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Deoiled algal biomass derived renewable sugars for bioethanol and biopolymer production in biorefinery framework

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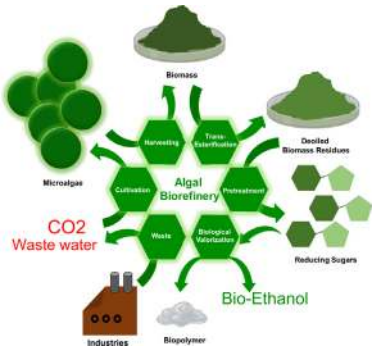
GRAPHICAL ABSTRACT



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ABSTRACT

The present study is designed to evaluate the potential of deoiled algal biomass (DAB) residue as an alternative resource for the production of bioethanol and biopolymers in a biorefinery approach. Hybrid pretreatment method resulted in higher sugar solubilization (0.590 g/g DAB) than the corresponding individual physico-chemical (0.481 g/g DAB) and enzymatic methods (0.484 g/g DAB). Subsequent utilization of sugars from hybrid pretreatment for bioethanol using *Saccharomyces cerevisiae* resulted in maximum bioethanol production at pH 5.5 (0.145 ± 0.008 g/g DAB) followed by pH 5.0 (0.122 ± 0.004 g/g DAB) and pH 6.0 (0.102 ± 0.002 g/g DAB). The experiments for biopolymer (PHB: polyhydroxybutyrate) production resulted in 0.43 ± 0.20 g PHB/g DCW. Extracted polymer on NMR and FT-IR analysis showed the presence of PHB. Exploration of DAB as an alternative renewable resource for multiple biobased products supports sustainability and also enables entirety use of DAB by addressing the DAB-residue allied disposal issues.

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1. Introduction

Microalgae sequester atmospheric CO_2 to accumulate carbon in the form of carbohydrates (starch and cellulose) and lipids (autographically produced) (Chng et al., 2017; Shokrkar et al., 2018). Microalgae (3G feedstocks) possess an edge over land-based plant biomass such as lignocellulosic substrates (2G feedstocks) with respect to biomass production rate which in case of microalgae is 5–10 times higher than lignocellulosics (Sanchez Rizza et al., 2017; Sivaramakrishnan and Incharoensakdi, 2018). Besides, the lack of lignin in microalgal biomass is the prime advantage relative to 2G counterpart where the access to carbohydrate pool is hindered by the presence of lignin mioties. This makes 2G renewables less attractive for commercial applications (Ho et al., 2013; Lee et al., 2015). This advantageous trait of microalgae is highly beneficial to the algal biorefineries as it can positively influence the minimum ethanol selling price (MESP) by omitting the additional step of lignin removal. Nevertheless, despite the high potential, large-scale cultivation of microalgae is hampered by high energy requirements, capital (CAPEX) and operational (OPEX) expenditures (Bibi et al., 2017; Velazquez-Lucio et al., 2018; Wu and Chang, 2019). Cultivation techniques that enable high biomass yield in open systems may possibly improve the economic feasibility of algal biorefinery, however, the cost-effective product recovery methods are indispensable (Venkata Mohan et al., 2016; Hwang et al., 2016; Chng et al., 2017; Venkata Mohan et al., 2019). Additionally, there are certain limitations associated with algal biofuel production such as lower biofuel yields, larger cultivation area requirement and high input cost specifically during downstream processing (Venkata Mohan and Prathima Devi, 2012; Laurens et al., 2015; Park et al., 2016). To overcome these limitations, algal bioprocess net energetic yield needs to be improved in terms of multiple product generation and entirety utilization without residual biomass disposal. An approach like entirety use of microalgal biomass ensures economical benefits to the existing algal refinery process and facilitates a considerable option besides the routine high biodiesel yield optimization strategies (Laurens et al., 2015). After lipid extraction, the biomass is termed as lipid extracted or deoiled algal biomass (DAB), which contains notable amount of reusable carbon (Subhash and Venkata Mohan, 2014). Currently, DAB residue is majorly dumped as waste or used directly as animal feed apart from use as a substrate for biomethane production. Alternatively, DAB residues could be used as a potential resource for renewable sugars production followed by its utilization for various biobased product synthesis such as biohydrogen, volatile fatty acids and bioethanol (Lee et al., 2015; Morrill et al., 2017).

Bioethanol is an eco-friendly and sustainable liquid biofuel that has been extensively investigated and commercialized (Hernández et al., 2015). However, ethanol commercialization is predominately based on first-generation (1G) feedstocks such as sugarcane, corn and sugar beet that led to the food versus fuel conflict as the food crops are being directed from food basket to biorefinery (Tesfaw and Assefa, 2014; Shokrkar et al., 2018). Therefore, to explore the alternative strategies, resource availability in a decentralized approach plays an important role. This is owing to the reason that the choice of biomass as a resource for ethanol production depends on several vital factors like socio-economic, environmental and industrial factors (Trivedi et al., 2015; Shokrkar et al., 2017). Although lignocellulosic biomass is present in large quantities, the extraction of simple fermentable sugars from these 2G feedstocks is challenging due to the high lignin content and the high cost involved in the biomass pretreatment (Prasad et al., 2019). Alternatively, the sugars derived from DAB can be considered as a renewable resource for bioethanol production (Venkata Mohan et al., 2019).

Besides bioethanol, DAB derived sugars can also be used for the production of multiple biological products using various biological processes. Biopolymers (PHA: polyhydroxyalkanoates) are the biodegradable polymers produced by microbiomes when subjected to stress conditions such as limitation of nitrates, phosphates, oxygen stress, etc (Venkata Mohan et al., 2010; Venkateshwar Reddy et al., 2015; Dietrich et al., 2019; Venkata Mohan et al., 2019). Since their first discovery by Lemogine in 1926, PHA's attracted a great deal of attention due to their biodegradability, chemical-diversity, biocompatibility, and their production from renewable carbon resources (Korkakaki et al., 2016; Venkateswar Reddy et al., 2015). The current commercial methods for PHA production use single strains, defined medium and are produced under aseptic conditions (Venkateswar Reddy and Venkata Mohan, 2015). However, the costs incurred due to these operational conditions increase PHA selling price, in turn, hindering their market potential to replace synthetic plastics. In order to be on par with synthetic plastics, the production cost of PHA has to be reduced which is majorly influenced by the cost of substrate (Amulya et al., 2015). Amongst the PHA types, polyhydroxybutyrate (PHB), a short-chain polymer is the most abundant and widely studied. Therefore, inexpensive and renewable substrates are being extensively evaluated for biopolymer production (Zhou et al., 2018).

The renewable sugars derived from microalgal biomass can effectively replace the current 2G based feedstocks and also invokes sustainability to algal refineries (Yadav and Sen, 2017; Khoo et al., 2019). The carbohydrate fraction in the DAB residue is majorly composed of polysaccharides like starch and cellulose along with the traces of monosaccharides and nutrients (Subhash and Venkata Mohan, 2014). The complex polysaccharide content of DAB limits its direct biological supplementation as a renewable resource. Hence, prior pretreatment is prerequisite to transform these recalcitrant sugar polymers into usable monomeric form. Studies are reported with various individual pretreatment methods like physical (autoclave, sonication), chemical (acid, base) and enzymatic (amylase, cellulases, the mixture of enzymes), that primarily aid in cell disruption and allow for extraction and structural modification of carbohydrate moieties by solubilization into the solute (Shokrkar et al., 2017; Chandra et al., 2019). Each of these pretreatment methods possess their own advantages and disadvantages, wherein the physical method is easy to operate but the resulting sugar yield is lower. On the other side, chemical method is suitable for effective degradation of polysaccharides, but the acid or base concentration needs to be optimized to avoid the inhibitor formation and moreover, the resulting sugar yields are low (Prajapati et al., 2015). Advantageously, enzymatic hydrolysis is more specific towards carbohydrate depolymerization with no/low inhibitors formation, but the major concern of this method is the requirement for longer reaction time apart from the high cost of enzymes. Therefore, there is a gap in the existing pool of knowledge on effective pretreatment methods for maximum sugar extraction from DAB and its entirety utilization as a renewable resource. In this context, the present study is an attempt to extract maximum sugars from DAB with various pretreatment strategies followed by utilization of sugar-rich hydrolysate for bioethanol and biopolymers production. *Saccharomyces cerevisiae* was used as a biocatalyst under various redox conditions to determine the best pH condition for maximum ethanol yield. Also, biopolymer production was evaluated using enriched mixed bacterial consortium as a biocatalyst by using feast and famine strategy. The developed technology is focused on the complete utilization of DAB with an aim to generate a range of sustainable biobased products in an integrated biorefinery approach.

2. Materials and methods

2.1. Algal biomass (deoiled)-pretreatment

In an attempt to figure out an integrated biorefinery in association with the previous study (Hemalatha et al., 2019), the lipid extracted or deoiled algal biomass (DAB) obtained was chanelled towards bioethanol and biopolymer production. DAB used in this study is dominated by Chlorella sp. and Scenedesmus sp. which were originally collected from Nacharam Lake, Hyderabad. Initially, the obtained DAB was

overnight dried at 40 ± 0.2 °C to remove the moisture content. The initial sugar content of DAB was 0.642 g/g, which was estimated as per the standard protocol (Laurens et al., 2015; de Farias Silva et al., 2018). To harness maximum sugars, DAB samples were subjected to various pretreatments like physicochemical, enzymatic and combined/ hybrid methods. Prior to pretreatment, 20 g of dried DAB powder was mixed with 1000 mL water in a Borosilicate bottle. Physicochemical pretreatment was evaluated by mixing the acid (1% v/v sulphuric acid) followed by autoclave (121 °C for 15 min) as per the reported studies (Naresh Kumar et al., 2018). While the enzymatic pretreatment (individual) was performed by loading the commercial grade α-amylase (400 IU/g, S D Fine-Chem Limited, India) and cellulase (10 IU/g, Sigma Aldrich, USA) enzymes. During the enzyme pretreatment, Erlenmeyer flasks were incubated in a shaker at 50 ± 0.2 °C for 72 h at 120 rpm. Hybrid pretreatment was evaluated by adding 20% of DAB (w/v) to water and autoclaved under acid-catalyzed conditions to enable the solubilization of sugars into water. Thereafter, the resultant was cooled to room temperature and allowed to enzymatic saccharification as mentioned above. Prior to enzymatic hydrolysis, pH of the media was adjusted to 5.8 ± 0.3 using diluted NaOH. Finally, the filtrate was collected and used as a substrate for bioethanol and biopolymer production.

2.2. Ethanol fermentation

Saccharomyces cerevisiae was maintained on YPD agar plate containing (g/L); glucose 20, yeast extract 10, peptone 20, agar 20 at different pH conditions (pH 5.0, 5.5 and 6.0) at 37 °C (Laurens et al., 2015). Yeast inocula for bioethanol production was prepared in sterile DAB hydrolysate by incubating at 37 °C for 24 h at 120 rpm. The fermentation of DAB hydrolysate was carried out separately in 250 mL Erlenmeyer flasks with a working volume of 100 mL. The DAB hydrolysate was inoculated with an overnight grown culture of *S. cerevisiae* (10% v/v) and the experiment was conducted using 0.590 g total sugars /g DAB composed of 83.05% glucose under three different pH conditions (pH: 5.0, 5.5 and 6.0). Samples were collected at different time intervals and centrifuged at 5,000 rpm for 5 min. The cell-free supernatant was evaluated for ethanol and residual sugar concentration.

2.3. Biopolymer production

Biopolymer (PHA: polyhydroxyalkanoates) production was evaluated using the DAB derived sugars as a carbon source (0.590 g total sugars /g DAB). The aerobic mixed inoculum was collected from pilot-scale sequential batch bioreactor operated with wastewater (Venkateswar Reddy and Venkata Mohan, 2015). Primarily the PHA producing microbial culture was enriched with the DAB derived sugars and external supply of sodium nitrate ($NaNO_3$) and dipotassium phosphate (K_2HPO_4) as a nutrient source in the ratio of 100:8:1. The initial enrichment is referred as the feast phase, where the nutrients along with the DAB sugars were given as substrate (Stage I). Subsequently, the enriched culture was fed with only DAB derived sugars and operated under nutrient-depleted conditions to induce the biopolymer accumulation, which is referred as famine stage (Stag-II). Experiments were performed in 100 mL borosilicate bottles by supplementing external oxygen with an air pump (DO: 1.2 ± 0.3 mg/L) (Amulya et al., 2015). The reactor was operated in the sequencing batch reactor mode with 48 h HRT and the samples were collected at different time intervals. Biopolymer was extracted according to the three-step optimized conditions as per the reported studies (Venkateswar Reddy et al., 2015). Initially, the collected bacterial pellet was washed twice using phosphate buffer (50 mM) at 4000 rpm for 10 min (28 ± 0.2 °C) followed by addition of equal amounts of sodium hypochlorite (10% NaOCl) and chloroform. Thereafter, the mixture was incubated for 3 h at 32 ± 0.5 °C and 120 rpm in a shaking incubator. Further, the solution was centrifuged at 6000 rpm for 10 min (32 ± 0.2 °C). Three different layers were observed from which the chloroform layer was collected and added into ice-cold methanol. The methanol precipitated polymer was collected and characterized. Enriched bacteria were stained with Nile red fluorescent dye and assayed with fluorescence-activated cell sorting (FACS) (Alves et al., 2017). Periodically samples were collected from the bioreactor and subjected to Nile red staining. The stained samples were assayed for fluorescent emission using Flow sight Amnis instrument (USA: Ideas 4.2) equipped with an excitation laser of 488 ± 20 nm and emission filter with a bandwidth of 585 ± 45 nm. The median difference represents the PHA positive and negative bacterial population.

2.4. Analysis

The sugar concentration, profiling and ethanol production were analyzed using high performance liquid chromatography (HPLC: Rezex Monosaccharides and organic acids RH^+ column: Flow rate 0.5 mL/min, oven temperature 75 °C) using 1 N sulphuric acid as a mobile phase (Sarkar et al., 2017). Prior to analysis, the samples were filtered through 0.2 µm membrane filter and 20 µL of filtered samples were injected into the column. Total suspended solids (TSS) and volatile suspended solids (VSS) were estimated using the standard protocols of APHA, 1998. Surface morphology of DAB before and after pretreatment was examined through FE-SEM, JOEL-JSM-7610F with an accelerating voltage of 2–15 kV. As the samples were non-conductive in nature, these were coated with a thin layer of platinum prior to examination (Harun et al., 2011).

To understand the molecular structure of extracted biopolymer, NMR analysis was performed. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded in Bruker AVANCE III 500 MHz NMR spectrometer at 20 °C. The extracted biopolymer sample was prepared by dissolving in deuterated chloroform prior to NMR analysis (Venkateswar Reddy et al., 2015).

FT-IR analysis of the extracted biopolymer was performed to understand the functional groups present in the biopolymer. The FT-IR spectrum was recorded using Thermo Nicolet Nexus 670 spectrophotometer in transmittance band mode within range of 400–4000 cm^{-1} using potassium bromide (KBr) by maintaining spectral resolution at 4 cm^{-1} (Venkateswar Reddy et al., 2015).

Thermal stability analysis of the extracted biopolymer was assayed using thermogravimetry analysis (TGA), differential scanning calorimetry (DSC) and differential thermal analysis (DTA). All the analysis were carried out using TA-SDT Q600 thermogravimetric analyzer. The runs were carried out at heating rate ranging from 30 °C to 600 °C with a 5 °C/min increment under nitrogen atmosphere (Mousavioun et al., 2013; Venkateswar Reddy et al., 2015).

3. Results and discussion

3.1. Deoiled algal biomass-sugars solubilization

3.1.1. Physicochemical pretreatment

Physicochemical pretreatment of DAB was evaluated using acid-catalyzed conditions (1% H_2SO_4), which resulted in maximum sugar solubilization of 0.481 g/g DAB (Table 1). Sugar distribution obtained from DAB physiochemical acid-catalyzed condition was majorly composed of glucose (0.219 ± 0.3 g/g DAB) followed by xylose (0.148 ± 0.01 g/g DAB) and arabinose (0.114 ± 0.02 g/g DAB). The total sugar yield obtained in this study (0.481 g/g DAB) is higher than that reported by Naresh Kumar et al., (0.26 g/g DAB) (Naresh Kumar et al., 2018). Acid-catalyzed condition with the thermal combination is reported to be effective for starch and cellulose-rich biomass (Laurens et al., 2015). In addition, acid concentration and temperature play a critical role on sugar yields, preferably mild acid (1%, v/v) concentration along with moderate temperature is efficient in harnessing maximum sugars by minimizing the inhibitor formation.

Table 1
Sugar profiles of DAB before and after pretreatment.

Parameter	Before Pretreatement	After Pretreatment		
		Physicochemical Method (PSC) (*AU + Sulphuric Acid)	Enzymatic	Combined (PSC followed by Enzymatic)
pH	7.0 ± 0.2	1.48 ± 0.2	5.8 ± 0.3	5.8 ± 0.2
Soluble Sugars (g/g DAB)	0.060	0.481	0.484	0.590
Glucose (g/g DAB)	0.040 ± 0.001	0.219 ± 0.3	0.484 ± 0.2	0.490 ± 0.3
Mannose (g/g DAB)	ND	ND	ND	ND
Galactose (g/g DAB)	ND	ND	ND	ND
Xylose (g/g DAB)	ND	0.148 ± 0.01	ND	0.045 ± 0.001
Arabinose (g/g DAB)	ND	0.114 ± 0.02	ND	0.060 ± 0.002

* AU: Autoclaved at 121 °C for 15 min, ND: Not Detected.

3.1.2. Enzymatic pretreatment of DAB

The major carbohydrate content in DAB is composed of starch and cellulose along with traces of other free sugars (Prajapati et al., 2015). Cellulose is made up of monomers of glucose linked together by β-1, 4 glycosidic bonds, as opposed to the α-1, 4 and α-1, 6 glycosidic bonds of starch. In the enzymatic pretreatment, cellulase specifically cleaves the β-1, 4 glycosidic bonds of algal cellulose (Shokrkar et al., 2018). Whereas, α-amylase functions to hydrolyze algal starch to monosaccharides through the cleavage of α-1, 4 and α-1, 6 glycosidic linkages. Accordingly, enzymatic pretreatment in this study was conducted with α-amylase and cellulase enzymes that resulted in total fermentable sugar recovery of 0.484 ± 0.2 g/g DAB. The obtained total sugars were majorly composed of glucose and no other sugars were observed due to specific enzymatic action (Table 1). The use of enzyme mix plays an important role in the sugar solubilization, wherein specific enzyme selection relative to a particular substrate is important (Shokrkar et al., 2017).

3.1.3. Hybrid pretreatment of DAB

Acid-treated biomass along with the sugar hydrolysate was cooled to room temperature and further subjected to enzymatic saccharification, this method is referred as hybrid pretreatment. The hybrid method yielded the maximum sugars (0.590 g/g DAB) than corresponding individual methods. In the case of individual acid-catalyzed conditions specific to starch and cellulose, biomass begins with the protonation of oxygen in a β-1,4-glycosidic bond or protonation of cyclic oxygen in a glucopyranose ring. Thereafter, glycosidic bond splitting occurs followed by ring-opening and transformation of polysaccharides to monosaccharides (Laurens et al., 2015). Further, the addition of enzymes (amylases/cellulases) effectively transformed the remaining unconverted polysaccharides resulting in higher reducing sugar yield than the corresponding individual methods. The distribution of sugars in hydrolysate obtained from hybrid pretreatment was majorly composed of glucose and a fraction of pentoses. Where, glucose was dominant (0.49 ± 0.3 g/g DAB) followed by arabinose (0.06 ± 0.002 g/g DAB) and xylose (0.045 ± 0.001 g/g DAB) which were relatively lower than glucose. The sugar yield plays an important role in the biobased product synthesis, wherein the utilization of DAB derived sugars as a resource allows sustainability and supports the biobased economy. The DAB was also examined with FE-SEM to understand the changes in surface morphology after pretreatment in comparison with the untreated algal biomass. Significant changes were observed in the morphology of DAB before and after pretreatment, wherein before pretreatment, the DAB particles were discrete and distinct (refer Supporting Information). Thereafter, with the course of pretreatment (hybrid method) significant agglomeration of DAB particles can be observed that relatively increased the particle size. This size increment may be ascribed to the course of heating under acid-catalyzed conditions that might have increased the tenderness of the DAB residue making it more prone to clumping as is evident from the treated image.

3.2. Bioethanol production from DAB derived sugars

Separate hydrolysis and fermentation method was studied to evaluate and optimize the bioethanol production from DAB derived sugars using *S. cerevisiae* as a biocatalyst (Hemalatha et al., 2019). To optimize the fermentation conditions, the effect of pH on ethanol production was investigated. The optimal pH for ethanol production ranged in between pH 4.0 to pH 6.0 depending on temperature, presence of oxygen, carbon composition and the yeast strain (Lee et al., 2015). Biosystem with pH 5.5 resulted in higher bioethanol production (0.145 ± 0.008 g/g DAB) followed by pH 5.0 (0.122 ± 0.004 g/g DAB) and pH 6.0 (0.102 ± 0.002 g/g). The ethanol concentration in the broth increased with due course of fermentation time, wherein biosystem operated at pH 5.5 resulted in 0.133 ± 0.004 g/g ethanol at 30 h. Further, the increment in the ethanol concentration was relatively marginal by the end (48 h) of the cycle. On the other hand, biosystem at pH 5.0 resulted in 0.095 ± 0.002 g/g bioethanol after 24 h and thereafter the trend was similar to pH 5.5. In the case of pH 6.0, the maximum ethanol production was observed at 30th h of cycle operation (0.079 ± 0.003 g/g), subsequently the increment was minimal. The obtained results confirmed that pH has a significant role in efficient bioconversion of carbohydrates to ethanol which is in agreement with the published report (Trivedi et al., 2015). During the growth phase of *S. cerevisiae*, it is important to maintain a constant intracellular pH as numerous enzymes work in synergy within the yeast cell to maintain growth and metabolism (Lagunas, 1993). Each enzyme works well at acidic pH due to the acidophilic nature of yeast, however, under deviating extracellular pH conditions from the optimal range, it becomes complex for the yeast cell to maintain constant intracellular pH, and the enzymes do not function favourably. Deactivation of enzymes leads to the decrement in efficiency of yeast cells to grow and produce ethanol (Lagunas, 1993; Venkata Mohan and Prathima Devi, 2012). This phenomenon is well co-related with the observation made in this study where ethanol concentration reduced when the initial pH was varied from 5.5 to 5.0 and 5.5 to 6.0. Moreover, it is reported that setting the experiments for ethanol production through yeast fermentation at optimum acidic pH resulted in both maximum ethanol yield and efficient control of bacterial contamination (Lee et al., 2015).

The bioethanol yield obtained in this study (0.145 g/g DAB) is relatively higher than that reported in the previous (0.116 g/g DAB) study (Hemalatha et al., 2019). This may be attributed to the higher release of glucose in the present study through hybrid pretreatment of DAB and the optimum pH condition (pH 5.5) maintained during *S. cerevisiae* mediated fermentation. However, there still exits scope for further improvement of bioethanol yield from DAB through process optimization by considering the vital process parameters viz., DAB loading, yeast inoculum concentration, co-factor concentration etc., to closely reach the theoretical yield.

3.2.1. Sugar consumption during bioethanol production

Pretreatment of DAB yielded both hexoses and pentoses namely

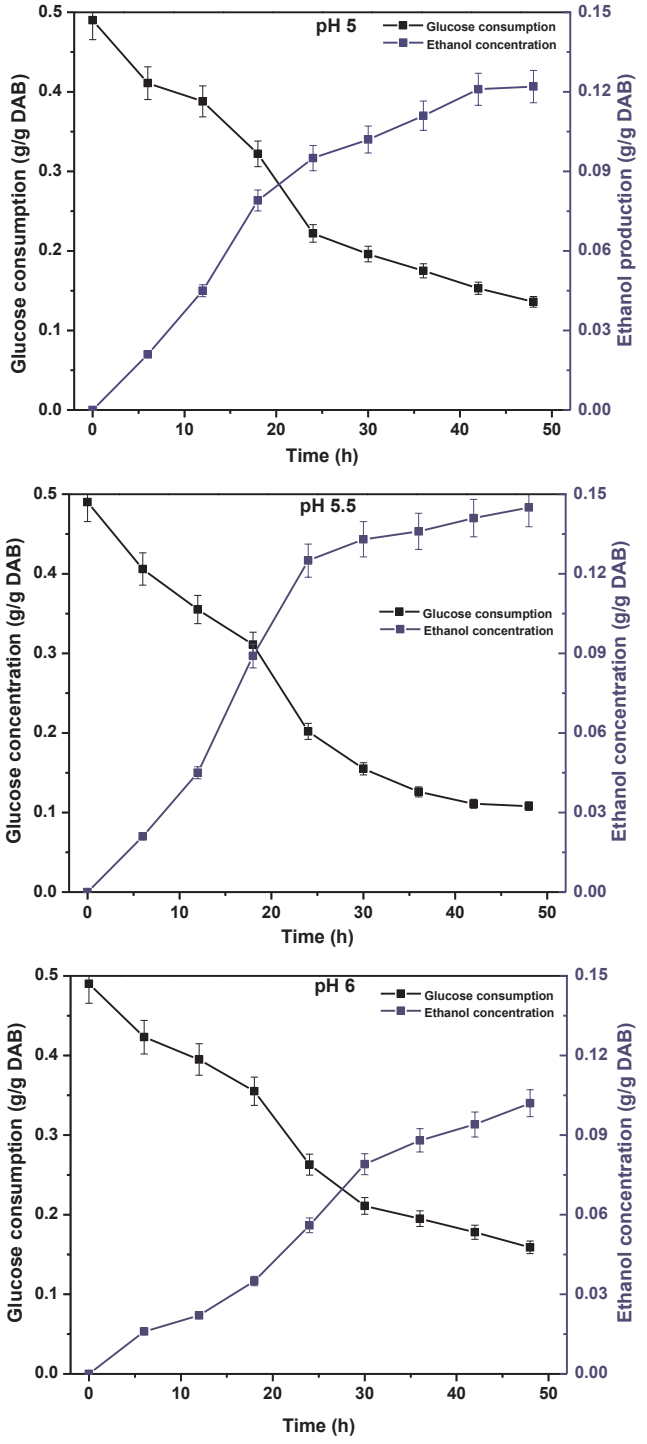

Fig. 1. Glucose consumption and bioethanol production (hybrid pretreatment method) with respect to pH variation.

glucose, xylose and arabinose. *S. cerevisiae* is the most commonly used fermenting organism for ethanol production, but it has the ability to consume selective sugars mostly glucose. Sugar consumption for all the studied experimental operations (pH 5- pH 6) showed similar depletion trend, but the consumption was higher in pH 5.5 (77.9%) followed by pH 5 (72.24%) and pH 6 (67.55%), which is also well synchronized with ethanol production profile studied within this pH range. The higher ethanol yield at pH 5.5 and pH 5 compared to pH 6 may be attributed to the acidophilic nature of *S. cerevisiae* with optimum pH for growth and fermentation commonly within the acidic range of pH 5–5.5. While, raise in extracellular pH (pH 6) far from the optimum intracellular pH might have affected the enzymatic activity of the yeast cells thus reducing the ethanol yield (Narendranath and Power, 2005; Zhao and Xia, 2010). Fig. 1 depicts the time course of glucose consumption (g/g DAB) and ethanol production profiles (g/g DAB) in the fermentation broth. Specifically, glucose consumption was only noticed in all the systems, this could be attributed to the use of wild type *S. cerevisiae* which utilizes glucose as a major carbon source than other sugars. During the course of fermentation, glucose was gradually consumed under all experimental conditions. The maximum consumption was noticed at pH 5.5 (0.382 g/g DAB) followed by pH 5.0 (0.354 g/g DAB) and pH 6.0 (0.331 g/g DAB). Although the sugar consumption showed marginal variation under pH 5.0 and pH 6.0, the respective ethanol yield was high in pH 5.0 than pH 6.0. The optimal pH condition is essential for maximum ethanol production, whereas acidic conditions (pH 2 to pH 4.5) alter the nutrient uptake capabilities and interfere with the cellular processes. On the other hand, alkaline pH (7.5 to 11) inhibits the cell growth and cell multiplication specifically in *S. cerevisiae*. This study shows that the initial pH showed a significant impact on individual sugar consumption. Nevertheless, the wild strain of *S. cerevisiae* lacks the ability to metabolise pentoses along with few hexoses when the media is specifically rich in glucose as a fermentative substrate (Lee et al., 2015; Lagunas, 1993).

3.3. Biopolymer production

3.3.1. Polyhydroxybutyrate (PHB)

Sugar-rich hydrolysate obtained from DAB was used as a substrate for biopolymer production. Fig. 2 illustrates the production of PHB with respect to operation time, wherein maximum PHB production of 0.43 ± 0.20 g PHB/g DCW was obtained in stage II famine mode of operation. This can be attributed to the depleted concentration of nutrient supplements in the DAB derived sugar hydrolysate that might have induced the accumulation of PHB. Whereas, at stage I substantial amount of nutrients present facilitated the biomass growth and carbon utilization rather than its storage. Initially during cycle 1, polymer yield was relatively low (0.32 ± 0.1 g/g DCW). Upon repeated cycle operations, a gradual improvement in the PHB production was achieved where maximum yield of 0.43 ± 0.2 g/g DCW was obtained in cycle 5. Use of DAB derived sugars for the production of biopolymers can be considered as one of the possible strategies to utilize the algal biomass residue on its entirety.

3.3.2. Fluorescence-Activated cell sorting (FACS)

PHB deposition in the bacterial cells (Nile red stained) was examined using flow cytometry analysis (Alves et al., 2017; Kim et al., 2019). FACS analysis allows the sorting and identification of a

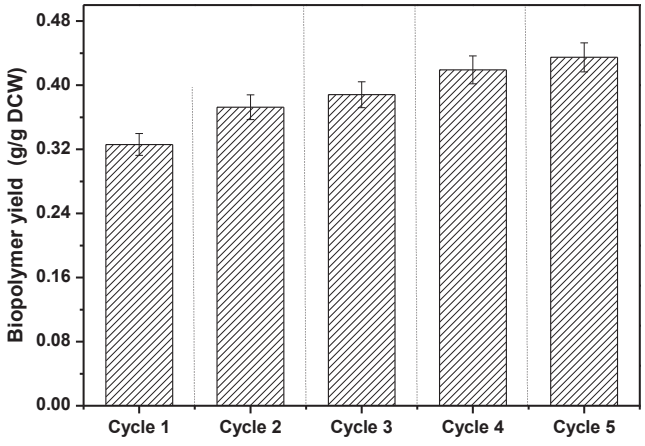

Fig. 2. Biopolymer production profile with respect to cycle operation.

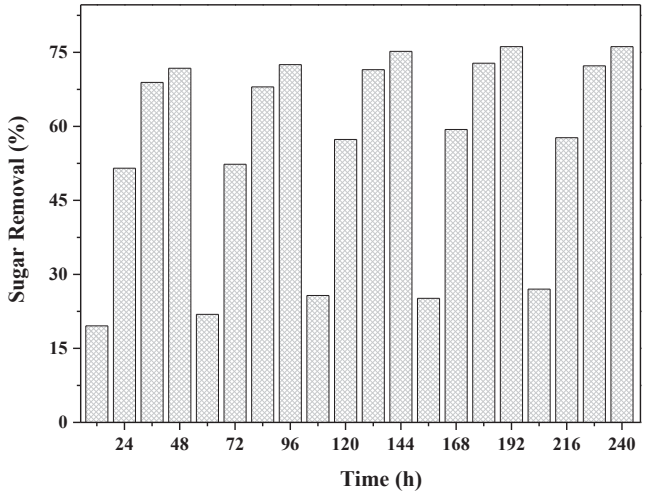

Fig. 3. Sugar removal profile during biopolymer production.

heterogeneous mixture of microbial cells based on the fluorescence emission and light scattering. Nile red dye specifically binds with PHB moieties in the bacterial cells and emits fluorescence upon its excitation (Kim et al., 2019). FACS analysis of PHB producing bacteria showed marked fluorescence emission than the corresponding non-PHB producing control consortium. It was observed that the PHB containing bacteria showed significant fluorescence emission with the median value of 688 (see Supplementary Information). On the contrary, non-PHB producing bacteria did not show fluorescence emission and accounted for lower median value of 48.87.

3.3.3. Sugar utilization during PHB production

DAB derived sugars resulted in a gradual decrement in the sugar concentration with respect to operation time, wherein, the maximum sugar removal was 76.17% (Cycle 5) (Fig. 3). Initially, during cycle 1 and 2 operation, the sugar removal was relatively lower i.e., 71.78% for cycle 1 and 72.52% in case of cycle 2, however, repeated batch operations increased the sugar consumption. It was observed that cycle 3 and cycle 4 accounted for the gradual increment in sugar metabolism and resulted in 75.21% and 76.15%, respectively. While, cycle 5 also resulted in 76.17% substrate removal, the corresponding polymer deposition was relatively higher i.e., 0.43 ± 0.2 g PHB/g DCW. This can be attributed to the nutrient stress that might have created a selective pressure on the microorganism which could have triggered good substrate removal along with PHB accumulation. Kucera et al., reported the PHB yield of 0.1 g PHB/g DCW using pure culture (Kucera et al., 2017). Whereas, this study with mixed microbial inoculum showed 4 times higher production of PHB (0.43 ± 0.20 g PHB/g DCW). Utilization of DAB derived sugars as a renewable resource for biopolymer production using mixed microbial inoculum allows sustainability to the algal biorefinery process and supports use of DAB on its entirety without any residue disposal issues.

The hybrid pretreatment involving acid-catalysed physiochemical method followed by enzymatic treatment resulted in considerable amount of usable sugars (0.590 g/g DAB). The total sugar consumption in the case of bioethanol was ~78% (pH 5.5) and total ethanol production was 0.145 g/g DAB. Utilization of these sugars for bioethanol production supports sustainability and accounts for additional benefits. Alternatively, the DAB derived sugars were channelled towards biopolymer production. It was found that on 76.17% sugar utilization 0.43 ± 0.2 g PHB/g dry cell weight (DCW) was produced which is equivalent to 0.021 g PHB/g DAB. The PHB extracted biomass can further be used as a soil additive or as a substrate for biomethanation process thereby reducing carbon footprint along with additional revenue generation. Though the yield of PHB per g DAB in the present study is lower than bioethanol yield (on per g basis), the former is a high value product compared to the latter. Thus both the conversion routes i.e., DAB sugars to bioethanol (route 1) and DAB sugars to PHB (route 2) are advantageous to the algal based biorefineries.

3.4. Biopolymer characterization

3.4.1. Functional group analysis

Functional group analysis of extracted biopolymer was conducted using FT-IR. Biopolymer (PHB) structurally contains C–H stretching, C–H bending ester groups (COOR) consisting of C=O and C–O vibrations (Venkateswar Reddy et al., 2015). The extracted biopolymer spectrum showed C–H vibrations corresponding to the methylene group at 2995 cm^{-1} (see Supplementary Information). The depicted absorbance at 1712 cm^{-1} corresponds to the stretching of the C=O bond, which is specific to the ester linkages that appear in the biopolymer. Additionally, a peak at 1292 cm^{-1} signifies the presence of C–O stretching. The depicted peaks are well in correlation with polyhydroxybutyrate (PHB) structure and are synchronizing well with the reported studies (Venkateswar Reddy et al., 2015; Korkakaki et al., 2016).

3.4.2. Molecular structural analysis

Molecular structural analysis of extracted biopolymer was carried out using NMR. The ^{1}H NMR results showed a peak signal at 1.25 ppm which corresponds to CH_3 side chain. In addition, a series of peaks from 2.28 ppm to 2.68 ppm are in agreement with the presence of CH_2 group while a peak at 5.28 ppm corresponds to CH group of PHB. In the case of ^{13}C NMR, four major peaks illustrating the presence of carbon atoms such as C = O, CH, CH_2 and CH_3 were noticed (see Supplementary Information). The ^{13}C NMR peak signals were obtained at 168.2 ppm (C_1), 68.4 ppm (C_2), 42.1 ppm (C_3) and 19.8 ppm (C_4). These peak signals are well in correlation with PHB structure. On the other side, the depicted peak signal between 74 and 78 ppm corresponds to $CHCl_3$. These NMR results are in agreement with the published biopolymer production reports (Venkateswar Reddy et al., 2015).

3.4.3. Thermal analysis

Extracted biopolymer thermal properties were examined by using TGA, DSC and DTA analysis. TGA curves represent the changes in weight loss of the samples with respect to temperature increment. While, the DSC analysis measures the amount of energy absorbed (endothermic) or released (exothermic) by the samples on heating or cooling (Mousavioun et al., 2013). Whereas, DTA curves illustrate the difference in temperature as exothermic or endothermic reactions in the examined samples. The extracted biopolymer weight loss initiated at 224 ± 2 °C that gradually increased until 296 ± 2 °C accounting to a total weight loss of 78% (see Supplementary Information). Interestingly, the DSC profiles also revealed the crystallization temperature (T_c) at 296 ± 2 °C. A small endothermic peak was observed at 172 ± 2 °C, this could be attributed to the presence of other copolymers. The DTA profiles also depict a similar trend with the glass transition temperature at 295 ± 2 °C.

4. Conclusions

The outcome of the study reveals the effective valorization of deoiled algal biomass (DAB) residue for bioethanol and biopolymer production. Hybrid pretreatment with acid-catalyzed physicochemical treatment followed by enzymatic pretreatment accelerated the sugar solubilization than corresponding individual methods. Consequent utilization of DAB derived sugars for bioethanol (0.145 ± 0.008 g/g DAB) and biopolymer (0.43 ± 0.20 g PHB/g DCW) production resulted in good yields. Utilization of DAB residue as an alternative resource for production of multiple biobased products can generate additional benefits to the existing algal refineries and also invokes sustainable

biobased economy in the biorefinery framework.

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Appendix A. Supplementary data

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