A simple process for recovery of a stream of products from marine macroalgal biomass

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HIGHLIGHTS

- High throughput and integrated process.
- Densification of product mixture with successive process progress.
- No acid and alkali application.
- Process comprises of maximum water extraction steps.
- Effective utilization of total biomass without any leftover solid waste.

GRAPHICAL ABSTRACT
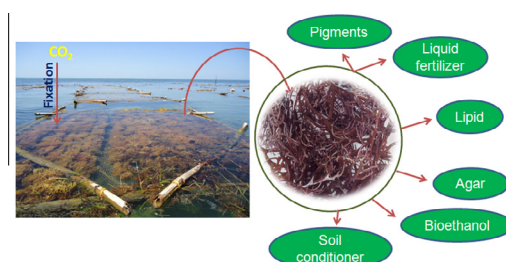
A schematic representation of seaweed cultivation for CO_2 sequestration coupled with bio-processing

ARTICLE INFO

ABSTRACT

The present study describes a simple process for recovering a stream of products sequentially including bioethanol from the fresh biomass of the red seaweed *Gracilaria corticata*. From processing of 100 g fresh biomass ($\sim$12.2 g dry), 166 ± 3 µg/g R-phycoerythrin, 126 ± 4 µg/g R-phycocyanin can be realized on fresh weight basis, and 1.41 ± 0.03% crude lipid, 22.45 ± 0.53% agar, 12.39 ± 0.85% soil conditioner, 2.89 ± 0.04% bioethanol on dry weight basis along with 318 ± 3 ml of mineral rich liquid with possible fertilizer applications. The advantages of this process are complete utilization of feedstock without compromising the yield and quality of products, reusability of solvents and no solid waste. Further, the products recovered from one ton fresh biomass were found to have an estimated market value of USD 1051 while processing cost including raw material as 241 USD, a fourfold value addition of feedstock.

1. Introduction

Oceans which occupy more than 70% of our planet represent vast, renewable resources that are quite suitable to satisfy the global needs of food, feed, medicine, chemicals and energy. There is a global effort to produce sustainable renewable fuels from plant biomass to supplement fast depleting fossil fuel reserves as well as to negate the global warming and climate change effects arising from burning of fossil fuels (Melero et al., 2012; Solomon, 2010). The U.S. Department of Energy targeted to replace 30% of the petroleum-based transportation fuel with biomass-based fuels by 2025 (International Energy Agency, 2012). Similarly, biofuel policy of India too targets 20% blending of transportation fuels by 2017. Ethanol is the most common biofuel in the U.S. (Yan et al., 2010) and the global demand has been projected to increase by 3.4 folds by 2035 (International Energy Agency, 2012). Most of the biofuel produced at present is from food crops (Aikawa et al., 2013). The diversification of food crops for biofuel would have adverse impact on human food supply chain and thus preclude their long term use

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(Aikawa et al., 2013). Therefore, a non-edible plant source like lignocellulose has been a preferred feedstock. The lignin which forms 20–30% (dry weight) of such feedstock is recalcitrant to most depolymerization processes employed for releasing of fermentable sugars for biofuel production. Consequently, the non-lignocellulosic sources such as marine macroalgae (seaweeds) have been explored as alternative potential feedstock for production of biofuel (Rajkumar et al., 2014) with low carbon footprint.

Seaweeds have worldwide distribution and are well known for their uses in human foods and in phycocolloid industries (McHugh, 2003). Over the past five decades, there has been phenomenal growth in global seaweed industry and annual worldwide production of seaweeds has been estimated at 26 million tons fresh (FAO, 2014), with a value USD 7.3 billion. Seaweed polysaccharides such as agar, carrageenan and alginate are one of the commercially valuable products extracted from seaweed resources and have a market value over USD 1 billion (Bixler and Porse, 2011). The seaweed phycocolloids industries uses only 15–30% of the total dry mass whiles the remainder 70–85% get degraded during the phycocolloids extraction process or drained out as a waste with effluents. However recent studies showed that the seaweeds are rich in several valuable metabolites such as natural pigments, protein, lipid, minerals and cellulose in addition to phycocolloids (Baghel et al., 2014a,b). There is a concerted research effort being directed to make required technological innovations in downstream process to convert macroalgal biomass to fuels and chemicals. Numerous research reports have unequivocally underlined the need for developing integrated technologies for biomass conversion to fuels and chemicals in order to overcome techno-economic barriers in production of biofuel (Baghel et al., 2014a; Jung et al., 2013; Kerton et al., 2013). Complete conversion of biomass to chemicals and fuels employing benign process with less or no effluents is the key determinant for the success of bioprocessing technology. The initial studies have mainly dealt with whole biomass conversion to fuel through hydrolysis followed by fermentation (Mutripah et al., 2014; Meinita et al., 2013; Park et al., 2012). Subsequently, synthetic microbial platforms were designed to convert either polysaccharide or the whole biomass into bioethanol (Enquist-Newman et al., 2014; Takeda et al., 2011; Wargacki et al., 2012). However, long term use of industrially important polysaccharides for bioethanol production could jeopardise the prevailing phycocolloid industry and allied markets worldwide. There have been a few reports on integration of biofuel production with recovery of either plant nutrient rich sap as plant growth stimulant (Khambhaty et al., 2012) or phycocolloid (Kumar et al., 2013) from seaweed biomass. Recently, Baghel et al. (2015), produced a stream of products along with bioethanol in integrated manner from fresh seaweed biomass in biorefinery approach. Nevertheless, this process involves utilization of various chemicals (ammonium sulfate, NaClO$_2$, NaOH and HCl) extensively while recovering products and subsequently be discharged along with effluents.

The present study describes a simple benign integrated process based on aqueous extraction steps for recovery of pigment, agar and mineral rich aqueous solution as liquid fertilizer from marine macroalgal biomass. The residual mass thus remained after agar extraction was converted into sugars through enzymatic hydrolysis and therafter bioethanol production following fermentation route.

2. Methods

2.1. Sample collection

Among the red seaweeds, *Gracilaria corticata* is most commonly and abundantly occurring taxon along the Indian shores but largely remained as untapped resources for agar. The fresh samples of

G. corticata were collected from Diu (N 20° 44.1′; E 70° 58.5′) on western coast of India and carried in cool pack to the laboratory. The sand and epiphytes were cleaned of from the thallus using brush in filtered seawater. The cleaned sample was then maintained for a week in the culture laboratory in filtered seawater for experimentation. The culture was maintained at 25 ± 1 °C under cool white fluorescent lamps at 15 μmol photons m^{-2} s^{-1} with a 12:12 h light: dark photoperiod.

2.2. Chemical composition analysis

For the determination of dry weight, fresh sample was blotted with tissue paper to remove excess external water and then dried in oven at 60 °C until a constant weight. Dry weight was calculated by subtracting the final weight from initial weight. Dry weight data was computed for expressing subsequent product yields (biorefinery process) on dry weight basis. The organic nitrogen content of sample was quantified using fine dry powder with instrument Elementar Analysensysteme GmbH vario MICRO cube, calibrated using sulfanilamide as a reference standard. The total protein contents were estimated by multiplying the nitrogen content by a factor of 6.25. Total lipid was extracted from 1 g dry algal sample following the method of Bligh and Dyer (1959) and gravimetrically determined the content. Agar and cellulose contents in the sample were determined following the method reported by Meena et al. (2008) and Mihranyan et al. (2004) respectively.

2.3. Extraction of stream of products from seaweed biomass in biorefinery approach

The sequence of extraction of different products from feedstock is presented in Fig. 1. In brief, a 100 g fresh *G. corticata* sample was homogenized in 200 ml distilled water using a mixer grinder followed by 12 h incubation at 4 °C. The incubated sample was mixed thoroughly and filtered through muslin cloth to separate crude pigment containing liquid fraction and residual mass. This step without incubation was repeated for further recovering pigment from the residual mass. The residual biomass thus obtained was used for extraction of other products following drying. The filtrates were mixed together and then subjected to ultra-membrane filtration (UMF) with 200 kDa cutoff poly sulfone membrane to separate the concentrated pigment mixture and mineral rich water. Pigments concentrate was diluted in distilled water and the
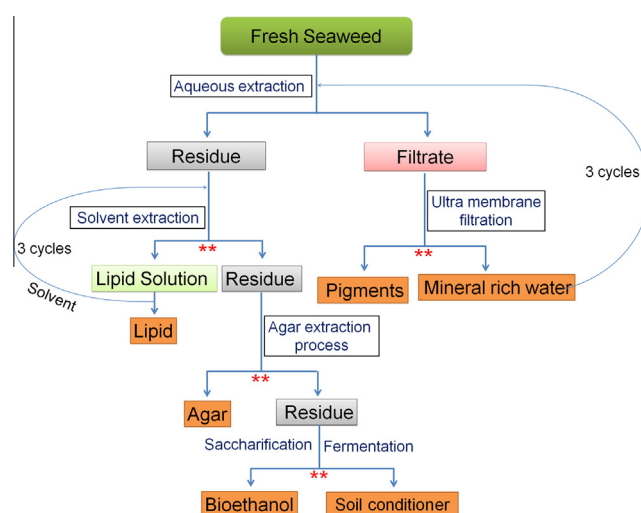

Fig. 1. Schematic presentation of biorefinery process. **Recyclability of water.

absorbance of solution was measured using a UV–Vis spectrophotometer (UV-160, Shimadzu, Japan) at 280, 564, 618 and 730 nm. The content of R-PE and R-PC pigments was calculated according to the following equation (Sampath-Wiley and Neefus, 2007):

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

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

The mineral rich water as obtained after ultra-membrane filtration was further reused for pigment extraction from at least two batches of fresh sample. The filtrate obtained at each time was analyzed for plant nutrients potential as growth stimulant. Both macro and micro-elements present in filtrate 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) was used for analysis with a concentration of 10 and 4 mg L^{-1} for macro and micro-elements analyzed.

The dried residual mass as obtained from pigment extraction was used for lipid extraction. The residue was mixed with 100 ml of chloroform–methanol (1:2 v/v) solvent mixture incubated at room temperature for 30 min and filtered through muslin cloth. The green lipid containing filtrate was collected and the residue was extracted repeatedly with the same solvent mixture till organic layer was colorless. All lipid containing fractions were combined and filtered through 44 µm Whatman cellulose filter paper. The lipid solution was washed by adding water, followed by mixing and separation using separating funnel. The upper aqueous layer and lower lipid layer were separated. The lipids were dried using a rotary evaporator and weighted. Further solvents (chloroform and methanol) from the lipid extraction were recovered using a rotary evaporator. The chloroform was recovered from greenish organic layer while methanol was recovered from the upper aqueous layer. The recovered solvents were recycled and used up to three times for lipid extraction with 100 g, 70 g and 50 g of FW G. corticata to ascertain the scope for recycling of solvents in lipid extraction as the solvent volume decreases progressively with each extraction cycle.

The dried residual mass remained after lipid extraction was used for agar extraction. Dried residue was mixed with distilled water at 1:35 ratio, cooked at 120 °C for 1.5 h in autoclave and the cooked hot slurry was homogenized in a grinder mixture and then centrifuged at 6300g for 6 min. The agar containing supernatant was collected and allowed to gel at room temperature. The gelled material was then frozen at −20 °C for 15 h and thawed to obtain the native agar which subsequently dried at 65 °C for 12 h and then characterized it using fourier transform infrared (FT-IR) spectroscopy (Perkin–Elmer Spectrum GX FTIR, USA). The FT-IR spectra of the sample compared with commercial Bacto Agar. Nikkansui type gel tester (Kiya Seisakusho, Tokyo, Japan) used to measure the gel strength of agar. For determination of gel strength, 1.5% solution of agar was prepared using Milli-Q water and kept at 10 °C for 12 h or overnight. The measurement was performed at 20 °C.

Residual pulp remained after agar extraction was dried and then hydrolyzed with 2% commercial enzyme cellulase 22086 (Novozyme, Denmark) in a fixed volume (50 ml) of sodium acetate buffer (pH 4.8) following the optimized condition as reported earlier (Trivedi et al., 2013). The reducing sugar in hydrolysate was measured spectrophotometrically using the 3,5-dinitrosalicylic acid (DNS) method reported by Miller (1959). Fermentation of sugars in hydrolysate into ethanol was carried out using the yeast Saccharomyces cerevisiae (MTCC No. 180, Institute of Microbial Technology, Chandigarh, India). The fresh yeast culture (10^9 CFU ml^{-1}) was then inoculated to the hydrolysate. Fermentation was carried out under optimized temperature of 28 ± 2 °C on an orbital shaker at 120 rpm for 12 h. The ethanol yield was analyzed by gas chromatography–mass spectroscopy (GC–MS).The residue remained after fermentation was subjected to solar drying and analyzed for CHNS contents to evaluate its potential as possible soil conditioner using the instrument, Elementar Analysensysteme GmbH vario MICRO cube, calibrated using sulfanilamide as a reference standard.

2.4. Costing of process and products

The costing of process as well as value of products as obtained from one ton fresh biomass was done in order to establish the superiority of the present process. The expenses towards processing include raw material, energy, solvents and manpower cost. The value of products was arrived based on price quoted in reputed commercial selling sites such as http://www.alibaba.com and http://www.angus-horticulture.co.uk.

2.5. Statistical analysis

All the analyses were performed in triplicate and the mean values were recorded.

3. Results and discussion

3.1. Proximate composition of G. corticata

The data on proximate composition provides fundamental information on various biochemical contents present in the biomass and forms basis for determining its possible effective utilization. Macroalgae, being the aquatic organisms, contains copious amounts of water in their body ranging from 75% to 90%, while the rest is largely represented by organic matter and to lesser extent minerals. The dry weight (DW) of G. corticata was found to be 12.2 ± 0.3%, while the reminder 87.8% accounting for water content. The water content in seaweeds widely varies with species and depends on the thallus architecture. The total protein and lipid content were found to be 13.85 ± 0.47 and 1.48 ± 0.09% on DW basis, respectively which were comparable to the earlier study by Baghel et al. (2014a). Among the carbohydrates, agar and cellulose content were found to be 23.01 ± 0.47 and 6.10 ± 0.16% DW, respectively. The agar content was similar to those reported earlier for G. corticata (Baghel et al., 2014a).

3.2. Bio-products and their quantitative and qualitative analysis

3.2.1. Pigments recovery

The content of pigments, R-phycoerythrin and R-phycocyanin in crude extract were 173 ± 5 µg/g and 137 ± 7 µg/g fresh weight (FW) respectively. The ultra membrane filtration of crude pigment with molecular weight cut-off poly sulfone membrane able to recover 166 ± 3 R-PE and 126 ± 4 µg/g FW R-PC with purity of 0.5 and 0.25. In general ammonium sulfate was used to precipitate the pigments from crude extract. In the previous study, 30% ammonium sulfate was used to precipitate pigments from crude extract, while in the present study ultra-membrane filtration was used for separation of pigments and the filtrate could be either reused for pigment extraction for another fresh batch of feedstock or used as plant nutrient rich extract for foliar applications. The pigments recovered from plant sources could be excellent substitutes for harmful synthetic pigments and can have wide range of application in the field of diagnostic, biomedical research, as food colorants in food industry, cosmetics and pharmaceutical applications (Pangestuti and Kim, 2011; Naidu et al., 1999).

Table 1
Progressive increase of mineral content (mg/100 ml) in liquid extract of *G. corticata* with each water recycling and use.

Minerals	Ist Cycle	IInd Cycle	IIIrd Cycle
B	1.03	1.66	2.21
Ca	8.66	18.01	31.68
Cu	0.01	0.01	0.01
K	153.2	341	529.33
Na	27.01	58.73	87.13
Mg	17.28	36.83	56.93
Mn	0.29	0.69	1.08
Se	0.49	0.60	0.74
Zn	0.15	0.20	0.31

3.2.2. Recovery of mineral rich liquid and its possible application

A 318 ± 3 ml liquid was obtained as filtrate from UMF of crude extract of pigment from the processing of total 3 batches each with 100 g fresh weight. The liquid contained copious amount of essential macro and micro-minerals/nutrients (K, Mg, Na, Ca and Fe, Zn, Cu etc.) of seaweed origin (Table 1). The minerals concentration increased proportionally from first batch to third batch of extraction indicating its gradual build up from each cycle (Table 1). In the recent decade, seaweed based minerals rich liquid extracts assumed considerable commercial value as plant growth stimulants. The recent studies have well established beneficial effects of seaweed extracts as organic fertilizer on seed germination and growth of various crops (Rao and Chatterjee, 2014; Akhtar et al., 2014; Shah et al., 2013). Thus, the mineral rich extract obtained in this process could be used as a liquid fertilizer for augmenting crop productivity and food production.

3.2.3. Crude lipid

Crude lipid extracted from the residual biomass remained after pigment extraction was 1.41 ± 0.10% DW (corresponds to 2.66 ± 0.02% of residual dry mass) which was comparable to that of 1.48 ± 0.09% DW recorded for direct extraction from the primary feedstock using conventional method (Table 2). Though lipid content of seaweeds is low, but the polyunsaturated fatty acid (PUFA) fraction is higher than those of terrestrial vegetables (Darcy-Vrillon, 1993). *G. corticata* contained low lipid content but rich in nutritionally important PUFAs 65.6 ± 2.5 of total FAs (Kumari et al., 2013). Lipids recovered from *G. corticata* in integrated process could be used as excellent food supplement in the nutraceutical industry. The reusability of solvents demonstrated successfully up to 3 cycles without significant negative effect on yields in each cycle (1.45, 1.43 and 1.38% DW) could significantly improve the economics of process. Additionally, replacement of centrifugation steps in pigment and lipid extraction with simple muslin cloth filtration would substantially contribute to the economics of overall process.

Table 2
Comparison of product yields of integrated biorefinery with those of obtained individually from primary biomass.

Products	% DW yield based on primary dry biomass	% DW yield based on residual biomass	% Yield based on individual extraction from primary biomass
Lipid	1.41 ± 0.03	2.66 ± 0.02	1.48 ± 0.09
Agar	22.45 ± 0.53	39.49 ± 1.08	23.01 ± 0.47
Bioethanol	2.89 ± 0.04	12.72 ± 0.14	–
Soil conditioner	12.39 ± 0.8	54.74 ± 0.38	–

3.2.4. Recovery of agar and its physical properties

The residual biomass resulted from lipid extraction was further processed for recovery agar. The agar yields were similar for both integrated and conventional process using primary biomass (Table 2). However, the agar content remained in residual biomass was remarkably higher (39.82 ± 0.49%) as compared to primary biomass (Table 2). The FT-IR spectra of agar obtained with the integrated bioprocess and commercial Bacto agar had characteristic bands at 931 cm^{-1} and 890 cm^{-1}, confirming the similarity with each other. Gel strength of agar was recorded ⩽100 g cm^{-2}.

3.2.5. Bioethanol production and CHNS composition of fermentation residues

The residues remained after agar extraction was accounted for 22.78 ± 0.16% of initial biomass DW. The enzymatic hydrolysis of pulp produced reducing sugars of 269 ± 2.6 mg/g residue. Fermentation of hydrolysate with *S. cerevisiae* produced bioethanol yield of 472 ± 4.9 mg per g reducing sugar corresponding to 92% conversion efficiency. The bioethanol yield was 12.72% based on residual mass. In this study, cellulose rich residual mass subjected to direct hydrolysis instead of cellulose extraction followed by hydrolysis. The direct utilization of pulp as substrate for hydrolysis and fermentation offers twin benefits. The first one is elimination of cellulose extraction step and thereby saving all those chemicals employed for its extraction while the second one dispenses the protein supplementation in fermentation broth as residual pulp consists of copious amounts of seaweed origin protein. The residue obtained after fermentation was found to be 12.39 ± 0.8% with the C:H:N:S composition of 42.8%:6%:9.7%:0.75%. Since residue remained at the end of process was rich in composition and could be a good soil conditioner.

The developed process successfully recovered 6 products of commercial value (Fig. 2). The findings reported in this study advances biorefinery process by substituting cumbersome ammonium sulfate precipitation of pigment with simple ultra filtration and dispensing cellulose extraction from residual biomass avoiding usage of corrosive chemicals (NaClO$_2$, NaOH and HCl) and their discharge in effluents. Instead, the residual mass (rich of cellulose) was enzymatically hydrolyzed directly to obtain reducing sugars which in turn fermented to produce ethanol. Therefore, all processing steps adopted in this study are aqueous based except lipid extraction where solvents used (chloroform and methanol).

4. Theoretical calculation and techno-economic feasibility of process

The global agar industries process 72,300 dry tons of agarophytic seaweeds (equivalent to 289,200 tons fresh weight assuming 75% water content) annually to produce 12,500 tons of agar (Bixler and Porse, 2011) and the remainder is lost as waste. However, processing of such biomass in biorefinery model can lead to recover a number of products as much as ⩾242.98 tons pigments (0.84 mg/g FW), ⩾867.6 tons lipid (1.2% DW), ⩾8676 tons soil conditioner (12% DW), ⩾2.85 million liter bioethanol (3% DW), ⩾86,760 tons liquid fertilizer (30% FW water recovery) along with 12,500 tons agar. These values were estimated based on average value calculated from previous study by Baghel et al. (2014a). The bioethanol produced from such residual biomass could be utilized for captive consumption by phycocolloids industries itself. As per our previous report (Baghel et al., 2015) and the present study, it is estimated that 15–40% of initial dry biomass always remained as residual biomass at the end of sequential extraction of multiple products and the cellulose content in it ranged from 27–35%. This value is quite comparable with those values reported for lignocellulosic biomass such as grasses (25–40%), hardwood barks

Fig. 2. Products recovered from biomass of *G. corticata* through biorefinery model and their possible applications.

(22–40%) and softwood barks (18–38%) (Balat, 2011). The production of bioethanol from seaweeds based residual mass is economically attractive option over woody biomass because it involves less unit operations (hydrolysis and fermentation) as it does not contain complex lignin which demands energy intensive chemical pre-treatment. Further, fermentation broth do not required any supplementation of nitrogen source as algal hydrolysate itself contains requisite protein for yeast growth. Yet another advantage of the present process is that the application of ultra-membrane filtration for separation of pigment (crude) from mineral rich aqueous solution, which could be viable at large scale production instead of using such high amount of ammonium sulfate (30–40%) for pigment precipitation. The direct utilization of residual biomass as obtained after agar extraction for bioethanol production also further avoids the need of cellulose extraction and associated chemical treatments to keep the process as benign as possible. It is also presumed that the water used at different extraction steps as shown in Fig. 1 can effectively be reused with RO filtration with ease at industrial scale. The concept of recovery of multiple products of commercial value in biorefinery model from feedstock not only results in complete utilization of biomass but also help to realize maximum value from crop which in turn benefit the communities engaged in seaweed farming. Seaweeds being marine origin do not compete with terrestrial plants for land, freshwater, fertilizer, pesticides, herbicides etc. and thus become the most promising feedstock for production of chemicals and biofuel. It is estimated from pilot scale trials (100 g fresh wt.) that one ton fresh biomass (~122 kg dry mass) yields 0.28–0.30 kg crude pigment (R-Phycoerythrin and R-Phycocyanin), 1.69–1.77 kg lipids, 26–28 kg agar, 3.49–3.59 kg of bioethanol, 14.9–15.3 kg of soil conditioner and 600–610 L of mineral rich liquid fertilizer. The raw material as well as processing cost of one ton fresh biomass was computed as 241 USD, while the market value of products estimated at 1051 USD. The breakup details of costing of process as well as value of products are presented in Table 3. It is evident from the costing exercise that if the biomass processed for recovery of agar alone, the ratio of processing cost and value

Table 3
Assessment of value of products as well expenses towards processing of one ton fresh biomass of *G. corticata* in biorefinery model.

Unit operation	Cost of unit operation (USD)	Products	Product value (USD)
1 ton fresh biomass	75.00	NA	NA
Aqueous extraction	13.5	600 L liquid fertilizer	600 (1USD/L)
		292 g natural colorants	65 (225USD/kg)
Lipid extraction	6.4	1.72 kg lipid	–
Agar extraction	44.1	27.3 kg agar	327.6 (12USD/kg)
Bioethanol production	2.1	4.47 L bioethanol	2.66 (0.64 USD/L)
		15.1 kg soil conditioner	55.8 (3.7USD/kg)
Manpower	100		
Total	241.1		1051.06

NA, not applicable

of product is 1:2 (169USD:327.6 USD) while corresponding ratio for multiproduct recovery is 1:4 (241USD:1051.9USD) which is far more beneficial and help to realize greater value from feedstock.

5. Conclusions

The biomass deconstruction process described in this study enables to realize the full potential of marine macroalgal feedstock for production of fuel and chemicals. The other advantage of the products that were obtained in this process can be used for further value addition studies for high end applications in nutraceutical, bio-medical and chemical industry. The ocean resources will play a greater role in meeting the food and energy security future mankind. The process disclosed in this study forms the basis for establishing sustainable macroalgal biorefinery.

Acknowledgements

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.2015.12.051.

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