Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Biorefining of marine macroalgal biomass for production of biofuel and commodity chemicals

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Abstract

The large scale production of marine macroalgae, mainly for human consumption, has given rise to their consideration for a nonlignocellulosic feedstock for the production of renewable fuels. However, making biofuel economical from algal biomass requires the co-production of additional useful biochemical components that are unique to algae and that have a proven market value. A viable and sustainable biorefining technology that maximizes the utilisation of feedstock for the production of chemicals along with fuel is, therefore, indispensable. Here, we for the first time demonstrate a tractable integrated process that facilitates sequential extraction of the major components of red algal biomass as commodity products such as pigments, lipid, agar, minerals and energy dense substrate (cellulose). The computed yield data from small-scale biorefinery trials suggest that a ton of fresh biomass supplies several valuable extracts - 0.3-0.7 Kg of R-phycoerythrin (R-PE), 0.1-0.3 Kg of R-phycocyanin (R-PC), 1.2-4.8 Kg of lipids, 28.4-94.4 Kg of agar, 4.4-41.9 Kg of cellulose and 3.1-3.6 of kilo liter mineral solution. The enzymatic hydrolysis and fermentation of cellulose thus obtained would yield 1.8-17.4 Kg ethanol. A distinct advantage of this process over direct extraction is the improved quality of agar (gel strength higher by 1.5-3 folds) without alkali and acid pretreatment of sample, the elimination of residue and the reduction by up to 85% in chemicals usage in cellulose extraction. The findings reported in this study forms the basis for starting new ocean-based bio-industries minimizing the dependence on the terrestrial resources for food, feed, energy and chemicals.

Keywords: Agar; Bioethanol; Biorefinery, Cellulose; Lipid; Pigments

Introduction

Currently, energy and environment are the two of the key issues given top priority while considering the new technologies for achieving sustainable economic growth and development in the world. The growing energy demand, fast depleting fossil fuel reserves and environmental effects of burning fossil fuel have collectively stimulated the search for sustainable alternative bioenergy sources and supplies[1]. To date, commercial biofuels production has been based on first-generation food crops, such as corn and sugarcane[2]. The continued production of biofuel from such resources has strained the food supplies and led to identification of diverse alternate biomass feedstocks as potential sources of renewable fuels. The biofuels from second-generation feedstock such as lignocellulosic biomass have met with limited success, due to high capital cost and technical challenges in downstream processes. In view of the inherent problems associated with respective feedstocks, an attention has been turned to marine macroalgae (seaweeds) as an alternate source of non-lignocellulosic feedstock for production of biofuels[3,4]. Seaweeds have been extensively utilised as food, feed, fertilizer and hydrocolloids[5,6] and thus farmed commercially in a number of countries, particularly in Asian regions. According to the latest information, the world production of seaweeds annually has been estimated at about 26 million tons (fresh) with China being the largest producer (13.5 million tons)[7]. Marine macroalgae have several distinct advantages for utilization as potential feedstock for production of renewable fuels[8-13]. Algae can achive high productivity with no agricultural inputs of arable land, freshwater and fertilizers, amenability for efficient depolymerisation, etc[4]. There is a worldwide research effort being directed to develop macroalgae as a biofuels feedstock by making required technological innovations in both cultivation technology and biomass conversion process technology for biofuel production at the needed scale[3]. Earlier studies on production of biofuels from marine macroalgal resources have followed typical conventional practices of

hydrolysis of biomass combining both chemical pretreateent and saccharification of biomass to sugars and their fermentation[14-16]. On the contrary, Kumar et al[17]demonstrated successfully production of bioethanol from algal waste (rich of holocellulose) following the revovery of agar from *Gracilaria verrucosa* (Hudson) Papenfuss. Nevertheless, recent R&D has successfully demonstrated the construction of an engineered microbial platform for direct production of bioethanol using either alginate alone[18] or whole biomass of brown macroalga *Saccharina japonica*[8,12]. Despite these technical advances, production of biofuel alone from macroalgal feedstocks is questionable from the context of sustainability and economic feasibility[9]. The 'biorefinery' concept, therefore, may improve the economics of sustainable production of biofuels with commodity products[13,19-21]. However, fractionation of biomass components in their near native state as platform molecules for subsequent transformation into value added chemicals, biomaterials and fuel is a bottleneck[9]. Furthermore, the by-products from the extended production of macroalgal polysaccharides for bioethanol on long term may jeopardise the multibillion seaweed hydrocolloid industry[22]. Therefore, fractionation and selective utilisation of biofuel substrate such as cellulose, a less utilized algal material, for bioethanol production from biomass would be the best option preventing any negative impacts on present hydrocolloid industry and associated markets worldwide. Present study describes a holistic tractable process towards a marine macroalgal biorefinery for sustainable complete utilization of feedstock for biofuel and a variety of natural products such as pigments, lipids, agar and mineral rich liquid in an integrated manner from fresh biomass, with little waste.

Experimental details

Sample collection.

Geliedella acerosa and *Gracilaria dura* were collected from the Adri (20.57°N and 70.16° E) and Veraval (20.55°N and 70.20° E) from west coast of India respectively. *Gelidium pusillum* was collected from Valinokam (9°.09' N; 78°.39' E) southeast coast of India. Samples were brought to the laboratory in cool condition. The sand and epiphytes were cleaned off by brushing in filtered seawater. The cleaned samples were then maintained in the laboratory in filtered seawater for experimentations.

Determination of dry weights.

Fresh samples of cleaned seaweeds were first blotted with tissue towels to remove external water content, weighted (initial fresh weight) and then dried in an oven at 60 °C until a constant weight. The dry weight of the sample was the percentage (%) of the oven dry weight calculated from the initial fresh weight. This data was used for calculating product yields (biorefinery process) on dry weight basis so as to compare with product yields obtained from dry biomass following conventional extraction methods.

Development of an integrated biorefinery process. The biorefinery process developed in this study containes several extractions in sequence as depicted in Fig 1. The details of each extraction are as follows:

A 50 g sample of fresh algal material was homogenized with chilled 100 ml of 0.1M phosphate buffer (pH 6.8) using mixer grinder and incubated for 12 h at 4 °C. The incubation samples with the phosphate buffer were then mixed thoroughly and centrifuged at 6300 g at 4^{o}C for 15 minute. The supernatant containing crude phycobilin pigments as protein was collected and the residue, with additional 50 ml of phosphate buffer, mixed thoroughly, centrifuged again and the two supernatants were combined. The residue remained after pigment extraction was saved for recovering the remaining products. The pigments purification were optimized using different concentration of ammonium sulphate (10, 20, 30,

40 and 50%). The absorbance of the solution was read using UV-Vis spectrophotometer (UV-160, Shmadzu, Japan) at 280, 455, 564, 618 and 730 nm. The content of R-PE and R-PC pigments were calculated according to following equation [23]:

$$R\text{–PC} = 0.154\ (A_{618} - A_{730})$$

$$R\text{–PE} = 0.1247((A_{564} - A_{730}) - 0.4583\ (A_{618} - A_{730}))$$

A 30% concentration of ammonium sulphate found suitable to precipitate higher yield and purity index was used for further large scale pigments purification. The supernatant obtained from 30% ammonium sulphate precipitation of pigments was analyzed for its nutritional potential for liquid fertilizer. The nutritional component of macroelements and trace elements were estimated using inductively coupled plasma atomic emission spectroscopy (Perkin-Elmer, Optima 2000, USA). The reference standard solution VIII (product no. 1.09492.0100, Merck, Germany) used for analysis with a concentration of 10 and 4 mg L^{-1} for macro- and micro-elements analyzed.

Lipids were extracted from the residues leftover from pigments extraction. The residues were mixed with 50 ml of a chloroform : methanol (1:2 v/v) solvent mixture, mixed and centrifuged at 2000 g at 4 ^{o}C for 20 minute. The green lipid layer was sipped out and the residue extracted repeatedly with the same solvent mixture, till greenish organic layer formed. The lipid containing solutions were combined and filtered through 44 µm Whatman cellulose filter paper. The lipid solution was washed by adding water, followed by centrifugation (2000 g, 4 ^{o}C and 5 min). The upper aqueous layer and lower lipid layer were separated. The lipids were dried using rotary evaporator and weighed. Further solvents (chloroform and methanol) from the lipid extraction were recovered using rotary evaporator. The chloroform was recovered from the greenish organic layer while methanol was recovered from the upper aqueous layer. The recovered solvents were recycled and used up to three

times in experiment with *Gracilaria dura* to acertain the scope for recycling of solvents in lipid extraction.

The residue remained after lipid extraction was mixed with distilled water at 1:5 ratio (based on primary biomass weight), autoclaved at 120 ^{o}C for 1.5 hours and homogenized entire content in a grinder mixture in hot condition and then centrifuged at 6300 g for 6 minute. The supernatant was collected and left to gel at room temperature. The gelled material was then frozen at −20 °C for 15 h and thawed to obtain the native agar. Agar obtained after thawing dried at 65 ^{o}C for 12 h. Characterization of agar samples was carried out by Fourier transform infrared (FT-IR) spectroscopy using Perkin-Elmer Spectrum GX FTIR (USA) and characteristic bands were compared with commercial Bacto Agar. The measurement of gel strength of extracted agar samples was carried out by a Nikkansui type gel tester (Kiya Seisakusho, Tokyo, Japan). For determination of gel strength 1.5% solution of agar was prepared in milli Q water and kept at 10 °C for 12 h. The measurement was performed at 20°C. The gelling and melting temperatures were measured[24]. To ascertain the applicability of agar for using in microbial culture media, nutrient agar plates were prepared using different concentrations of extracted agar and compared with the commercial Bacto agar (1.5%, gel strength 600 g/cm^2) as standard. The concentrations of agar investigated in this study included 1.5% , 0.75% and 0.75% of agar extracted from *G. dura* (gel strength 546 g/cm^2), *G. acerosa* (gel strength 1240 g/cm^2) and *G.pusillum* (gel strength 1150 g/cm^2) respectively. The freshly revived *E.coli* strain was inoculated on agar plates and incubated at 37 °C for 24 hrs.

The residual masses remained after agar extractions were used for cellulose extraction. Residual pulps were soaked in acetate buffer (1:15 w/v) containing 36% $NaClO_2$ of residual biomass for bleaching at 60 °C for 8 h. The bleached samples were washed with water till neutrality. Thereafter, the samples were treated with 0.5 M NaOH solution (1:6 w/v) at 60 °C for 12 h. The alkali treated mass was washed with water till neutrality. The neutralized biomass were re-suspended in 5% v/v hydrochloric acid (1:4 w/v) and heated up to boiling. The resultant slurry was kept overnight at room temperature,

followed by washing with water to remove the excess acid and dried to obtain cellulose. Characterization of celluloses was carried out by Fourier transform infrared (FT-IR) spectroscopy using Perkin-Elmer Spectrum GX FTIR (USA) and characteristic bands were compared with Whatman filter paper cellulose.

Enzymatic hydrolysis of cellulose and bioethanol production

Cellulose was hydrolysed with commercial enzyme cellulase 22086 (Novozyme, Denmark). The process was optimized with respect to enzyme dosage and incubation period. A 0.5 g extracted sample of cellulose (*Geliedella acerosa*) was mixed with concentrations of 1%, 2% and 5% v/v cellulase in a fix volume (30 ml) of sodium acetate buffer (pH 4.8), and incubated for hydrolysis for 48 h at 45 °C on an orbital shaker. The optimal production of sugars was determined by measuring sugar yields in samples collected at at 12 h intervals. The reducing sugar was measured spectrophotometrically using the 3, 5-dinitrosalisylic acid (DNS) method[25]. The optimized conditions were further used for hydrolysis of cellulose extracted from *Gracilaria dura* and *Gelidium pusillum*. Further, to check the efficiency of cellulase 22086, the enzymatic hydrolysis of Whatmann filter paper was also studied as a control experiment under the same optimized conditions. The hydrolysed samples were subjected to HPLC (Shimadzu, Japan) for determination of hydrolysates. Fermentation of the cellulose hydrolysate for ethanol was carried out using the yeast *Saccharomyces cerevisiae,* (strain MTCC No. 180, Institute of Microbial Technology, Chandigarh, India). The hydrolysate obtained after enzymatic hydrolysis was enriched with peptone (5 g/L) and yeast extract (3 g/L). The fresh yeast culture (109 CFU/mL) was then inoculated to the fermentation broth. Fermentation was carried out at a temperature of 28 ± 2 °C on an orbital shaker at 120 rpm for 48 h. Sub samples were withdrawn at 12 h interval and analyzed for ethanol yield and residual reducing sugars by GC–MS and DNS method, respectively.

Process scale up.

The consistency of process was evaluated with a batch processing with 500 g fresh weight *Gelidiella acerosa* biomass. All experimental components were increased proportionately to biomass. Pigments were extracted with 1L and 0.5L of phosphate buffer in two cycles. Total lipid was recovered using repeated extraction with 0.5L of solvent Chloroform:Methanol (1:2) till greenish organic layer appeared. The residue obtained after lipid extraction was mixed with 2.5L distilled water and autoclaved at 120 ^{o}C for 1.5 hours followed by centrifugation and freezing and thawing. Residual pulp (31.25 g) remained after agar extraction was bleached with 11.25 g $NaClO_2$ in 0.47L of sodium acetate buffer at 60 °C for 8 h. The bleached samples were washed with water till to neutrality. Thereafter, the samples were treated with 0.19L of 0.5 M NaOH solution at 60 °C for 12 h. The alkali treated mass was washed with water till neutrality. The neutralized biomass was re-suspended in 0.125L of 5% v/v hydrochloric acid and heated up to boiling. The resultant slurry was kept overnight at room temperature, followed by washing with water to remove the excess acid and dried to obtain cellulose

Extraction of total lipid, agar and cellulose using conventional methods.

The yield and properties of products obtained with biorefinery process were compared with extraction from primary biomass of native agar, lipid and cellulose using different conventional methods[26-29]. Ten g of dry seaweeds samples were used for agar and cellulose extraction, while 500 mg dry weight samples used for total lipid extraction.

Determination of CO_2 consumptions.

Fresh seaweed samples were dried at 60 °C to a constant weight and final weights were measured. CO_2 consumption was calculated based on C%. Dry samples were grounded in

fine powder with liquid nitrogen using pestle and morter. C% content was analyzed with Elementar Analysensysteme GmbH vario MICRO cube, calibrated using sulfanilamide as a reference standard. and CO_2 consumption was calculated using previously reported formula[30] with modification as follows:

CO_2 consumption (in kg CO_2) for production of one ton of fresh seaweed = Amount of C (kg) per ton of fresh seaweed * 3.666

Results and discussion

Dry weights.

Macroalgae contain 75-85% water in total body weight, while the rest is represented by organic contents and minerals. The dry weight (DW) fraction of the three red algae as *Gelidiella acerosa*, *Gelidium pusillum* and *Gracilaria dura* investigated in this study were 25.39 ± 0.14%, 38.08 ± 0.25% and 12.24 ± 0.09% respectively.

Qualitative and quantitave determination of products recovered from biorefinery process.

The concentration of pigments of R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) in crude extract of *G. acerosa*, *G. pusillum* and *G. dura* were 449 ± 20, 341 ± 4; 754 ± 11, 108 ± 10 and 358 ± 10, 177 ± 10 µg/g Fresh weight (FW) respectively. The precipitation of pigments with 30% ammonium sulphate was found suitable for all species with highest purity ratio for both pigments R-phycoerythrin and R-phycocyanin (Supplementry Fig. 1). The yields of R-PE for *G. acerosa, G. pussilum and G. dura* were 419 ± 3, 715 ± 5 and 340 ± 5 µg/g FW respectively, while corresponding values for R-PC were 303 ± 4, 99 ± 12 and 160 ± 5 µg/g FW respectively (Fig. 2). The purity ratio of R-PE and R-PC for *G. acerosa, G. pusillum* and *G. dura* were 0.45:0.25, 0.58:0.19 and 0.59:0.24 respectively. Both R-PE and R-

PC (phycobiliproteins) known to have excellent spectroscopic properties, high absorption coefficients, and high quantum yields which make them suitable for using in diagnostics as fluorescent markers and a wide range of applications in biomedical research[31] and as non-toxic natural colorants for food and cosmetics[32]. Natural pigments besides being non-toxic, may possess various biological activities such as anticancer, antioxidant, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective activities that make their use attractive, compared with synthetic toxic pigments in food, cosmetics and pharmaceutical application[32,33].

Liquid obtained as supernatant- 153 ± 3 ml for *G. acerosa*, 157 ± 3 ml for *G. pusillum* and 180 ± 4 ml for *G. dura*- after pelleting out pigments from respective samples contained phosphate (from 0.78 ± 0.02 to 0.92 ± 0.02 mg/100 ml) and ammonium sulphate (from 25.39 ± 0.10 to 25.79 ± 0.08 g/100 ml) applied exogenously for pigment extraction and pricipatation. The liquid also contained additional essential macro- and micro-minerals/nutrients (K, Mg, Na, Ca and Fe, Zn, Zn, Cu etc.) of seaweed origin (Table 1). Recent studies have showed an effective application of different macroalgae mineral-rich extract as a plant growth stimulant on various crops such as wheat and rice[34-37]. The liquid rich of ammonium sulphate, phosphate and minerals implicates its possible applicability as fertilizer for various crops. The chemicals such as ammonium sulphate, phosphate and potassium are considered as major fertilizer. Therefore, the recovery of liquid with all chemicals used for extraction of pigments can effectively be employed for agriculture applications as fertilizer. The liquid byproduct of the biorefinery may therefore serve as foliar plant nutrient spray for various crops. Alternatively, the ammonium sulphate precipitation step in pigments extraction can be replaced with ultra membrane filtration and the mineral water thus obtained could be reused for subsequent pigment extraction from fresh batch of sample.

Lipids were extracted from the residual biomass recovered following pigment extraction. Total lipid content ranged from 0.94 ± 0.05 to 1.41 ± 0.10 % DW. The corresponding values for a direct extraction from the primary feedstock were ranged between 1.03 ± 0.1% and 1.53 ± 0.26% DW. The yields obtained with both sources were comparable (Table 2). Though lipid content of these specific algae is low, PUFA fraction in them has often been higher than in terrestrial vegetables[38,39]. The algal lipid contains nutritionally important polyunsaturated fatty acids (PUFAs) as high as 10-70% of total FAs with nutritionally beneficial n6/n3 ratio (0.1:1–3.6:1)[40]. Lipids recovered through integrated biorefinery process from different macroalgae could be used as excellent food additives in nutraceutical industry. Moreover, the solvents employed for lipid extraction in *G. dura* were also recovered and reused up to three cycles without significant negative effect on yields in each cycle (0.94, 0.91, and 0.85% DW) indicating the cost effectivity of the entire process.

Agar was extracted from the residual biomass samples following lipid extraction. The agar yields obtained by integrated bioprocess and by the conventional extraction method from primary biomass were similar (Table 2). The FT-IR spectra of agar obtained with integrated bioprocess and commercial Bacto agar had characteristic bands at 931 cm^{-1} and 890 cm^{-1}, confirming the similarity with each other (Fig. 3). Interestingly, the gel strength of agar from the integrated bioprocess was 1.5-3.0 fold higher than agar obtained by conventional extraction from primary biomass (Table 2), and superior to many commercially marketed agars (typically 250-850 g/cm^2). The environmentally damaging (and costly) pretreatment of biomass with alkali and acid may become redundant in this new integrated process. Also, the tests carried out to find out the suitability of extracted agar in microbial culture media preparations showed no differences in growth patterns between those of prepared using commercial Bacto agar preparation (Supplementry Fig. 2). This experiment also confirmed that lower concentrations of extracted agar (*G. acerosa* & *G. pusillum*) could be used for

microbiological application due to their high gel strength property, compared with present commercial products.

The residual pulp (15-40% of the originl biomss on a DW basis) remained after agar extraction contained 3.57 ± 0.10 and 11.01 ± 0.7% cellulose on DW which accounts for 27 - 35% of pulp wt. The cellulose extracted from residual pulp of respective samples were comparable with those of obtained with primary biomass using conventional methods (Table 2). The characterization of cellulose with FTIR presented spectra with characteristic peaks, matching with that of cellulose of Whatman filter paper (Fig. 4). Cellulose extraction from such residual biomass minimizes the usage of chemicals by up to 60 to 85% and eliminates the environmentally damaging decoloration and defatting treatments, usually followed in conventional cellulose extraction. Alternatively, wet pulp of *G. acerosa* itself processed without drying which yielded as much as 8.7 ± 0.5% cellulose, similar to that obtained with dry pulp and circumventing the operations involved with dry pulp preparation.

Cellulose hydrolysis and fermentation

The cellulose obtained in the present study was enzymatically hydrolysed with commercial cellulase 22086 using optimized dosage (2%), hydrolysis period (36 h) and temperature (45 °C) (Supplemantry Table 1). The hydrolysis of cellulose extracted from *G. acerosa*, *G. pussilum* and *G. dura* produced reducing sugars of 920 ± 5 mg/g, 930 ± 5 and 910 ± 3 mg/g cellulose respectively which corresponds to 83.63%, 84.54% and 82.72% conversion efficiency respectively. A parallel experimented conducted on hydrolysis of WFP (Whatmann filter paper) showed reducing sugars increasing proportionally with hydrolyzing period. The sugar yields at 36 h was found to be 807±4.9 mg/g cellulose with 73.36% saccharification efficiency while at 60 h yielded 938±4.9 with 85.25% saccharification efficiency (Supplemantry Table 1). Though the saccharification efficiency of enzyme was

found to be almost similar for both algal and WFP cellulose, the total hydrolysis period for algal cellulose was remarkably short (36 h) than WFP cellulose (60 h). The HPLC analysis of hydrolysate confirmed the formation of monosaccharides indicating the conversion of both algal and WFP cellulose to glucose (Supplementary Fig. 3). Fermentation of respective hydrolysates with *Saccharomyces cerevisiae,* (MTCC No. 180) for 12 h (optimal period) produced bioethanol 418 ± 3, 416 ± 4.5 and 411 ± 5 mg/g reducing sugar corresponding to a conversion efficiency of 89.08%, 87.70% and 88.65% respectively (Table 3). The conversion efficiency of algal sugars to ethanol obtained in this study remained between 87 and 89 % which is higher than those values of 80 to 83% reported for brown marcoalgae using an engineered microbial platform[8,12]. Further, the fermentation efficiency of WFP sugars was found to be 90%, a value quite similar to that obtained for algal sugars (87-89%).

Process scale up.

The scaling up of biorefinery process with 500 g fresh feedstock of *G. acerosa* yielded pigments R-PE 403 µg/g FW, R-PC 297 µg/g FW, total lipid 1.45%, agar 23.44% cellulose 9.6 % on DW basis and 1550 ml mineral rich liquid. The agar had gel strength of 1200 g/cm^2 with gelling and melting temperature 41˚C and 92 ˚C respectively. The yields and properties of all products were nearly the same as those realized from 50 g scale biorefining process confirming the scope and feasibility for developing scale-up process. The computed yield data from bench-scale trials reveal that once the process is engineerd for commercial scale, 0.3-0.7 Kg of R-phycoerythrin, 0.1-0.3 Kg of R-phycocyanin, 1.2-4.8 Kg of lipids, 28.4-94.4 Kg of agar, 4.4-41.9 Kg of cellulose and 3.1-3.6 of kilo liter mineral solution can be realized from the processing of one ton fresh biomass of all three investigated species. The enzymatic hydrolysis and fermentation of cellulose thus obtained would yield 1.8-17.4 Kg ethanol.

Phycocolloids (agar, carrageenan and alginate) are the major seaweed extractives produced commercially worldover. The current value of phycocolloids in global market has been estimated at over US$ 1 billion[22]. In 2009, total 72300 tons (dry) agarophytic seaweed resource has been utilised for production of 12500 tons agar[22]. This means that on an average 17% of raw material only contributed to product while the larger chunk (83%) has been dumped as a waste or put to use for low value applications. The disposal of such residul biomass along with seaweed industry effluents might generate considerable environmental concerns. It is now well established in this study that it is possible to recover a number commercial products such as natural pigments, minerals, lipid, and energy dense substrate cellulose from red algal resources. To the best of our knowledge, there is no agar processing industry at present attempting to recover any such products.

The principal advantage of the present biorefinery process is the siginificant co-production of value added byproducts from feedstock, thereby defraying the high production cost of ethanol. The agar industry in India annually harvests about 1990 tons FW (490 tons DW) of *G. acerosa* from wild stocks for agar extraction. The processing of this feedstock in biorefinery enables to realize a stream of products- 6.1 million litre liquid fertilizer, 0.8 ton R-PE, 0.59 ton R-PC, 7.1 ton lipid, 47.04 ton cellulose- besides 114.85 ton agar. The cellulose in turn can be used for producing 19.66 ton bioethanol. Macroalgae being photosynthetic plants, use substantial amount of atmospheric CO_2 for synthesis of organic biomass. For example, *G. acerosa* uses about 320 Kg equivalent CO_2 for attaining one ton fresh biomass while *G. pusillum* and *G. dura* uses 530 and 120 Kg CO_2 respectively. The National Biofuel Policy in India aims to target 20% blending of biofuels by 2017. The earlier data on nearshore pilot scale cultivation of *Gelidiella* sp. suggests that a total 46 tons FW ha^{-1} y^{-1} can be produced in two harvesting cycle. Based on this value, it is estimated that 460 million tons fresh biomass y^{-1} be harvested over 10 million hectare area (correspons to about

5 % of total Exclusive Economic Zone of India). Upon biorefining this quantity could generate 6.66 billion liters bioethanol, which is adequate enough for meeting the targeted blending of petrol by 2017 in India along with the co-production of substantial amounts of agar, lipid and mineral liquid having immense trade value in established seaweed markets which are continuously growing worldwide with discovery of additional new products and new applications.

Conclusions

The biorefining process, the next generation biomass processing technology, developed for red algae in the present study, enables to realize the potentials offered by seaweed resources to the fullest extent. To meet the bioethanol targets, vast sea front has to be farmed with seaweeds for producing several hundred million tons of biomass for feeding the biorefinery. Seaweed farming is manpower intensive and thus creats new additional employment and sustainable income sources, improving the livelihoods and socio-economic status of economically underprivileged coastal communities. The large scale farming of macroalgae creates ocean-based industry and sustainable income streams, mitigates coastal eutrification minimizing the formation of macroalgal blooms and also mitigates the global warming and climate change affects arising from GHG emissions by burning of fossil fuels, in addition to freeing the dependency on terrestrial resources for food, feed, water, chemicals and energy.

Acknowledgements

The financial support received from BioWALK4Biofuel (grant No. 241383) is gratefully acknowledged. Authors would also like to thank CSIR-CSMCRI for providing encouragement and facilities required for carrying out this work.

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Figure captions

Figure 1 Schematic presentation of developed biorefinery process.

Figure 2 UV-spectra of pigments of different macroalga precipitated with 30% ammonium sulphate. R-PE, R-Phycoerythrin and R-PC, R-Phycocyanin.

Figure 3 FTIR spectra of agar extracted from different macroalga using integrated process and standard agar. a, Bacto agar , b, *Gelidium pusillum*, c, *Gelidiella acerosa,* d, *Gracilaria dura*.

Figure 4 FTIR spectra of cellulose extracted from different macroalga using integrated process and standard cellulose. a, Whatman Cellulose, b, *Gelidium pusillum*, c, *Gelidiella acerosa,* d, *Gracilaria dura*.

Supplementry Figure 1 Contents of pigment R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) with 10-50% ammonium sulphate precipitation.

Supplementry Figure 2 *E. coli* grown in nutrient broth supplemented with commercial agar and agar extracted from different agarophytes. a, 1.5% Bacto agar, b, 1.5% *G. dura* agar, c, 0.75% *G. acerosa* agar , d, 0.75% *G. pusillum* agar.

Supplementry Figure 3 HPLC chromatogram of a) standard glucose, b) algal hydrolysate (36 h), c) Whatmann filter paper hydrolysate (36 h) and d) Whatmann filter paper hydrolysate (60 h).

Table 1 Composition of liquid extract of different seaweeds (mg/100 mL)

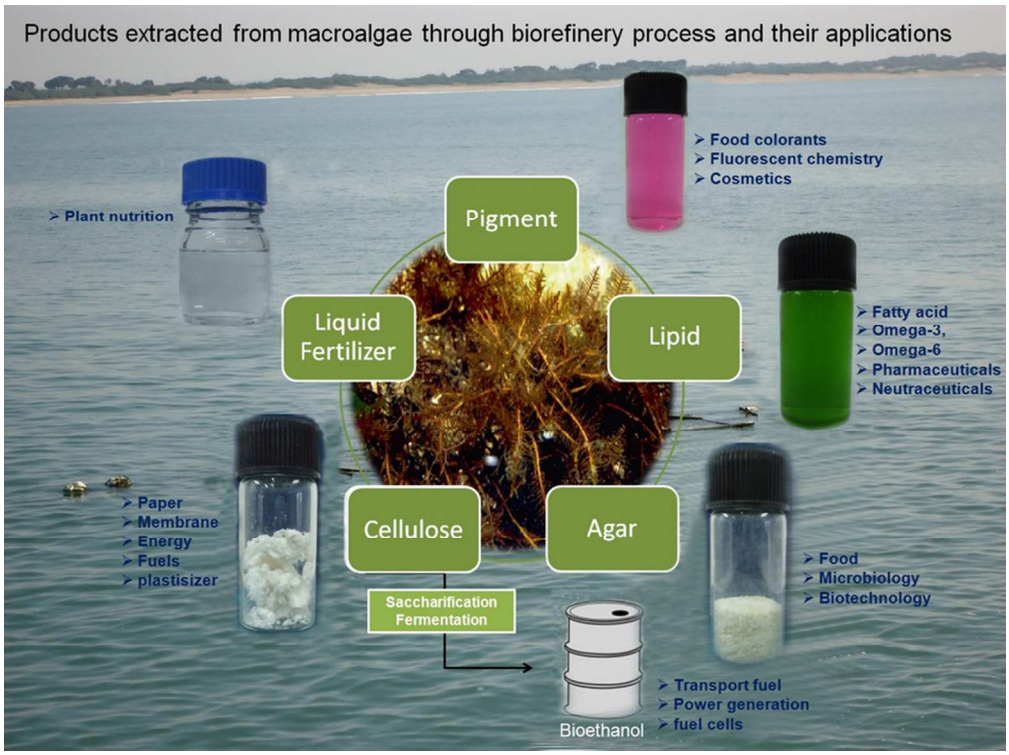
Mineral	*Gelidiella acerosa*	*Gelidium pusillum*	*Gracilaria dura*
Al	0.07±0.01	0.23±0.16	0.08±0.02
Ca	10.38±2.25	14.07±3.82	7.48±3.03
Cr	0.05±0.03	0.14±0.10	0.04±0.02
Cu	0.03±0.02	0.07±0.04	0.01±0.01
Fe	0.09±0.01	1.59±1.31	0.11±0.02
K	132.34±2.88	71.31±7.75	96.37±0.62
Mg	12.88±2.43	12.73±1.58	11.61±4.21
Mn	0.06±0.05	0.19±017	0.16±0.05
Na	12.70±0.49	18.60±0.94	12.32±0.32
Ni	0.01±0.01	0.03±0.02	0.01±0.01
Se	0.63±0.33	0.57±0.32	0.42±0.31
Zn	1.76±0.32	1.03±0.05	0.30±0.26
$(NH4)_2SO_4$ (g)	25.68±0.08	25.79±0.08	25.39±0.10
PO_4^{3-} (g)	0.92±0.02	0.90±0.02	0.78±0.02

Table 2 Comparison of yields of different products and physical properties of agar obtained through biorefinery process and direct extraction using conventional methods from red algal species

	Gelidiella acerosa		*Gelidium pusillum*		*Gracilaria dura*	
Product Yield (%)	**Integrated process**	**Direct extraction**	**Integrated process**	**Direct extraction**	**Integrated process**	**Direct extraction**
Dry weight (%)	25.39 ± 0.14		38.08 ± 0.25		12.24 ± 0.09	
Lipid (%)	1.41 ± 0.10	1.53 ± 0.26	1.26 ± 0.05	1.34 ± 0.08	0.94 ± 0.05	1.03 ± 0.1
Agar (%)	23.04 ± 1.09	24.50 ± 0.70	24.78 ± 0.94	25.23 ± 0.50	23.24 ± 0.55	25.15 ± 0.78
Gel strength (g/cm^2)	1240 ± 20	423 ± 15	1150 ± 50	750 ± 30	546 ± 25	250 ± 10
Gelling temp.(˚C)	41 ± 1	38.5± 0.5	45 ± 0.5	44 ± 0.5	35 ± 1	33 ± 0.5
Melting temp. (˚C)	92.5 ± 0.5	84.5 ± 0.5	96 ± 0.5	94 ± 0.5	86.5 ± 0.5	82 ± 0.5
Cellulose (%)	8.84 ± 0.5	9.97 ± 0.23	11.01 ± 0.7	12.20 ± 0.45	3.57 ± 0.10	3.70 ± 0.13

Table 3 Laboratory scale data on saccharification and bioethanol production from cellulose extracted from different seaweeds using biorefinery process

Species	Total sugar (mg/g)	Unfermented Sugar (mg)	Ethanol yield (mg/g sugar)	Theoretical yield (mg)	Efficiency (%)
Gelidiella acerosa	920 ± 5	42	418 ± 3	469.2 ± 2.5	89.08
Gelidium pusillum	930 ± 5	56	416 ± 4.5	474 ± 2.6	87.70
Gracilaria dura	910 ± 3	60	411 ± 5	464.1 ± 1.5	88.65



Products extracted from macroalgae through biorefinery process and their applications
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