PTJ's Evaluation Committee

Name : PROF AHMAD ZUHAIRI ABDULLAH

Date : 08/04/2017

11/4/17

Signature and Stamp of Dean/ Director of PTJ

Name : PROFESOR AZLINA HARUN @ KAMARUDDIN
Dekan
Pusat Pengajian Kejuruteraan Kimia
Kampus Kejuruteraan
Universiti Sains Malaysia, Seri Ampangan
14300 Nibong Tebal, Seberang Perai Selatan
Pulau Pinang

Date :

RU GRANT
FINAL REPORT CHECKLIST

Please use this checklist to self-assess your report before submitting to RCMO.
Checklist should accompany the report.

NO.	ITEM	PLEASE CHECK (✓)		
		PI	JKPTJ	RCMO
1	Completed Final Report Form	✓		✓
2	Project Financial Account Statement (e-Statement)	✓		✓
3	Asset/Inventory Return Form *(Borang Penyerahan Aset/Inventori)*	N.A.		~~N.A~~
4	A copy of the publications/proceedings listed in Section D(ii) (Research Output)	✓		✓
5	Comprehensive Technical Report	✓		✓
6	Other supporting documents, if any	N.A.		
7	Project Leader's Signature	✓		✓
8	Endorsement of PTJ's Evaluation Committee	✓		✓
9	Endorsement of Dean/ Director of PTJ's			✓

Purchase Requisition ▸ Purchase Order ▸ Suppliers ▸ Maintenance ▸ Financials ▸ Coda Info ▸ Reports ▸ Admin ▸

UserCode: AIN / USMKCTLIVE / PJKIMIA **Program Code: Votebook9100** **Current Program : Votebook (Header)**

Current Date : 06/04/2017 10:12:04 AM **Version: 15.125, Last Updated at 03/01/2017** **DB: 13.00, 9/18/2010 VB: 13.01, 3/14/2011** **Switch Language : English /Malay**

Wildcard : eg. Like 100%, Like 10%1, Like %1

Element 1: 1001 Element 2: % Element 4: PJKIMIA

Element 5: 814187 Year: 2017

Detail	Excel	Budget Rule	Budget Control	Account Description	Budget Account Code	Roll over	Budget	Cash Received	Advanced	Commit	Actual	Available	Percentage
Detail	Excel	1458	T	Projek Kumpulan Wang Uni Penyelidikan	1001.111.0.PJKIMIA.814187	38,039.87	0.00	0.00	0.00	0.00	0.00	38,039.87	0.00%
		1458	T	SubTotal		38,039.87	0.00	0.00	0.00	0.00	0.00	38,039.87	0.00%
Detail	Excel	1459	T	Projek Kumpulan Wang Uni Penyelidikan	1001.221.0.PJKIMIA.814187	-1,797.99	0.00	0.00	0.00	0.00	0.00	-1,797.99	0.00%
Detail	Excel	1459	T	Projek Kumpulan Wang Uni Penyelidikan	1001.223.0.PJKIMIA.814187	-317.55	0.00	0.00	0.00	0.00	0.00	-317.55	0.00%
Detail	Excel	1459	T	Projek Kumpulan Wang Uni Penyelidikan	1001.224.0.PJKIMIA.814187	-1,298.83	0.00	0.00	0.00	0.00	0.00	-1,298.83	0.00%
Detail	Excel	1459	T	Projek Kumpulan Wang Uni Penyelidikan	1001.225.0.PJKIMIA.814187	-1,001.98	0.00	0.00	0.00	0.00	0.00	-1,001.98	0.00%
Detail	Excel	1459	T	Projek Kumpulan Wang Uni Penyelidikan	1001.227.0.PJKIMIA.814187	-23,723.65	0.00	0.00	0.00	0.00	-0.35	-23,723.30	0.00%
Detail	Excel	1459	T	Projek Kumpulan Wang Uni Penyelidikan	1001.228.0.PJKIMIA.814187	-2,051.90	0.00	0.00	0.00	0.00	0.00	-2,051.90	0.00%
Detail	Excel	1459	T	Projek Kumpulan Wang Uni Penyelidikan	1001.229.0.PJKIMIA.814187	-50,856.62	0.00	0.00	0.00	1,100.00	0.00	-51,956.62	0.00%
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		1460	T	SubTotal		46,000.00	0.00	0.00	0.00	0.00	0.00	46,000.00	0.00%
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		9999		GrandTotal		1,680.68	0.00	0.00	0.00	1,100.00	-0.35	581.03	0.00%

Carbohydrate Polymers 94 (2013) 561–566

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journal homepage: www.elsevier.com/locate/carbpol

Hydrolysis of macroalgae using heterogeneous catalyst for bioethanol production

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ABSTRACT

Utilization of macroalgae biomass for bioethanol production appears as an alternative source to lignocellulosic materials. In this study, for the first time, Amberlyst (TM)-15 was explored as a potential catalyst to hydrolyze carbohydrates from *Eucheuma cottonii* extract to simple reducing sugar prior to fermentation process. Several important hydrolysis parameters were studied for process optimization including catalyst loading (2–5%, w/v), reaction temperature (110–130 °C), reaction time (0–2.5 h) and biomass loading (5.5–15.5%, w/v). Optimum sugar yield of 39.7% was attained based on the following optimum conditions: reaction temperature at 120 °C, catalyst loading of 4% (w/v), 12.5% (w/v) of biomass concentration and reaction time of 1.5 h. Fermentation of the hydrolysate using *Saccharomyces cerevisiae* produced 0.33 g/g of bioethanol yield with an efficiency of 65%. The strategy of combining heterogeneous-catalyzed hydrolysis and fermentation with *S. cerevisiae* could be a feasible strategy to produce bioethanol from macroalgae biomass.

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1. Introduction

Focus on the use of biomass as an alternative energy feedstock to fossil fuels is intensifying in these recent years due to its significant role in reducing CO_2 emissions to the atmosphere when combusted as fuel (Schmidt, Leduc, Dotzauer, Kindermann, & Schmid, 2010). While development of fuels from biomass continues apace, first-generation bioethanol derived from edible crops have come under serious controversy because of food versus fuel feud (Nigam & Singh, 2011). Second-generation bioethanol which is mainly derived from lignocellulosic biomass offers an alternative option due to its abundant availability and do not compete with food production (Jegannathan, Chan, & Ravindra, 2009). Nevertheless, problems in removing lignin from lignocellulosic materials have impeded the commercialization potential of this renewable source (Karthika, Arun, & Rekha, 2012; Zakzeski, Bruijnincx, Jongerius, & Weckhuysen, 2010). Thus, interest has now diverted to third-generation bioethanol which is derived from aquatic sources such as macroalgae (Ross, Jones, Kubacki, & Bridgeman, 2008). Generally, macroalgae grows faster compared to terrestrial crops and does not compete with agricultural land area for mass cultivation. In addition, macroalgae contains high carbohydrate content which is rich in polysaccharides and more importantly, does not contain lignin (Park et al., 2011).

The utilization of macroalgae as bioethanol feedstock is still scattered in literature. Goh and Lee (2010) have reported that if *Eucheuma* spp. is used as a feedstock for bioethanol production, the estimated bioethanol yields could reach up to 110,000 tonnes annually. The carbohydrate is composed of the two monosaccharides: D-galactose and 3,6-anhydro-galactose, 56.2% and 43.8%, respectively (Lin, Tako, & Hongo, 2000). After hydrolysis process, these monosaccharides are suitable to be used as substrate during fermentation process for bioethanol production.

Up to now, the uses of enzyme and homogeneous acid-catalyst have been reported for the hydrolysis of carbohydrates from various biomass to fermentable sugar (Monavari, Galbe, & Zacchi, 2011; Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011; Seguin, Marinkovic, & Estrine, 2012). However, enzyme catalyzed processes face several challenges such as difficulty in recovering the enzyme from the products and requiring long hydrolysis time. On the other hand, Meinita et al. (2011), Jeong et al. (2012) and Khambhaty et al. (2012) have reported bioethanol production from *Kappaphycus alvarezii* (cottonii) using sulfuric acid as the catalyst. Nevertheless, it was found that the homogeneous acid hydrolysis process also suffers from several problems such as formation of large amount of hazardous compounds and the catalyst cannot be recovered for subsequent use. Therefore, the need of easily separable and reusable heterogeneous acid catalyst is considered essential for the hydrolysis process, in term of economical and environmental perspective. In order to ensure efficient process, the heterogeneous catalysts should be water-tolerant (Busca, 2007), have strong acidic sites and high surface area (Vigier & Jérôme, 2010). Recently, several studies have reported on the use of various types of heterogeneous acid catalyst for the hydrolysis of disaccharides (cellobiose and sucrose) with the aim to ascertain its activity (Dwiatmoko, Choi, Suh, Suh, & Kung, 2010; Nasef, Saidi, & Senna, 2005).

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http://dx.doi.org/10.1016/j.carbpol.2013.01.042

Table 1
Properties of Amberlyst (TM)-15 catalyst.

Property	Amberlyst (TM)-15
Surface area (m^2/g)	34.85
Particle size (μm)	600–800
Capacity (meq/gm)	4.2
Average pore dia (Å)	260
Supplier	Rohm & Haas, France

Thus, the objective of this study is to develop a new method of saccharification for raw macroalgae extract using a strong acidic heterogeneous catalyst, Amberlyst (TM)-15. Process variables such as catalyst loading, temperature, biomass concentration and reaction time were thoroughly optimized to attain the highest simple sugar yield. Then, the simple sugar was further explored for possible bioethanol production.

2. Material and methods

2.1. Raw materials and chemicals

Eucheuma cottonii which was used in all the experiments was purchased from Futt Put Enterprise (north coast of Sabah, Malaysia). The *E. cottonii* sample was washed with distilled water, dried at 40 °C, pulverized, and filtered with 300-mesh filters. The sample obtained was analyzed for proximate composition and kept in an air tight container until being used for the preparation of macroalgae extract.

Amberlyst (TM)-15, Amberlyst (TM)-A21, Calcium hydroxide, standard D-galactose, D-glucose and *Saccharomyces cerevisiae* (YSC2, type II) were purchased from Sigma–Aldrich (USA). Sulfuric acid, potassium dihydrogen phosphate and ethanol were purchased from Fisher Scientific (UK). All reagents were of analytical grade. Table 1 shows the properties of Amberlyst (TM)-15 catalyst.

2.2. Preparation of macroalgae extracts

120 g of dry *E. cottonii* (less than 300 mesh) was soaked in 4 L water for 30 min. Then, it was grounded to form pulp, followed by boiling at 90 °C for 2 h. The hot extracts were filtered (45 μm mesh size), dried to a constant weight at 40 °C and pulverized.

2.3. Proximate composition analysis

Crude protein content in *E. cottonii* was determined according to (Method 988.05) Association of Official Analysis Chemists (AOAC, 2000). Crude fat was determined by the Soxhlet extraction method (Method 920.30) (AOAC, 2000). Ash content was determined by heating the samples at 550 °C for 1 h. The total carbohydrate content of *E. cottonii* was determined according to a modified method that is based on the National Renewable Energy Laboratory (NREL, Golden, CO) analytical methods for biomass (Sluiter et al., 2008) using a two-step acid hydrolysis procedure. The sample was initially subjected through a primary 72% sulfuric acid hydrolysis, followed by a secondary dilute-acid hydrolysis. The content of sugar was quantified with high performance liquid chromatography.

2.4. Hydrolysis of E. cottonii

2.4.1. Heterogeneous-catalyzed hydrolysis

The heterogeneous-catalyzed hydrolysis process using Amberlyst (TM)-15 was investigated by varying different process parameters: amount of Amberlyst (TM)-15 (2–5%, w/v), temperature (110–130 °C), biomass loading (solid/liquid ratio: 5.5–15.5%, w/v) and reaction time (0–2.5 h). The dried macroalgae extract was subjected to distilled water to form different solid/liquid ratios. The mixture was then mixed with different amount of Amberlyst (TM)-15 and incubated at different temperature in an autoclave reactor. Stirring speed for all experiments was maintained at 370 rpm while the internal pressure of the autoclave was kept constant at 10 bars. After a specific hydrolysis time, the samples were cooled to room temperature and the residue was separated from the liquid by filtration using filter paper. For comparison, instead of using macroalgae extracts, raw *E. cottonii* (s/l: 12.5) without prior extract (Section 2.2) was directly subjected to hydrolysis. The raw biomass was directly treated with 4% (w/v) Amberlyst (TM)-15 at 120 °C for 1.5 h. All experiments in the current work were performed in triplicates, and the data reported are the average of the three replications.

2.4.2. Homogeneously-catalyzed hydrolysis

The performance of the heterogeneous-catalyzed hydrolysis was compared with conventional sulfuric acid hydrolysis. 12.5% (w/v) macroalgae extract was treated with 0.2 M sulfuric acid at 120 °C for 1.5 h.

2.5. Neutralization of hydrolysate

The acidic hydrolysate was neutralized with appropriate amount of Amberlyst (TM)-A21 or lime until pH 6.3–6.8 was attained. After neutralization, the hydrolysate was filtered and being measured for sugar content and subsequently fermented for ethanol production.

2.6. Bioethanol fermentation by yeast

S. cerevisiae was used for fermentation. The hydrolysate was concentrated by rotary evaporator before transferring to a basal medium which consist 0.175% (w/v) KH_2PO_4 at pH 5. The volumetric ratio of hydrolysate to basal medium used was 1:2. The medium was poured into a 250 mL Erlenmeyer flask and was sterilized at 121 °C for 15 min. The mixture was then incubated in a shaking incubator at 34 °C with a shaking speed of 135 rpm for a total time of 144 h. 1.5 mL of samples were withdrawn at different interval time during fermentation and was centrifuged at 10,000 × *g* for 10 min. The supernatant obtained after centrifugation was then analyzed for bioethanol and residual glucose and galactose content.

2.7. Analytical method

The sugar concentration was quantified with an Agilent series 1200 infinity high performance liquid chromatography (HP-LC) system equipped with a 385-ELSD and a 300 mm × 7.7 mm Hi-Plex Ca column. The mobile phase used is deionized water at a flow rate of 0.6 mL min^{-1} and injection volume of 20 μL. Purified nitrogen was used as carrier gas (70 psi) for the detector. The spray chamber temperature was set at 40 °C whereas detector temperature at 80 °C. Samples were diluted 100 times with deionized water and filtered with 0.20 μm syringe filter (Nylon membrane, Fisher Scientific) prior to HP-LC analysis. The presences of various compounds in the sample were identified by comparing the retention time of individual peaks with those of standard compounds (glucose and galactose, Sigma). Calibration curve for different types of sugars were obtained for quantitative analysis. The sugar yield was calculated as:

$$\text{Yield}\% = \frac{\text{Concentration (g/l) of sugar at time of } t}{\text{Initial concentration (g/l) of substrate}} \times 100\% \quad (1)$$

Bioethanol concentration was quantified by gas chromatography (GC) using a 5890 Series II chromatography equipped with

Table 2
Composition of the macroalgae species *Eucheuma cottonii*.

Component	Composition % (w/w)
Carbohydrate	35.2
Protein	2.2
Lipid	3.7
Ash	26.1
Moisture	21.7
Others	11.1

flame ionization detector (FID) (Hewlett Packard, Palo Alto, CA). The column used was 2.0 m in length and 0.2 cm ID, 80/120 mesh Carbopack B-DA/4% Carbowax 20M (Supelco, USA). The operating condition was as follows: detector temperature of 225 °C; injector temperature of 225 °C; oven temperature was programmed to increase from 100 (2.0 min) to 175 °C at 10°CC min^{-1} For each GC analysis, 2 μL of sample was injected. Helium was used as the carrier gas while 0.5% (v/v) 2-pentanone was used as internal standard. Bioethanol yield and percent theoretical yield were calculated based on the following equations, respectively (Keating, Robinson, Bothast, Saddler, & Mansfield, 2004):

$$Y_{P/S} = \frac{[EtOH]_{max}}{[Sugar]_{ini}} \quad (2)$$

$$Y_{\%T} = \frac{Y_{P/S}}{0.51} \times 100\% \quad (3)$$

where $Y_{P/S}$ = ethanol yield (g/g), $[EtOH]_{max}$ = highest ethanol concentration achieved during fermentation (g/L), $[Sugar]_{ini}$ = total initial sugar concentration at onset of fermentation (g/L), $Y_{\%T}$ = percent theoretical yield (%), 0.51 is the maximum ethanol yield per unit of hexose sugar from glycolytic fermentation (g/g), P = product and S = substrate.

At least three samples were used in all analytical determinations, and data are presented as the mean of three replicates.

3. Results and discussion

3.1. Raw material composition

Table 2 shows the composition of *E. cottonii* used in this study. The carbohydrates content in the biomass is 35.2% (w/w), which consists of 25% (w/w) galactose and 10.2% (w/w) glucose. The remaining components are protein, lipid, ash, moisture, and other, which occurred to be 2.2%, 3.7%, 26.1%, 21.7% and 11.1%, respectively.

3.2. Hydrolysis of *E. cottonii*

3.2.1. Effect of the catalyst loading

The effect of catalyst loading on hydrolysis of *E. cottonii* extract is shown in Fig. 1. It can be seen that the yield of galactose generally increases with time for all catalyst loading. This result indicated that Amberlyst (TM)-15 has the potential to hydrolyze galactans extracted from *E. cottonii* to galactose. The type of galactans in *E. cottonii* are mainly κ-carrageenans that can be extracted using hot water (Estevez, Ciancia, & Cerezo, 2004). The structure of κ-carrageenans was reported as alternating 3-linked β-D-galactose 4-sulfate and 4-linked 3,6-anhydro-α-D-galactose units connected through glycosidic linkages (Estevez, Ciancia, & Cerezo, 2000) which contains the basic unit of D-typed galactose that can be fermented by yeast (Meinita et al., 2011). Fig. 1 also shows that higher loading of Amberlyst (TM)-15 accelerates the hydrolysis rate of macroalgae extract, which can be explained due to the increase in the total number of available active catalytic sites for the reaction (Rinaldi, Meine, vomStein, Palkovits, & Schüth, 2010). The highest

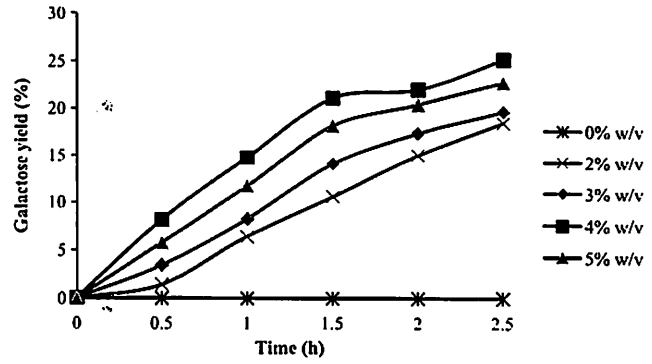

Fig. 1. Effects of catalyst concentrations on the hydrolysis of seaweed extract in the presence of a heterogeneous catalyst Amberlyst (TM)-15. (Conditions: s/l ratio: 5.5%, reaction temperature 110 °C, reaction time: 0–2.5 h.)

galactose yield of 25.2% was attained at a catalyst loading of 4% (w/v). However, the galactose yield decreased when the amount of catalyst loading was increased beyond 4% (w/v). The reaction produces a negative sugar output rate as degradation is evident. This is because higher acid concentration might degrade the sugar compounds and produce more by-product inhibitors such as 5-hydroxy-methyl-furfural and organics acids (Park et al., 2011). In order to further elucidate on the role of catalyst, hydrolysis was carried out without catalyst, whereby no reducing sugar was detected even after 2.5 h of reaction (Fig. 1). The results indicated that catalyst plays a crucial role in hydrolyzing the galactans to galactose. The mechanism which is widely accepted for the acid catalyzed hydrolysis of carrageenan is based on the protonation of the glycosidic oxygen. High temperature and acidic medium are reported to promote hydrolysis of the acid-sensitive (1 → 3) glycosidic linkages (Khambhaty et al., 2012). 4% (w/v) of Amberlyst (TM)-15 was chosen as the optimal catalyst loading.

3.2.2. Effect of the reaction temperature

The effect of reaction temperature was also investigated in this study. According to Fig. 2, an increase in temperature from 110 to 120 °C resulted in a sharp increase in the formation of galactose. This shows that temperature has a significant effect on the hydrolysis yield of macroalgae extract. Higher temperature was also reported to result in improved hydrolysis rate relative to low temperature (Meinita, Hong, & Jeong, 2012). After 1.5 h at 120 °C, the highest galactose yield of 27.7% was attained. However, beyond 1.5 h, there is a slight drop in the yield of galactose. This is probably caused by conversion of galactose to other chemicals such as HMF or levulinic acid (Jeong & Park, 2010). For the hydrolysis conducted

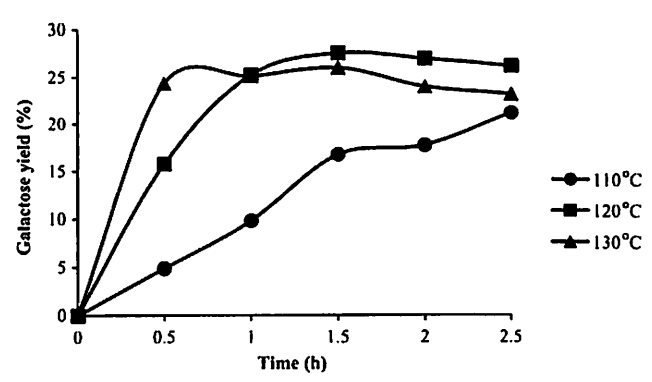

Fig. 2. Effect of reaction temperature on the hydrolysis of seaweed extract in the presence of a heterogeneous catalyst, Amberlyst (TM)-15. (Conditions: s/l ratio: 5.5%, catalyst loading: 4%, w/v, reaction time: 0–2.5 h.)

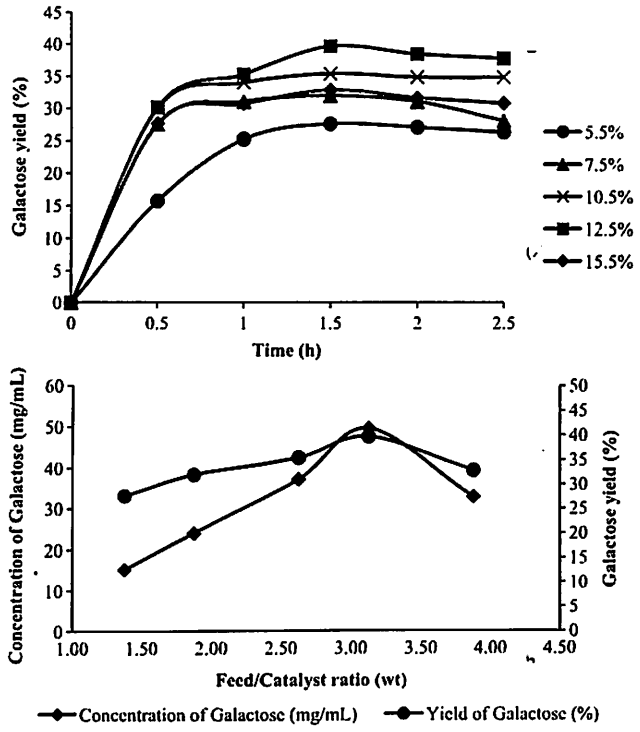

Fig. 3. Effect of biomass concentration on the hydrolysis of seaweed extract in the presence of a heterogeneous catalyst, Amberlyst (TM)-15. (Conditions: reaction temperature: 120 °C, catalyst loading: 4%, w/v, reaction time: 0–2.5 h). (A) Solid/liquid ratio (5.5–15.5%). (B) Concentration (mg/mL) and yield (%) of galactose after 1.5 h.

at 130 °C, it can be seen that initially, at 0.5 h, the galactose yield obtained surpassed the yield obtained at 110 °C and 120 °C. However after 0.5 h, instead of increasing, the galactose yield was found to stabilize at 25% with a slight drop, then after. This result indicates that extended reaction time or high reaction temperature can cause a negative effect on the galactose yield due to degradation of sugars in hydrolysis reactions and formation of undesirable by-product (Harun & Danquah, 2011b). In addition, Amberlyst (TM)-15 may also be deactivated at higher hydrolysis temperature due to low thermal stability of ion-exchange catalyst. Furthermore, high temperature could also cause the reduction of catalyst surface area as well as the number of H^+ active site (Morales, van Grieken, Martín, & Martínez, 2010). Therefore, 120 °C was chosen as the optimum hydrolysis temperature for this study.

3.2.3. Effect of the biomass concentration

Fig. 3(A) shows the effect of different biomass concentrations on the formation of galactose. When hydrolysis was conducted at 120 °C for 2.5 h, the galactose yield was found to increase gradually with an increase in biomass concentration up to 12.5% (w/v); beyond that loading, there was reduction in galactose yield. This result is expected because, at fixed catalyst loading, increasing biomass concentration resulted in more carbohydrate available for hydrolysis. In order to facilitate a more meaningful result, the data in Fig. 3(A) was used to plot the correlations between the concentration and yield of galactose to the weight ratio of biomass to catalyst as shown in Fig. 3(B). This will allow the analysis on the effective biomass to catalyst ratio so that suitable amount of biomass can be used for fixed amount of catalyst. Note that biomass concentration of 5.5%, 7.5%, 10.5%, 12.5% and 15.5% correspond to 1.38, 1.88, 2.63, 3.13 and 3.88 biomass/catalyst ratio, respectively. At 1.5 h, the highest galactose yield of 39.7% was obtained at 3.13 g biomass/g catalyst ratio and the lowest yield of 27.7% was obtained at 1.38 g biomass/g catalyst ratio. The results can be explained by the total amount of carbohydrates present in the biomass. Higher biomass loading constituted higher carbohydrates content with more fermentable sugars that can be hydrolyzed by the catalyst Amberlyst (TM)-15 (Harun & Danquah, 2011a). One interesting observation can be seen from Fig. 3(B). The gradient for the concentration of galactose in the reaction mixture curve is much steeper than the galactose yield. This indicates that with an increase in biomass loading/biomass to catalyst ratio, the increase in galactose yield is not as fast as the increase in the concentration of galactose in the reaction mixture. This result reveals that there may be a retention of sugars in the biomass due to higher hydrolysate viscosity (Miranda, Passarinho, & Gouveia, 2012). From these results, it could be concluded that the optimum conditions for hydrolysis of *E. cottonii* extract were: 4% (w/v) of Amberlyst (TM)-15, 12.5% (w/v) of biomass concentration, reaction temperature at 120 °C and reaction time of 1.5 h.

3.3. Comparison with other hydrolysis methods

The effectiveness of Amberlyst (TM)-15 (a type of solid catalyst) was compared to a more common type of catalyst, 0.2 M H_2SO_4 (liquid catalyst) in the hydrolysis of *E. cottonii*. The results of the comparison as shown in Table 3 clearly indicate the superiority of Amberlyst (TM)-15 as compared to H_2SO_4. For *E. cottonii* with extract, the yield of galactose obtained using Amberlyst (39.7%) was slightly higher than those using H_2SO_4 (34.6%). Recently, Khambhaty et al. (2012) have reported the production of bioethanol from *E. cottonii* extracts, in which the hydrolysis process was catalyzed by 0.9 N sulfuric acid. The maximum reducing sugar yield was merely 26.2% (Table 3). This comparison shows that Amberlyst 15 is a far more superior catalyst than sulfuric acid and has the same activity as sulfuric acid. The acid resins were merely an acidifier of the aqueous slurries, having a similar effect as an aqueous solution of sulfuric acid (Rinaldi, Palkovits, & Schüth, 2008). In addition, Amberlyst (TM)-15 is more environmental friendly because it can be easily separated from the products after hydrolysis process and can be recycle use.

One additional experiment was conducted using the optimum condition obtained for *E. cottonii* extract but using the un-treated macroalgae (fresh seaweed) and the results are shown in Table 3. Based on the result attained in Table 3, the formation of reducing sugars obtained from fresh *E. cottonii* (24.4%) was relatively lower than that of *E. cottonii* extract (39.7%). This is because pre-extraction of carrageenans from macroalgae can remove impurities that can hinder the hydrolysis of galactans, thus facilitate hydrolysis process and subsequently increase the sugar concentration. In addition to that, fresh *E. cottonii* was also subjected to H_2SO_4 and as expected, a lower yield of 20.6% was obtained as shown in Table 3. All the results show the superiority of Amberlyst (TM)-15 for the hydrolysis of *E. cottonii* extract.

3.4. Neutralization

The hydrolysate obtained after hydrolysis of *E. cottonii* extract needs to be neutralized before fermentation (Canilha, de Almeida e Silva, & Solenzal, 2004). During the hydrolysis reaction, it does not only de-polymerize the polysaccharide, but it also hydrolyzes the sulfate group attached to the galactans. Thus, the concentration of total dissolved solids in the hydrolysate before neutralization is as high as 69,000 mg/L. In this study, the effectiveness of two types of neutralization agent was tested; Amberlyst (TM)-A21 and lime (Table 4). After neutralization, concentration of total dissolved solids with Amberlyst (TM)-A21 and lime were 3280 mg/L and 7320 mg/L, respectively. Although the sulfate group could potentially be neutralized by the addition of lime, however it resulted

Table 3
Comparison with other hydrolysis methods of *Eucheuma cottonii*.

Raw material	Reaction conditions					Yield of carbohydrates (%)	References
	Temperature (°C)	Type of catalyst	Catalyst loading	Biomass loading	Reaction time (h)		
Heterogeneous acid catalyst							
Seaweed extract	120	Amberlyst (TM)-15	4% (w/v)	12.5% (w/v)	1.5	39.7	This work
Fresh seaweed	120	Amberlyst (TM)-15	4% (w/v)	12.5% (w/v)	1.5	24.4	This work
Homogeneous acid catalyst							
Seaweed extract	120	H_2SO_4	0.2 M	12.5%	1.5	34.6	This work
Seaweed extract	100	H_2SO_4	0.9 N	5.0%	5 cycles (1 cycle/h)	26.2	Khambhaty et al. (2012)
Fresh seaweed	120	H_2SO_4	0.2 M	12.5%	1.5	20.6	This work

Table 4
Data on neutralization of acidic hydrolysate with Amberlyst-A21 and lime.

Sample	TDS (ppm)	Conductivity (mS)	pH	Sugar (mg/mL)	Color of solution
Hydrolysate without treatment	69,000	137.9	0.80	19.0	Deep yellow
Hydrolysate after treated with Amberlyst (TM)-A21	3280	6.54	6.40	15.0	Yellowish
Hydrolysate after treated with lime	7320	14.67	6.70	18.0	Brown

to the formation of inorganic salt. High concentration of salt in the hydrolysate can hamper fermentation process (Khambhaty et al., 2012). Thus an additional step is required to reduce the level of dissolved salts before fermentation. Instead, when using Amberlyst (TM)-A21 (a weak basic ion-exchange resins), the sulfate group is removed while leaving the sugar concentration relatively unaffected. The role of anion-exchange resins is to substitute the sulfonic anions with hydroxyl ions that can lead toward neutralization. In addition, treatment of the acidic hydrolysate with ion-exchange resin can be one of the most efficient methods for removing inhibiting compounds prior to fermentation (Nilvebrant, Reimann, Larsson, & Jönsson, 2001).

3.5. Fermentation

The utilization rate of fermentable sugar toward bioethanol concentration was monitored during the fermentation process in order to understand the relationship between both variables. As shown in Fig. 4, there were significant differences in the fermentation performance with different inoculum levels. Bioethanol production rate increases with increase in inoculum levels up to certain level and the bioethanol production rate decreases beyond that level. The lowest yeast inoculum levels of 8 mg/mL resulted in very low consumption of sugar resulted with a long lag phase which produces lower bioethanol concentration of 3.48 mg/mL after 144 h. Higher bioethanol production was obtained with an inoculums amount of 16.0 mg/mL. The bioethanol production rate in the early phase of the culture was relatively slow but increased after 24 h and reached a maximum after 72 h of fermentation. The increase in inoculum levels increases bioethanol yield (65%) due to better utilization of sugars and this results in extracellular bioethanol production (Nurgel, Erten, Canbaş, Cabaroğlu, & Selli, 2002). However, after a certain point level, further increase in inoculum levels did not improve the bioethanol production (Fig. 4(C)). The high amount of inoculums can adversely affect bioethanol production due to the fact that high inoculums level decreases the viability of yeast population (Arifa Tahir, 2010). A decrease in bioethanol production was observed after exponential phase due to the depletion of galactose in the fermentation broth. As a result, the produced bioethanol acts as a carbon source and inevitably being consumed by yeast in the same manner as the original simple sugar, thus decreasing the bioethanol concentration during fermentation (Harun & Danquah, 2011b).

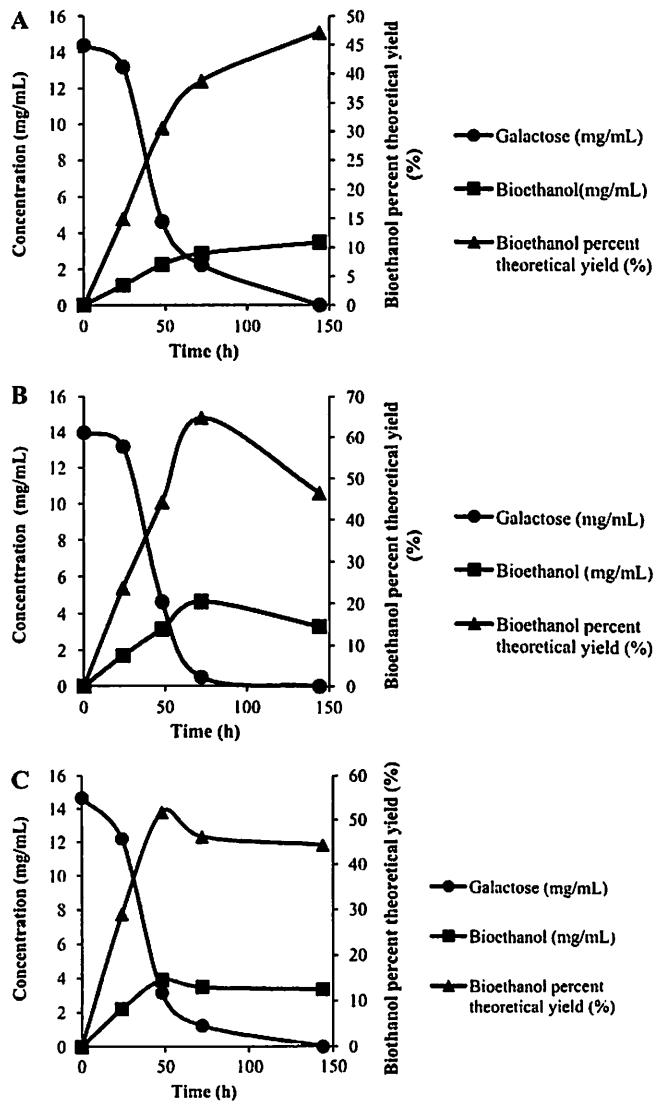

Fig. 4. The effect of different *Saccharomyces cerevisiae* inoculums amounts on bioethanol production. (A) 8 mg/mL (B) 16 mg/mL (C) 24 mg/mL.

4. Conclusion

From the results, it could be concluded that *E. cottonii* could be a potential feedstock for bioethanol production. Heterogeneous catalyst Amberlyst (TM)-15 can act efficiently as solid acid catalyst in the hydrolysis of sugar. This simple but effective method could facilitate an energy-efficient and cost-effective conversion of macroalgae biomass into bioethanol.

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Enzymatic hydrolysis and fermentation of seaweed solid wastes for bioethanol production: An optimization study

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ABSTRACT

Studies on bioethanol produced from a variety of non-edible feedstocks have recently gained considerable attention because such production can avoid the conflict between food and fuel. In this study, an attempt was made to investigate the production of bioethanol using low-cost feedstock, namely, seaweed solid wastes obtained after the extraction of κ-carrageenan. The utilisation of seaweed solid wastes will simultaneously help to overcome its disposal problem. Two different processes were used: the SHF (separate hydrolysis and fermentation) process and the SSF (simultaneous saccharification and fermentation) process. For the SHF process, enzymatic hydrolysis was conducted by varying three process variables, substrate concentration, pH and temperature, but a constant enzyme dosage was maintained. The highest glucose yield of 99.8% was obtained at pH 4.8, a temperature of 50 °C and a substrate concentration of 2% (w/v) seaweed solid wastes. With subsequent fermentation, a bioethanol yield of 55.9% was obtained. In contrast, for the SSF process, a yield of 90.9% bioethanol was obtained. From these results, it was determined that the SSF of seaweed solid wastes with *Saccharomyces cerevisiae* has several advantages over SHF because the former is a simple one-step procedure that can save time, cost and energy consumption while achieving a high yield of bioethanol.

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1. Introduction

In recent years, the occurrence of global warming and an ever-increasing demand for liquid fuels has positioned bioethanol derived from renewable resources as a potential replacement for gasoline [1–4]. First-generation bioethanol that is made from edible crops has raised many societal issues, including competition with food sources and disruption in the food-to-population ratio [5]. To overcome these issues, the focus on bioethanol has shifted to the development of second-generation bioethanol that is derived from lignocellulosic wastes. However, the delignification of lignocellulosic biomass is still a barrier that must be overcome before the commercialisation of second-generation bioethanol can become a reality [6–11], and thus a more sustainable feedstock should be developed to overcome these barriers. Therefore, third-generation bioethanol that is derived from seaweed or macroalgae is considered to be a more viable feedstock that does not have the major drawbacks associated with first- and second-generation bioethanol [12–16].

One typical example of macroalgae that can be found abundantly in Malaysia and South East Asia is *Eucheuma cottonii* (*Kappaphycus alvarezii*). *E. cottonii* is a type of red seaweed and the major commercial source of κ-carrageenan. The κ-carrageenan extracted from this seaweed is an important ingredient used in food, health and personal care products [17]. Due to the extract's wide range of applications, the carrageenan industry has grown rapidly, and Bixler & Porse [18] reported that carrageenan production reached 50,000 tons in 2009, with a total value of US$ 527 million.

Many countries in the world, including Malaysia, have great interest in *E. cottonii*, which has been largely cultivated on the east coast of Sabah (Malaysia) [19]. Because the content of κ-carrageenan in seaweed only varies from 25 to 35% (on a dry weight basis), during the extraction process, an enormous amount of waste products is left behind, which is usually referred to as seaweed solid wastes. At the moment, only a small portion of seaweed solid wastes are utilised as organic fertiliser, whereas most are discarded as waste, eventually causing environmental pollution. Seaweed solid wastes are mainly composed of cellulosic materials with a low lignin content, which can be a potential third-generation feedstock for bioethanol production. Thus, the utilisation of seaweed solid wastes for bioethanol production is an innovative step toward minimising seaweed waste disposal and adding value to seaweed waste biomass [20]. Such

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production can also lead to the development of a seaweed-based biorefinery for simultaneous carrageenan and bioethanol production that can enhance the sustainability of the seaweed industry. The utilization of a low-value byproduct for bioethanol production will pave the way towards the development of an economical biorefinery with zero-waste production.

Although there have been many studies reported in the literature on the production of third-generation bioethanol, but most of these studies focussed on raw seaweed as feedstock using mainly physicochemical method [12,14,21], which is commonly used for the saccharification of terrestrial biomass. Dilute acid hydrolysis is a typical physicochemical method used to treat raw macroalgae biomass with 0.3–0.9 N sulphuric acid at 100–140 °C [12,14]. Nevertheless, it was widely reported that homogeneous acid hydrolysis faces several limitations such as formation of toxic hydrolysates which will inhibit microbial fermentation. Thus there is a need of a detoxification process which is still scarcely reported in the literature. Apart from the environmental and economic merits of macroalgae, many challenges still exist. One of the challenges is that macroalgae have unique carbohydrates composition. Besides agar, starch and cellulose, it may also contain mannitol and laminarin which are distinctively different from terrestrial biomass. Due to this difference, methods used for terrestrial based-biomass cannot be directly applied to macroalgae biomass and the selection of appropriate microorganisms are pivotal for successful bioethanol fermentation. Kim et al. [21], reported the conversion of raw macroalgae (*Laminaria japonica*) to bioethanol via dilute-acid pre-treatment followed by simultaneous saccharification and fermentation with *Saccharomyces cerevisiae*. It was reported that merely 7.0–9.8 g/l bioethanol was produced. The low bioethanol yield obtained was because *S. cerevisiae* could only consume glucose but not mannitol, which was found to be 81% of the total sugars in the hydrolysates.

As the interest in utilizing macroalgae for bioethanol production are growing rapidly, there is a need for intense research for efficient utilization of this biomass. Currently, the use of macroalgae to produce fuel bioethanol faces significant technical and economic challenges and its success depends on the development of a highly effective enzyme systems and efficient microorganisms to convert simple sugars to bioethanol. One possible method for converting macroalgae to bioethanol is via enzymatic hydrolysis followed by fermentation of the simple sugar produced. Enzymatic hydrolysis is a more effective approach for producing sugars from cellulosic biomass because of higher conversion yield and the production of lower amount of toxic hydrolysates compared to acid hydrolysis process [22]. The enzymatic hydrolysis of seaweed solid wastes can be carried out by using a mixture of cellulase and β-glucosidase. Unlike terrestrial lignocellulosic biomass that contains lignin, the production of bioethanol from seaweed solid wastes is a straightforward process that does not require pretreatment. This method has the advantage of not requiring neutralization step or water treatment and therefore minimizing the operational costs.

SSF (simultaneous saccharification and fermentation), which combines enzymatic hydrolysis and fermentation into a single step, is another alternative for the production of bioethanol from cellulosic materials. SSF is usually preferred over the SHF (separate hydrolysis and fermentation) process because the inhibition of cellulase can be reduced and the rapid conversion of glucose into bioethanol by yeast results in a faster production rate and lower capital costs [23,24]. To the best of the author's knowledge, the use of SSF technology for producing bioethanol using seaweed solid wastes from *E. cottonii* has not been reported in the literature. Apart from the technical aspects of bioethanol production from macroalgae, this study will also look into the production cost. Cost estimates for cellulosic ethanol production have been widely reported by many researchers in order to evaluate the economic viability of cellulosic ethanol production [25–27]. However, whether bioethanol produced from seaweed solid wastes will be competitive with gasoline price is still questionable as no data has been reported in the literature.

Therefore, the objective of this study is to produce bioethanol from seaweed solid wastes using two different technologies: SHF (separate hydrolysis and fermentation) and SSF (simultaneous saccharification and fermentation). For the SHF process, the effects of substrate concentration, pH and temperature on the enzymatic hydrolysis of seaweed solid wastes was studied. Besides that, these laboratory-derived primary data have been used to estimate the production cost of bioethanol from seaweed solid wastes.

2. Material and methods

2.1. Raw materials and chemicals

E. cottonii was purchased from Futt Put Enterprise (north coast of Sabah, Borneo). Initially, 80 g of *E. cottonii* was washed with tap water to remove sand and salt. The samples were then soaked in 1.6 L distilled water for 30 min. They were then blended and boiled at 90 °C with mechanical stirring for 1 h until the algae disintegrated. Subsequently, the carrageenan extract was filtered with a pressure pump. The extract residue was then washed with water and dried at 40 °C in a drying oven until a constant weight was recorded. The raw material, seaweed solid wastes, was milled and screened through 20–80 mesh sieves and stored in a desiccator at room temperature until further use.

The enzymes used for hydrolysis were Novozyme 188 (263 CBU/g) and Celluclast 1.5 L (798 EGU/g) (Novozyme, Denmark), which were obtained from Science Technics Sdn Bhd. Calcium hydroxide, standard D-glucose, *S. cerevisiae* (YSC2, type II), yeast extract, peptone and dextrose were purchased from Sigma–Aldrich (USA). Acetic acid, sodium acetate, sulphuric acid, phosphoric acid and ethanol were purchased from Fisher Scientific (UK). All reagents used were of analytical grade. Table 1 shows the properties of the enzymes used in this study.

All experiments were performed in triplicate. However, for clarity, only the average values are reported.

2.2. Cell cultivation

S. cerevisiae was used to ferment the sugars formed from the hydrolysis process into bioethanol. Yeast was precultured overnight in 50 mL of YEPD (yeasts extract peptone dextrose) medium (1.0% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) in a 250-mL Erlenmeyer flask at pH 5.0. The medium was then autoclaved for 20 min at 121 °C before being used [28]. The preculture was incubated at 35 °C with shaking at 130 rpm for 24 h in a shaking incubator (Benchmark Scientific Inc., New Jersey). Yeast cells were then harvested by centrifugation at 10,000 × *g* for 2 min and washed with phosphoric acid (1% v/v). The washing and centrifugation steps were repeated three times to remove the residual sugars in the medium.

Table 1
Characteristics of enzymes.

Enzymes	Enzyme activity	Optimal pH	Optimal temperature °C
Cellulase	82.08 FPU/mL[a]	4.5–5.0	55–65
β-glucosidase	326.12 CBU/mL[b]	4.5–5.0	55–65

[a] One international filter paper unit (FPU) was defined as the amount of enzyme that releases 1 μmol glucose per minute during hydrolysis reaction.
[b] One unit of β-glucosidase activity was defined as the amount of enzyme converting 1 μmol of cellobiose to produce 2 μmol of glucose in 1 min.

2.3. SSF (separate hydrolysis and fermentation)

2.3.1. Enzymatic hydrolysis of seaweed solid wastes

Hydrolysis of the seaweed solid wastes to glucose was performed in a 50 mM citrate buffer with a cellulase (celluclast 1.5 L, Sigma Aldrich) loading of 15 FPU/g substrate. Fungal β-glucosidase (Novozyme 188, Denmark) was used to supplement the β-glucosidase activity with an enzyme loading of 52 CBU/g substrate for 72 h in a water-bath shaker operated at an agitation speed of 150 rpm. The biomass was incubated with the enzyme solution at different substrate concentrations (0.4–2.8% w/v), pH values (2.8–7.8) and temperatures (30–60 °C). Each hydrolysis process was conducted at a working volume of 50 mL in a 100-mL shake flask. The flasks were sealed with rubber stoppers equipped with needles for CO_2 venting. At selected time intervals, a 1.5-mL sample was withdrawn and immediately heated for 5 min in a boiling water bath to terminate the enzymatic reaction. The hydrolysis mixture was centrifuged at 12,000 × *g* for 5 min to remove solid matter. The supernatants were preserved at −2 °C and submitted to reducing sugar analysis to determine the percentage of hydrolysis by HP-LC (high-performance liquid chromatography).

2.3.2. Fermentation

After the enzymatic hydrolysis (optimum condition from Section 2.3.1), yeast was added to the enzymatic hydrolysate, which was concentrated by rotary evaporation at pH 5. The mixture was then incubated in a shaking incubator at 35 °C at a shaking speed of 130 rpm for 6 h. Three-hundred-microlitre samples were withdrawn at 2-h intervals and centrifuged at 10,000 × *g* for 15 min at 5 °C. The supernatant obtained after centrifugation was analysed for ethanol and residual glucose.

2.4. Prehydrolysis and SSF (simultaneous saccharification and fermentation)

Seaweed solid wastes were prehydrolysed for 6 h in citrate buffer (50 mM, pH 4.8) at 50 °C using a 250-mL Erlenmeyer flask (Fig. A1) with a working volume of 50 mL. The enzyme loading used was exactly the same as that used in the SHF process (Fig. A2). The temperature was subsequently reduced to 37–46 °C for a further 3 h to allow for SSF after yeast inoculation. The experiments were performed in a shaking incubator (Benchmark Scientific Inc., New Jersey) at 130 rpm. Three-hundred-microlitre samples were withdrawn at 0.5-h intervals and centrifuged at 10,000 × *g* for 15 min at 5 °C. The supernatant was stored in a freezer at −2 °C and submitted to sugar and bioethanol analysis. Apart from that, bioethanol production was also scaled-up to a 2 L fermenter (Labfors®, Infors AG, Switzerland) using the optimized conditions identified from shake flask experimentations. 1.5 mL of sample was withdrawn at different interval time during SSF and was centrifuged at 10,000 × *g* for 15 min. The supernatant was stored in −2 °C freezer and subjected for sugar and bioethanol analysis.

2.5. Chemical compositional analysis of seaweed solid wastes

The cellulose, lignin and hemicellulosic fractions of pulverised seaweed solid wastes were determined by using a two-step acid hydrolysis procedure, a modified method that is based on analytical methods developed at the National Renewable Energy Laboratory (NREL, Golden, CO) [29]. The sample was initially subjected to a primary 72% sulphuric acid hydrolysis, followed by a secondary dilute-acid hydrolysis. The content of sugar was quantified by HP-LC (high-performance liquid chromatography).

2.6. Analytical methods

2.6.1. Determination of sugar concentration

The hydrolysates were diluted 50 times with deionised water and filtered with a 0.20-μm syringe filter (Nylon membrane, Fisher Scientific). The concentration of glucose in the filtrate was analysed by using an Agilent Series 1200 Infinity HP-LC (high-performance liquid chromatography) system equipped with a 385-ELSD (evaporative light-scattering detector) and a 300 mm × 7.7 mm Hi-Plex Ca column. The mobile phase used was deionised water at a flow rate of 0.6 mL min^{-1} and an injection volume of 20 μL. Purified nitrogen was used as a nebuliser gas at a pressure of 70 psi for the ELSD detector. The spray chamber temperature was set to 40 °C, whereas the ELSD detector temperature was set to 80 °C. The sugar concentration was evaluated using a calibration curve generated from HPLC-grade sugar. The glucose yields were calculated as follows:

$$\text{Yield}\% = \frac{\text{Concentration}(g/l) \text{ of sugar at time of } t}{\text{Initial concentration}(g/l) \text{ of substrate}} \times 100\% \quad (1)$$

2.6.2. Determination of bioethanol concentration

Bioethanol concentrations were determined by GC (gas chromatography) using a 5890 Series II chromatograph equipped with a FID (flame ionisation detector) (Hewlett Packard, Palo Alto, CA). The column used (80/120 mesh Carbopack B-DA/4% Carbowax 20M, Supelco, USA) measured 2.0 m in length and 0.2 cm in I.D. The operational temperature at the injection port and detector was 225 °C. The initial temperature of the oven was set to 100 °C for 2 min, then increased to 175 °C at a rate of 10 °C min^{-1} and maintained at that temperature for 9.5 min. For each GC analysis, 2 μL of sample was injected. Helium was used as the carrier gas, and 0.5% (v/v) 2-pentanone was used as an internal standard. Bioethanol yield and percent theoretical yield were calculated based on the following equations, respectively:

$$Y_{P/S} = \frac{[\text{EtOH}]_{max}}{[\text{Sugar}]_{ini}} \quad (2)$$

$$Y_{\%T} = \frac{Y_{P/S}}{0.51} \times 100\% \quad (3)$$

where $Y_{P/S}$ = ethanol yield (g/g), $[\text{EtOH}]_{max}$ = highest ethanol concentration achieved during fermentation (g/L), $[\text{Sugar}]_{ini}$ = total initial sugar concentration at onset of fermentation (g/L), $Y_{\%T}$ = percent theoretical yield (%), 0.51 is the maximum ethanol yield per unit of hexose sugar from glycolytic fermentation (g/g), P = Product and S = Substrate.

2.7. Scanning electron microscopy analysis

The structures of untreated and treated enzyme hydrolysed seaweed solid waste samples were observed by SEM (scanning electron microscopy). Dried samples were placed on an aluminium stub. Images were obtained using a Fei Quanta 450 FEG (Eindhoven, NL).

2.8. Estimation of bioethanol production cost from seaweed solid wastes in Sabah, Malaysia

The processing cost of bioethanol from seaweed solid wastes (with 2009 as the reference year) can be calculated based on the estimated feedstock cost as well as capital cost, operating and maintenance costs. Thus, the unit cost of bioethanol production can be expressed using the following equation;

$$C_{Et} = C_F + C_{O\&M} + C_I \quad (4)$$

where C_{Et} is the bioethanol production cost (RM/l); C_F is feedstock cost (RM/l); $C_{O\&M}$ is operating and maintenance cost (RM/l); and C_I is investment cost (RM/l).

2.8.1. Estimation of seaweed solid wastes feedstock costs

Feedstock cost includes the cost for transporting seaweed solid waste by truck to the assumed bioethanol plant which the distance of transportation should be less than 100 km in radius from the collection area. The transportation cost was estimated to be RM (Ringgit Malaysia) 0.20/tonne km [49].

2.8.2. Operating cost

The operating cost includes variable and fixed operating cost. Variable cost includes all consumable materials for production such as raw material costs, electricity, machine repair and maintenances, administration, etc., were estimated and adjusted to the prices in the base year 2009. The operating cost per litre of the bioethanol can be calculated by dividing the total annual operating costs by the annual capacity of the bioethanol production plant (See details in Table A1 in Appendix A).

2.8.3. Estimation of capital investment cost

The total capital investment comprises all the initial cost of machines and equipment procurements including their installation. The installed equipment cost is estimated by scaling up or down the same equipment that was reported in the NREL process with known cost, but of a different size using the following equation [30]:

$$\text{Cost}_{\text{unknown}} = \text{Cost}_{\text{known}} \left(\frac{\text{Size}_{\text{unknown}}}{\text{Size}_{\text{known}}} \right)^{f_{\text{scale}}} \quad (5)$$

where f_{scale} is a characteristic scaling exponent adopted from Humbird et al. [31]. All major items of the investment costs are summarized and shown in Table A2 (Appendix A). The capital cost per litre of the bioethanol was then calculated by amortization, recovered over the life time of the plant. This can be obtained by a common annualized capital cost equation:

$$A = P\left[\frac{i(1+i)^n}{(1+i)^n - 1}\right] \quad (6)$$

where A is the annual payments (RM/year); P is the present worth of the first investment cost (RM); i is the annual interest rate; and n is the project life in years. The annualized capital investment cost is then divided by annual amounts of bioethanol produced to obtain the capital cost per litre of the bioethanol.

2.8.4. Assumptions for the estimation of bioethanol production cost in this study

In this study, seaweed solid wastes are assumed to be the feedstock for bioethanol production with a plant capacity of 2.8×10^5 l/day to produce anhydrous fuel grade alcohol. The plant is assumed to operate 330 days/year. The project life is assumed to be 20 years, and the interest rate of this investment is 6%/annum. Table 2 shows the yield of bioethanol from seaweed solid waste and other technical assumptions.

Table 2
Estimated annual production of bioethanol in Sabah, Malaysia and technical assumption in this study.

Feedstock	Unit	Value
Eucheuma spp. production[a]	ktonnes/year	125.00
Solid seaweed waste	ktonnes/year	81.25
SSF yield	%	90.9
Bioethanol production	ktonnes/year	73.9
Plant's capacity	L/day	283658.8
Operation days	days	330
Project life[b]	years	20
Interest rate	%/annum	6
Year for cost basis	year	2009

[a] Based on statistics reported by Sabah Dept. Fisheries (Malaysia) [53].
[b] From Rakopoulos et al., 2007 [54].

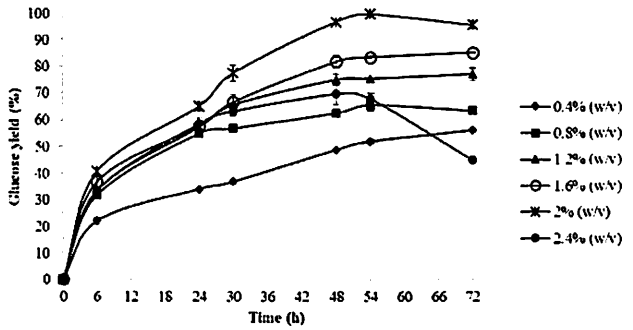

Fig. 1. Effect of substrate concentration on enzymatic hydrolysis. (Conditions: pH 4.8, temperature 50 °C.)

3. Results and discussion

3.1. Composition of seaweed solid wastes

The cellulose fraction constituted 64% of the seaweed solid wastes. The results showed that glucose was the only simple sugar detected. There was no hemicellulose or acid-insoluble lignin detected in the sample, thus making it a very promising substrate for ethanol production.

3.2. SHF (separate hydrolysis and fermentation)

3.2.1. Optimisation of enzymatic hydrolysis parameters

Fig. 1 shows the yield of glucose obtained from seaweed solid wastes subjected to enzymatic hydrolysis with durations ranging from 0 to 72 h at various substrate concentrations. The glucose yield was generally observed to increase at longer hydrolysis durations because more time is allowed for the enzyme to hydrolyse the cellulose, producing more glucose. When the substrate concentration was gradually increased from 0.4% (w/v) to 2% (w/v), the glucose yield was also observed to gradually increase. This observation was expected because a higher substrate concentration indicates the availability of more cellulose that can be hydrolysed to glucose. At 2% (w/v) substrate loading, the theoretical maximum glucose yield was achieved at 54 h, with a glucose concentration of 19.97 mg/mL. Extending the hydrolysis duration beyond 54 h caused a slight drop in the glucose yield, possibility due to the degradation of glucose and formation of undesirable by-products [32].

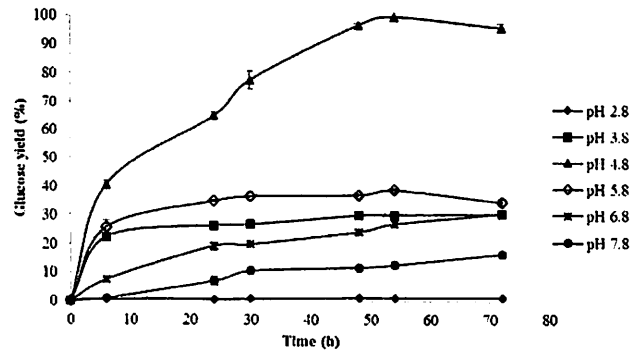

Fig. 2. Effect of pH on enzymatic hydrolysis of seaweed solid wastes. (Conditions: substrate concentration 2% (w/v), temperature 50 °C.)

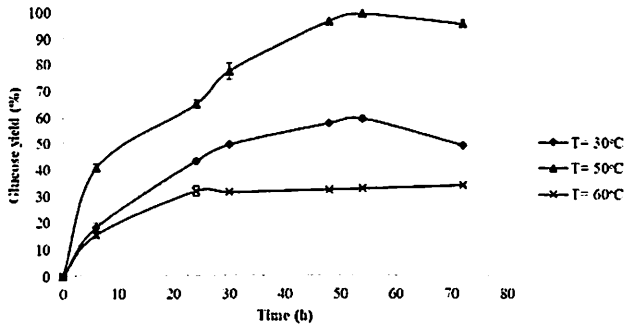

Fig. 3. Effect of temperature on the enzymatic hydrolysis. (Condition: substrate concentration 2% (w/v), pH 4.8.).

When the substrate concentration was increased from 2% (w/v) to 2.4% (w/v), there was a significant drop in the glucose yield. The detection of an optimum substrate concentration has also been reported by other researchers [32,33]. This optimum concentration occurs because when the substrate concentration is increased beyond its optimum value, the fixed amount of enzyme used then becomes a limiting factor, subsequently causing end-product inhibition and mass transfer limitations within the reaction mixture due to the high viscosity of the slurry, which leads to a low glucose yield [34].

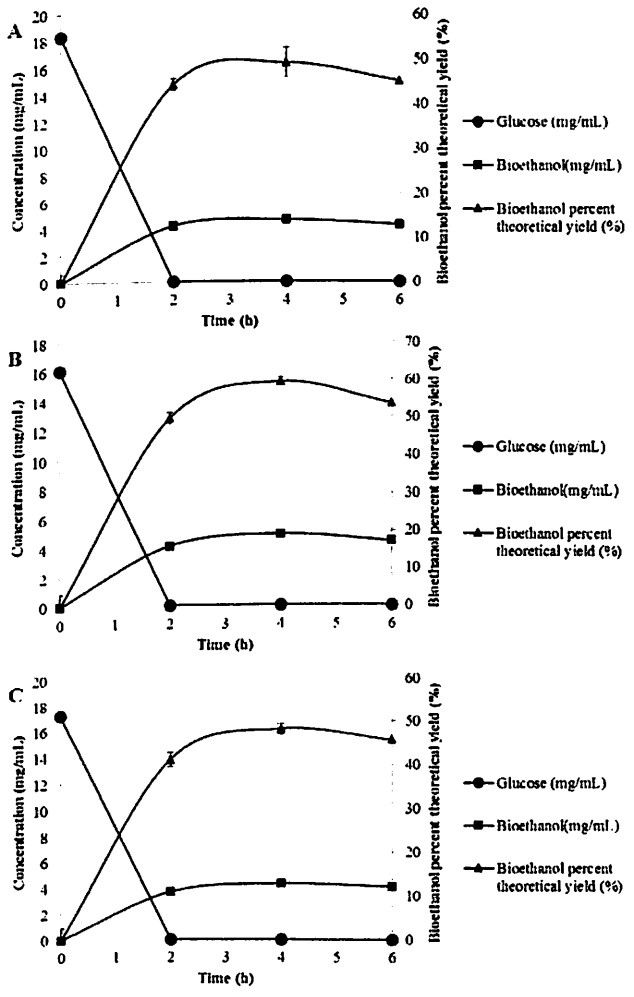

Fig. 4. The effect of different *Saccharomyces cerevisiae* inoculums amounts on bioethanol production. (A) 5.8 mg/mL (B) 17.5 mg/mL (C) 23.3 mg/mL.

The effect of pH on the hydrolysis of seaweed solid wastes was investigated, and the results are illustrated in Fig. 2. When the pH of the medium was increased from 2.8 to 3.8, the glucose yield obtained increased from 1.1% to 30.4%. When the pH of the medium was further increased to 4.8, the highest glucose yield of 99.8% was obtained after 54 h of hydrolysis. This finding is in agreement with the results reported by Ingesson et al. [34], who obtained the optimum glucose concentration at a pH ranging from 4.5 to 5.0. However, with the subsequent increase in pH, the glucose yield dropped drastically. The observation of the effect of pH on glucose yield can be explained as follows. When the pH was increased from 2.8 towards the optimum pH value for cellulase, the enzyme's activity increased and more cellulosic biomass was hydrolysed. At pH 4.8, the enzyme exerted its highest activity, resulting in the optimum glucose yield. Subsequently, when the pH was increased above its optimum value, the enzyme's activity gradually decreased. Changes in pH value affect the electrostatic bonding between the enzyme and substrate during the hydrolysis process [32]. Therefore, when enzyme charges are disrupted, the enzymes undergoes changes in its 3D structure, thus making the active site no longer suitable to catalyse the hydrolysis reactions [35].

Temperature is another variable that has a significant effect on enzyme activity. The hydrolysis of seaweed solid wastes was carried out at temperatures ranging from 30 to 60 °C. As illustrated in Fig. 3, it

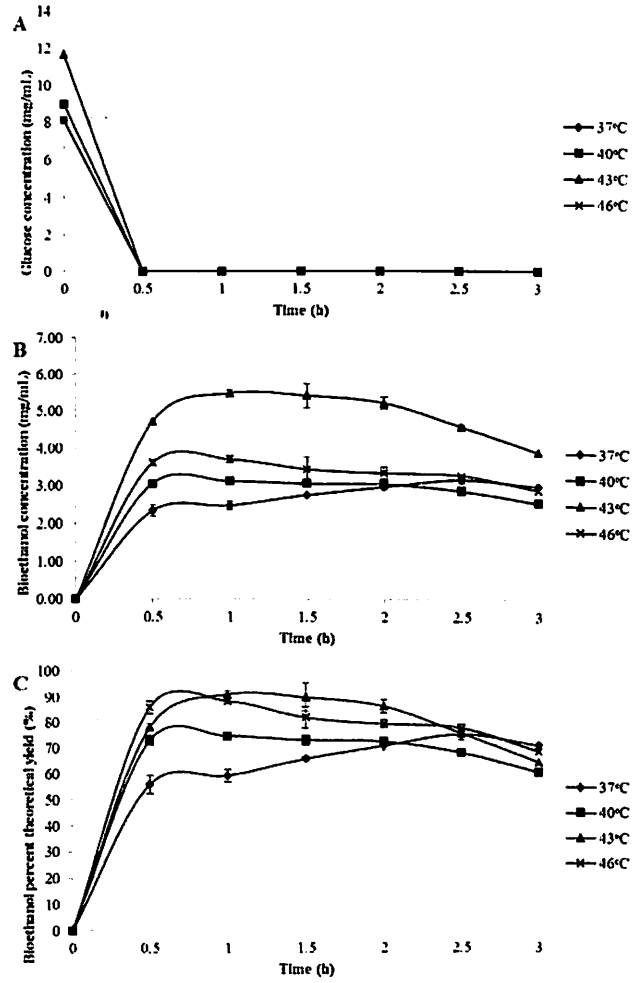

Fig. 5. Time course of bioethanol production and glucose consumption during 3 h SSF of the seaweed solid wastes at different reaction temperature. SSF: 2% (w/v) solid loading, pH 4, enzyme loading of 15 FPU/g of cellulase and 52 CBU/g β-glucosidase, and with *Saccharomyces cerevisiae*. (A) glucose concentration, (B) bioethanol concentration, and (C) bioethanol percent theoretical yield (%).

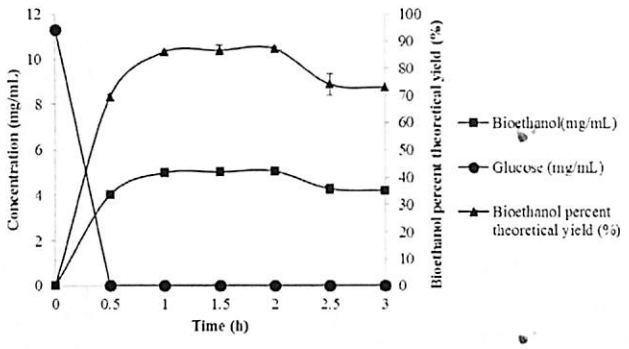

Fig. 6. SSF process in a 2 L fermenter at 43 °C, 2% (w/v) solid loading, pH 4, enzyme loading of 15 FPU/g of cellulase and 52 CBU/g β-glucosidase, and with *Saccharomyces cerevisiae*.

was observed that after 72 h of hydrolysis, the yield of glucose initially ascended to 95.9% with an increase in temperature from 30 to 50 °C and then descended to 34% with a further increase in temperature to 60 °C. The optimum enzymatic hydrolysis temperature was 50 °C, reflecting the highest glucose yield of 99.8%. This temperature has also been observed to be the optimum temperature for the enzymatic hydrolysis of different sources of cellulosic biomass [36,37]. At temperatures below 50 °C, a low glucose yield was recorded, which may have occurred because low temperature inhibits the enzyme's activity. However, when the temperature was increased to 60 °C, the glucose yield attained was much lower compared to the yields obtained at lower temperature. Increases in temperature affect the kinetic energy of enzymatic reactions, which increases the frequency of collision between a substrate and the active sites of an enzyme. Thus, thermal agitation may cause enzymes to denature, thereby reducing the availability of active sites [35].

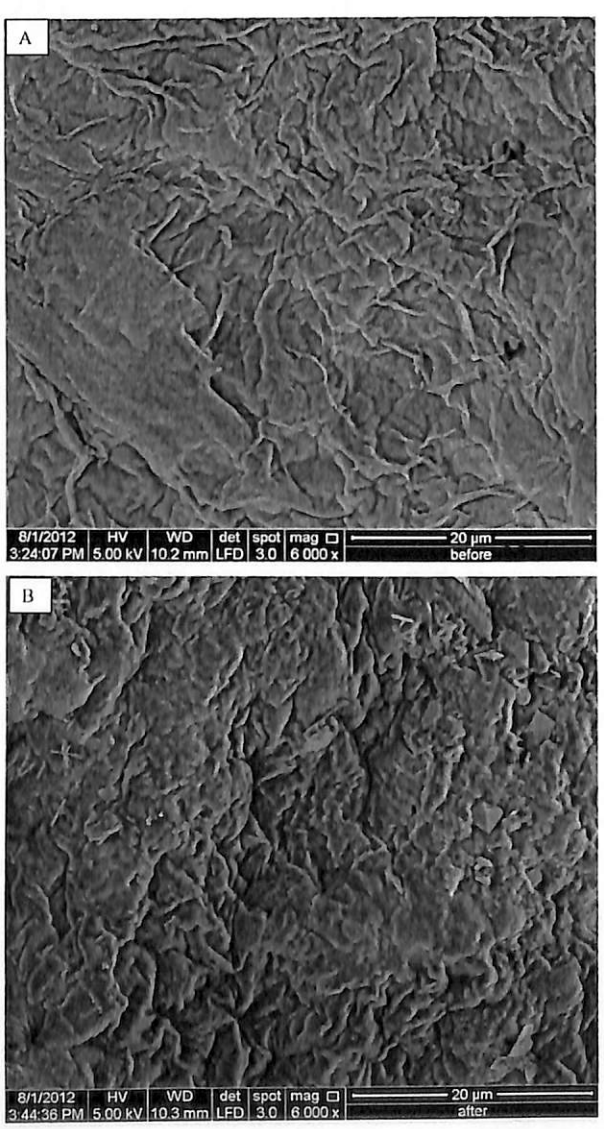

Fig. 7. (A) Scanning electron micrographs of (A) untreated, (B) treated seaweed solid wastes enzymatically hydrolysed under optimized conditions.

3.2.2. Fermentation of enzyme hydrolysate

In the present study, fermentation of the glucose obtained from the enzymatic hydrolysis of seaweed solid wastes was performed to determine the potential for this waste to be utilised for bioethanol production. The yeast inoculum size is a very important parameter in the industrial exploitation of bioethanol fermentation [38]. As shown in Fig. 4, significant differences in the fermentation performance were observed at different inoculum levels. In general, the bioethanol production rate rapidly increased and reached an optimum value after 4 h of fermentation. For *S. cerevisiae*, the most important and common carbon source is glucose. In this study, the glucose content was exhausted after 2 h, which indicates a rapid consumption of glucose by the yeast during the fermentation process. Higher bioethanol production was obtained with an inoculum concentration of 17.5 mg/mL (59%) compared to that obtained with a concentration of 5.8 mg/mL (49%). As reported in the literature, an increase in inoculum size will cause an increase in bioethanol production due to better utilisation of sugars, which results in extracellular bioethanol production [38,39]. However, the final bioethanol concentration decreased as the yeast inoculum concentration was further increased to 23.3 mg/mL (Fig. 4C). This result demonstrates that after reaching the optimum inoculum level, high inoculum concentration can adversely affect bioethanol production by reducing the viability of the yeast population [40].

3.3. SSF (simultaneous saccharification and fermentation)

The simultaneous saccharification and fermentation (SSF) of solid seaweed wastes was evaluated based on the effect of various temperatures (37–46 °C) on glucose consumption, bioethanol concentration and yield for a period of 3 h, as shown in Fig. 5. As indicated in Fig. 5A, it was observed that the glucose liberated during the early stage was rapidly fermented, and after 0.5 h, no glucose was detected in the broth. Simultaneously, the bioethanol concentration for SSF carried out at all temperatures showed a sharp increase in the first 0.5 h. However, beyond 1 h at 40–46 °C, there was a slight drop in the concentration of bioethanol (Fig. 5B). This decrease is probably caused by the loss of bioethanol due to evaporation during the SSF process [41], and bioethanol may also be consumed by yeast as a carbon source [42,43], thus explaining the decreasing trend of the bioethanol concentration. According to Ramon-Portugal et al. [44], with the accumulation of bioethanol in a medium, the microbial population can adapt itself to consume sugar and bioethanol simultaneously. However, for SSF conducted at 37 °C, the bioethanol concentration increased slightly and steadily throughout the experiment and only decreased beyond 2.5 h, probably because the low temperature was insufficient to activate the enzyme, thus resulting in a slower release of glucose. Thus, the consumption rate of glucose in the SSF process was higher than its production rate.

After 0.5 h at 37, 40 and 43 °C, the SSF bioethanol concentrations were 2.33, 3.05 and 4.70 mg/mL, respectively, whereas a bioethanol concentration of 3.60 mg/mL was obtained from SSF at 46 °C, lower

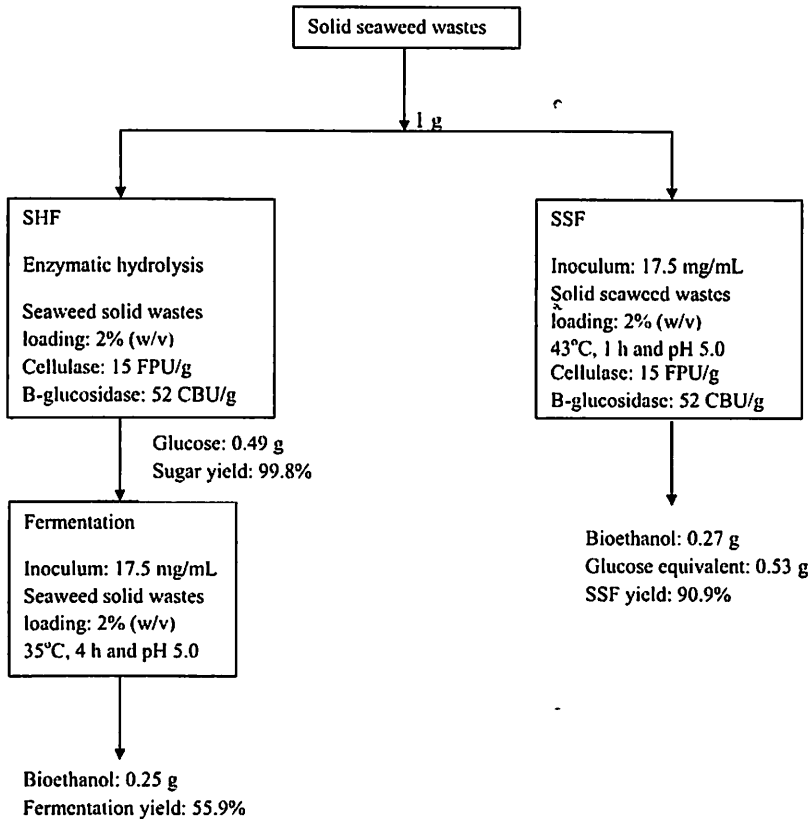

Fig. 8. Mass balance based on glucose amounts for bioethanol production by SHF and SFF.

than that obtained by SSF at 43 °C. It was observed that the concentration of bioethanol increased with temperature to an optimum value. This result is because increasing the temperature increases the reaction rate. After 1 h at 43 °C, the highest bioethanol concentration and bioethanol yield of 5.47 mg/mL and 90.9%, respectively, were achieved (Fig. 5C). The optimum temperature is usually limited by yeast cell viability; above the optimum temperature (43 °C), yeast cells can still survive but produce significantly lower bioethanol concentrations [45]. Temperature is a crucial factor for SSF because of the differences in the optimum temperature for hydrolysis (45–50 °C) and that of the yeast (35 °C) [46]. It is very important to operate SSF at a temperature close to the optimum temperature of the enzymatic hydrolysis process. In this study, operating the SSF at a temperature of 43 °C enhanced the hydrolytic enzyme activities because the temperature is close to the enzyme's optimum temperature, and simultaneously, at this temperature, the yeast cells are metabolically active. These observations are consistent with findings reported by other authors [47,48]. Therefore, it was concluded that the optimum temperature was 43 °C.

The results for SSF (simultaneous saccharification and fermentation) carried out in a 2 L fermenter is shown in Fig. 6. The conditions used for operating the 2 L fermenter is based on the optimum conditions identified using shaking flasks. The results showed that 87% bioethanol yield can be achieved, which is only slightly lower than the yield achieved in shaking flasks at 90.9%. This result indicated that the fermentation process using seaweed solid wastes could be easily scaled-up to large fermenter without compromising its performance.

Table 3
Estimation of bioethanol production cost from seaweed solid wastes in Sabah, Malaysia.

Feedstock	Seaweed solid wastes	
	RM/l	Share of total cost (%)
Feedstock cost (C_F)[a]	0.017	1.11
Net operating cost ($C_{O\&M}$)	1.255	82.29
Investment cost (C_I)	0.253	16.60
Total bioethanol cost per litre (C_{Et})[b]	1.525	100
Total bioethanol cost per litre (USD$/l)[c]	0.44	

[a] RM 0.20 per kilometre per tonne as the price of the transportation cost [55].
[b] $C_{Et} = C_F + C_{O\&M} + C_I$.
[c] The exchange rate of Ringgit Malaysia (RM) against the US dollar was approximately Rm 1 to 3.40 US dollar on December 31, 2009 (Bank of Malaysia website, www.bnm.gov.my).

3.4. Scanning electron microscopy

SEM (scanning electron microscopy) analysis revealed ultrastructural changes in seaweed solid wastes during enzymatic hydrolysis. The images were magnified by a factor of 5000. As shown in Fig. 7A, the surface of untreated samples was smooth and continuous. In addition, the untreated samples exhibited rigid and highly ordered fibrils. However, after enzymatic hydrolysis, the structure became loose and rough (Fig. 7B). This finding confirmed

Table 4
Fuel bioethanol production costs for different feedstocks.

Raw material	Production cost (US$/l)	Reference
Seaweed solid wastes	0.44	This work
Coffee cut-stems	0.75–077	[26]
Corn stover	0.66–80	[51,52]
Wheat residue	0.51	[56]
Spruce chips	0.72	[57]

the disintegration of the samples' cellulosic structure due to the enzymatic conversion of cellulose to its constituent sugars [49].

3.5. Overall mass balance

A detailed mass balance for each step of the SHF and SSF processes are shown in Fig. 8. In this study, 1 g of solid seaweed wastes was subjected to two different processes. For the SHF process, after enzymatic hydrolysis, 0.49 g of glucose was released, corresponding to an enzymatic hydrolysis yield of 99.8%. With subsequent bioethanol fermentation, 0.25 g of bioethanol was produced, corresponding to an SHF yield of 55.9%. For the SSF process, 0.27 g of bioethanol was directly produced, which is equivalent to 0.53 g of glucose being fermented, corresponding to an SSF yield of 90.9%. These results demonstrated that the SSF process is superior to the SHF process for enzymatic hydrolysis-based bioethanol production because apart from being a single-step reaction procedure, it also requires a shorter reaction time to achieve a higher bioethanol yield. SSF was observed to be approximately 35% more effective for bioethanol production than SHF.

3.6. Comparison with other studies

In this work, seaweed solid wastes were used as feedstock for bioethanol production. Interestingly, the bioethanol yield obtained from solid seaweed wastes was comparable with previously reported bioethanol yields obtained from various macroalgae materials such as brown algae by J. ye Lee et al. [48]. In their study, a maximum bioethanol concentration and a maximum bioethanol yield of 6.65 g/L and 67.39%, respectively, were achieved by using *S. cerevisiae* as the fermentation agent after 48 h of SSF. In another study by Lee and Lee [50] using *S. cerevisiae* (KCCM50550) as the fermentation agent, a bioethanol concentration of only 2.7 g/L was attained after 7 days of fermentation. This result demonstrates that the highest bioethanol yield obtained from seaweed solid wastes via the SSF process (90.9%) in this study is significantly higher than that reported by several other studies and can be obtained in a much shorter time. Thus, the operating costs of bioethanol production can be significantly reduced. The results obtained in this study demonstrate a clear improvement in the fermentation process, indicating that solid seaweed wastes can be an alternative feedstock for bioethanol production.

3.7. Economy feasibility study

The bioethanol production cost using seaweed solid wastes is shown in Table 3. It can be seen that the operating cost accounts for the highest share (82%) of the total unit cost of bioethanol production. On the other hand, feedstock cost constitutes only 1% and the capital cost constitutes another 16% of the total cost. Compared to other feedstocks, bioethanol production cost from seaweed solid wastes are competitive and economically viable as shown in Table 4. However, it should be noted that the reported cost for bioethanol production varies significantly depending on the conversion technologies, feedstock and allocation methods. According to C. F. Triana et al. [26], ethanol derived from coffee cut-stems has a high production cost due to the high utilities cost involved in utilizing liquid hot water and diluted acid as the pretreatment technologies. The feedstock cost was the second highest contributing factor to the production cost. The relatively high cost of feedstock may be due to the absence of a proper logistic system for collecting the feedstock to a central collection point for its utilization. Other feedstock such as corn stover and wheat stalks may require additional cropping practices that leads to additional cost [51,52].

4. Conclusion

This study demonstrated that seaweed solid wastes, an abundant by-product of carrageenan processing, is a potential and novel biomass feedstock for the production of bioethanol by fermentation. Bioethanol production by the SSF process was observed to be more effective than the SHF process, producing 5.47 mg/mL of bioethanol within 1 h, with a theoretical yield of 90.9%. Overall, seaweed solid wastes was an attractive feedstock for be bioethanol production from both the economic standpoints and environmentally friendly.

Acknowledgements

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Appendix A

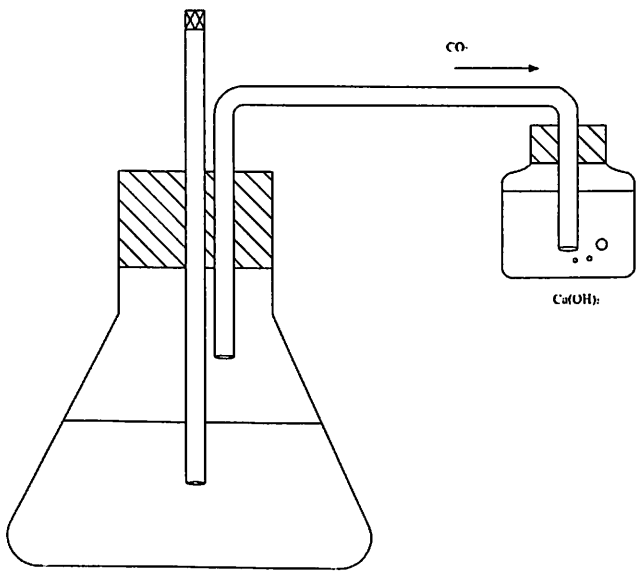

Fig. A1. Schematic drawings of shake flasks used in the present study.

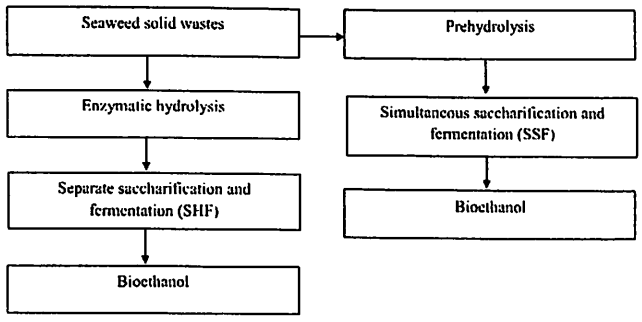

Fig. A2. Schematic illustration showing differences between separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

Table A1
Net operating cost of seaweed solid wastes.

Item	Tonnes/day	Unit price (RM/tonnes)	Seaweed solid waste		Reference
			Annual expense (RM/year)	Unit cost (RM/l)	
I. Raw material preparation and prehydrolysis process					
Water	12,311	2.7	10,968,750.00	0.12	[58]
Acetic acid	13	1724.1	7,347,359.91	0.08	[59]
Sodium hydroxide	9	515.4	1,570,359.38	0.02	[60]
Enzymes	44	5517.2	80,109,744.00	0.86	[27]
Subtotal			19,886,469.28	1.07	
II. SSF					
Water	12,311	2.7	10,968,750.00	0.12	
Yeast and YEPD	830	16.4	4,491,960.00	0.05	[31]
Subtotal			15,460,710.00	0.17	
III. Utilities					
Electricity	12,800	0.337	1,423,488.00	0.02	
Subtotal				0.02	
iV. Labor, supplies and overhead costs of direct labors	Daily wage (RM/day)	Operator/shift			
Operators	34.62	5	171,369.00	0.002	
Maintenances	34.62	2	68,547.60	0.001	
Administration salaries	40% of O & M labor		47,793.10	0.001	
Operating supplies	0.75% of annual capital		48,962.50	0.001	
Maintenances supplies	1% of annual capital		65,283.36	0.001	
General and administrative	60% of total labor		100,365.52	0.001	
Insurance	0.75% of annual capital		48,962.50	0.001	
Subtotal			551,283.58	0.006	
Total			37,321,950.86	1.255	

Table A2
Estimation of the investment cost of a bioethanol plant in Sabah, Malaysia [31].

Installed capital costs	RM
Feed handling	18,458,718.90
SSF	49,384,779.19
Distillation	17,471,352.46
Storage	7,186,234.21
Boiler	129,325,611.04
Utilities	18,841,654.56
Solids/syrup separation	31,516,949.49
Total capital investment	271,185,299.85
Annual capital investment cost	23,643,170.19
Investment cost (RM/l)	0.253

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Immobilization of β-glucosidase from *Aspergillus niger* on κ-carrageenan hybrid matrix and its application on the production of reducing sugar from macroalgae cellulosic residue

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HIGHLIGHTS

- Novel glutaraldehyde activated κC-PEI polyelectrolyte complex beads were prepared.
- β-glucosidase immobilized on hybrid matrix beads retained high activity.
- The immobilized β-glucosidase showed enhanced activity and good reusability.

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ABSTRACT

A novel concept for the synthesis of a stable polymer hybrid matrix bead was developed in this study. The beads were further applied for enzyme immobilization to produce stable and active biocatalysts with low enzyme leakage, and high immobilization efficiency, enzyme activity, and recyclability. The immobilization conditions, including PEI concentration, activation time and pH of the PEI solution were investigated and optimized. All formulated beads were characterized for its functionalized groups, composition, surface morphology and thermal stability. Compared with the free β-glucosidase, the immobilized β-glucosidase on the hybrid matrix bead was able to tolerate broader range of pH values and higher reaction temperature up to 60 °C. The immobilized β-glucosidase was then used to hydrolyse pretreated macroalgae cellulosic residue (MCR) for the production of reducing sugar and a hydrolysis yield of 73.4% was obtained. After repeated twelve runs, immobilized β-glucosidase retained about 75% of its initial activity.

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1. Introduction

The advantages of macroalgae biomass as feedstock for third-generation bioethanol production over previous generations have been discussed in several studies (Borines et al., 2013; Goh and Lee, 2010). Macroalgae biomass contain large amount of carbohydrate that can be readily fermented into bioethanol. Besides that, macroalgae do not compete land area with terrestrial crops and fresh water is not required for their cultivation. One type of red macroalgae, *Eucheuma cottonii*, is largely cultivated on the east coast of Sabah (Malaysia). The major polysaccharide constituents of red macroalgae are κ-carrageenan (κC), which can be easily obtained through extraction or dissolving them into an aqueous solution. κC is widely used as agent for thickening, gelling in food industries, and potential as raw material for hydrogel synthesis. However, after extracting κC, an enormous amount of residual materials is left behind, which is usually called macroalgae cellulosic residue (MCR). The residual biomass can be utilized for the production of bioethanol because it contains large amount of cellulosic materials. The utilization of MCR as an energy resource would pave the way for converting waste material into a product that has high commercial value.

In general, bioethanol production from biomass involves pretreatment, enzymatic hydrolysis, and fermentation. During enzymatic hydrolysis of cellulose, increasing cellobiose concentration has an inhibitory effect on the endo- and exo-glucanases, and thus β-glucosidase are considered the rate-limiting factor of most cellulolytic systems (Miletić et al., 2012). However, free β-glucosidase are relatively unstable, costly and difficult to recover after industrial process. In order to make the bioethanol production process from biomass more economical, improvement on the enzyme assisted technology is necessary. The immobilization of β-glucosidase on/in a solid matrix support increases the prospect of lower

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production cost by increasing efficiency, easy separation from the product in the reaction mixture, thermal stability and possible recycling of enzymes. Various techniques have been developed for β-glucosidase immobilization, including entrapment in polymeric gels, covalent linking to insoluble supports, adsorption, or a combination of these methods (Radva et al., 2011). β-glucosidase has been immobilized on different supports, such as alginate (Keerti et al., 2014; Ortega et al., 1998), silica gel (Matthijs and Schacht, 1996), magnetic chitosan microspheres (Zheng et al., 2013), and Eupergit C (González-Pombo et al., 2011). In the field of enzymes immobilization, κ-carrageenan is one of the popular choices of support using entrapment techniques that is inexpensive but unfortunately not efficient (Hernández-Rodríguez et al., 2014; Nakagawa et al., 2013). This is mainly because of the weak mechanical stability and leakage of enzymes over time, which can be attributed to the larger pore size of κ-carrageenan matrix (Sankalia et al., 2006). Moving forward, enzyme crosslinking with bi-functional reagents like glutaraldehyde (GA) followed by entrapment in natural polymers can be an attractive way to reduce leakage. Immobilization of lipase by crosslinking through glutaraldehyde and subsequent entrapment in alginate-carrageenan hybrid matrix was reported to show 89% immobilization yield (Abdulla and Ravindra, 2013), while immobilization in chitosan-based matrices and crosslinking through glutaraldehyde showed 5.6% recovered activity of β-galactosidase (Vieira et al., 2013). However the leakage of immobilized enzyme due to low thermal stability resulted in low recovery of enzyme activity. Thus, there is still room for improvement and this can be obviated by immobilization of the enzyme through adsorption on charge polymeric support.

Polyethyleneimine (PEI), a highly aminated hydrophilic polymer is effective in immobilizing cells due to its role as a crosslinking agent (Chu et al., 2009). However, there is very little work on the use of PEI for immobilization of β-glucosidase. Thus, this study attempted to utilize glutaraldehyde crosslinked κ-carrageenan for the immobilization of β-glucosidase using the covalent method via polyethyleneimine and glutaraldehyde. To the best of our knowledge, the immobilization of β-glucosidase on this hybrid matrix and its subsequent use for enzymatic hydrolysis of macroalgae cellulosic residue (MCR) has not been reported in the literature. Initially, a hybrid bead was formulated by evaluating the effect of PEI concentration, activation time, and pH of the PEI solution on the immobilization efficiency and catalytic activity. Characterization of all the formulated beads was carried out using Fourier transform infrared spectroscopy (FTIR), elemental analyzer (EA), scanning electron microscopy (SEM), and simultaneous thermal analyzer (STA) techniques. The effects of pH, temperature, and reusability on the catalytic activity of immobilized β-glucosidase were also investigated. Finally, immobilized β-glucosidase were applied to the hydrolysis of MCR, and their performance was compared with that of free enzymes.

2. Methods

2.1. Materials

Red macroalgae (*E. cottonii*) used in the present work was purchased from Futt Put Enterprise (north coast of Sabah, Malaysia). Upon receiving, the macroalgae was washed with distilled water and dried at 40 °C. After drying, the sample was pulverized, screen through 300-mesh and stored in a clean air-tight container until being used for subsequent experiments.

Novozyme 188, β-glucosidase from *Aspergillus niger*, was used for immobilization studies. Celluclast, a commercial cellulase preparation from *Trichoderma* sp. was used for hydrolysis of macroalgae cellulosic residue (MCR). Both enzymes were obtained from Science Technics Sdn. Bhd. The activities of cellulase were reported by the manufacturer as 82.08 filter paper unit (FPU)/mL. Glutaraldehyde solution (GA) (25%) was purchased from FLUKA (Switzerland), polyethyleneimine (PEI), standard cellobiose, standard D-glucose, potassium chloride, acetic acid, sodium hydroxide, Dowex (TM) Dr-G8, sodium acetate, and bicinchoninic acid (BCA) kit were purchased from Sigma–Aldrich (USA).

2.2. kappa-carrageenan (κC) extraction

3% (w/v) of dry *E. cottonii* was boiled at 90 °C for 2 h and the kappa-carrageenan (κC) extract was separated from residue, dried to a constant weight at 40 °C and pulverized (Tan et al., 2013). Then, the extract residue was boiled for another 30 min with 2 L distilled water and filtered with pressure pump. It was then dried at 40 °C, pulverized, screened through 80 mesh and used for subsequent experiments. The residue is now named as macroalgae cellulosic residue (MCR).

2.3. Preparation of beads

2.3.1. κappa-carrageenan beads (κC)

κ-Carrageenan (2% w/v) solution was prepared by heating up powdered κC with distilled water at 70 °C and with continuous stirring to completely dissolve the κC. The κC solution was transferred into a 50 mL burette and added dropwise to an agitated potassium chloride solution (0.3 M). This method produced approximately 450 uniform κC beads of approximately 2.5 mm in diameter for every 50 mL of the κC mixture. The beads formed were kept at 25 °C for 3 h in the potassium chloride solution for it to harden. Finally, the beads obtained were filtered and rinsed twice with distilled water.

2.3.2. Preparation and modification of crosslinked κappa-carrageenan hybrid beads

Initially, κC (2% w/v) solution was prepared in distilled water at 70 °C. The mixture was heated and stirred for 20 min to ensure a homogeneous solution was obtained. Then 0.25% glutaraldehyde (GA) was added to the resulting solution as a crosslinker. The solution was maintained in a thermostatic shaking water bath at 50 °C for 24 h. After that, it was transferred into a 50 mL burette and the subsequent steps are exactly the same as reported in Section 2.3.1 for obtaining uniform beads.

The beads obtained were further modified using polyelectrolytes (PEI) followed by a mediator (GA) to bind β-glucosidase enzyme via covalent bond. The beads were modified by varying the formulation variables (PEI concentration, activation time, and pH) listed in Table 1. The modified beads were thoroughly washed with distilled water and soaked in 1% glutaraldehyde (GA) solution for 2 h. The role of GA is to activate the beads for immobilization. The beads were then washed with distilled water to remove unreacted GA. After that, the activated beads were subjected to the immobilization steps. The activated beads were loaded with β-glucosidase by soaking in 50 mL of diluted β-glucosidase solution (pH 4.8) with an enzyme activity of 1.28 U/mL for 24 h. The immobilized β-glucosidase beads were washed thoroughly three times with distilled water to remove unbound enzyme and stored in 50 mM sodium acetate buffer solution at 4 °C until being used. The effects of different formulation variables on the immobilization yield and enzyme activity were thoroughly evaluated. In all the above experiments, the immobilized β-glucosidase with the highest immobilization yield and activity (U/g) were chosen for further experiments as an optimum formulation. All the optimization experiments were performed in triplicate (standard deviations were in all cases <5%), with average data reported.

Table 1
Formulation design.

Variables	Values	Constants		
		PEI concentration (% w/v)	Activation time (h)	pH
PEI concentration (% w/v)	0	–	0	0
	1.0	–	2	8
	2.0	–	2	8
	3.0	–	2	8
	4.0	–	2	8
	5.0	–	2	8
Activation time (h)	0.5	2	–	8
	1.0	2	–	8
	2.0	2	–	8
	3.0	2	–	8
	4.0	2	–	8
	5.0	2	–	8
	6.0	2	–	8
	8.0	2	–	8
	20.0	2	–	8
pH	7.0	2	5	–
	7.5	2	5	–
	8.0	2	5	–
	8.5	2	5	–
	9.0	2	5	–

2.4. Assay of β-glucosidase activity

The activity of immobilized β-glucosidase was assayed by hydrolysis of cellobiose. Cellobiose (0.5% w/v) and immobilized β-glucosidase were mixed with 50 mL of sodium acetate buffer (50 mM, pH 4.8). After keeping the mixture in a shaking water bath at 50 °C for 45 min, the immobilized β-glucosidase was separated from the buffer solution and the glucose concentration from cellobiose hydrolysis was analysed with high performance liquid chromatography (HP-LC). One unit (U) of the enzyme activity was defined as the amount of the enzyme that releases 2 μmol glucose from cellobiose per min under the assay conditions (Zheng et al., 2013). The immobilized β-glucosidase activity (U/g of support) was calculated by dividing the enzyme activity by the total dry weight of support. The relative activity was calculated using Eq. (1).

$$\text{Relative activity} = \frac{\text{activity}}{\text{maximum activity}} \times 100\% \quad (1)$$

2.5. Immobilization of β-glucosidase and soluble protein determination

The amount of enzyme immobilized was determined by subtracting the amount of protein in the supernatant after immobilization from the total amount of protein used for immobilization. The immobilized yield was calculated based on the following equation (Borges et al., 2014):

$$\text{Immobilization yeild } (\%) = \frac{C_i V_i - C_f V_f}{C_i V_i} \times 100\% \quad (2)$$

where C_i represents the initial protein concentration, V_i is the initial volume of enzyme solution, C_f is the protein concentration in the total filtrate, and V_f is the total volume of the filtrate. The protein concentration was measured as described in Section 2.6.2.

2.6. Analytical methods

2.6.1. Analysis of sugars

The sugar concentration was quantified with an Agilent series 1200 infinity high performance liquid chromatography system which was equipped with a 385-ELSD (evaporative light scattering detector) and a 300 mm × 7.7 mm Hi-Plex Ca column. Distilled-deionised water was used as the mobile phase with a flow rate of 0.6 mL/min and injection volume of 20 μL. Purified nitrogen was used as carrier gas (70 psi) for the detector. The HPLC-ELSD's spray chamber temperature was set at 40 °C whereas detector temperature at 80 °C. For quantification, the peak areas were determined, and the concentration calculated with an external calibration curve. The yield of glucose was calculated as follows:

$$\text{Yield } (\%) = \frac{\text{Concentration (g/l) of glucose at time of } t}{\text{Initial concentration (g/l) of substrate}} \times 100\% \quad (3)$$

2.6.2. Analysis of protein

The solution obtained from Section 2.3.2 was diluted to 5 mL using 50 mM sodium acetate buffer. After centrifuging the solution at 10,000g for 2 min, 1 mL of the diluted supernatant was transferred to a test tube, and 1 mL of QuantiPro Working Reagent (bicinchoninic acid reagent) was added. The test tube was vortex gently for thorough mixing, left standing for 1 h in water at 60 °C and then cooled to room temperature. The absorbance of the samples was measured at 562 nm using a spectrophotometer (Agilent Technologies carry 60 UV–Vis). The protein concentration of the samples was calculated from a bovine serum albumin (BSA) standard curve.

2.6.3. Zeta potential measurement

Zeta potential (ξ-potential) measurements for PEI solutions were performed using the Zetasizer Nano-ZS (Malvern Instruments, UK). PEI solutions with different concentration and pH values was used in this study. The pH values were adjusted using concentrated acetic acid or sodium hydroxide.

2.7. Biophysical characterization of immobilized β-glucosidase

Beads obtained from each step in Section 2.3.2 was characterized. The beads are named as formulation 1 (κC/K^+) as control gel beads, formulation 2 (κC/GA/K^+), formulation 3 (κC/GA/K^+/PEI^+), formulation 4 (κC/GA/K^+/PEI^+/GA), and formulation 5 (κC/GA/K^+/PEI^+/GA/β-glucosidase). All the beads prepared as described above were subject to freeze-drying.

2.7.1. FTIR spectroscopy

FTIR spectroscopy was used to detect the presence of functional group in the beads. The infrared spectra of all formulations were obtained using a Fourier transform infrared spectroscopy (IR-Prestige-21, Shimadzu, Japan). FTIR spectra of the samples pressed in KBr pellet were obtained at a resolution of 2 cm^{-1} between 4000 and 500 cm^{-1} at ambient temperature. The characteristic peaks were recorded.

2.7.2. Elemental analysis (EA)

EA was used to detect the presence of PEI in the beads. The elemental analysis of samples was performed using an elemental analyzer (2400 series II CHNS/O, Perkin Elmer Instruments model, USA). Helium gas was used as carrier (20 psi) and oxygen gas (15 psi) as fuel with compressed air and pneumatic gas.

2.7.3. Scanning electron microscopy (SEM)

The morphology and the physical structure of all formulations were observed by scanning electron microscopy (SEM) using a Fei Quanta 450 FEG (Eindhoven, NL) instrument together with energy-dispersive X-ray spectral (EDX) analysis at the required magnification at room temperature. The beads were mounted on brass stubs using carbon paste. All images were taken at a magnification of 10,000× and observed using a voltage of 10 kV, with the secondary electron image (SEI) as a detector.

2.7.4. Simultaneous thermal analyzer

The thermal stability of beads were characterized by thermogravimetry (TGA) and differential scanning calorimetry (DSC) using a simultaneous thermal analyzer (STA 6000, Perkin Elmer Instruments model, USA). A sample weight of 6 mg was crimped in a standard aluminium pan and heated from 30 to 550 °C at a constant heating rate of 10 °C/min under constant purging with nitrogen at 20 mL/min.

2.8. Biochemical characterization of immobilized β-glucosidase

Optimum modified formulation of immobilized β-glucosidase (36 U/g of support) and free β-glucosidase (64 U/g protein) were used for this test. All enzymatic hydrolysis experiments were performed in triplicate (standard deviations were in all cases <5%), with average data reported.

2.8.1. Effect of pH on the immobilized β-glucosidase

The optimal pH of free and immobilized β-glucosidase was analysed by incubating the immobilized enzyme at pH values ranging from 2.8 to 8.8 using sodium acetate buffer in the reaction mixture while other conditions were similar as reported in Section 2.4. The optimum pH is as 100% activity and the relative activity at each pH is expressed as a percentage of the 100% activity.

2.8.2. Effect of temperature on the β-glucosidase

The effect of incubation temperature on free and immobilized β-glucosidase was determined by performing the enzyme reaction (Section 2.4) at different temperature ranging from 40 to 70 °C. The optimum temperature is taken as 100% activity and the relative activity at each temperature is expressed as a percentage of the 100% activity.

2.8.3. Operational stability of immobilized β-glucosidase

Operational stability test was carried out by repeating 12 batch of experiments using the following method: immobilized enzyme was incubated in 0.5% (w/v) of cellobiose solution (50 mM sodium acetate buffer pH 4.8) for 3 h at 50 °C. After each cycle, the immobilized enzyme was washed with distilled water and re-used. The reaction medium was then replaced with fresh medium. Aliquots were removed and assayed for hydrolysis yield as earlier specified. The activity of immobilized enzyme in the first run was defined as the control and attributed a relative activity of 100%.

2.9. Hydrolysis of MCR

4% (w/v) of macroalgae cellulosic residue (MCR) was pretreated using 4% (w/v) Dowex (TM) Dr-G8. 4% (w/v) MCR at 120 °C for 45 min. Enzymatic hydrolysis of the 2% (w/v) pretreated macroalgae cellulosic residue (MCR) to glucose was performed in a 50 mM sodium acetate buffer with a cellulase (celluclast 1.5 L, Sigma Aldrich) loading of 45 FPU/g substrate in a total working volume of 50 ml. At the same time, free (64 U/g protein) or immobilized β-glucosidase (36 U/g of support) was added to the mixture to assist the hydrolysis at 50 °C for 72 h in a water-bath shaker operated at an agitation speed of 150 rpm. Samples were taken from the reaction mixture periodically during incubation for sugar analysis.

3. Results and discussion

3.1. Optimization of the immobilization process

The influences of several key immobilization conditions were investigated. Fig. 1a revealed that PEI concentration plays a crucial role on the immobilization yield and enzyme activity. The activities of the immobilized β-glucosidase was determined using cellobiose as substrate. The increase of PEI concentration from 0% to 2% led to an increase in the immobilization yield from 38.7% to 92.8% and the enzyme activity from 0.46 to 31 U/g. These results can be explained by the increase of aldehyde groups available to bind with the amine groups of the enzyme with a covalent bond. At higher PEI concentration, more amine groups will be available in the support to react with GA (Adriano et al., 2008). In general, the amount of enzyme immobilized on carriers have a strong influence on the performance of the immobilized enzyme (Zhang et al., 2013; Zheng et al., 2013). In this study, a 2.4-fold increase in the immobilization yield of β-glucosidase leads to a more than 10-fold increase in the enzyme activity. The exponential increase in enzymatic activity with immobilization yield was in agreement with the study reported by Zhang et al. (2014). However, further increase in PEI concentration led to a decrease in immobilization yield and enzyme activity. The zeta potential of the PEI solution at a concentration from 1% to 5% was measured to explain this phenomenon (Fig. S1a). Zeta potential decreases from +27.5 to +24.5 mV when PEI concentration was increased from 2% to 5% due to the increasing ionic strength of the suspension and intermolecular interactions. When PEI concentration was increased beyond 2%, the increasing concentration of macro-ions and repulsive forces between the same charges particles caused only a part of the polymer chain can bind to the surface of the negatively charged hybrid beads (Pan et al., 2011). As a consequence, the apparent activity of the immobilized β-glucosidase did not increase, implying that the amount of amine groups in the support decreased. This limits the amount of enzyme binding which is consistent with previously reported data by Li et al. (2014). In additional, at PEI concentration of 4% and 5%, the enzyme activity dropped to almost nil even through the immobilization yield was 56.3% and 54.6%, respectively. This is because at high PEI concentration, the adsorption of enzyme into the porous support occurs very fast and the covalent bond act as a barrier for substrate diffusion leading to a lower enzyme activity. Therefore, PEI concentration of 2% was taken as the optimized value and was used for the optimization of the activation time as shown in Fig. 1b (Zhang et al., 2014; Li et al., 2014).

The amount of immobilized β-glucosidase is correlated closely to the availability of active sites on the hybrid beads. The activation time for κC beads-PEI polyelectrolyte complex formation is an important parameter affecting the number of available active sites. The activation time was studied from 0.5 to 20 h to evaluate its effect on the amount of β-glucosidase loaded on the activated beads and activity. As expected, initial increase in activation time resulted in a significant increase in the activity of immobilized β-glucosidase. The activation time attained optimum within 4 h where maximum immobilization yield (98.4%) and activity (36 U/g) were obtained. Beyond 4 h, there was a moderate decrease in the immobilized yield with significant change in the enzyme activity. This could be because longer activation time provides more time for cross-linking in the matrix and hardening of the bead surface, which led to formation of compact beads and pronounced gel bead shrinkage, leading to lower immobilization yield and activity (Das and Ng, 2010). Thus, the substrate is inaccessible to enzyme as a result of steric hindrance or decrease in the enzyme active site. Since a prolonged activation time might cause some damage to the hybrid matrix, and the enzyme loading amount was almost saturated at an activation time of 4 h, further experiments were conducted under this condition to optimize other operating parameters.

Fig. 1c displays the effect of PEI solution pH values on the synthesis of immobilized β-glucosidase hybrid beads. The immobilized β-glucosidase showed higher activity at pH 8 than that at below and above pH 8. The zeta potential of the PEI solution at

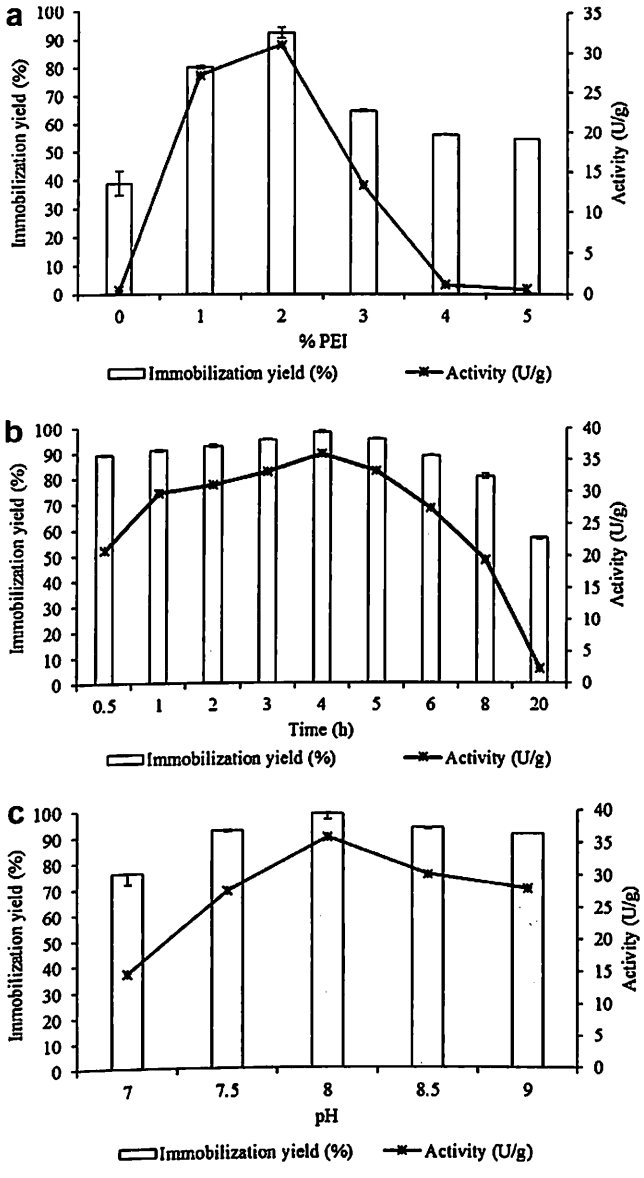

Fig. 1. Effect of (a) PEI concentration, (b) activation time, and (c) pH of PEI solution on the immobilization yield and enzyme activity.

pH 6.5–10 was measured to explain this phenomenon (Fig. S1b). When the pH value was increased from 7 to 8, the number of protonated amino groups of PEI increased, so more ionic interaction between protonated amino groups and sulphate groups in crosslinked κC hybrid beads resulted to a stronger polyelectrolyte complex formed. Simultaneously the unprotonated part will react with GA forming a Schiff's base and creating a free aldehyde terminal group to bind β-glucosidase with a covalent bond. However, when the pH value was further increased from 8 to 9, the positive charge on PEI decreased (27.5–23.8 mV), thereby weakening the ionic interaction between the crosslinked κC hybrid beads and PEI solution (Wu et al., 2014). In the present study, the pH of κC solution was observed at around 6.7 with a negative zeta potential value (−28.7 mV, data not shown). When crosslinked κC hybrid come into contact with positive PEI chains, the electrostatic repulsion will be eliminated by the neutralization of charges. More importantly, the addition of GA to κC solution at a pH of 4.1 enhanced the negative potential from −28.7 to −47.8 mV (data not shown), thus drawing more PEI molecules towards the interface to form electrostatic complexes with GA and leading to the increased stability of κC hybrid beads.

3.2. Characteristic of immobilized β-glucosidase

3.2.1. Fourier transform infrared (FTIR) spectra

FTIR spectroscopy is a powerful technique to investigate the existence of the new functional group in the beads. Fig. S2a–e shows the FTIR spectra of formulation 1 (κC/K^+) as control beads, formulation 2 (κC/GA/K^+), formulation 3 (κC/GA/K^+/PEI^+), formulation 4 (κC/GA/K^+/PEI^+/GA), and formulation 5 (κC/GA/K^+/PEI^+/GA/β-glucosidase), respectively. Based on Fig. S2a which shows the FTIR spectra of formulation 1, the bands observed at 849, 925, 1080, and 1260 cm^{-1} are attributed to D-galactose-4-sulphate, 3,6-anhydro-D-galactose, glycoside linkage and sulphate ester stretch of κC, respectively. The broad band at 3200–3600 cm^{-1} is due to stretching of —OH groups of the polysaccharide. The —OH bending absorption appears as medium band at about 1628 cm^{-1}. It was found that the infrared spectra of extracted carrageenan (formulation 1) from *E. cottonii* exhibited the features of kappa-carrageenan (Pereira et al., 2009). For formulation 2, the occurrence of chemical reaction between the hydroxyl groups of κC and GA as the crosslinking agent was confirmed by FTIR measurement. Fig. S2b shows a relatively slight decrease in the absorption bands of hydroxyl group in formulation 2. The reduction indicates that reaction between GA and κC has occurred whereby hydroxyls had been consumed during the crosslinking reaction. Previous researchers had reported that hydroxyls from polymer can easily react with aldehyde forming hemi acetal structure (Chen et al., 2012; Mansur et al., 2008). However, the peak ascribed to acetal groups did not appear in the observed FTIR spectra perhaps due to the low amount of acetal groups in the beads. The low concentration of acetal groups could possibly be beyond the detection limit of spectroscopic analysis.

Notably, after PEI loading, the IR spectrum of formulation 3 exhibited a significant decrease in the intensity of the peak at 1080 cm^{-1} and disappearance of peak at 849 cm^{-1} (Fig. S2c). Theoretically, the presence of amine group should have been detected at 3422 cm^{-1} (N—H stretching) and 1628 cm^{-1} (N—H bending) (Yilmaz et al., 2011). Unfortunately, both bands overlapped with the broad band of the —OH group of κC. This indicates the successful interaction between protonated amine groups of PEI and sulphates groups of κ-carrageenan which led to the formation of PEI-κC polyelectrolyte complex. As for κC/GA/K^+/PEI^+/GA formulation, GA is expected to react with primary amine groups of PEI, forming "Schiff's base" (Fig. S2d) that leads to the detection of peak at 3422 cm^{-1} (N—H stretching) and weaker peak at 1628 cm^{-1} (N—H bending). The C=N stretching band is located from 1625–1665 cm^{-1}, so it is assumed that generating the Schiff's base (—C=N—) influenced the appearance of the adjacent peak. Although the intensity of the Schiff's base is relatively low and the peak is not clear in these spectra, however, the formation of Schiff's base was clearly indicated by the change of colour from yellowish to red colour (Fig. S3a and b). Besides that, the unreacted GA side (bifunctional spacer arm) created a free aldehyde groups for the covalent immobilization of β-glucosidase. These aldehyde groups appeared as a slight indentation at 1350 cm^{-1}. Fig. S2e shows that the aldehyde groups diminished in formulation 5, and this indicated that the free aldehyde groups have coupled with the amino groups of β-glucosidase to form covalent bonds during the immobilization process.

Elemental analyses of the different formulations were performed in order to provide the actual composition of C, H, N and S (Table S1) in the beads. The elemental analysis data shows that the composition for formulation 1 were 28.5% (C), 4.3% (H), 0% (N), and 2.7% (S), whereas for formulation 3 were 32.2% (C), 9.1% (H), 7.6% (N), and 3.2% (S). The absence of nitrogen in formulation

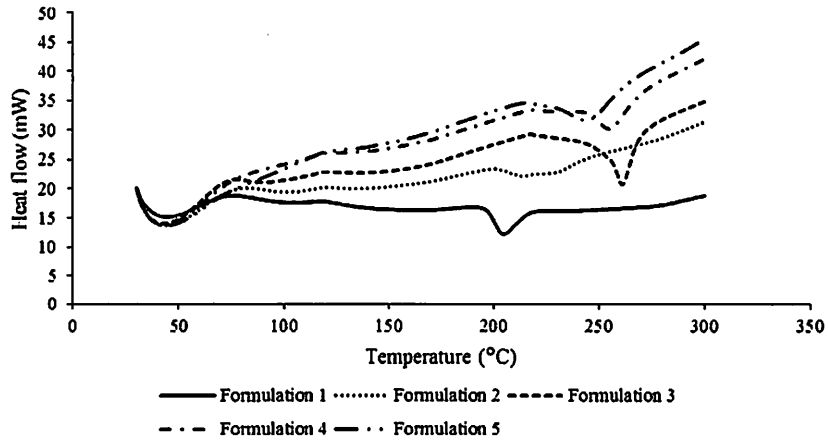

Fig. 2. DSC thermograms of formulation 1 (κC/K⁺) as control beads, formulation 2 (κC/GA/K⁺), formulation 3 (κC/GA/K⁺/PEI⁺), formulation 4 (κC/GA/K⁺/PEI⁺/GA), and formulation 5 (κC/GA/K⁺/PEI⁺/GA/β-glucosidase).

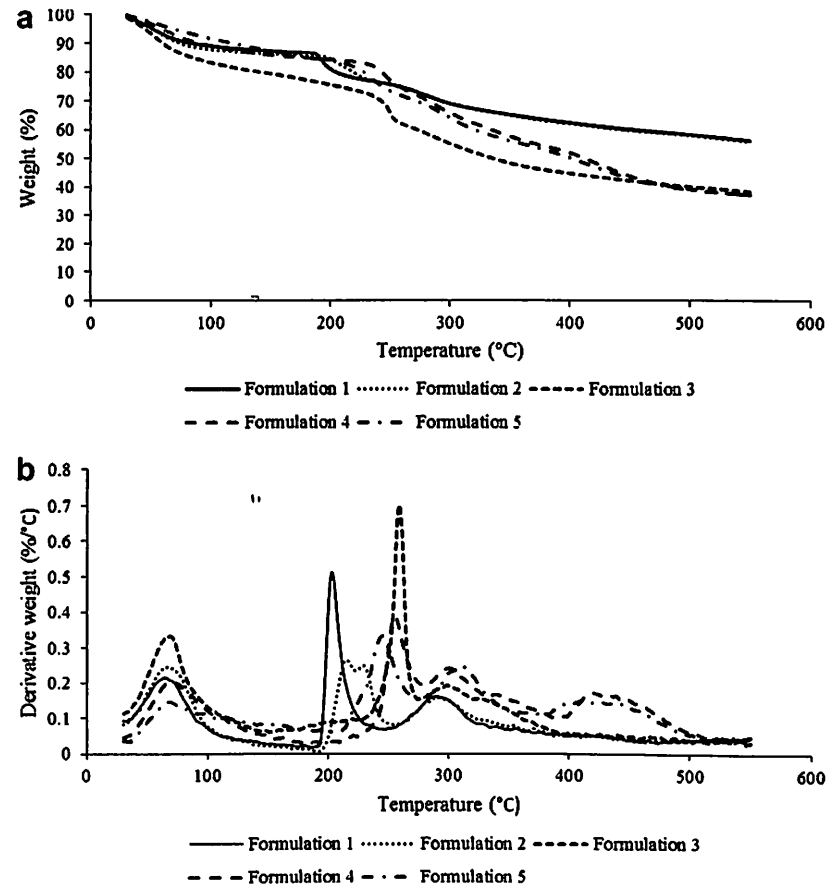

Fig. 3. Thermogravimetric weight loss curves. (a) TGA and (b) DTG of formulation 1 (κC/K⁺) as control beads, formulation 2 (κC/GA/K⁺), formulation 3 (κC/GA/K⁺/PEI⁺), formulation 4 (κC/GA/K⁺/PEI⁺/GA), and formulation 5 (κC/GA/K⁺/PEI⁺/GA/β-glucosidase).

1 while its presence in high concentration in formulation 3 proved the presence of PEI in the carrageenan beads. These conclusions were further verified by SEM and thermogravimetric analysis (TGA) results which will be presented in the following sections.

3.2.2. Thermal behaviour of hybrid β-glucosidase

In order to reduce the leakage of immobilized enzyme, κ-carrageenan was treated with polyethyleneimine and glutaraldehyde to improve the thermal stability of hybrid beads. Simultaneous thermal analyzer was performed to prove the formation of a strong polyelectrolyte complex by assessing the thermal stability of the prepared hybrid beads. DSC thermograms of formulation 1–5 are shown in Fig. 2. All formulations show one broad endothermic transition at lower temperature corresponding to the loss of water associated to the hydrophilic groups of polymers. The figure also revealed a gradual increase in the endothermic bands from 204.7 °C (ΔH = 48.9 J/g) to 213.5 °C (ΔH = 35.8 J/g), by crosslinking κC with GA (formulation 2). However, this endothermic peak was

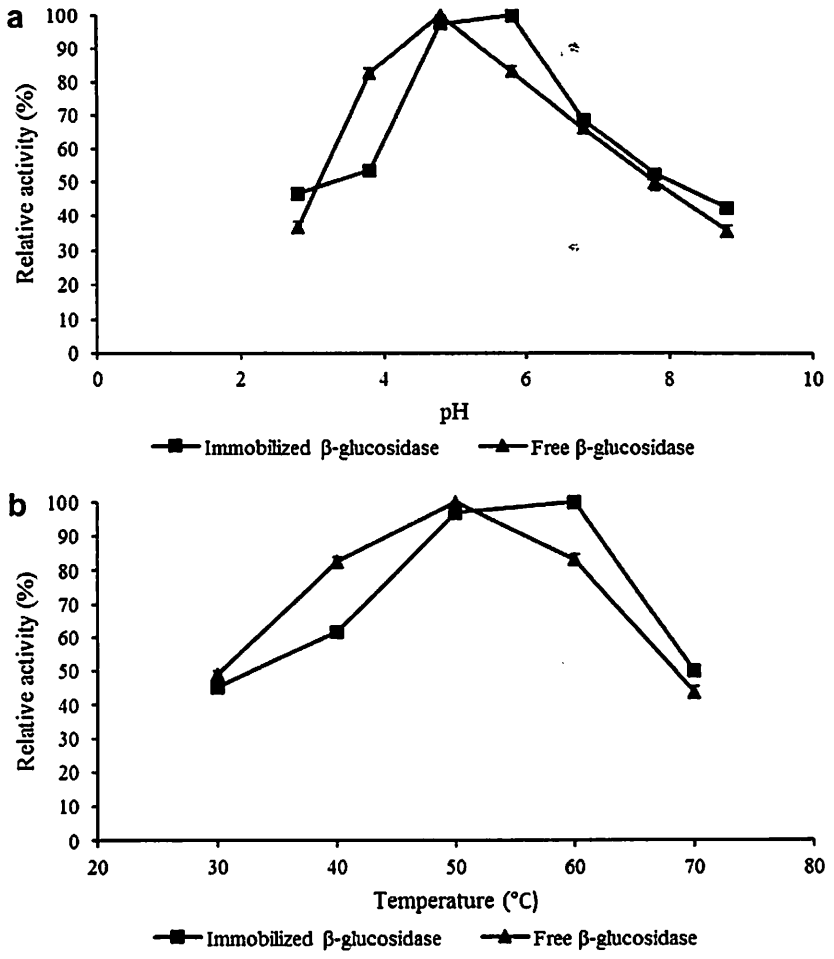

Fig. 4. Effect of pH (a) and (b) temperature on the activity of free and immobilized β-glucosidase. (Modified formulation of 2% (w/v) κC crosslinked with 0.25% GA and soaked in 2% (w/v) PEI at pH 8 for 5 h followed by 1% (w/v) GA for 2 h. The hybrid beads were then soaked in β-glucosidase for 24 h.)

less pronounced because the GA bound to the polymer is amorphous, which implies comparable but reduced relative crystallinity due to crosslinking. The shift of this peak to higher temperature indicated that the crosslinking treatment improves thermal stability of hybrid beads as a result of this interaction (Chen et al., 2013). Furthermore, the treatment of hybrid beads with PEI and then with GA showed single peaks at 261.2 °C (ΔH = 218.8 J/g) and 255.4 °C (ΔH = 202.4 J/g), respectively. This indicated the formation of a strong polyelectrolyte complex due to polyelectrolyte interaction between the polyanions ($-OSO_3^-$) and the polycations ($-NH_3^+$). When beads with formulation 3 is being crosslinking with GA, the endothermic peaks were less pronounced due to the crosslinking which will disturb the intra and inter molecular hydrogen bonds. Therefore, the decrease in the enthalpy values (ΔH) when using GA has to be attributed to an increase in the extent of crosslinked network formation which breaks exothermically. In addition, the lower transition temperature can be explained by hypothesizing an 'inhibition effect' exerted by the GA, which prevented the recovery of the structurally ordered microcrystalline domains to the advantage of a more amorphous final molecular structure (Farris et al., 2011). DSC thermogram of enzyme loaded beads was similar to that of formulation 4 except all corresponding peaks were shifted to lower temperature (245.6 °C) and higher enthalpy values (228.2 J/g). This might be due to the presence of β-glucosidase.

The temperature values of the decomposition peak of formulation 1–5 were verified by TGA (Fig. 3) and the thermal degradation data are illustrated in Table S2. Upon heating, all curves show a weight loss at temperature ranging from 30 to 120 °C, mainly due to the loss of absorbed water through hydrogen bonds. At higher temperature, formulation 1–5 exhibited two to three peaks (Fig. 3) indicating mass loss in several steps. The sharp peak in the DTA curve (denoted as T_2) indicated removal of sulphur dioxide and fragmentation of carbohydrate backbone (Vinceković et al., 2010). The treatment of κC with PEI followed by GA showed a gradual and obvious improvement in the temperature of maximum decomposition (T_m). Besides that, the increasing trend of weight loss from materials indicates that the decomposition of PEI and GA. A fourth event for bead with formulation 4 and 5 is due to the subsequent decomposition process which was verified at T_4 (438.7 °C). The decomposition process at higher temperature for formulation 4 and 5 was due to its crosslinked nature, since it is well known that crosslinking leads to increased thermal stability.

3.3. Optimum pH and temperature of the hybrid immobilized β-glucosidase

Fig. 4a shows the relative activity of immobilized and free β-glucosidase at various pH. For the free β-glucosidase, 100% relative activity was attained at an optimum pH value of 4.8. While for the immobilized β-glucosidase, 100% relative activity was attained from pH 4.8–5.8. This result shows that the immobilized β-glucosidase was able to tolerate a broader range of pH value. This might be due to the structural and conformational stability of β-glu-

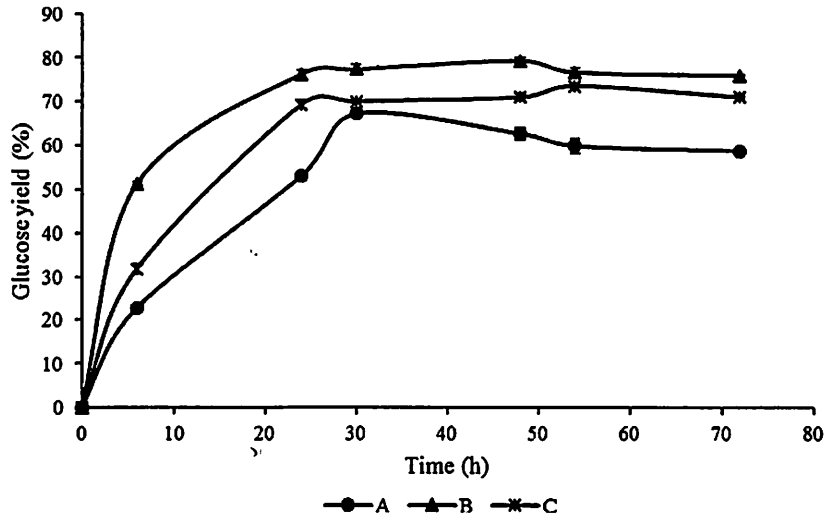

Fig. 5. Hydrolysis of pretreated macroalgae cellulosic residue (MCR) under different conditions. Enzymatic hydrolysis of pretreated MCR was performed as described in Section 2.9. (A) Cellulase, (B) cellulase with free β-glucosidase, (C) cellulase with immobilized β-glucosidase.

cosidase within the microenvironment of hybrid matrix (Verma et al., 2013).

Relative activity as a function of temperature is shown in Fig. 4b. For the free β-glucosidase, the relative activity was initially found to increase with temperature up to 50 °C. Beyond 50 °C, the relative activity began to drop because the β-glucosidase start to denature. Similar trend was found for the immobilized β-glucosidase up to 50 °C. However, the immobilized β-glucosidase was found to be able to sustain almost 100% relative activity when the temperature was further increase to 60 °C. Beyond 60 °C, then only the relative activity began to drop. Possible reasons for the increased thermal stability could be because immobilization of β-glucosidase increased the rigidity of the enzyme and the formation of covalent bond between hybrid matrix and enzyme further enhance it to withstand higher temperature (Srivastava and Anand, 2014). This advantage is very important as it allows the enzyme to be used at higher temperature that may be very favourable to the industry to achieve faster reaction rates. Enhanced thermal stability of immobilized enzyme was also reported in other works (Abdulla and Ravindra, 2013; Piñuel et al., 2011; Verma et al., 2013).

3.4. Reusability of the hybrid immobilized β-glucosidase

The ability to be used repeatedly without significant drop in activity is one of the most important characteristics of immobilized enzymes. This is because most of the enzyme used in the industry are very expensive. The residual activity of immobilized β-glucosidase for 12 cycles and each cycle was operated for 3 h. After ten cycles, the residual activity of immobilized β-glucosidase was 84.9% of the initial activity. No sharp decrease in the activity of immobilized β-glucosidase was detected in the entire course of operation, and 75.6% of its original activity was maintained even after 12th operation cycle. This indicates a very high operational stability of the immobilized β-glucosidase. The relative higher reuse stability of immobilized β-glucosidase in this study could reduce the operating cost and thus is more promising for industrial applications.

3.5. Hydrolysis of macroalgae cellulosic residue

When conducting the immobilization process under optimum formulation, an immobilized β-glucosidase with an enzyme activity of 36 U/g of support could be obtained. The immobilized β-glucosidase was further used to test their suitability for enzymatic hydrolysis of macroalgae cellulosic residues (MCR). The hydrolysis of pretreated MCR by cellulase was evaluated after adding either free or immobilized β-glucosidase and is shown in Fig. 5. In the absence of β-glucosidase, cellulase could hydrolyse 2% w/v of pretreated MCR to produce glucose with a yield of 62.8% in 48 h. However, the addition of free or immobilized β-glucosidase increased the yield of glucose to 79.4% or 71%, respectively. Supplementation with β-glucosidase could evidently relieve product inhibition when the level of sugar produced was more than 30 g/l (Tan and Lee, 0000). Although the enzymatic hydrolysis activity of immobilized β-glucosidase was slightly less than that of free β-glucosidase, but one must remember that immobilized enzyme facilitates easier recovery for repeated used. The lower activity for immobilized β-glucosidase could be because its spherical shape reduce homogeneous distribution within the reaction mixture than that of free β-glucosidase. It could also be due to higher mass transfer resistance that reduce the yield of glucose produced using immobilized compared with free enzyme under identical reaction conditions.

4. Conclusion

β-Glucosidase was successfully immobilized on glutaraldehyde activated κC-PEI polyelectrolyte complex beads through crosslinking method with 98.4% of immobilization yields and 36 U/g of enzyme activity. The enzymatic stability of β-glucosidase against pH and thermal stresses was improved by immobilization. This study provides a novel process for β-glucosidase immobilization and demonstrates an alternative process for sugar production by hydrolysing the cellulosic biomass from macroalgae cellulosic residue.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.10.146.

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

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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 °C) followed by enzymatic hydrolysis (45 FPU/g of cellulase, 52 CBU/g of β-glucosidase, 50 °C, pH 4.8, 30 h). The yield of sugar obtained was found more superior than conventional pretreatment process using H_2SO_4 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.

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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, Lee, Ha, & Park, 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, Mirhosseini, Taip, Ling, & Tan, 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, Sutanto, Huynh, Ismadji, & Ju, 2013; Kootstra, Beeftink, Scott, & Sanders, 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

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

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

2. Methods

2.1. Raw materials and chemicals

E. cottonii were obtained from Futt Put Enterprise (north coast of Sabah, Borneo). The macroalgae was first washed with tap water to remove impurities and dried at room temperature for 24 h. Next 3% (w/v) of *E. cottonii* was boiled at 90 °C for 1 h 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 °C, pulverized, screened through 80 mesh and used for subsequent experiments.

The enzymes used in the enzymatic hydrolysis were commercial cellulase (Celluclast 1.5 L, 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, *S. 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).

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

Property	Dowex (TM) Dr-G8
Shape	Bead
Particle size (μm)	300–1200
Capacity (mequiv./gm)	4.5
Particle density (g/mL)	1.22
Functional group	Sulfonic acid

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 *S. 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 °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 °C for 60 min. In the second step, the reaction mixture was diluted to 4% sulfuric acid and autoclaved at 121 °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 °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 °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 °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 °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 °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 °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, Maache-Rezzoug, Sannier, Rezzoug, & Maugard, 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 °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 °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 °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 °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 to pH 5.0 by 5 M NaOH. The flasks were sealed with rubber stoppers equipped with needles for CO_2 venting. 1.5 mL of sample was withdrawn at different interval time during SSF and was centrifuged at 10,000 × g for 15 min. The supernatant was stored in −2 °C freezer and subjected for sugar and bioethanol analysis.

2.7. Analytical methods

2.7.1. Sugar, by-product and bioethanol analysis

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

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

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 20 M (Supelco, USA). The temperature of the injection unit and detector were 225 °C. The oven was heated to 100 °C for 2 min and the temperature was raised

to 175 °C at a rate of 10 °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, Robinson, Bothast, Saddler, & Mansfield, 2004):

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

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 "$0.51 \times f \times [\text{Biomass}] \times 1.111$" 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

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

2.7.3. Scanning electron microscopy (SEM) analysis

The morphology and the physical structure of the untreated MCR and the MCR treated with solid acid catalyst were observed by scanning electron microscopy (SEM) using a Fei Quanta 450 FEG (Eindhoven, NL). The samples were dried at 50 °C to constant weight and coated with Au/Pd film. All images were taken at a magnification of 10,000× and observed using a voltage of 5 kV.

2.7.4. Brunauer–Emmett–Teller (BET)

The Brunauer–Emmett–Teller (BET) surface area, pore size and pore volume of the untreated and treated MCR was determined using nitrogen adsorption/desorption isotherms at −196 °C in a surface area analyzer (ASAP 2020, Micromeritics Co., USA). Prior to analysis, the sample was degassed for 8 h at 120 °C under vacuum (5 mmHg) to remove moisture and any contaminants. BET surface area was calculated from these isotherms using the BET method. Total volume of pores was determined by single point adsorption total pore volume of pores with P/P_o at 0.984. The average pore size was calculated by Density Functional Theory (DFT) method (Seaton, Walton, & Quirke, 1989) and software provided by Micromeritics Instrument Corporation.

2.7.5. Ammonia temperature programmed desorption (NH_3-TPD)

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

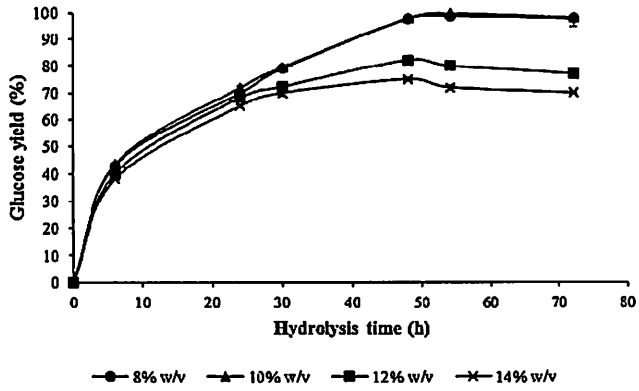

Fig. 1. Effect of biomass loading on enzymatic hydrolysis of MCR. Pretreatment conditions: pretreatment time: 30 min, reaction temperature: 120 °C, catalyst loading: 4% (w/v). Enzymatic hydrolysis conditions: 2% (w/v) pretreated MCR, 15 FPU/g of cellulase, 52 CBU/g of β-glucosidase, pH 4.8, 50 °C.

3. Results and discussion

3.1. Macroalgae cellulosic residue (MCR) composition

Cellulose and acid insoluble lignin were analyzed using the method of two-step acid hydrolysis. The results showed that cellulose fraction comprised 68% and there was 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 °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, Jeong, Shin, & Kim, 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 °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)

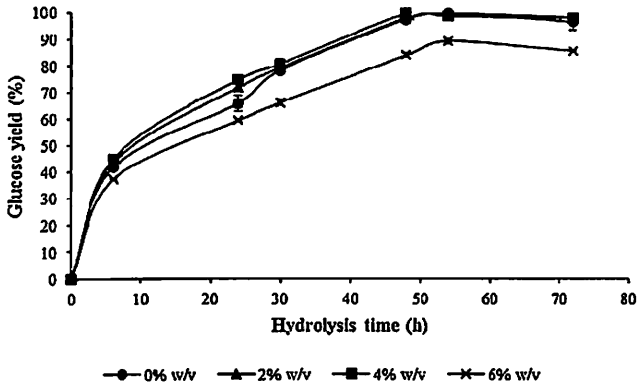

Fig. 2. Effects of catalyst loadings on the pretreatment of MCR in the presence of a heterogeneous catalyst Dowex (TM) Dr-G8. Pretreatment reaction conditions: biomass loading: 10% (w/v), pretreatment time: 30 min, reaction temperature: 120 °C. The enzymatic hydrolysis conditions were the same as that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

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 °C and subsequent enzymatic hydrolysis (50 °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 was increased from 15 min to 30 min. This is because longer pretreatment duration allows sufficient time for the solid acid catalyst to degrade MCR biomass and thus more cellulose site is available for enzymatic

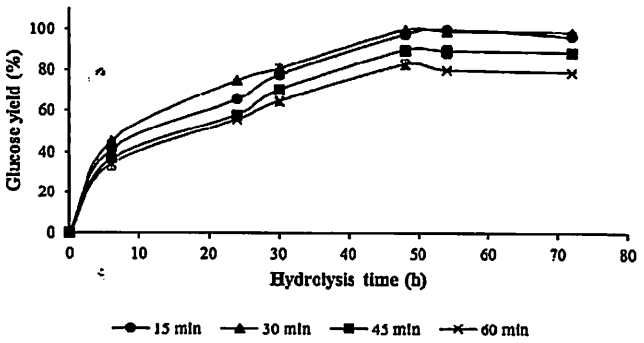

Fig. 3. Effect of the pretreatment time on enzymatic hydrolysis of MCR. Pretreatment conditions: biomass loading: 10% (w/v), reaction temperature: 120 °C, catalyst loading: 4% (w/v). The enzymatic hydrolysis conditions were the same as that in Fig. 1.

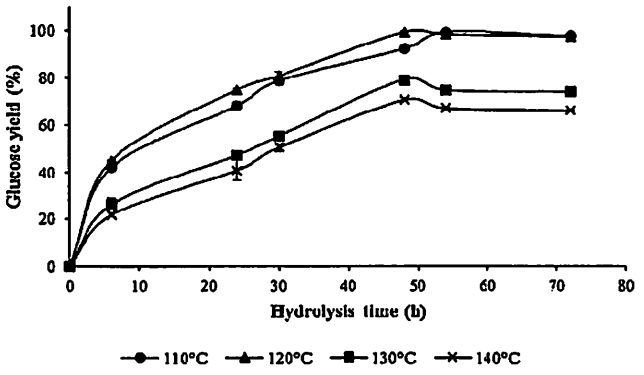

Fig. 4. Effect of the pretreatment temperature on enzymatic hydrolysis of MCR. Pretreatment conditions: biomass loading: 10% (w/v), pretreatment time: 30 min, catalyst loading: 4% (w/v). The enzymatic hydrolysis conditions were the same as that in Fig. 1.

hydrolysis. However, further increase in pretreatment time gave a negative effect on the glucose yield. The glucose yield decrease to 82.8% (48 h hydrolysis time) when the pretreatment time was prolonged to 60 min. This is because longer pretreatment time might degrade the cellulose and release soluble glucose from MCR during the pretreatment process itself, which led to less amount of cellulose that is available for enzymatic hydrolysis and hence a decrease in glucose yield. This finding is supported by Harun and Danquah (2011), where they reported that increasing pretreatment time up to 60 min does not increase the hydrolysis process of microalgae but instead pose a risk in reducing the production of bioethanol. Besides that, shorter duration for pretreatment process is actually favorable because it has a positive impact on energy consumption. Therefore, 30 min of pretreatment time was chosen as the optimum value for treating macroalgae cellulosic residue (MCR) (Harun & Danquah, 2011).

3.2.4. *Effect of pretreatment temperature*

The effect of pretreatment temperature on the enzymatic hydrolysis is presented in Fig. 4. When the pretreatment temperature was increased from 110 to 120 °C, the glucose yield of pretreated macroalgae cellulosic residue (MCR) improved significantly. This shows that MCR pretreated at higher temperature was more acquiescent to enzymatic hydrolysis. After 48 h of enzymatic hydrolysis, the glucose yield attained was 99.8% after being treated at 120 °C, which increased over 7.2% in comparison with that at 110 °C. This phenomenon could be explained as high pretreatment temperatures facilitate cellulose fibers to dissolve faster and making more cellulose accessible to enzyme attack. In other words the adsorption rate of enzyme onto the

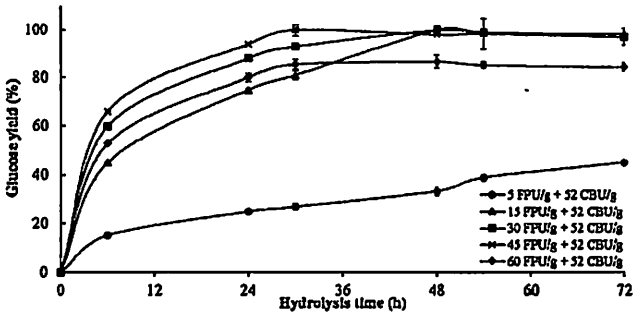

Fig. 5. Effect of different enzyme loadings on hydrolysis of pretreated MCR. Enzymatic hydrolysis conditions: 50 °C, 72 h, 120 rpm at 2% (w/v) of pretreated MCR in 50 mM sodium acetate buffer at pH 4.8.

macroalgae cellulosic residue surface increased at higher pretreatment temperature which leads to higher hydrolysis rate. However, when the pretreatment temperature was increased more than 120 °C, the glucose yield decreased significantly. For example, at 140 °C pretreatment temperature, the glucose yield obtain was only 66.5% even at the longest hydrolysis time. This is again possibly due to partial cellulose degradation during pretreatments under harsh conditions. Harun and Danquah (2011) also reported that acid pretreatment temperature above 140 °C gave a significant decrease in the amount of reducing sugars from microalgae biomass. Therefore, 120 °C is the optimum pretreatment temperature and it is worthwhile to note that this optimum temperature is relatively milder compared to that of terrestrial biomass (Jung, Kim, Kim, & Kim, 2013; Tucker, Kim, Newman, & Nguyen, 2003). Up to this point, the optimum pretreatment condition for macroalgae cellulosic residue (MCR) is 4% (w/v) Dowex (TM)-Dr G8, 120 °C, and 30 min. At this condition, no degradation of cellulose was detected.

3.3. Enzymatic hydrolysis

3.3.1. Effect of enzyme loading

For evaluation on the effect of enzyme loading on MCR pretreated with Dowex (TM) Dr-G8, samples were subjected to enzymatic hydrolysis with 5–60 FPU/g of 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 contribute to a lower glucose yield (Singh, Kumar, Bishnoi, & Bishnoi, 2009; Zheng, Pan, Zhang, & Wang, 2009). This was supported by the findings shown in Fig. 5 whereby at hydrolysis time of 30 h, glucose yield decreased by 14.4% when the cellulase loading was increased from 45 to 60 FPU/g.

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

3.3.2. Effect of different pretreatment methods

In order to further understand the digestibility of the macroalgae cellulosic residue (MCR) pretreated with Dowex (TM) Dr-G8, MCR was also pretreated with two other methods (H_2SO_4 and NaOH) and the content of protein in the supernatant and glucose yield were determined, as summarized in Table 2. It is well established that the adsorption of cellulase on substrate is a prerequisite for enzymatic hydrolysis of cellulose to occur. Among the different pretreated substrates evaluated in this study that were subjected for enzymatic hydrolysis, the MCR pretreated with Dowex (TM) Dr-G8 were observed to be more vulnerable to enzymatic hydrolysis and gave the highest glucose yield (99.8%). Therefore, it could be speculated that for the same cellulase loading, MCR pretreated with Dowex (TM) Dr-G8 would adsorb significantly more cellulase than MCR 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, Chamorro, Romano, Felissia, & Area, 2013; Sambusiti, Ficara, Malpei, Steyer, & Carrère, 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 Section 3.3.3.

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

Substrates	T (°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

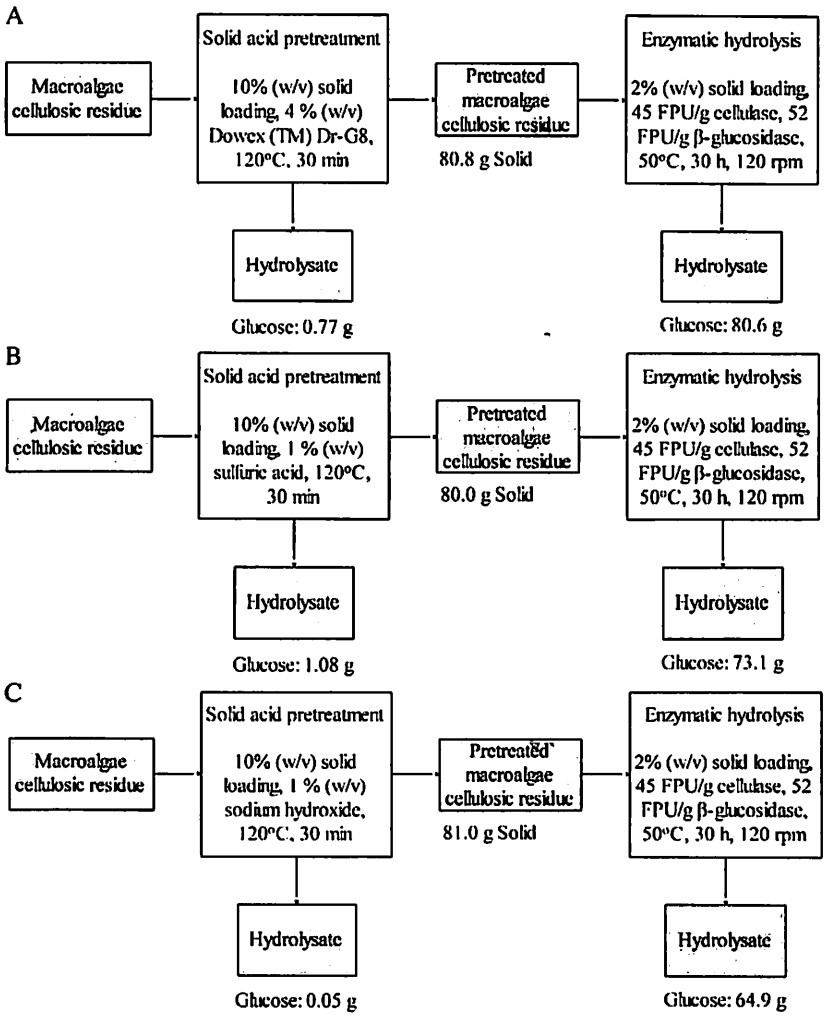

Fig. 6. Overall mass balance for (A) solid acid, (B) dilute acid, and (C) sodium hydroxide pretreatment.

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

3.4. *Simultaneous saccharification and fermentation (SSF)*

In the previous section, the effect of pretreatment using Dowex (TM) Dr-G8 was only evaluated up to the stage of glucose yield. Thus, in this section, the use of SSF process for the production of

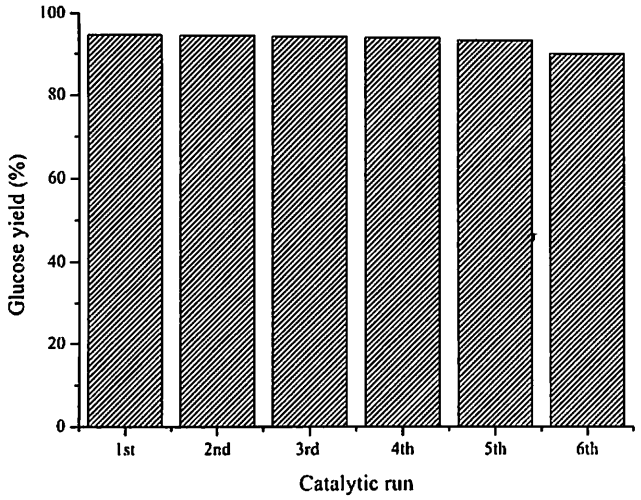

Fig. 7. Performance of the recycled catalyst at repeated runs. Pretreatment conditions: catalyst loading: 4% (w/v), s/l ratio: 4% (w/v), pretreatment time: 30 min, reaction temperature: 120 °C.

bioethanol from MCR pretreated with Dowex (TM) Dr-G8 will be presented. For any bioethanol from biomass production process, it is important to produce high concentration of bioethanol due to the energy intensive nature of distillation and dehydration process (Ahmed, Nguyen, Huynh, Ismadji, & Ju, 2013). In order to achieve a high final bioethanol concentration, a high substrate loading, and hence a high cellulose content, is crucial for the SSF process to be economically viable. The effect of solid loading (pretreated MCR) on bioethanol production using the SSF process with prehydrolysis was carried out at 43 °C and the results are shown in Fig. 8. It is important to differentiate between conventional SSF and SSF process with prehydrolysis. SSF with prehydrolysis has the potential to significantly improve the bioethanol yield because this will facilitate more effective stirring at the beginning of the process (Hoyer, Galbe, & Zacchi, 2013). Fig. 8 indicates that the glucose accumulated during prehydrolysis was rapidly consumed after the inoculation of *S. cerevisiae*. In general, the glucose concentration accumulated during prehydrolysis increased with the higher solid loading, 37.8, 65.3, 85.5, and 110.6 g/L corresponding to solid loading of 4%, 6%, 8% and 10% (w/v), respectively. The glucose concentration was found to decrease as the SSF process progressed which was accompanied by a rapid increase in the fermentation product. The glucose was totally consumed within 3 h after inoculation and then remained at a low level for all solid loading, indicating that fermentation by the yeast was much faster that enzymatic hydrolysis. Therefore, the rate-limiting step in SSF process for bioethanol production is the enzymatic hydrolysis of the pretreated macroalgae cellulosic residue (MCR) into glucose, rather than the fermentation of the reducing sugar to bioethanol by yeast. Although the bioethanol concentration was found to generally increase with higher solid loadings, but the percentage of theoretical yield was found to generally drop with higher solid loadings. At the highest loading of 10% (w/v), the highest theoretical yield achieved was only 31.1%. In fact, it was found that the lag phase (results not shown) for the highest solid loading of 10% (w/v) reached almost 4 h. This lag phase is due to the adaptation of the yeast to fermentation conditions and its duration is related with the solid loading. The low theoretical yield at high solid loading could be due to insufficient enzyme and furthermore the high initial sugar concentration at higher solid loading could inhibit the enzyme leading to low cellulose hydrolysis. In addition, high solid loading can also cause mixing problems due to higher viscosity of the mixture that lead to mixing problem at the beginning of the SSF process. A similar result was also reported for pretreatment of macroalgae by Lee et al. (2013). The substrate loading could not be increased beyond the maximum of 6% due to high hygroscopic properties of macroalgae. Thus for this study, SSF process loaded with 4% (w/v) pretreated

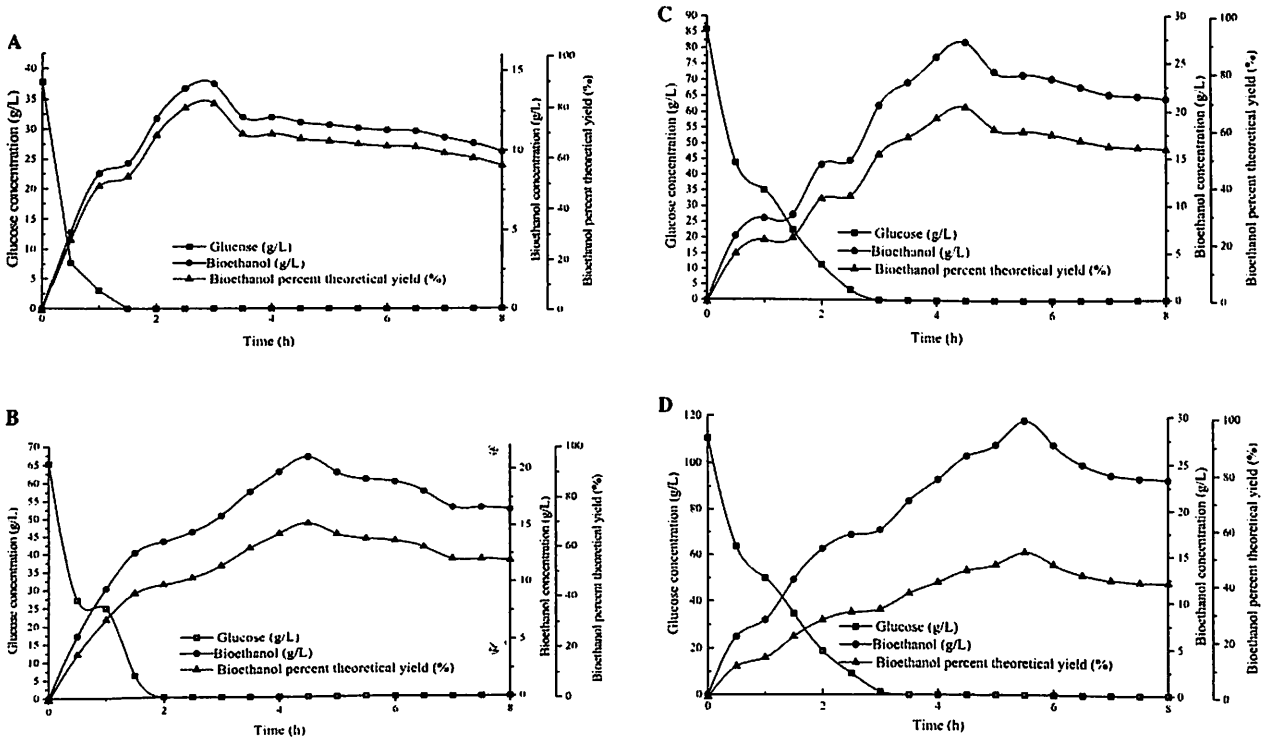

Fig. 8. Profile of bioethanol production and glucose consumption for 8 h SSF of pretreated MCR suspended in deionized water at different solid loadings. (A) 4% (w/v), (B) 6% (w/v), (C) 8% (w/v) and (D) 10% (w/v). Conditions: enzyme loading of 45 FPU/g of cellulase and 52 CBU/g of β-glucosidase, 50 °C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

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

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

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

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

3.5. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) analysis revealed the morphological features and surface characteristic of MCR pretreated with Dowex (TM) Dr-G8 compared with the untreated MCR. For the untreated MCR, there were highly ordered 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).

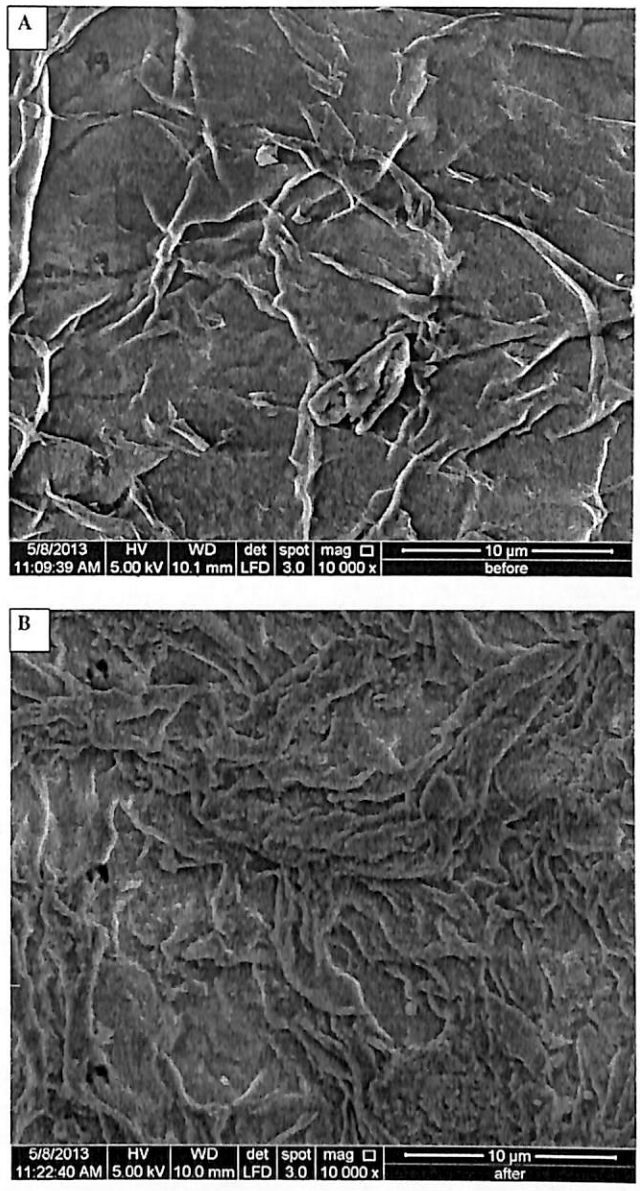

Fig. 9. Scanning electron micrographs of (A) untreated, (B) solid acid pretreated MCR under optimized conditions.

3.7. NH_3-TPD measurement

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

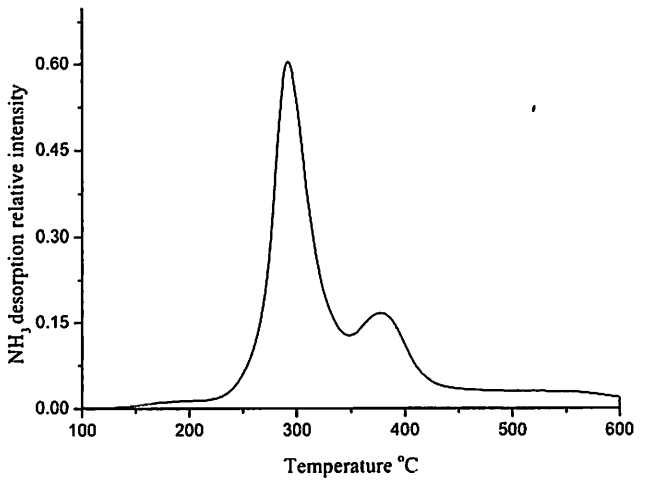

Fig. 10. Temperature programmed desorption (TPD) profiles of NH_3-TPD for fresh Dowex (TM) Dr-G8 (B).

4. Conclusion

This study successfully demonstrated a novel pretreatment method coupled with SSF process for the conversion of macroalgae cellulosic residue (MCR) to bioethanol. The conversion of MCR to bioethanol would simultaneously provide a more sustainable waste management system. The catalyst was found capable of being used repeatedly with minimal drop in activity making it a green process. Thus, this study have pave the way for utilizing macroalgae cellulosic residue by converting it to third-generation bioethanol.

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Comparison of different process strategies for bioethanol production from *Eucheuma cottonii*: An economic study

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HIGHLIGHTS

- Red macroalgae *Eucheuma cottonii* was used as feedstock for bioethanol production.
- A combined chemical and enzymatic process is more effective than other hydrolysis schemes.
- A techno-economic evaluation shows the feasibility of the process.

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ABSTRACT

The aim of this work was to evaluate the efficacy of red macroalgae *Eucheuma cottonii* (EC) as feedstock for third-generation bioethanol production. Dowex (TM) Dr-G8 was explored as a potential solid catalyst to hydrolyzed carbohydrates from EC or macroalgae extract (ME) and pretreatment of macroalgae cellulosic residue (MCR), to fermentable sugars prior to fermentation process. The highest total sugars were produced at 98.7 g/L when 16% of the ME was treated under the optimum conditions of solid acid hydrolysis (8% (w/v) Dowex (TM) Dr-G8, 120 °C, 1 h) and 2% pretreated MCR (P-MCR) treated by enzymatic hydrolysis (pH 4.8, 50 °C, 30 h). A two-stream process resulted in 11.6 g/L of bioethanol from the fermentation of ME hydrolysates and 11.7 g/L from prehydrolysis and simultaneous saccharification and fermentation of P-MCR. The fixed price of bioethanol obtained from the EC is competitive with that obtained from other feedstocks.

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1. Introduction

Concerns about fluctuations in oil prices, greenhouse gas emissions, depletion of fossil fuels and energy insecurity have stimulated global efforts in searching for more sustainable energy sources (Ogunkoya et al., 2015). Bioethanol can be derived from biomasses via different reaction pathways and has been recognized as a potential alternative to petroleum-based transportation fossil fuels (Vohra et al., 2014). First-generation bioethanol that is mainly made from crop biomass has significantly affected the world economy because of the competition for energy and food (Nigam and Singh, 2011). Second-generation bioethanol can be produced from lignocellulosic wastes. However, the delignification of lignocellulosic waste has become an obstacle to be solved. Different chemical pretreatment methods are used to increase cellulose accessibility (López-Linares et al., 2015; Monavari et al., 2011). Therefore, production of reducing sugars from lignocellulosic biomass tends to be complex and capital intensive. Thus, terrestrial biomass-based bioethanol seems to not be sustainable at present because of environmental as well as economic impacts. Interestingly, third-generation bioethanol based on algae biomass is considered to be a more viable feedstock over previous generations (Goh and Lee, 2010).

Recently, red macroalgae biomass has been proposed as a candidate for a promising alternative renewable energy source to substitute fossil fuels because of their special characteristics including the absence of or a very low lignin content and high carbohydrates (Jeong et al., 2012; Kim et al., 2015; Tan and Lee, 2015). Moreover, macroalgae cultivation does not invade arable land. *Eucheuma cottonii*, a red macroalgae, is one of the most abundant species along the east coast of Sabah (Malaysia). It has a high carbohydrate content and can be hydrolyzed to fermentable sugars such as glucose and galactose, which are then fermented to ethanol. However, the conversion technology for the production of bioethanol from a macroalgae biomass is at an early stage. Thus, the carbohydrate content in macroalgae requires new economic treatment methods to make the monomers available for fermentation.

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The conventional methods used to convert polysaccharides into reducing sugars include chemical and enzymatic method. In general, dilute acids are typically used as the catalyst for chemical hydrolysis, while enzymes are used as the catalyst for enzymatic hydrolysis. Since the performance of hydrolysis process is affected by chemical compositions (in particular carbohydrate content) of biomass, it is of great importance to obtain its composition to effectively use them as carbon source for bioethanol production. For example, *E. cottonii* (EC) contains both cellulose and κ-carrageenan. The cellulose content of these red macroalgae can be easily converted into reducing glucose via the enzymatic hydrolysis process by using a mixture of cellulase and β-glucosidase (Tan and Lee, 2014). However, κ-carrageenan content cannot be easily hydrolyzed by the enzyme because of their ability to bind with water effectively, thus forming weak water gels which are very stable against enzymatic degradation. Recently, several studies reported bioethanol production from *Kappaphycus alvarezii* (*E. cottonii*) using sulfuric acid as the catalyst (Abd-Rahim et al., 2014; Hargreaves et al., 2013). Nevertheless, these methods have major drawbacks in product separation, reactor and equipment corrosion, and waste effluent treatment (Guo et al., 2012). Many catalytic systems have not been commercialized because of the difficulty in separating homogeneous catalysts from product solutions. The utilization of a solid acid catalyst has the potential to overcome some of the above limitations because of the ease of catalyst separation. To date, some reviews concerning the hydrolysis of cellulose using solid acid catalysts have been reported (Huang and Fu, 2013). Apart from the technical aspects of bioethanol production from macroalgae, this work also investigates production costs. Economic analysis has been used as a promising tool to assist the biofuels research community in identifying key cost drivers, evaluating novel technologies and assessing new process configurations (Tunà and Hulteberg, 2014). However, whether bioethanol produced from macroalgae will be economically feasible remains unknown.

The main objective of this research study is to minimize inhibitor formation and enhance reducing sugar production; thus, a combined process of solid acid and enzymatic hydrolysis was evaluated. Accordingly, we report a practical approach for using a strong acidic solid catalyst, Dowex (TM) Dr-G8, to hydrolyze EC (whole biomass or macroalgae extract), pretreatment of macroalgae cellulosic residue (MCR) and enzymatic hydrolysis of fractionated EC (f-EC) or pretreated MCR (P-MCR) to produce a high quality hydrolysate. The subsequent work is to compare different fermentation process configurations for the production of bioethanol from EC hydrolysate. Finally, these laboratory-derived primary data have been used to estimate the production cost of bioethanol from EC.

2. Methods

2.1. Raw materials and chemicals

The *E. cottonii* (red macroalgae) that was used in this study was purchased from Futt Put Enterprise (north coast of Sabah, Malaysia). The red macroalgae was first washed in water to remove salts and dirt and then was dried at 40 °C and milled through a 300-mesh screen. The sample obtained was stored in a clean airtight container at room temperature until further use.

Strong acid cation-exchange resin Dowex (TM) Dr-G8, calcium hydroxide, standard D-galactose, D-glucose, 5-hydroxyl-methyl furfurals (5-HMF), *Saccharomyces cerevisiae* (YSC2, type II) yeast extract, peptone, and dextrose were purchased from Sigma–Aldrich (USA). Sulfuric acid, potassium dihydrogen phosphate and ethanol were purchased from Fisher Scientific (UK). The enzymes used for hydrolysis were Novozyme 188 (263 CBU/g) and Celluclast 1.5 L (798 EGU/g) (Novozyme, Denmark), which were obtained from Science Technics Sdn. Bhd (Malaysia). All reagents were of analytical grade. Table S1 shows the properties of Dowex (TM) Dr-G8.

All experiments were performed in triplicate, and the data reported are the average of the three replications.

2.2. Biomass preparation

Three percent (w/v) of dry *E. cottonii* (EC) was boiled at 90 °C for 2 h, and the hot extract (macroalgae extract) was filtered (45 μm mesh size) from residue, dried to constant weight at 40 °C and pulverized (Tan et al., 2013). Then, the residue was boiled for 30 min with 2 L of distilled water and then was filtered using a pressure pump. The residue is now named as macroalgae cellulosic residue (MCR) was then dried at 40 °C, pulverized, screened through an 80-mesh screen; the result was used for subsequent experiments.

2.3. Chemical compositional analysis

The total carbohydrate content of *E. cottonii* (EC), macroalgae extract (ME), and macroalgae cellulosic residue (MCR) was determined according to a modified method that is based on analytical methods developed at the National Renewable Energy Laboratory (NREL, Golden, CO) using a two-step acid hydrolysis procedure (Sluiter et al., 2008). Samples of approximately 300 mg (dry basis) were initially subjected to a primary 72% sulfuric acid hydrolysis, followed by a secondary dilute-acid hydrolysis. The resulting concentrations of dissolved reducing sugar were determined by HP-LC (high-performance liquid chromatography), as described in Section 2.6.1. Crude protein content in EC, ME and MCR was determined according to (Method 988.05) from the Association of Official Analysis Chemists (AOAC, 2000). Crude fat was determined by the Soxhlet extraction method (Method 920.30) (AOAC, 2000). Ash content was determined by weighing samples before and after heating in a furnace at a temperature of 550 °C for 1 h. Moisture content was also determined using analytical methods established by the National Renewable Energy Laboratory (NREL, Golden, CO) (Sluiter et al., 2008).

2.4. Hydrolysis of macroalgae biomass

An overview of the experimental works carried out in this study is illustrated in Fig. S2.

2.4.1. Solid acid hydrolysis

The hydrolysis reaction was carried out in a Teflon-lined stainless steel autoclave (200 mL). Fig. S1 shows a schematic diagram of the experimental apparatus for the hydrolysis. Optimization of the thermal solid acid hydrolysis conditions was carried out with a 2–10% (w/v) Dowex (TM) Dr-G8, 110–140 °C reaction temperature, 8–20% (w/v) biomass loading (solid/liquid ratio), and 0–2.5 h reaction time. The dried intact EC or ME were subjected to distilled water to form different solid/liquid ratios. The mixture was then mixed with different amounts of Dowex (TM) Dr-G8. The autoclave reactor was then closed and heated by an electrical heater to the desired temperature. The temperature in the autoclave reactor in each experiment was controlled by a PID temperature controller. The stirring speed for all experiments was maintained at 370 rpm while the internal pressure of the autoclave was kept constant at 10 bars. After completion of the reaction, the autoclave reactor was cooled to room temperature. The catalyst and residue substrates were separated by filtration. The filtrate was analyzed for soluble sugar and by-product content using high-performance liquid chromatography (HPLC). After optimization of the solid acid

hydrolysis conditions, the residue substrates were washed with water until a neutral pH of the wash water was obtained; then, the residue substrates were dried at 60 °C in a hot air oven until a constant weight was attained. The dried fractionated EC (f-EC) was then used for further enzymatic hydrolysis under the conditions that were optimized earlier (Tan and Lee, 2014). Similarly, dilute-acid and alkali hydrolysis methods were also individually employed to hydrolyze the mentioned feedstocks for comparison (Section 2.3.).

2.4.2. Pretreatment and enzymatic hydrolysis

Macroalgae cellulosic residue (MCR) was cellulose extracted from the same macroalgae (EC). About 10% (w/v) of MCR was pretreated using 4% (w/v) Dowex (TM) Dr-G8 at 120 °C for 45 min. Once the pretreatment process was completed, the residues were separated from liquid by filtration using filter paper. Subsequently, the residual substrates is now named as pretreated macroalgae cellulosic residue (P-MCR) was then dried at 40 °C, pulverized, screened through an 80-mesh screen. EC biomass pretreated with optimum reaction conditions described in the Section 2.4.1 or P-MCR were selected for enzymatic hydrolysis. The f-EC or P-MCR were hydrolyzed enzymatically under the conditions optimized earlier (Tan and Lee, 2014). The biomass was hydrolyzed in a citrate buffer (pH 4.8, 50 mM) with a solid consistency of 2% (w/v). The enzyme loading of cellulase and β-glucosidase were 45 FPU/g and 52 CBU/g, respectively. All enzymatic hydrolysis runs were performed in shake flasks with 50 mL working volumes; the flasks were placed in a shaking incubator at 50 °C at 150 rpm for 72 h. At different time intervals, samples were withdrawn, boiled at 100 °C for 10 min to de-activate the enzyme, and centrifuged at 10,000 rpm for 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 (HPLC).

2.4.3. Combined severity factor (CSF)

For solid acid catalyzed hydrolysis, a calculated combined severity factor (CSF) was used to integrate the effects of catalyst loading, reaction temperature and times into a single parameter, calculated as in Eq. (1) (Chum et al., 1990):

$$\mathrm{CSF} = \log\left\{t \cdot \exp\left[\frac{T_H - T_R}{14.75}\right]\right\} - \mathrm{pH} \quad (1)$$

where t is the reaction time (h), T_H is the reaction temperature (°C), T_R is a reference temperature of 100 °C, 14.75 is the fitted value of the arbitrary constant, and pH is the acidity of the aqueous solution in terms of solid acid concentration.

2.5. Fermentation process configurations

2.5.1. Cell cultivation

S. cerevisiae (YSC2, type II) was used for fermentation assays. Yeast inocula were grown in a 250 mL Erlenmeyer flask with 50 mL of sterile culture medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) galactose, and 0.175% (w/v) potassium dihydrogen phosphate. The preculture was incubated at 35 °C in a shaking incubator (Benchmark Scientific Inc., New Jersey) with shaking at 130 rpm for 24 h. The liquid cultures had an initial cell dry weight ranging from 16 mg/mL to 17.5 mg/mL. Yeast cells were then harvested by centrifugation (10,000 × g, 15 min, 5 °C) and were washed with 1% v/v phosphoric acid.

2.5.2. Two streams model

The fermentation of macroalgae extract (ME) hydrolysates obtained from solid acid hydrolysis (optimum condition from Section 2.4.1) was performed in a 250 mL Erlenmeyer flask. The basal medium consisted of 0.175% KH_2PO_4. The fermentation broth consisted of the hydrolysate and basal medium at a ratio of 1:2; the broth was adjusted to pH 5 with sodium hydroxide. The aforementioned mixture was incubated with 16 mg/mL (initial cell dry weight) of the inocula that was prepared above. The fermentation was conducted in a shaking incubator at 35 °C with gentle shaking at 130 rpm for a total time of 96 h.

Prehydrolysis and simultaneous saccharification and fermentation (PSSF) medium was prepared with the following steps: 5% (w/v) of pretreated macroalgae cellulosic residue (P-MCR) (see Section 2.4.2) was prehydrolyzed for 24 h in citrate buffer (50 mM, pH 4.8) at 50 °C in a 250 mL Erlenmeyer flask with a 50 mL working volume. Cellulase and β-glucosidase were applied as described for enzymatic hydrolysis (Section 2.4.2). Afterwards, the temperature was decreased to 43 °C for an additional 8 h to allow for the SSF process. SSF was started by adding yeast inoculum (initial cell dry weight 17.5 mg/mL) and 0.175% KH_2PO_4. The flask was incubated in a shaking incubator at 130 rpm.

At selected time intervals, samples taken from the fermentations were centrifuged at 10,000 × g for 15 min at 5 °C. The supernatant obtained after centrifugation was determined for ethanol and residual sugar concentration using HP-LC and GC, respectively.

2.5.3. Integrated model

The prehydrolysis and simultaneous saccharification and co-fermentation (PSSCF) experiment was carried out in a sterilized 250 mL Erlenmeyer flask in a shaking incubator at 130 rpm. Prehydrolysis was carried out before inoculation for 24 h at 50 °C. The galactose-containing hydrolysate (optimum condition from Section 2.4.1) was diluted 2-fold with citrate buffer (50 mM, pH 4.8) that consisted of 0.175% KH_2PO_4. The 5% (w/v) P-MCR (see Section 2.4.2) was suspended in the solution. The cellulase and β-glucosidase were applied as described for enzymatic hydrolysis (Section 2.4.2). Afterward, the temperature was decreased to 43 °C and the flask was inoculated with harvested yeast at the same concentration as in the PSSF process for 96 h.

Samples for measuring sugar content and bioethanol concentration were obtained at set times during fermentation. The sugar and bioethanol concentrations were determined by using HP-LC and GC, respectively.

2.6. Experimental analysis

2.6.1. Analysis of hydrolysis products

The glucose, galactose, and fermentation inhibitors concentrations such as 5-hydroxymethylfurfural (5-HMF) in the hydrolysate were determined using an Agilent series 1200 infinity high performance liquid chromatography system (HPLC) outfitted with a Hi-Plex Ca column (300 mm × 7. 7 mm) and a 385-ELSD (evaporative light scattering detector). The analysis was performed with distilled–deionised water in the mobile phase at an isocratic flow rate of 0.6 mL/min and injection volume of 20 μL. Purified nitrogen was used as the carrier gas (70 psi) for the detector. The HPLC-ELSD's spray chamber temperature was set at 40 °C and the detector temperature was set at 80 °C. All samples were properly diluted and filtered through a 0.20 μm syringe-filter before analysis to remove particles. Quantitative analysis was performed using a calibration with external standards of known concentrations. The yields of the acid hydrolysis (Eq. (2)) and enzymatic hydrolysis (Eq. (3)) were calculated as follows:

$$\text{Yield } \% = \frac{\text{Concentration(g/l)of sugar at time of } t}{\text{Initial concentration(g/l)of substrate}} \times 100\% \quad (2)$$

$$Y_E\ (\%) = \frac{\Delta S_g}{C} \times 100 \quad (3)$$

where Y_E is the glucose yields of enzymatic hydrolysis (%), ΔS_g is the glucose increase (g/L) during enzymatic hydrolysis, C is the initial concentration of the substrate (g/L).

2.6.2. Analysis of bioethanol

The bioethanol that was produced was analyzed by a gas chromatography-flame ionization detector (GC-FID) (5890 Series II, Hewlett Packard, Palo Alto, CA). The column used was an 80/120 mesh carbopack B-DA/4% Carbowax 20 M (Supelco, USA) that measured 2 m in length and 0.2 cm in internal diameter. Detector and injection port temperatures of 225 °C each were used during the operation of the GC. The oven temperature was set to 100 °C for 2 min and then was increased to 175 °C at a rate of 10 °C/min. The carrier gas was helium, and an injection volume of 2 μl was used while 0.5% (v/v) 2-pentanone was used as the internal standard. Bioethanol yield and percent theoretical yield were calculated based on the following equations, respectively (Keating et al., 2004):

$$Y_{P/S} = \frac{[EtOH]_{max}}{[Sugar]_{ini}} \quad (4)$$

$$Y_{\%T} = \frac{Y_{P/S}}{0.51} \times 100\% \quad (5)$$

where $Y_{P/S}$ = ethanol yield (g/g), $[EtOH]_{max}$ = highest ethanol concentration achieved during fermentation (g/L), $[Sugar]_{ini}$ = total initial sugar concentration at onset of fermentation (g/L), $Y_{\%T}$ = percent theoretical yield (%), 0.51 is the maximum ethanol yield per unit of hexose sugar from glycolytic fermentation (g/g), P = Product and S = Substrate.

2.6.3. FTIR spectroscopy

The raw and pretreated EC biomasses were characterized for changes in structural composition. An FTIR spectrometer (IR-Prestige-21, Shimadzu, Japan) was used to characterize the samples, which were prepared by mixing a biomass sample and KBr in a ratio (w/w) of 1:100. The mixtures were ground well, and the spectra were recorded in the range of 500–4000 cm^{-1} using 200 mg of biomass + KBr mixture in the form of pellets.

2.7. Techno-economic aspects of bioethanol production from E. cottonii (EC) biomass in Sabah, Malaysia

An economic analysis (with 2009 as the reference year) was performed to evaluate the production of bioethanol from EC by using a two streams model (Section 2.5.1). Processing costs of bioethanol from EC can be categorized into four groups: feedstock cost, capital cost, operating costs and maintenance costs. The unit cost of bioethanol production can be calculated by the equation:

$$C_{Et} = C_F + C_{O\&M} + C_I \quad (6)$$

where C_{Et} is the bioethanol production cost (RM/L), C_F is the feedstock cost (RM/L), $C_{O\&M}$ is the operating and maintenance cost (RM/L) and C_I is the investment cost (RM/L).

2.7.1. Estimation of E. cottonii feedstock costs (C_F)

The average price of *E. cottonii* (EC) during the year was found to be RM 0.25/kg. With the yield of bioethanol from EC biomass at 393198.1 L/day as shown in Table S2, the cost of EC for 1 L of bioethanol produced can be estimated. It was found that the EC feedstock cost for bioethanol production was RM 0.24/L of bioethanol. The raw materials cost is the single largest expense item of the bioethanol production process. Hence, an accurate estimate of this value is indispensable to predict the total bioethanol cost.

2.7.2. Operating cost ($C_{O\&M}$)

In this study, the operating cost includes variable and fixed operating costs. Variable costs including all consumable materials for production such as raw material costs, electricity, machine repair and maintenances, administration, etc., were estimated and adjusted to the prices in the base year 2009. The administration expenses were assumed to be a fixed percentage of the total labor cost. For operating and maintenance costs and the plant's insurance, each item could also be assumed as a fixed percentage of the annualized capital cost. The operating cost per liter of bioethanol can be calculated by dividing the total annual operating costs by the annual capacity of the bioethanol production plant (see details in Table S2).

2.7.3. Estimation of capital investment cost (C_I)

The capital investment cost comprises all the initial costs of machines and equipment procurements and their installations. The installed equipment costs were estimated by scaling the base equipment costs from the same equipment that was reported in the NREL process that was based on the appropriate metric and applying an appropriate scaling factor:

$$Cost_{new} = Cost_{base}\left(\frac{Capacity_{new}}{Capacity_{base}}\right)^{scaling\ factor} \quad (7)$$

where the scaling factor is the scale exponent adopted from Humbird and Aden (2009). A summary of the main equipment costs that were used in this study is given in Table S3 of Supplementary data. A breakdown of the total capital investment cost of the bioethanol as a unit cost was then calculated by amortization that was recovered over the lifetime of the plant. This can be obtained by a common annualized capital cost equation:

$$A = P\left[\frac{i(1+i)^n}{(1+i)^n - 1}\right] \quad (8)$$

where A is the annual payments (RM/year), P is the present worth of the first investment cost (RM), i is the annual interest rate and n is the project life in years. The annualized capital investment cost is then divided by the annual amounts of bioethanol that are produced to obtain the capital cost per liter of the bioethanol.

2.7.4. Calculation assumptions

The basic economic analysis that was performed was based on the following assumptions. Fresh EC is assumed to be the feedstock for bioethanol production by a bioethanol plant with a capacity of 3.9×10^5 L/day to produce anhydrous fuel grade alcohol. The plant is assumed to operate 330 days/year. The project life is assumed to be 20 years, and the interest rate of this investment is 6%/annum. Prices in the year 2009 were chosen as the base year for the analysis in this study. Table S4 shows the yield of bioethanol from EC (MCR and ME) and other general technical assumptions that were used in this study.

3. Results and discussion

3.1. Proximate analysis of red macroalgae

The chemical composition of raw *E. cottonii* (EC), macroalgae extract (ME), and macroalgae cellulosic residue (MCR) are summarized in Table S5. The initial composition of raw EC was determined to be: 8.20 ± 0.52% cellulose (presented as glucose), 51.15 ± 0.32% κ-carrageenan, 5.25 ± 0.39% protein, 0.23 ± 0.91 lipid, and 20.40 ± 0.41% ash. The carbohydrate, protein, lipid, and ash

contents of ME were found to be 75.70 ± 0.38%, 3.81 ± 0.12%, 0.23 ± 0.22%, and 20.26 ± 0.24%, respectively. MCR was found to contain a total carbohydrate content of 99.8 ± 0.15%, 0.06 ± 0.53% of protein, and 0.14 ± 0.13% of ash. Total carbohydrates accounted for approximately 59.35–99.80% the dry material, which makes EC a very promising substrate for bioethanol production.

To provide a more precise characterization of the polysaccharide fractions in EC, FTIR analysis was performed in the range of 400–4000 cm^{-1}. The FT-IR spectra of raw EC are shown in two bands in the 4000–2000 cm^{-1} range, a broadly intense stretched peak at approximately 3415 cm^{-1} and a weak band at approximately 2926 cm^{-1}; these are characteristic absorption bands of hydroxyl groups and C–H bonds in the polysaccharide molecule, respectively (Fig. S3a). The peaks located at 1621 cm^{-1} correspond to the amide I band and are attributable to protein. The peaks located at 1450 cm^{-1} correspond to the O–H bend of carboxylic acids. Distinct and intense peaks that are observed at 1260 and 1159 cm^{-1} could be attributed to the C–O stretchings of alcohol and ester, respectively. The intense band of α-1,3 and β-1,4-glycosidic bonds in κ-carrageenan occurred at 1065 cm^{-1}. A relatively strong band at approximately 930 cm^{-1} indicated the presence of 3, 6-anhydro-D-galactose (DA) in raw EC (Pereira et al., 2009), while those at 845 cm^{-1} corresponded to galactose-4-sulfate (G4S) with moderate intensity in the FTIR spectra. These two absorption bands are characteristically found in FTIR spectra of κ-carrageenan standards (Sahu et al., 2011). Thus, these results confirm that EC is essentially a carrageenophyte with κ-carrageenan.

The ME obtained by hot water extraction from EC is composed by major quantities of strongly gelling κ-carrageenan. In this work, the FTIR spectra of macroalgae extract show the main features of κ-carrageenan as summarized by Pereira et al. (2009). The spectra of these samples also show the bands at approximately 930 and 845 cm^{-1}, with the same intensity pattern as in EC (Fig. S3b). The chemical composition previously studied in the red macroalgae *E. cottonii* (EC) suggests that the structure of κ-carrageenan contains the basic unit of D-typed galactose with galactose that can be fermented by yeast (Meinita et al., 2012). FTIR spectra obtained in the present study would confirm the presence of galactose in raw EC.

As shown in Fig. S3 (c), the characteristic bands of MCR were found to be similar to earlier reported FTIR spectra of cellulose in the literature (Siddhanta et al., 2011). A strong band at 1437 cm^{-1} defined the cellulose allomorph as cellulose I (Dayal et al., 2013). The absorbance band at these bands is assigned to symmetric CH_2 bending vibrations. An increase in the intensity of OH in plane bending vibration at the 1387 cm^{-1} band is observed; this band is specific to cellulose (Bodirlau et al., 2008). The presence of absorption bands at 1159 cm^{-1} in the FTIR spectra of MCR results from C–O–C stretching vibrations at the β-(1, 4)-glycosidic linkages of cellulose.

3.2. Production of galactose by solid acid hydrolysis reaction

3.2.1. Effect of Dowex (TM) Dr-G8 loading

Catalyst loading is an important parameter that needs to be optimized to increase galactose yield. Figs. 1(a) and 2(a) show the effect of catalyst loading on hydrolysis of EC and ME as a function of reaction time, respectively. From the figures, when the hydrolysis reaction was carried out without a catalyst, it was noted that no galactose was produced even after 2.5 h of the reaction for both types of biomass. This indicates that the critical role of the solid catalyst is to catalyze the hydrolysis reaction.

In EC hydrolysis, as shown in Fig. 1(a), it was apparent that the yield of galactose gradually increases over time for all catalyst loading. Moreover, one can observe that higher Dowex (TM) Dr-G8 loading resulted in a higher hydrolysis rate. This effect was substantial, and galactose yield increased from 28.7% to 35.1% after the reaction of 2.5 h when the catalyst loading increased from 2% (w/v) to 6% (w/v). A possible explanation for these appearances is that the increase in catalyst loading is expected to enhance the opportunity of contact between the cellulose and SO_3H groups of the catalyst, which can be attributed to an increase in the total number of catalytically active sites that led to a faster reaction rate to promote the conversion of EC to galactose (Xiong et al., 2014). However, galactose yield decreased when catalyst loading increased beyond 6%. It is considered that beyond this level, the reaction produces a negative sugar output rate because degradation was evident (Hargreaves et al., 2013). In general, during the dilute sulfuric acid hydrolysis of biomass, the system would have excessive acidity because 5-HMF is generated by the degradation of hexoses in parallel with the formation of sugars (Park et al., 2011). From these results, fermentation inhibitors such as 5-hydroxy-methyl-furfural (5-HMF) were generated by solid acid hydrolysis of EC (see Supporting information, Figure S4a). Thus, 6% (w/v) catalyst loading showed the best performance because no 5-HMF was detected even after 2 h of reaction time.

In ME hydrolysis, comparable with those that occurred in EC as shown in Fig. 2(a), the yield of galactose also tended to increase with increasing catalyst loading but slightly decreased in a more acidic system. At the initial stage, the increasing catalyst loading contributed to the increasing galactose yield, but the values were slightly lower than those obtained in EC hydrolysis. These observations were reasonable when compared with EC (fresh macroalgae without treatment), indicating that the ME contains mostly κ-carrageenan. It is obvious that galactose was the main product produced from the solid acid hydrolysis reaction. Other monosaccharides were present in trace amounts or were not detectable (data not shown). Thus, hydrolysis of ME may need a longer reaction time to reach the equilibrium yield. The hydrolysis rate increased remarkably when the amount of Dowex (TM) Dr-G8 increased from 2% to 6% (w/v). A reaction time of 2.5 h was necessary to obtain the optimum galactose yield of 40.3% with 6% (w/v) of catalyst loading. The formation of galactose obtained from ME is relatively higher than that of fresh EC. This is because pre-extraction of κ-carrageenan from EC can remove fibers that can hinder the hydrolysis of galactans, thus enhancing the hydrolysis process and subsequently increasing galactose production. Furthermore, by increasing the catalyst loading to 8%, a short reaction time of 1.5 h was enough to result in an optimum galactose yield of 41.1%. Galactose yield gradually decreased after 1.5 h. This phenomenon could be explained because the higher the Dowex (TM) Dr-G8 loading, the lower the hydrolysis reaction activation energy required; thus, the maximum galactose yield could be reached after a short reaction time. However, as the hydrolysis reactions proceeded, the degradation of monosaccharides predominated. Similarly, overly high solid catalyst loading would lead to the further conversion of galactose to by-products (see Supporting information, Figure S5a).

6% and 8% (w/v) of Dowex (TM) Dr-G8 were chosen as optimal catalyst loadings for hydrolysis of EC and ME, respectively.

3.2.2. Effect of reaction temperature

The effect of reaction temperature on the formation of galactose from *E. cottonii* (EC) and macroalgae extract (ME) for 0–2.5 h were investigated in this study. According to Fig. 1(b), hydrolysis of EC barely occurred at 110 °C, and galactose yield reached 35.1% after 2.5 h. By increasing the reaction temperature to 120 °C, only 1 h was required to obtain the optimum galactose yield of 35.7%. However, the main reason was that the "physical" barrier for the hydrolysis activation energy of EC could be overcome with increasing reaction temperature. In addition, a higher reaction

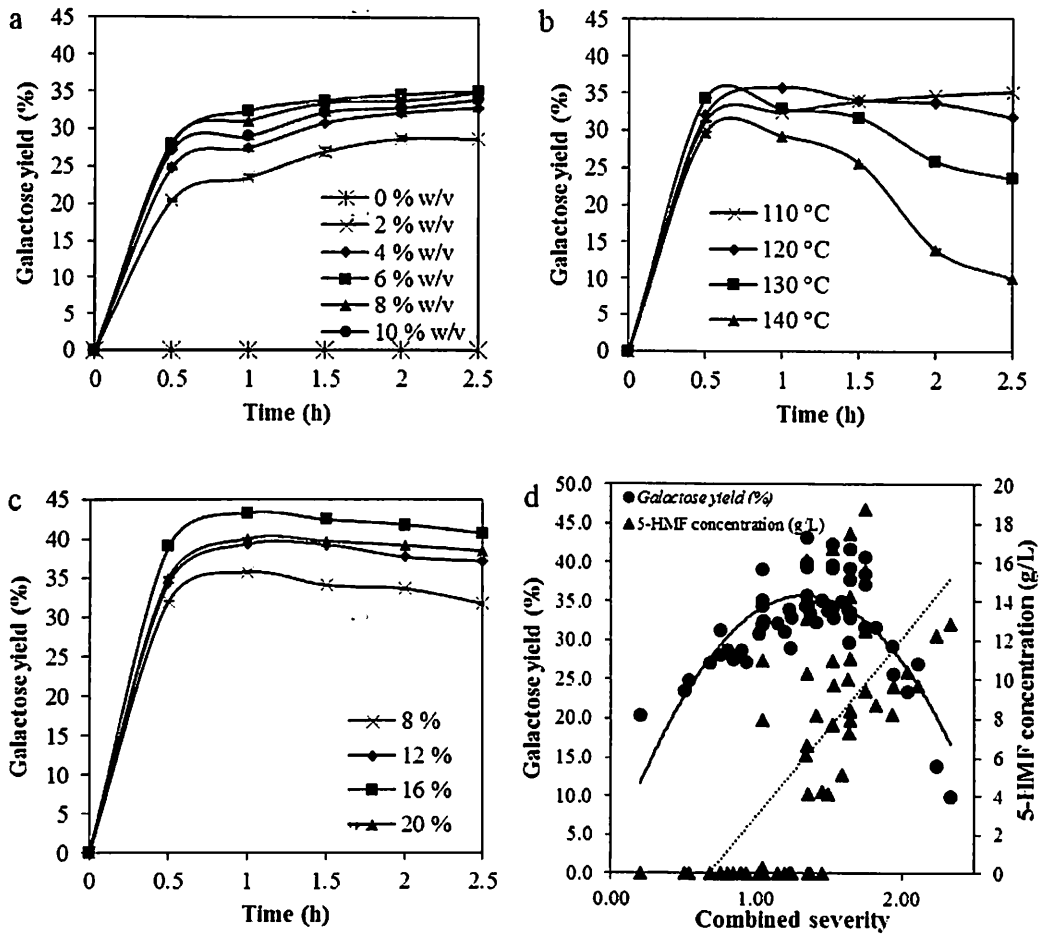

Fig. 1. Effect of (a) catalyst loading, (b) reaction temperature, (c) biomass loading, and (d) combined severity on the hydrolysis of *Eucheuma cottonii* (EC) in the presence of a solid catalyst Dowex (TM) Dr-G8.

temperature can significantly enhance the mass transfer rate between the catalyst sites and β-glucosidic bonds of the galactan chain as a result of the low viscosity of the system. In contrast, a lower reaction temperature will slow down the hydrolysis reaction rate, while a higher reaction temperature also increases the degradation of galactose. By increasing the reaction temperature to 140 °C, only 0.5 h was required to obtain the optimum galactose yield of 29.7%. However, it decreased sharply from 27.9% at 0.5 h to 10% at 2.5%. This trend was attributed to the decomposition of galactose. Fig. S4(b) shows the effect of reaction temperatures and time on the 5-HMF concentration. The 5-HMF concentration increased with reaction time in all experiments, and a higher reaction temperature led to the faster decomposition of galactose. The 5-HMF was 4.2 g/L after 2.5 h, while the optimum concentration of 5-HFM reached approximately 12.8 g/L in 2.5 h at 140 °C.

In addition, ME yielded similar results compared to EC. From Fig. 2(b), the galactose yield increased with increasing reaction temperature from 110 to 120 °C. The highest galactose yield was obtained at 120 °C after 1 h of hydrolysis with a 42.4% yield. After reaching the optimum yield, the galactose yield slowly decreased for the following hour. The galactose yield also declined at 140 °C, indicating that the galactose that was formed was not stable at higher temperature and degraded into 5-HMF. There is no doubt that reaction temperature has a great influence on the rate of the hydrolysis reaction and the stability of the sugars produced. These results were consistent with the trend of galactose yield from the hydrolysis of EC at 120 and 140 °C. Fig. S5(b) shows the concentration of 5-HMF at various reaction temperatures. It is observed that reaction time and reaction temperature have a significant influence on the formation of 5-HMF. For example, when the reaction temperature increases from 110 to 140 °C, the concentration of 5-HMF increases from 8.6 g/L to 17.3 g/L for a 1 h reaction time. In fact, it has been reported that in the acidic hydrolysis of marine algae, such a harsh condition results in over-decomposition of sugars that are formed by-products such as 5-HMF, levulinic acid, formic acid and char (Jeong et al., 2011). The galactose yield in ME hydrolysis was higher than that in EC. This result can explain the fact that the different structure and the presence of other compounds in EC give different results for sugar content compared to ME. Moreover, the ME that is mainly composed of κ-carrageenan is easier to hydrolyze into galactose.

As a by-product, 5-HMF has an inhibiting effect on the fermentation of sugars for bioethanol production. Therefore, a higher reaction temperature and shorter reaction time should be considered for macroalgae biomass hydrolysis to prevent the degradation of galactose. Hence, a reaction temperature of 120 °C was determined to be the optimum value for hydrolysis of EC and ME in this study.

3.2.3. Effect of biomass loading

The effect of this parameter was investigated because process economics and environmental friendliness can be improved if higher biomass dosages were used with a constant amount of catalyst (Figs. 1c and 2c). It was found that the galactose yield increased in the first 1 h, and then decreased for all biomass

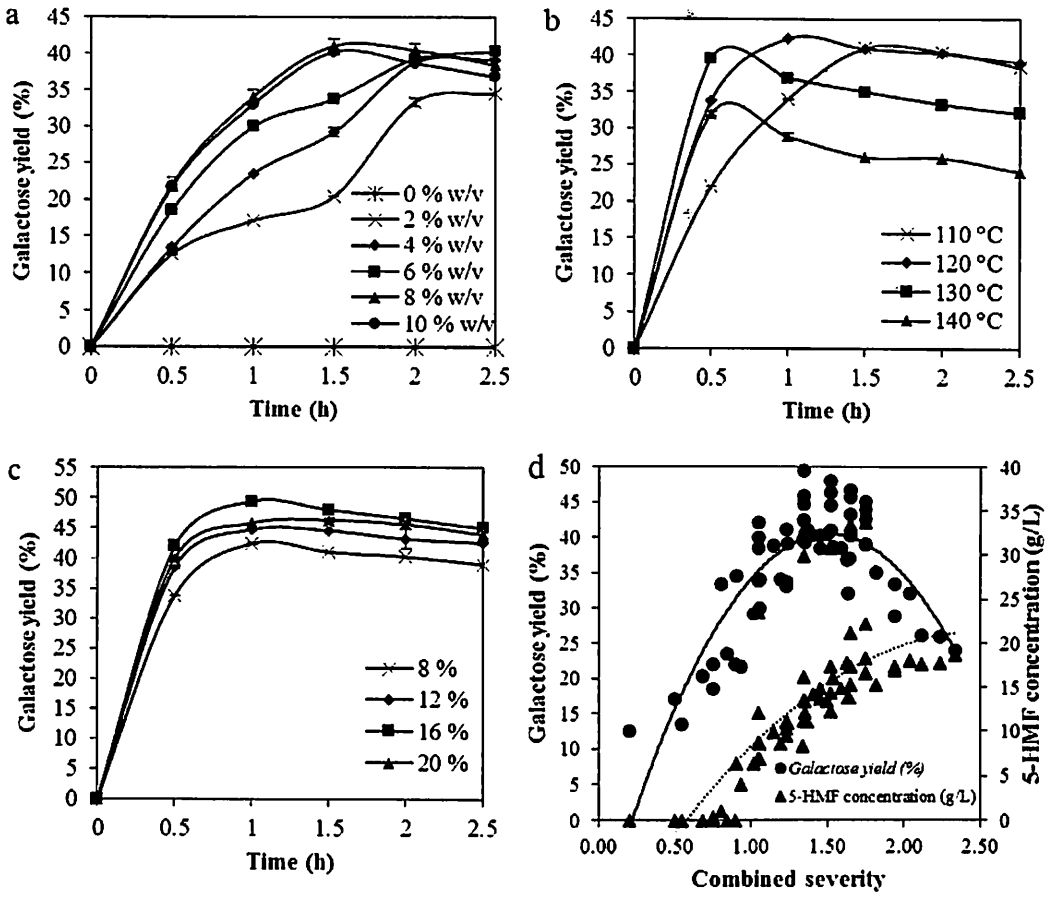

Fig. 2. Effect of (a) catalyst loading, (b) reaction temperature, (c) biomass loading, and (d) combined severity on the hydrolysis of macroalgae extract (ME) in the presence of a solid catalyst Dowex (TM) Dr-G8.

loadings in both types of biomass. Also shown in Figs. 1(c) and 2(c), the galactose yield was increased by increasing the s/l (solid/liquid) ratio of biomass (EC and ME) until 16% and then the galactose production decreased. This phenomenon could be a result of the total amount of carbohydrates that are present in the biomass. A higher biomass loading would increase the carbohydrate content with more galactose that can be hydrolyzed by the catalyst Dowex (TM) Dr-G8 (Harun and Danquah, 2011). However, the recovery of galactose was slightly lower at 20% than that at 16% at the same reaction time point. The decrease may be interpreted as follows. The high solid content led to the high viscosity, which caused the difficulty in handling the slurry (Kim et al., 2014). In addition, part of the galactose converted into 5-HMF (see Supporting information, Figures S4c and S5c). The rate of galactose production is not as fast as the rate of galactose degradation. Moreover, the other reason might be because of insufficient catalyst for the additional biomass loading. Hence, the optimum catalyst and biomass loading for optimum galactose yield have to be scrutinized from an economical aspect.

The highest value of galactose yield was obtained at 43.2% in EC and 49.4% in ME. It shows that Dowex (TM) Dr-G8 can hydrolyze ME with a higher yield compared to EC as a substrate. As mentioned earlier, the EC fiber content with higher crystallinity might also affect the hydrolysis process (Khambhaty et al., 2012). Optimal conditions for the hydrolysis of macroalgae biomass were catalyst loading: 6% (w/v) (EC) and 8% (w/v) (ME), temperature 120 °C, 1 h of reaction time and 16% of biomass loading.

3.2.4. Effect of combined severity factor

The effect of the combined severity (CS) on galactose yield and 5-hydroxy-methyl-furfural (5-HMF) concentration in hydrolysis of EC and ME was investigated (Figs. 1d and 2d). CS value is a representative parameter for the strength of a hydrolysis process. The severity factor is the combined catalyst loading, reaction temperature, and reaction time in acid hydrolysis. The biomass decomposition in solid acid mainly occurs by two mechanisms: hydrolysis of galactan and decomposition of galactose. Careful control of the reaction condition can minimize the degradation of galactose into by-production (5-HMF). As shown in Fig. 1(d), the galactose yield of EC was slightly lower than those from ME over the entire CS range, and they exhibited a similar tendency. Higher galactose yields of approximately 39–45% occurred at more severe CS ranging from 1.05 to 1.74 in both types of biomass. They were increased as CS was raised from 0.2 to 1.35 and then decreased gradually with further CS increases. This result indicated that a higher severity factor can cause over-degradation of carbohydrates. The highest galactose yields from the hydrolysis of EC and ME were 43.2% and 49.4%, respectively. Jeong et al. (2012) obtained a glucan yield of 33.05% in the hydrolysate from pretreated *Gelidium amansii* (red macroalgae) at a CS of 2, and Kim et al. (2014) achieved a maximum total reducing sugar yield of 8% when *Enteromorpha intestinalis* (green macroalgae) was hydrothermally hydrolyzed (pretreatment) at a CS of 3.84. The hydrolysis yield can vary as a result of the influence of catalyst loading, reaction temperature and reaction time that can differ even at the same CS.

Sugars were degraded quicker according to the severity of the reaction conditions. It is important to obtain galactose solutions with low concentrations of by-product (5-HMF) if the hydrolysate of EC and ME were used as the carbon source in fermentation media. As shown in Figs. 1(d) and 2(d), 5-HMF formation was clearly affected by the severity factor at lower biomass loading (<16% s/l ratio); a higher severity factor will lead to a higher 5-HMF formation. The concentration of 5-HMF increased drastically when harsher hydrolysis conditions were applied. For example, solid acid hydrolysis reaction conditions at CS between 1.94 and 2.33 were 6–8% (w/v) Dowex (TM) Dr-G8, 130–140 °C, and 2–2.5 h. The 5-HMF concentration in the ME hydrolysate was the highest at 18.6 g/L (CS = 2.33), compared to the EC hydrolysate, which was the highest at 12.8 g/L (CS = 2.33). However, at higher biomass loading (>16% s/l), a higher 5-HMF concentration of approximately 12.1–33.7 g/L occurred at more severe CS ranging from 1.05 to 1.74 in both types of biomass. Therefore, the authors recommend using solid-acid hydrolysis at CS below 1.35 and biomass loading below 16% s/l ratio.

3.3. Enzymatic hydrolysis

Water insoluble pretreated residue (f-EC) from the optimal solid acid hydrolysis reaction and the pretreated macroalgae cellulosic residue (P-MCR) were subjected to an enzymatic hydrolysis test by a cellulase complex (Celluclast 1.5 L) supplemented with β-glucosidase (Novozyme 188). The content of glucose released was detected to evaluate the effectiveness of the pretreatment using Dowex (TM) Dr-G8. As shown in Fig. S6, the amount of the glucose increased gradually and reached 0.649 g/g of fractionated EC, which corresponds to a 64.9% hydrolysis yield after 48 h. The figure showed that, over time, the amount of glucose increased, and after 48 h, there was an insignificant decrease in the amount of glucose that was observed in the same experiments. Similarly, in the first 24 h of enzymatic hydrolysis of P-MCR, more sugars were obtained, and the levels then sluggishly increased before reaching a maximum at 30 h. It is clear that there is a significant difference in the glucose yield between the f-EC and P-MCR. The MCR pretreated with Dowex (TM) Dr-G8 were observed to be more vulnerable to enzymatic hydrolysis and the conversion of cellulose was almost complete, with a glucose yield of 99.8% after only 30 h of reaction, which was 1.56 times greater than when using f-EC as the substrate. 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. In addition, it was estimated that as a result of degrading part of the cellulose and releasing soluble glucose from EC during the solid acid hydrolysis process itself, a smaller amount of cellulose was available for enzymatic hydrolysis and hence resulted in a lower glucose yield.

3.4. Combination methods for hydrolysis at conditions giving high sugar yields

The hydrolysis of macroalgae in this process can be carried out chemically by e.g., solid acid hydrolysis or enzymatically. While it is possible to obtain cellulose hydrolysis close to 100% by enzymatic hydrolysis, it is difficult to achieve such a high yield with acid hydrolysis. Thus, chemical and enzymatic hydrolysis processes have been combined to more effectively obtain reducing sugars from macroalgae. In this study, the total reducing sugars available for fermentation from EC are the summation of the galactose and glucose monomers that are found in both the solid acid hydrolysate and the enzymatic hydrolysate (Table S6). Based on our study, the highest total sugars were produced at 98.7 g/L when the 16% of ME treated under the optimum conditions of solid acid hydrolysis (8% (w/v) Dowex (TM) Dr-G8, 120 °C, 1 h) and 2% P-MCR treated by enzymatic hydrolysis (50 °C, pH 4.8, 30 h). In addition, with 16% of EC (whole biomass) treated under the optimum conditions of solid acid hydrolysis (6% (w/v) Dowex (TM) Dr-G8, 120 °C, 1 h) and 2% f-EC treated by enzymatic hydrolysis (50 °C, pH 4.8, 48 h), the total sugars released in the hydrolysate were 82.1 g/L. This result was slightly lower compared with the feedstock by using ME and P-MCR.

The use of the combination of chemical and enzymatic hydrolysis methods for converting polysaccharides of macroalgae into fermentable sugars have been studied and reported. Based on Table S6, our study shows a better result, with the concentration of sugars at 98.7 g/L, compared with Abd-Rahim et al. (2014). The concentration of reducing sugars after combining both methods reached 49.9 g/L when EC (whole biomass) was hydrolyzed with sulfuric acid followed by enzymatic hydrolysis. In addition, a recent study showed that hydrolysis of red macroalgae *Gracilaria sp.* by acid hydrolysis (0.1 N H_2SO_4, 121 °C, 1 h) followed by enzymatic hydrolysis (pH 4.5, 50 °C, 100 rpm, 6 h) resulted in a hydrolysate containing galactose (26.8 g/L) and glucose (6.1 g/L) (Wu et al., 2014). This yield is slightly lower than the yield obtained in the current work. The results showed that the solid acid catalyst (Dowex (TM) Dr-G8) effectively catalyzes the hydrolysis of cellulosic reactants into monosaccharides, similarly to H_2SO_4. Considering the requirement for corrosion resistant equipment and the handling of acid waste disposal during acid hydrolysis, solid acid hydrolysis is preferred for the production of reducing sugars.

3.5. Fermentation of macroalgae extract hydrolysate

As shown in Fig. 3, fermentation of ME hydrolysate was evaluated by bioethanol concentration and yield and galactose consumption during 96 h. At the start of the fermentation, the concentration of galactose was 35 g/L. After 48 h, most of the galactose was consumed while the bioethanol concentration reached 7.93 g/L. After 72 h, 97.1% of galactose was consumed and the optimum bioethanol concentration was 11.6 g/L, corresponding to a yield of 64.6%. The fermentation efficiency obtained in this study was found to be comparatively higher than earlier reported values. Recently, Meinita et al. (2012) reported that bioethanol production from hydrolysates obtained from acid pretreatment of *E. cottonii* resulted in 1.7 g/L (41% theoretical yield) of bioethanol (Meinita et al., 2012).

3.6. Prehydrolysis and simultaneous saccharification and fermentation (PSSF) of P-MCR

The chemical reaction involves the enzymatic hydrolysis of P-MCR followed by fermentation of the simple sugars. Therefore, the conversion performance was examined to investigate the macroalgae bioethanol production process in accordance with the fermentation time using commercial yeast. After 24 h of prehydrolysis, the glucose concentration was 27.5 g/L at 4% (w/v) substrate loading. The aim of prehydrolysis was to promote the hydrolysis of P-MCR by maintaining the optimum temperature for the enzymes in the early hours of the SSF process. As seen in Fig. 4(a), after addition of the yeast cells, the glucose concentration dropped rapidly while the bioethanol concentration increased rapidly, reaching the highest bioethanol concentrations of 11.7 g/L, corresponding to 92.7% of the theoretical yield based on the glucose content of the substrate. The yeast used here produced optimum bioethanol from enzymatic hydrolysate of P-MCR after 3.5 h of SSF and it declined thereafter. The decline in bioethanol production after 3.5 h of fermentation could be attributed to consumption of accumulated bioethanol by the yeast as was observed

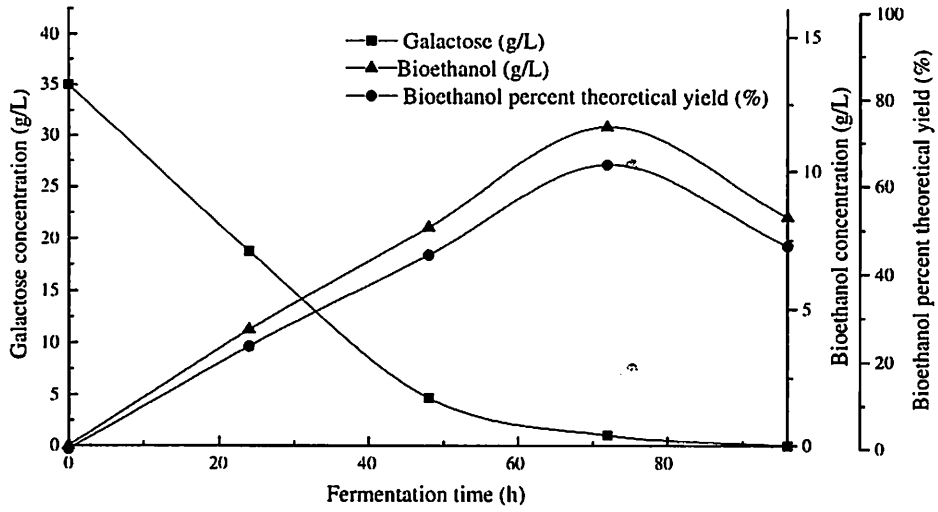

Fig. 3. Bioethanol production and galactose content in fermentation medium using hydrolysates of macroalgae extract (ME) in batch fermentation.

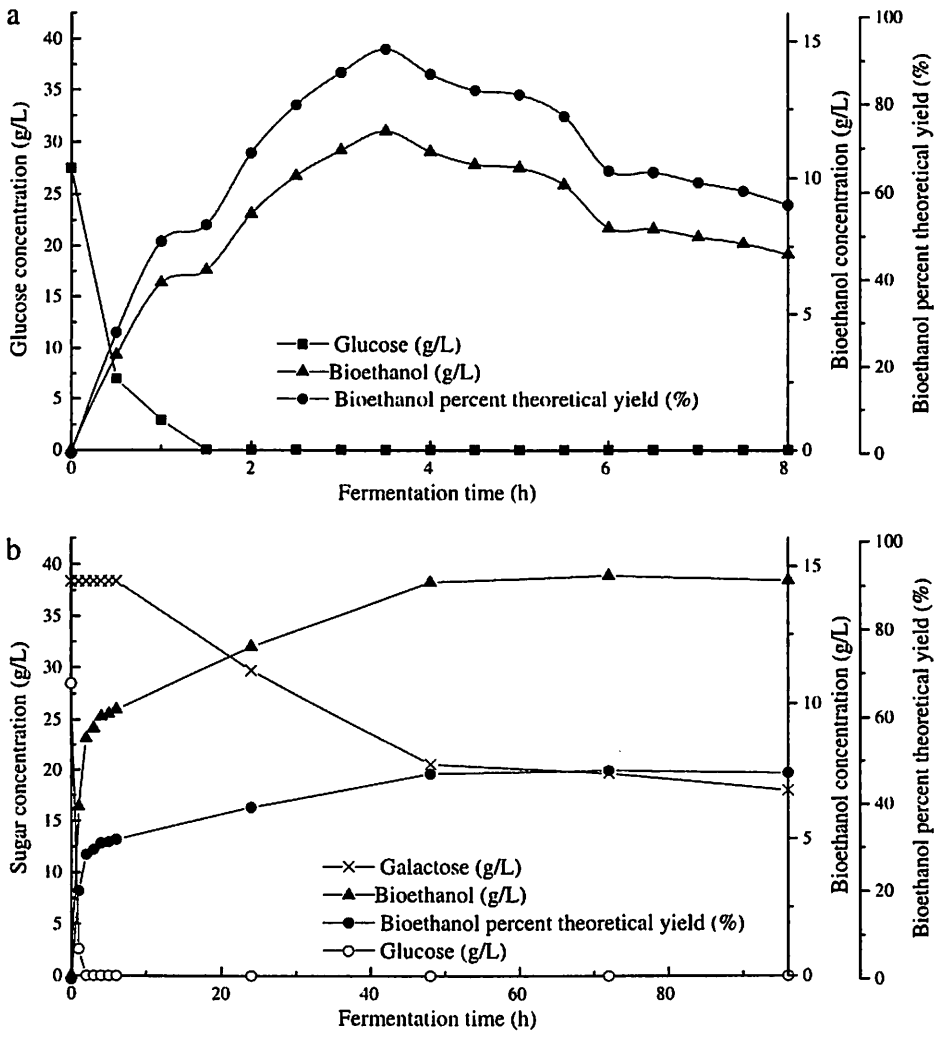

Fig. 4. Profile of bioethanol production and glucose consumption for SSF of P-MCR suspended in (a) citrate buffer and (b) macroalgae extract hydrolysate. Conditions: enzyme loading of 45 FPU/g of cellulase and 52 CBU/g of β-glucosidase, 50 °C for prehydrolysis, and 43 °C for SSF.

during our earlier studies (Tan and Lee, 2014). Interestingly, the bioethanol yield from P-MCR was also found to be comparable with the previously reported ethanol yields from various macroalgae biomasses such as *Saccharina japonica* (67.4% theoretical yield) (Lee et al., 2013) and *G. amansii* (84.9%, theoretical yield) (Kim et al., 2015).

3.7. Prehydrolysis and simultaneous saccharification and co-fermentation (PSSCF)

The ME hydrolysate (galactose containing hydrolysate) was assembled with the P-MCR in a concentration of 4% (w/v) followed by enzymatic prehydrolysis for 24 h; afterwards, the cultured yeast was inoculated for bioethanol fermentation (Fig. 4b). For successful utilization of this macroalgae for bioethanol production, it requires that both sugars (galactose + glucose) should be efficiently converted to bioethanol in high yield. The glucose utilization rate was not affected by the existence of galactose. It was observed that glucose was consumed faster than galactose, glucose was completely utilized in 3 h, and no galactose was consumed in the same time period; in addition, approximately 19.7 g/L of galactose remained unutilized from 72 h until the end of fermentation. In addition, the presence of glucose decreased the galactose utilization rate compared to the utilization rate observed in the galactose-only condition (Fig. 3). Moreover, even after glucose was completely consumed, the galactose utilization rate could not be recovered to the level of the galactose-only conditions. This is consistent with the fact that while *S. cerevisiae* can ferment both glucose and galactose, it ferments glucose and galactose sequentially with a diauxic lag period; in addition, the bioethanol yield from galactose is lower than the yield from glucose. It is known that galactose metabolism is an energy-intensive, inductive process that requires the synthesis of numerous enzymes. Thus, the rate of flux through the galactose utilization pathway is lower than that of glucose utilization. The bioethanol concentration reached the optimum at 72 h of incubation; it remained more or less a plateau beyond this point indicating that no advantage was seen in extending fermentation. After 72 h of fermentation, an overall 45.9 g/L of mono sugars were consumed and the final bioethanol concentrations of 14.7 g/L (47.4% theoretical yield) were obtained.

3.8. Economic analysis

Table S7 shows the cost structure of a unit cost of bioethanol production from EC. The feedstock cost accounted for approximately 17.06% of the total cost, while the net operating cost constituted 73.39% and the investment cost took another 9.55% of the total cost. It is obvious that the net operating cost accounts for the highest share of the total unit cost of the bioethanol production. The results of this study deviate considerably from a number of previous techno-economic assessments of cellulosic bioethanol production. Considering the bioethanol production costs from different feedstocks depicted in Table S8, the fixed price of bioethanol obtained from EC biomass (0.41 USD $/L) is competitive with that obtained from other feedstocks, such as coffee cut-stems, wheat residue, and corn stover.

4. Conclusion

In this study, we have demonstrated the feasibility of producing bioethanol from *E. cottonii* through different process strategies: the two-stream model and the integrated model. The two-stream model was shown to be the best strategy, resulting in 11.6 g/L of bioethanol from the fermentation of ME hydrolysates and 11.7 g/L from prehydrolysis and simultaneous saccharification and fermentation of P-MCR. Considering the production efficiency and environmental protection, solid acid is the better choice for reducing sugar production. The designed process and its economic assessment suggest that *E. cottonii* could be considered a profitable feedstock for bioethanol production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.08.008.

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