Moroccan Journal of Biology
Number 12 (2015)

http://www.fst.ac.ma/mjb
e-ISSN: 2351-8456 - p-ISSN: 1114-8756

Bioethanol production from acid hydrolysate of *Ulva rigida* C. Agardh by using *Pachysolen tannophilus* and *Zymomonas mobilis*

M. El Harchi, F.Z. Fakihi Kachkach, N. Elmtili

Laboratory of Biology and Health, Department of Biology, Faculty of Sciences, Abdelmalek Essaadi University Tetouan, Morocco

Abstract

In order to evaluate the possibility of bioethanol production from marine algae, (*Ulva rigida* C. Agardh), the fermentability of sugar hydrolysate acid of algal biomass, as a substrate, was investigated. *Pachysolen tannophilus* Boidin & Adzet, and *Zymomonas mobilis* (Lindner) De Ley & Swings were compared as fermenting microorganisms. The fermentation experiments were carried out in Erlenmeyer flasks maintained in a rotator shaker at 30°C and 120 rpm for 96h. It was shown that the hydrolysates presented different fermentability capacities. Ethanol yield obtained were 0.2 and 0.3 g/g of (sugar consumed) for respectively *Z. mobilis* and *P. tannophilus* strains in the same order. Under the optimized fermentation conditions, the method adaptation was an important strategy to improve ethanol productivity, allowing a maximum ethanol yield of 0.37 g/g for *P. tannophilus*. These preliminary results indicate a potential use of the macroalgae namely *U. rigida* for bioethanol production.

Keywords: *Ulva rigida*, hydrolysate, fermentation, bioethanol, *Pachysolen tannophilus*, *Zymomonas mobilis*, adaptation.

Introduction

Climate change, depletion and escalating price of petroleum, fuel security and economic development have led to search for replacing nonrenewable fossil fuel by a sustainable and eco-friendly renewable energy (Foody B, 1988; Bai *et al*., 2008). Bioethanol has been considered as a candidate of alternative energy of fossil resources (Farrell *et al.*, 2006). Indeed, it is a clean alternative fuel source due to its low toxicity, biodegradability, and its ability to effectively blend with petrol/gasoline without any engine modification (Harun *et al*., 2010).

Bioethanol has been produced from agricultural feedstock and lignocellulosic biomass in many countries (Demirbas, 2005; Nigam *et al*., 2011). The production of bioethanol from sugars and starch-containing materials (first generation of ethanol) could interfere with food security. Conversely, the second generation of bioethanol, using lignocellulosic materials as feedstock, would be without direct negative impact on food resources, although it may indirectly use agricultural lands (Harun & Danquah, 2011). Due to the recalcitrant structure of lignocellulosic materials, the delignification of biomass is still a barrier that must be overcome before the commercialization of the second-generation bioethanol (Gupta *et al*., 2009; Shafiei *et al*., 2013; Kim & Kim, 2014). Otherwise, the third-generation bioethanol, derived from macroalgae, has recently been considered a promising source of bioethanol whilst avoiding major disadvantages associated to the production of first- and second-generation bioethanol (Goh *et al*., 2010; john *et al*., 2011).

Marine algae are attractive renewable energy resources due to their abundance, high photosynthetic efficiency (Luning & Pang, 2003), and lignin-free composition (Jones & Mayfield, 2012). They do not require arable land, fertilizer, or fresh water, and consequently, they would not compromise food supply or cause an environmental major problem (John *et al*., 2011). Marine algae are

CONTACT N. Elmtili ✉ elmtili@hotmail.com 🖃 Abdelmalek Essaadi University PO Box 2121, 93002 Tetouan, Morocco

classified into three broad groups based on pigmentation: Brown (Phaeophyceae), red (Rhodophyceae) and green (Chlorophyceae) algae. Green algae, in particular *Ulva* species, are considered opportunistic seaweeds and proliferate in eutrophicated coastal waters (Teichberg *et al*., 2010; Borowitzka, 1972). They have traditionally been a part of local diets due to their high nutritional value (Bobin Dubigeon *et al*., 1997). *Ulva* spp. are used as food in Japan, and a source of the commercialized product "Aonori" or "green laver" (Nisizawa *et al*., 1987). At present, the potential use of these algae is still poorly explored (Chattopadhyay *et al*., 2007), and the *Ulva* species are commercially important in terms of hydrocolloids, but they are an important source of complex polysaccharides (Hernandez-Garibay *et al*., 2011).

The green algae contain various types of glucans, polysaccharides which can be hydrolyzed to fermentable sugars (Percival, 1979). Many sugars are not freely available and belong to the structural and storage carbohydrates, and consequently the acid hydrolysis is widely used to release fermentable sugars with maximum yield and purity (Moiser *et al*., 2005; Chandel *et al*., 2007a,b). In this regard, the resulting hydrolysate contains varying amounts of reducing sugar and broad range of substances due to the reaction of by-products from sugar and lignin degradation (furans, phenols and organic acids…). Many of these substances are known to inhibit the ethanol producing microorganisms in the subsequent fermentation steps (Almeida *et al*., 2007; Jonsson *et al.*, 2013). To circumvent the negative effects of acid pretreatment, the detoxification process and the adaptation methods, have been proposed to improve the ethanol fermentation (Prekha *et al*., 1986; Parawira & Tekere, 2010). The Fermentation process is the decomposition of organic compounds into simpler compounds attended by microorganisms (Hogg, 2005). Microorganisms such as bacteria and yeasts have the capability to ferment sugars for the production of bioethanol. The yeast *Pachysolen tannophilus* (Fanta *et al.*, 1984; Seo *et al*., 2009) and bacterium *Zymomonas mobilis* (Roger *et al*., 1997; Kasthuri *et al*., 2012) are widely used for fermentation of biomass hydrolysate.

In this context, the objective of this paper is to investigate the possibility of using the macroalgae *U. rigida* as raw materials for ethanol production by fermentation. Since the hydrolysates produced from this marine biomass contain reducing sugars, the ability of (*P. tannophilus* and *Z. mobilis*) to metabolize their sugars and convert them to ethanol was evaluated and compared.

Materials and methods

Raw materials

The biomass of *U. rigida* (Chlorophyceae) was harvested in September 2012 from the Tetouan coastal region, Azla, Morocco (Figure 1). The macroalgae species (*Ulva rigida* C. Agardh) was kindly identified by Pr. Riadi Hassan and Pr. Kazzaz Mohamed (Laboratory of Diversity and Conservation, Abdelmalek Essaâdi University, Morocco). After washing with tap water to remove salt and debris, it was dried in an oven at 60°C for 24h and ground into powder using a blender.

Figure 1. *U. rigida* from sea to laboratory.

Bioethanol fermentation of *U. rigida* hydrolysate

Acid hydrolysate preparation

The hydrolysis reaction of 10 % (by mass per volume) of green algae was conducted using 4 %(v/v) H_2SO_4 (95-97%, d=1.83) for a final volume of 180 ml, and heated in an autoclave at 120° C for 1h according to El harchi *et al.* (2015). The resulting hydrolysate was neutralized with NaOH pellets (pH to 7), and separated from the insoluble residues by filtration.

Then, 120 ml of the hydrolysate were supplemented with yeast extract, 10 g/l and peptone, 10 g/l, pH=6 (medium for *P. tannophilus* fermentation). In addition, 60 ml were supplemented with 10 g/l yeast extract, 1 g/l KH_2PO_4, 0,5 g/l $MgSO_4,7H_2O$ and 1 g/l $(NH_4)_2SO_4$ pH=6 (medium for *Z. mobilis* fermentation). A volume of 30 ml of hydrolysate was placed in six Erlenmeyers of 125 ml designed for anaerobic fermentation (four flasks for parent and adapted strain of *P. tannophilus* and two flasks for the bacterium *Z. mobilis*). Afterward, the hydrolysate was sterilized at 111°C for 15 min, and after cooled in room temperature.

Culture conditions and yeast adaptation

The *Z. mobilis* CP4 was gently supplied by Pr. Anna Irini Koukkou, University of Ioannina, Greece. The bacterial strain was grown at 30°C and 120 rpm in the synthetic medium fermentation (SMF) which contained the following ingredients (in g/l): glucose (20), yeast extract (10), KH_2PO_4 (1), $(NH_4)_2SO_4$ (1), $MgSO_4$.7H2O (0.5). This medium was sterilized by autoclaving at 121°C for 15 min before use.

The *P. tannophilus* MUCL 27787 was generously supplied by Pr. Philippe Thonart, University of Liege, Belgium, and used in this study as ethanol producer. The strain culture was performed in a 125 ml Erlenmeyer flask with 30 ml of YEPD medium (yeast extract, 10 g/l; peptone, 10 g/l; glucose, 20 g/l) in a shaking incubator at 30°C and 120 rpm. Media were sterilized by autoclaving at 120°C for 15 min. The culture was maintained at 4°C and renewed every five weeks.

P. tannophilus was adapted against *U. rigida* acid hydrolysates. Adaptation procedure was performed by sequentially transferring and growing cells in media containing the hydrolysate. This method utilizes the microorganism of each experiment as the inoculum of the next one. A concentration of 5% (v/v) of the strain culture in the YEPD medium was added to fermentation media which contains macroalgae hydrolysate (prepared in the same condition above). Incubation lasted 16 h with agitation at 120 rpm and 30°C. A 5% of the broth was then sub-cultured into the hydrolysate fresh media and incubated for the second adaptation cycle. This cycle repeated until 10 times to obtain ‘adapted strain’.

Ethanol fermentation experiments

The hydrolysate obtained from acid hydrolysis process was used as a fermentation medium for bioethanol production. Strains were first activated in YEPD broth and hydrolysate for parent and acclimated yeast and in medium SMF for the bacteria *Z. mobilis* respectively in a shaking incubator at 120 rpm, 30 °C.

Each two flasks, prepared previously, were aseptically inoculated with 5% (v/v) of seed culture of parent and adapted yeast and the bacterium strain respectively to achieve an initial optical density of 0.1 absorbance units at 600 nm. The fermentation hydrolysates were incubated at 30 °C and agitated at 120 rpm for 4 days.

During the fermentation process, sample was withdrawn from the media and centrifuged, and the supernatants were filtered through a 0.45 mm cellulose acetate filter prior to analysis. The cell concentration was determined by dry cell weight. The amount of bioethanol and residual reducing sugars were estimated by using the Boehringer Mannheim enzymatic kit reference number 10139068035 and

DNS method respectively (Miller, 1959). Assays were performed in duplicates.

Calculation of Kinetic and Yield Parameters

The ethanol yield ($Y_{P/S}$, g/g) was defined as the ratio of the maximum ethanol concentration (g/l) to the total sugars consumed (g/l). The ethanol volumetric productivity (Q_P, g/l h) was calculated as the maximum ethanol produced divided by the time to achieve maximum ethanol production (h). The fermentation efficiency (FE, %) was calculated by the ratio of the average produced ethanol to the ethanol theoretically produced in the biochemical conversion of the sugars consumed.

Results and Discussion

The acid hydrolysate from *U. rigida* was readily fermented to ethanol by using parent and adapted *P. tannophilus* yeasts. As shown in fig.2, significant differences in the ethanol production profiles were observed between strains.

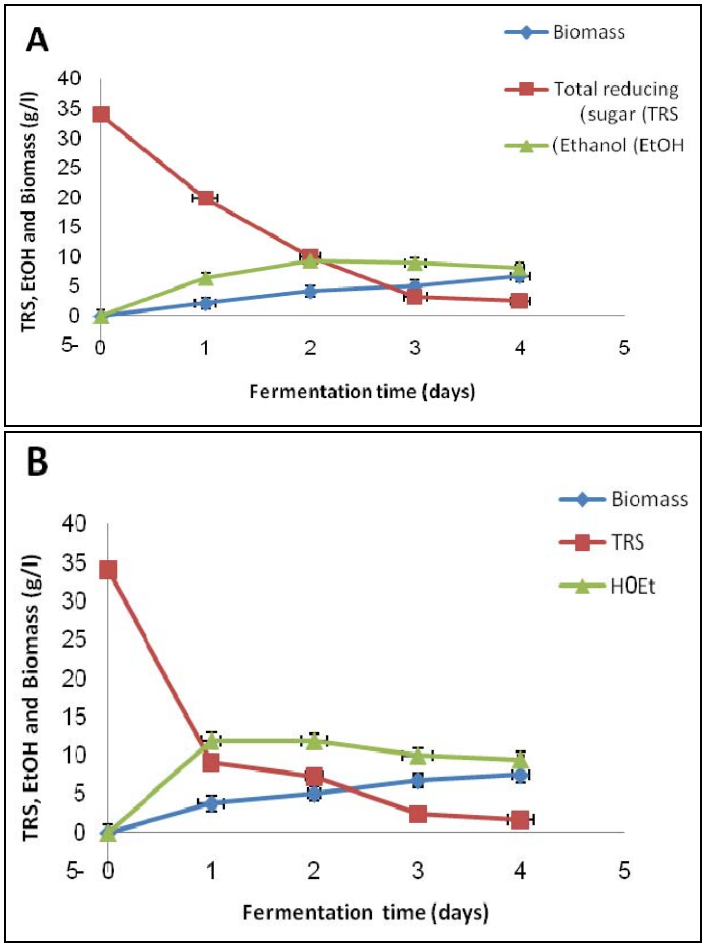

Figure 2. Ethanol production from hydrolysate derived from *U. rigida* using *P. tannophilus* strain. (A) Non-acclimated *P. tannophilus* (Parent) and (B) acclimated *P. tannophilus* yeast to hydrolysate. Data represents the average results of duplicate fermentations with error bars displaying the standard deviation from the mean. For data points without errors bars, the errors were smaller than the size of the symbols.

At the beginning of fermentation, the reducing sugars (34 g/l) in the fermentation medium were decreased and coincide with an increase of cells growth and the ethanol production (Figure 2A). This is due to the use of sugars by cells for their growth and ethanol synthesis. At 48 h of fermentation, the sugar depleted and the ethanol reached its maximum concentration (9.26 g/l), with a yield of 0.3 g ethanol/g sugar consumed and a fermentation efficiency of 53.3%. As the fermentation period increases, the ethanol level decreased approximately, while cells continued growing. This behavior may be attributable to the utilization of sugars for growth and metabolism.

The cells mass production was higher in adapted strain than found in parent one (Figure 2B). It should be noted that during hydrolysis process, a range of toxic compounds (lignin and sugars degradation products) are formed and inhibited ethanol fermentation (Klinke *et al.*, 2003). Our results showed that after several fermentation cycles, the adapted yeast resistance against existent inhibitory compounds present in the fermentation broth has increased.

In addition, the consumption of reducing sugars was faster than parent strain and all sugars had been completely utilized after 24 of inoculation, coinciding an ethanol concentration of 11.92 g/l, an ethanol yield of 0.37 g/g sugars consumed and a fermentation efficiency of 68,8%. The adaptation of the hydrolysate detoxification method seems to increase the fermentative competence and give a better yield of ethanol. The resulting yield of ethanol was lower than of previous studies. Ismail *et al*. (2012) and Beall *et al*. (1992) have reported yields of ethanol from 0.475 g/g to 0.51 g/g for the fermentation of the Wheat Straw and corn cobs, and hulls acid hydrolysate respectively. In other studies using green algae, Trivedi *et al*. (2013), Ge *et al*. (2011), and Wu *et al*. (2014) obtained an ethanol yield of 0.45 g/g from *U. fasciata*, 0.44 g/g from *Laminaria japonica* and of 0.47 g/g from hydrolysate *Gracilaria* spp.

For the strain *Z. mobilis*, the sugar and ethanol concentration changed during fermentation process. The reducing sugar concentration was 34 g/l at the start of fermentation and the concentration of ethanol increased proportionally to the gain of fermentation time and bacterium biomass. As shown in the Figure 3, the fermentation time extension from 2 to 4 days had no effect on converting the sugar to ethanol, and biomass was continuously increasing in the broth. The maximum ethanol production was 6 g/l obtained after 48h of incubation.

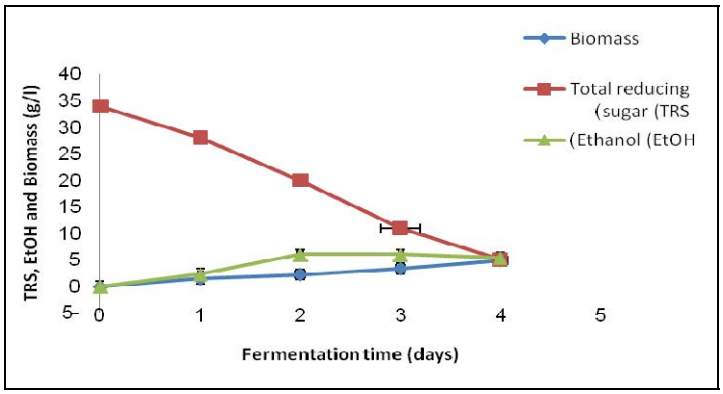

Figure 3. Ethanol production by *Zymomonas mobilis* CP4 on *Ulva rigida* hydrolysate. Data represents the average results of duplicate fermentations with error bars displaying the standard deviation from the mean.

The resulting yield of ethanol was equivalent to 0.207 g/g of sugars consumed with ethanol volumetric productivity and fermentation efficiency of 0.125 g/l/h h and 34.6 % respectively. The results are comparable with previous studies. Using the acid hydrolysate from the seaweed *Kappaphycus alvarezii*, Meinita et *al*. (2012) reported an ethanol yield of 0.21 g/g. Ferreira et *al*. (2011) reported a yield of 0.19 g/g from bagasse hydrolysate. Using Rice Straw hydrolysate and the yeasts cells Kocher & Kalra (2013) obtained an ethanol yield of 0.11 g/g when fermented by the yeasts cells. However, the ethanol yield achieved in this study (0.21g/g) was considerably lower than that reported by Kumar *et al*. (2013), i.e. 0.43 g/g from the residue of *Gracilaria verrucosa*. Parekh *et al*. (1986) have found an ethanol yield of 0.45 g/g using the wood hydrolysate. The resulting overall low ethanol production may have basis in the presence of substantial amount of fermentation inhibitors in the popular hydrolysates (sugar and lignin degradation products) which inhibited the growth and fermentation activity of the bacteria. Also, this poor fermentability should be attributed to the some sugars as pentose sugars that not readily fermented by the *Z. mobilis* CP4 (Zhang *et al*., 1995).

Conclusion

The results of the present study showed a significant potential of *U. rigida* as a promising raw material for bioethanol production. It was concluded that the adapted yeast showed a better efficiency

than the non-adapted one, considering fermentation yield and time required for the process. Fermentation of acid hydrolysate by adapted yeast cells generate a maximum ethanol (11.92 g/L) with an ethanol yield of about (~0.37 g/g) and fermentation efficiency of 70%. Further studies related to process of hydrolysis and fermentation will be carried out to improve bioethanol production.

References

Almeida JRM, Modig T, Petersson A, Hahn-Hagerdal B, Liden G, Gorwa-Grauslund MF. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol. **82**: 340-349.

Bai FW, Anderson WA, Moo-Young M (2008) Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv. **26**: 89–105.

Beall D, Ingram LO, Ben Bassat A, Doran JB, Fowler DE, Hall RG, Wood BE (1992) Conversion of hydrolysates of corn cobs and hulls into ethanol bv recombinant Escherichia coli B containing integrated genes for ethanol production. Biotechnology Letters **14**: 857-862.

Bobin Dubigeon C, Lahaye M, Barry JL (1997) Human colonic bacterial degradability of dietary fibres from sea-lettuce (*Ulva* sp). J. Sci. Food Agric. **73**: 149–159.

Borowitzka MA (1972) Intertidal algal species diversity and the effects of pollution. Aust. J Mar.Freshw.Res. **25**: 73–84.

Chandel AK, Chan EC, Rudravaram R, Narasu ML, Rao LV, Ravindra P (2007a) Economics and Environmental Impact of Bioethanol Production Technologies: An Appraisal. Biotechnol. Mole. Biol. Rev. **2**: 14-32.

Chandel AK, Kapoor RK, Narasu ML, Viswadevan V, Kumaran SSG, Ravinder R, Rao LV, Tripathi KK, Lal B, Kuhad RC (2007b) Economic evaluation and environmental benefits of biofuel: an Indian perspective. Int. J. Global Energy Issues. **28**: 357-381.

Chattopadhyay K, Mandal P, Lerouge P (2007) Sulphated polysaccharides from Indian samples of *Enteromorpha compressa* (Ulvales, Chlorophyta): Isolation and structural features. Food Chemistry **104**: 928-935.

Demirbas A (2005) Bioethanol from cellulosic materials: a renewable motor fuel from biomass. Energy Sources **27**: 327-337.

Fanta GF, Abbott TP, Herman AI, Burr RC, Doane WM (1984) Hydrolysis of wheat straw hemicellulose with trifluoroacetic acid. Fermentation of xylose with *Pachysolen tannophilus*. Biotechnol Bioeng. **26**: 1122-1125.

Farrell AE , Plevin RJ,Turner BT, Jones AD, Hare MO and Kammen DM (2006) Ethanol can contribute to energy and environmental goal. Science **311**: 506-508.

Ferreira AD, Mussatto SI, Cadete RM, Rosa CA, Silva SS (2011) Ethanol production by a new pentose-fermenting yeast strain, *Scheffersomyces stipitis* UFMG-IMH 43.2, isolated from the Brazilian Forest. Yeast **28**: 547-554.

Foody B (1988) Ethanol from Biomass: The Factors Affecting it's Commercial Feasibility. Iogen Corporation, Ottawa, Ontario, Canada.

Ge L, Wang P, Mou H (2011) Study on saccharification techniques of seaweed wastes for the transformation of ethanol. Renew. Energy **36**: 84-89.

Goh CS, Lee KT (2010) A visionary and conceptual macroalgae-based third-generation bioethanol (TGB) biorefinery in Sabah, Malaysia as an underlay for renewable and sustainable development. Renew. Sust. Energ. Rev. **14**: 842-848.

Gupta R, Sharma KK, Kuhad RC (2009) Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic

ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. Bioresour Technol. **100**: 1214-1220.

Harun R, Danquah MK, Forde GM (2010) Microalgal biomass as a fermentation feedstock for bioethanol production. J. Chem.Technol. Biotechnol. **85**: 199-203.

Harun R, Danquah M.K (2011) Influence of acid pre-treatment on microalgal biomass for bioethanol production. Process Biochem. **46**: 304-309.

Hernández-Garibay E, Zertuche-González JA, Pacheco-Ruiz I (2011) Isolation and chemicals characterization of algal polysaccharides from the green seaweed *Ulva clathrata* (Roth) C. Agardh. Journal of Applied Phycology **129**: 491-498.

Hogg, S (2005) Essential Microbiology. John Wiley & Sons Ltd, England.

Ismail WA, Braim RR, Ketuly KA, Awang Bujang DSS, Arifin Z (2012) Production of Biocellulosic Ethanol from Wheat Straw. Acta Polytechnica **52**: 28-34.

John RP, Anisha GS, Nampoothiri KM, Pandey A (2011) Micro and macroalgal biomass: A renewable source for bioethanol. Bioresour. Technol. **102**: 186-193.

Jones CS, Mayfield SP (2012) Algae biofuels: versatility for the future of bioenergy. Curr. Opin. Biotechnol **23**: 346-351.

Jonsson LJ, Alriksson B, Nilvebrant NO (2013) Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol. Biofuels **6**: 1-10**.**

Kasthuri T, Gowdhaman D, Ponnusami V (2012) Production of ethanol from water hyacinth (*Eichhornia crassipes*) by *Zymomonas mobilis* CP4: Optimization studies. Asian Journal of Scientific Research **5**: 285-289.

Kim TH, Kim TH (2014) Overview of technical barriers and implementation of cellulosic ethanol in the U.S. Energy **66**: 13-19.

Klinke HB, Olsson L, Thomsen AB, Ahring BK (2003) Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: wet oxidation and fermentation by yeast. Biotechnol Bioeng. **81**: 738-747.

Kocher GS, Kalra KL (2013) Optimization of pretreatment, enzymatic saccharification and fermentation conditions for bioethanol production from rice straw. Ind J Appl Res. **3**: 62-64.

Kumar S, Gupta R, Kumar G, Sahoo D, Kuhad RC (2013) Bioethanol production from *Gracilaria verrucosa*, a red alga, in a biorefinery approach. Bioresource Technol. **135**: 150-156.

Luning K, Pang S (2003) Mass cultivation of seaweeds: current aspects and approaches. J. Appl. Phycol. **15**: 115–119.

Meinita MDN, Kang JY, Jeong GT, Koo HM, Park SM, Hong YK (2012) Bioethanol production from the acid hydrolysate of the carrageenophyte *Kappaphycus alvarezii* (cottonii). J. Appl. Phycol. **24**: 857-862.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry **31**: 426-428.

Moiser N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Biores. Technol. **96**: 673-686.

Nigam PS, Singh A (2011) Production of liquid biofuels from renewable resources. Prog. Energ. Combust. **37**: 52-68.

Nisizawa K, Noda H, Kikuchi R, Watanabe T (1987) The main seaweed foods in Japan. Hydrobiologia **151**: 5–29.

Parawira W, Tekere M (2010) Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit. Rev. Biotechnol. (In Press).

Parekh SR, Yu S, Wayman M 1986 Adaptation of *Candida shehatae* and *Pichia stipitis* to wood hydrolyzates for increased ethanol production. Appl. Microbiol. Biotechnol. **25**: 300-304.

Percival E (1979) The polysaccharides of green, red and brown seaweeds: their basic structure, biosynthesis and function. Bri. Phycol. J. **14**: 103-117.

Rogers P, Joachimsthal E, Haggett K (1997) Ethanol from lignocellulose: Potencial for *Zymomonas*-based process. Austral. Biotechnol. **7**: 304-309.

Seo, HB, Kim S, Lee HY, Jung KH (2009) Improved Bioethanol Production Using Activated Carbon-treated Acid Hydrolysate from Corn Hull in *Pachysolen tannophilus* **37** : 133-140.

Shafiei M, Zilouei H, Zamani A, Taherzadeh MJ, Karimi, K (2013) Enhancement of ethanol production from spruce wood chips by ionic liquid pretreatment. Appl Energ. **102**: 163-169.

Teichberg M, Fox MT, Olsen YO, Valiela I, Martinetto P, Iribarne O, Muto EY, Petti MAV, Corbisier TN, Soto-Jiménez M, PáezOsuna F, Castro P, Freitas H, Zitelli A, Cardinaletti M, Tagliapietra D (2010) Eutrophication and macroalgal blooms in temperate and tropical coastal waters: nutrient enrichment experiments with *Ulva* spp. Glob Change Biol. **16**: 2624-2637.

Trivedi N, Gupta V, Reddy C, Jha B (2013) Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga *Ulva fasciata* Delile. Bioresour. Technol. **100**: 6658-6660.

Wu FC, Wu JY, Liao YJ, Wang MY, Shih IL (2014) Sequential acid and enzymatic hydrolysis in situ and bioethanol production from *Gracilaria biomass*. Bioresour. Technol. **156**: 123-131.

Zhang M, Eddy C, Deanda K, Finkelstein M, Picataggio S (1995) Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. Science **80**: 240-243.