



## Starch from the sea: The green macroalga *Ulva ohnoi* as a potential source for sustainable starch production in the marine biorefinery

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### ABSTRACT

The growing population, decreasing arable land and fresh water supply questions the sustainability of terrestrial agriculture for securing safe nutrients supply, particularly starch- an essential ingredient for all staple foods. Here, we report the isolation, characterization and offshore production assessment of native starch from green seaweed *Ulva ohnoi* cultivated in seawater. Starch content varied from 1.59% to 21.44% depending on growth conditions and seasons. Our results show that nutrient starvation significantly increased the starch concentration up to 21.4% on dry weight basis. The extracted fraction contained 75.45% starch, and the starch extraction yield from the *U. ohnoi* biomass was 50.37%. *Ulva* starch granules are spherical, ovoid and irregularly shaped, 5–7 μm in size. Their gelatinization temperature is 69 °C and they are susceptible to α-amylase and amyloglucosidase digestion. *U. ohnoi* biomass cultivated offshore for 13 months showed an average starch yield of 3.43 ton/ha/year (t·ha<sup>-1</sup>·y<sup>-1</sup>). This study encourages the potential use of offshore produced biomass for sustainable starch supply as an alternative to current agricultural products, the production of which requires arable land and fresh water.

### 1. Introduction

Starch is a major food carbohydrate and a critical staple for humans. Extracted starch production is about 65 million ton per year, and it constantly increases by 2–3% per year, with a predicted market of 77\$ billion for 2018 [1,2]. For the majority of humans (~90%), starch and its derivatives are the major energy sources, accounting for > 50% of world's daily calorie intake [1]. Indeed, rice alone supplies 23% of calories consumed by humans [3]. Moreover, starches, including modified starches are important ingredients of various industries and are used for multiple applications including food, fermentation, textile, cosmetics, pharmaceutical, packaging, synthetic polymer industries and in other technological developments [4]. Modified starches and different native starch-based blends are an attractive bio-based alternative to biodegradable synthetic polymers. Starch-based bioplastics represent 80–85% of marketed bioplastic [2]. In addition, starch from cereals is used for biofuel production for last 10 years in the United States and has also become prominent in other parts of the world [1]. These competing

applications cause shortage and price increase of cereal-based food starch [5].

Current strategies for starch production rely on the classic land-based agriculture [6]. The commercial starch is extracted from terrestrial biomass sources such as maize, rice, wheat, potato, and tapioca [4]. However, as indicated by the Food and Agriculture Organization (FAO), in the light of growing world population, there is growing pressure on the supply of food which raises a question regarding the sustainability of production of cereals and other conventional starch sources in the future [7,8]. The concerns over decreasing the availability of land and potable water, and the environmental risks associated with the further expansion of agricultural lands, put into question the sustainability of future terrestrial agriculture to supply stable, safe and secure food starch [5,9,10]. Current starch demands large amount of freshwater in production process and in-turn in the cultivation of these crops [11,12]. Furthermore, the pandemic problem of obesity and the metabolic syndrome recently triggered programs for finding new starches with improved properties for human health [13].

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Due to the above-mentioned problems, it is a need of the hour to look for alternative sources of food starch, with small land and water footprint and positive impact on human health.

Marine macroalgae (seaweeds) are highly productive and thrive in the photic zone along the coastlines. Macroalgae have been a source of food back to 2700 BCE in China and this practice remains widespread currently in Eastern countries such as Japan, Korea, China, etc. [14,15], and are also common in certain Western countries [16]. Several species have been reported to have high nutritional values including protein, carbohydrates, fats, crude fiber, minerals, essential amino acids and antioxidants [9,17] which makes them excellent candidates for a healthy food for human nutrition [14,18]. Seaweeds may overcome several adverse environmental issues associated with the cultivation of terrestrial crops for food, e.g., arable land, fertilizers, and freshwater usage [19]. These advantages make them attractive feedstock for sustainable food, fuel, and chemical generation [20,21]. Such sustainable utilization of marine biomass, known as marine biorefinery [22], can thereby strengthen the future maritime economies and low carbon societies [6].

Starch is a major storage carbohydrate in various species of seaweeds [23,24]. In *Cladophora* the starch is stored inside their chloroplast [24], whereas in red algae such as *Gracilariopsis* [25,26] and other *Rhodophyta* [19,23] they are found in the cytoplasm as granules. In green seaweeds such as *Ulva*, starch plates are found surrounding pyrenoids and starch granules among thylakoid membranes of the chloroplasts similar to plants [27–30]. Starch content fluctuates seasonally and reaches 32% of the DW in *Ulva rigida* [31]. Moreover, starch concentration can be increased significantly when seaweeds are cultured under nutrient stress [29,31–34] and with blue light [35]. Use of this carbohydrate was reported for bioenergy production [21,36,37] however, whole biomass hydrolysis was done to produce monosaccharides for fermentation. However, multiple of mentioned biorefinery applications require native starch granules, isolation of which for biorefinery application has not been reported.

The goal of this work was to develop a new, sustainable pathway for edible starch production, using offshore cultivated biomass. We show the presence of starch in the *U. ohnoi*, and report the method for extraction in native granular form, followed by characterization of the extracted starch granules in terms of thermo-chemical properties. We also report strategies to increase starch concentration in *U. ohnoi* biomass through altering growth conditions. We further assessed the impact of seasonal variation on *U. ohnoi* starch yield in biomass produced by offshore cultivation in the coastal water of Tel Aviv, Israel. Our work suggests a new sustainable pathway for starch production offshore. Such an approach is expected to reduce the environmental burden of modern industrial agriculture on arable land and fresh water and improve food security in multiple coastal areas.

## 2. Materials and methods

### 2.1. *Ulva* collection, molecular identification and biomass production

A culture of frond-like *Ulva* species was procured from an outdoors seaweed culture collection at Israel Oceanography and Limnological Research, Ltd. (IOLR), Haifa, Israel. The *Ulva* specimen originated from the random collection conducted over the past years along the Israeli Mediterranean Sea [38]. Genomic DNA from this *Ulva* culture was extracted using CTAB extraction protocol as described by Coat et al. [39]. Polymerase chain reaction (PCR) was run on the extracted genomic DNA to amplify the *rbcl* gene using primers: RH1 and 1385R, as designed by Hayden & Waaland [40]. The PCR product was sequenced at Macrogen's sequencing service (Macrogen Inc.). The sequences search and alignment of the obtained sequence was performed with the database sequences available in NCBI database using BLAST (Basic Local alignment search tool) [41]. The BLAST results showed that the gene sequence from *Ulva* cultivated at IOLR, matched 98% with

*Ulva ohnoi* as published by Hiraoka et al. [42].

This macroalgae, hereafter referred as *U. ohnoi* throughout the article, was grown in our laboratory under controlled conditions using cylindrical, sleeve-like macroalgae photo-bioreactors (MPBR) (Polytiv, Israel, Length 100 cm, thickness 200  $\mu\text{m}$ , width 40 cm) [43] under natural irradiance in July 2017. Artificial seawater (ASW) having a total salinity of 37‰, prepared using distilled water containing dissolved dried Red Sea salt (Red Sea Inc., IS) was used as a cultivation medium. Nutrients were supplied by adding ammonium nitrate ( $\text{NH}_4\text{NO}_3$ , Haifa Chemicals Ltd., IS) and phosphoric acid ( $\text{H}_3\text{PO}_4$ , Haifa Chemicals Ltd., IS) to maintain 6.4  $\text{g m}^{-3}$  of nitrogen ( $\text{N}_2$ ) and 0.97  $\text{g m}^{-3}$  of phosphorus (P) in the ASW. Initially, 20 g fresh *U. ohnoi* was inoculated in each sleeve with total volume of 40.4 L in a reactor. All other conditions such as pH, temperature and flow rate were maintained as mentioned in our previous study [43]. After a growth period of 2 weeks, biomass was collected from the MPBR and washed three times with distilled water to remove the seawater and surface salts. The washing was followed by draining the water using a salad spinner and all the remaining surface water was removed using blotting paper [44]. This biomass defined as wet weight (WW) was used for microscopic observation, starch estimation, and starch extraction. Dry weight (DW) was determined after drying the washed fresh biomass at 105 °C until constant weight was obtained using moisture analyzer (BM-50-5, Biobase Biotechnology (Shandong) Co. Ltd., China).

### 2.2. Microscopic observation of *Ulva ohnoi*

Thalli of *U. ohnoi* were observed under light microscope (Nikon ECLIPSE TE2000-S, Nikon Instruments, NY, USA) with or without iodine staining of the starch, confocal microscope (ZEISS LSM 510META, Carl Zeiss, Oberkochen, Germany), phase contrast microscope (Carl Zeiss (Suzhou) Co. Ltd., Suzhou, China), and transmission electron microscope (TEM, JEM-1400 plus electron microscope, Peabody, MA, USA). Starch granules morphology inside the thallus was observed using TEM.

### 2.3. Determination of starch content in *Ulva ohnoi*

Harvested *U. ohnoi* biomass was dried to a constant weight at 40 °C in an oven, ground to fine powder using a mortar and pestle with the help of liquid  $\text{N}_2$ . Starch content in *U. ohnoi* was determined using a total starch assay kit (K-TSTA-100A, Megazyme, Ireland) with minor modification. In short, 10 mg DW sample ( $n = 3$ ) were weighed in 2 ml tube and washed twice in 200  $\mu\text{l}$ , 80% (v/v) ethanol to remove any glucose present. Two hundred microliters of 2 M potassium hydroxide was then added and the tubes were incubated horizontally for 30 min at 37 °C and 150 rpm. After incubation, the mixture was heated in boiling water bath for 1 min to completely dissolve the starch. Tubes were cooled at 23 °C for 5 min. and 0.8 ml sodium acetate buffer (1.2 M, pH 3.8) was added. Immediately, 0.01 ml  $\alpha$ -amylase, 0.01 ml amyloglucosidase were added and mixed using a vortex mixer. The mixture was incubated for 1.5 h at 50 °C and 150 rpm. Following incubation, the tubes were centrifuged at 1800 g for 10 min (Eppendorf centrifuge 5424, Hamburg). The glucose released was measured at 510 nm, by reacting 0.01 ml supernatant with 0.3 ml glucose oxidase-peroxidase (GODPOD) for 20 min. Starch concentration in percentage of DW was calculated with the molar mass conversion from glucose to any-hydroglucose (the starch monomer unit) of 0.9.

### 2.4. Effect of nutrients, light, and temperature on starch content in *Ulva ohnoi*

*U. ohnoi* was grown in the ASW with MPBR (Reactors no. 1–5) as mentioned earlier under natural irradiance. Nutrients were supplied by adding  $\text{NH}_4\text{NO}_3$ , and  $\text{H}_3\text{PO}_4$  as mentioned in Section 2.1. Reactor 1 did not contain any nutrient for 2 weeks. Remaining reactors were supplied

with nutrients for 2 weeks, except in reactor 3, the concentration of nutrients was increased to  $2\times$ . To give low light stress, the intensity of the daylight in reactor 3, 4 (for 2nd week) and 5 (for both the weeks) was reduced to  $\sim 50\%$  by covering the reactor from outside with black nylon netting. The sole  $\text{CO}_2$  supply was from the bubbled air. Other conditions such as pH (8.2), salinity, and flow rate (2–4 L/min) were maintained uniformly in all reactors. Temperature and light intensity, inside and outside of the reactor was measured by using Hobo pendant temp/light data logger (UA-002-64, Onset, MA, USA). After 2 weeks, *U. ohnoi* samples were harvested, and biomass gain (WW) was recorded. DW content of the biomass was obtained as mentioned in Section 2.1. Starch content was determined by using Megazyme total starch assay kit as explained before in Section 2.3.

In a separate experiment, *U. ohnoi* was cultivated in MPBR to study the effect of seasonal light and temperature variation on starch content for two weeks during August, November and December 2017. *U. ohnoi* was grown in ASW with nutrients (as in Section 2.1) for 1 week. Following this, the medium was replaced with ASW without any nutrients for 1 week to induce nutrient starvation. The light and temperature inside and outside the reactor were monitored as above. Starch concentration was analyzed before and after starvation.

### 2.5. Extraction and purification of starch granules from *Ulva ohnoi*

The washed biomass of *U. ohnoi* (100 g) was mixed with cold distilled water (1:20 (w/v)) and homogenized to fine particulate suspension using homogenizer (HG-300, Hasigtai Machinery Industry Co., Ltd., Taiwan). The obtained mixture was filtered through nylon filter with 100  $\mu\text{m}$  pore size to remove larger particles. The filtrate was then sequentially passed through 50 and 10  $\mu\text{m}$  pore size nylon filters. The filtrate obtained at the end was centrifuged at 1811 g for 6 min (Rotanta 46 RSC, Hettich Instruments, LP, Germany), to obtain the pellet containing starch granules. This pellet was washed three times with absolute ethanol to remove pigment and lipid contamination. For purification of starch extracted from 100 g fresh *U. ohnoi* biomass, 300 ml of absolute ethanol was used, which was later recovered using rotary evaporator. The obtained off-white pellet was dried at room temperature for 24 h before being used for further characterization.

### 2.6. Microscopic studies, size distribution and purity of starch granules

Starch granules were studied for their morphology by using light microscope and transmission electron microscopy. The size distribution of extracted starch granules was analyzed by laser diffraction using Mastersizer (Mastersizer 3000, Malvern Instruments Ltd., Malvern, UK). Morphology of *U. ohnoi* starch (US) granules was observed using TEM and surface morphology was observed using scanning electron microscopy (SEM) and atomic force microscopy (AFM). SEM image was obtained using Quanta 200 FEG ESEM, Oregon, USA) after fixing the sample on silicon tape and then coating with gold using sputter coater. AFM scans were obtained using NanoWizard II Bio-AFM (JPK Instruments, Germany), at 25 °C. Samples were imaged in air, in an intermittent contact mode, using NSC21/NoAl silicon tips (NT-MDT, spring constant between 8.5 and 33 N/m). The purity of the extracted starch powder was measured using Megazyme's total starch assay kit as described in Section 2.3. The starch extraction yield was calculated as, the fraction of starch extracted using the extraction procedure in comparison to the total starch present in the initial *Ulva* biomass, measured with Megazyme's total starch assay kit.

Presence of minor impurities in starch such as cellulose, ash, lipid, and ulvan monomers were analyzed. Protein content was determined by Lowry method [45]. Cellulose content was measured by aseptically reacting approximately 10 mg sample (washed with 70% ethanol and dried) with 10  $\mu\text{l}$  cellulose ((C2730 Sigma-Aldrich, Israel) in 1 ml sodium acetate buffer (500 mM, pH 4.5) for 24 h, at 50 °C [46]. The glucose released was measured by GODPOD assay and values were

converted to percent cellulose. Ash content was measured by weighing approximately 500 mg of the moisture free starch sample into a crucible, burning the sample for 3 h in a furnace at 550 °C. The crucibles were cooled to 105 °C, resultant weight of the sample was recorded and the difference in weight is taken as the ash content [47]. Lipid content was measured by following protocol of Bligh and Dyer [48]. Briefly, 500 mg of starch was taken in glass vial and mixed with 10 ml methanol and 5 ml chloroform. Mixture was vortexed for 3 min and kept for 24 h at 25 °C. Mixture was vortexed for 2 min and 5 ml of chloroform was again added and then it was shaken vigorously for 1 min. Distilled water (10 ml) was added and vortexed again for 2 min. The layers were separated by centrifugation for 10 min at 2000 rpm. The volume of chloroform layer was recorded and it was filtered through Whatman filter paper No.1 into a clean glass vial (W1). Chloroform was evaporated using boiling water bath and the residue was further dried at 105 °C for 1 h. The weight of the vial was again recorded (W2) and the difference in the weight was used to calculate the total lipid content in percentage of starch DW used.

### 2.7. High-performance ionic chromatography (HPIC)

The HPIC analysis was carried out to determine monomer composition of the extracted starch using enzymatic and acid hydrolysis method with slight modification [49]. Starch powder ( $\sim 20$  mg) was autoclaved at 121 °C for 10 min and after cooling to 25 °C it was treated with 1  $\mu\text{l}$  amyloglucosidase and  $\alpha$ -amylase ( $\geq 260$  U/ml, A7095 Sigma) ( $\geq 250$  U/g, A7595 Sigma) in 1 ml sodium acetate buffer (200  $\mu\text{M}$ , pH 4.8) for 36 h at 45 °C. Acid hydrolysis of starch was carried out based on Jiang et al. [50]. Briefly,  $\sim 20$  mg starch was mixed with 500  $\mu\text{l}$  of 2% sulfuric acid. The mixture was autoclaved at 121 °C for 10 min and cooled to 25 °C. After hydrolysis, the hydrolysates were appropriately diluted and analyzed for monosaccharides using HPIC (Dionex ICS5000 platform, Thermo Fischer Scientific, MA, USA) equipped with Dionex AminoPac PA-10 column and its corresponding guard column using rhamnose, arabinose, galactose, glucose, xylose and glucuronic acid as standards ( $\geq 99\%$  purity, Sigma). The program used by Robin et al. [20] was followed to separate the monosaccharides including glucuronic acid. The column temperature was kept at 30 °C and the flow rate was set to 0.25 mL  $\text{min}^{-1}$ . Electrochemical detector with AgCl as a reference electrode was used for detection.

### 2.8. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectrum was recorded for dry *Ulva* starch (US) powder using Bruker Platinum ATR-FTIR spectrometer from 390 to 4000  $\text{cm}^{-1}$  and compared with the potato starch (PS) (Sigma, Israel). Starch sample was directly placed onto the diamond crystal for analysis.

### 2.9. Thermogravimetry and differential scanning calorimetry (TGA/DSC)

Thermogravimetric and differential scanning calorimetric (TG-DSC) analyses were carried out to study the thermal properties of starch granules using a Jupiter STA 449 F5 instrument (NETZSCH, Germany). Dry starch powder (5 mg) was subjected to a temperature range of 30 to 900 °C with the increase in temperature at 5 °C/min under nitrogen ( $\text{N}_2$ ) atmosphere [51]. The empty aluminum crucible was used as the reference. Thermal analysis of US was compared with that of standard PS (Sigma-Aldrich, 33615).

To study the gelatinization temperature of the starch, DSC was performed following the procedure of Malumba et al. [51]. Starch (2.5 mg DW) was weighed into an aluminum crucible and deionized water (7.5  $\mu\text{l}$ ) was added. The crucibles with starch–water slurry were hermetically sealed with a lid having a hole in the center. After 1 h of storage at room temperature for equilibration, measurements were

performed over a temperature range of 30 to 200 °C. Samples were scanned at a temperature increase rate of 5 °C/min., under N<sub>2</sub> against empty crucibles as a reference. Thermal parameters of gelatinization including onset (To), peak (Tp), conclusion (Tc) temperatures, gelatinization range (ΔT) and gelatinization enthalpy (ΔH) were recorded. The onset temperature and peak temperatures were computed using NETZSCH Proteus Thermal analysis software (NETZSCH, Germany). ΔH was calculated by dividing the integrated peak area with heating temperature rate (K/s) and further with the weight of the starch slurry in milligram.

### 2.10. Offshore *Ulva ohnoi* biomass cultivation and annual starch production

*U. ohnoi* was grown in a coastal area near Tel Aviv for 13 months, from March 2016 to April 2017. Flat cultivation reactor with an area of 0.045 m<sup>2</sup> and surrounded by a netted cage were used for cultivation at the same location in the coastal sea of Tel Aviv as mentioned in our previous report [52]. Three reactors were used in the study and results are reported as mean values. Samples were taken on various occasions during the year for starch analysis. Daily growth rate (DGR) was calculated using Eq. (1) [52]:

$$DGR = \frac{1}{N} \cdot \frac{(M_{ww-f} - M_{ww-i})}{M_{ww-i}} \quad (1)$$

where N (d) is the number of days between measurements,  $M_f$  is the final wet weight (WW) (g) measured at the end of each growth period, and  $M_i$  is the WW (g) of the inoculum.

Total starch production (TSP) from dry *U. ohnoi* biomass per year was calculated as per Eq. (2).

$$TSP = \sum_{i=1}^n (M_{wwf} - M_{wwi}) \cdot \frac{DW}{WW} \cdot S \quad (2)$$

where,  $M_f$  is the final wet weight (WW) (g<sub>ww</sub>/m<sup>2</sup>) measured at the end of each growth period *i*, and  $M_i$  is the initial WW (g<sub>ww</sub>/m<sup>2</sup>) of the inoculum, *DW* is the dry weight percentage of *U. ohnoi* biomass, *S* is amount of starch in grams per gram of dry weight of *U. ohnoi* biomass, and *d* is the cultivation time in days. The value obtained was converted to t ha<sup>-1</sup> yr<sup>-1</sup>.

### 2.11. Statistical analysis

Statistical analysis of the data obtained was performed using Excel (ver. 13, Microsoft, WA, USA). The significance of the differences was determined using One-way analysis of variance (ANOVA) at significance level  $\alpha = 0.05$  and when comparing only two treatments for each variable, student's *t*-test ( $\alpha = 0.05$ ) was used. Graphs were plotted using Excel and Origin 8. All experiments and controls were done at least in triplicates unless otherwise stated.

## 3. Results and discussion

### 3.1. Microscopic observation of *Ulva ohnoi* and starch content determination

Total starch content in *U. ohnoi* grown for two weeks (July 2017), in ASW supplied with nutrients (6.4 g m<sup>-3</sup> of N<sub>2</sub> and 0.97 g m<sup>-3</sup> of P) was 4.5% of the DW. As in Fig. 1, starch granules were seen inside the *U. ohnoi* cells. Blue starch granules inside cells with brown thallus were seen when stained with Lugol's Iodine solution. The whole cytoplasm and the starch granules were seen to be shifted in one side of the cell when seen under a confocal microscope, probably due to dehydration of the thallus. Spherical, ovoid, pear-shaped and irregularly shaped starch granules were visible inside *U. ohnoi* cells as seen under TEM. Starch granules were present inside the chloroplast and embedded in the

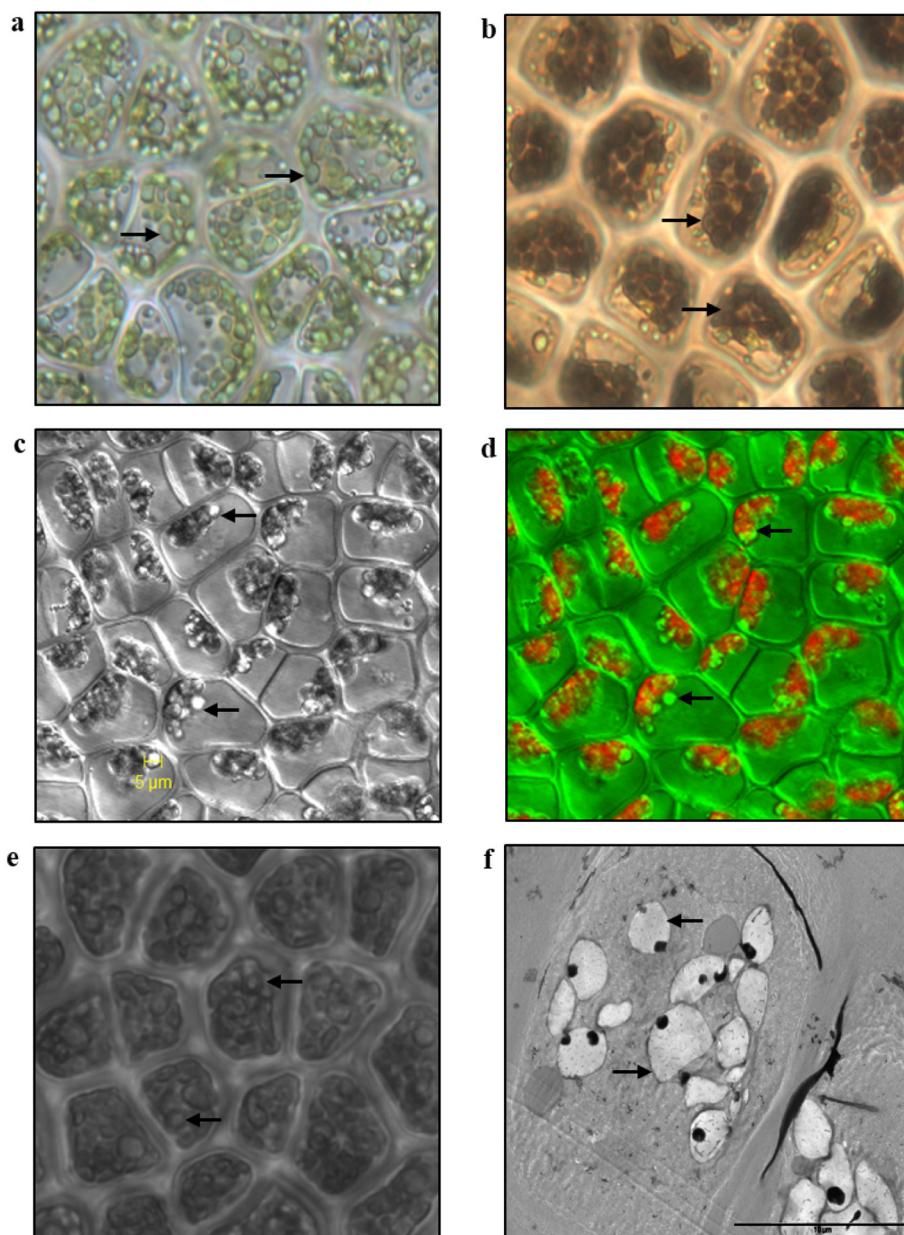
thylakoid membrane similar to that in terrestrial plants [53]. Starch plates surrounding pyrenoid had been also observed before [27,29]. TEM also indicated the size of granules to be about 5–7 μm.

### 3.2. Effect of nutrient, light, and temperature on starch content

In an experiment carried out in August 2017, to see the effect of nutrient addition on starch production in *U. ohnoi*, the alga in a reactor supplied with no nutrients (Table 1) showed the highest concentration of starch (7.33% DW) compared to that in all other reactors supplied with nutrients (6.4 g m<sup>-3</sup> of N<sub>2</sub> and 0.97 g m<sup>-3</sup> of P) (Fig. 2a). ANOVA analysis indicated results to be highly significant (*P*-value ≤ 0.0001). *U. ohnoi* supplied with nutrients for two weeks showed 3.5% starch whereas *U. ohnoi* supplied with nutrients and only 50% light (Table 2) in the second week showed 2.34% starch. *U. ohnoi* supplied with nutrients but 50% light stress for one week and for both the weeks, showed the same amount of starch (2.34%). *U. ohnoi* supplied with 2 × nutrient concentration of nutrients (12.8 g m<sup>-3</sup> of nitrogen and 1.94 g m<sup>-3</sup> of phosphorus) and 50% less light (Table 2) in the second week showed the least amount of starch (1.59%). Less starch and biomass was seen in the *U. ohnoi* when 50% less light was given (Fig. 2a, reactor 3, 4 and 5) compared to biomass with no light stress (reactor 1 and 2). Furthermore, the biomass yield, when supplied with 2 fold nutrients in the second week (reactor 4) compared to 1 × nutrients (reactor 3) was higher by only 1.5%, as both the reactors got 50% less light. This shows that even when the nutrient concentration was increased to 2 fold, there was a marginal increase in biomass due to less light availability, as also observed by Rosenberg and Ramus et al. [34]. Light is an important abiotic factor for growth and photosynthesis in *U. ohnoi* [34]. With less light, there is decreased photosynthesis rate leading to less glucose production rate and hence decreased growth and less starch accumulation as observed in our study and as well as observed by other authors [26,54]. At the same time, stored energy is utilized for growth, as observed in our results (Fig. 2a). ANOVA analysis also indicated that there was significance difference among biomass supplied with 50% light stress and no light stress (*P*-value = 0.01).

The reactor supplied with nutrients showed 16.83% biomass increase in 2 weeks compared with initial biomass concentration whereas only 4% biomass increase from initial concentration was observed in *U. ohnoi* under nutrient starvation. These results indicated that in the presence of nutrients, *U. ohnoi* shows more biomass growth but contains a low concentration of starch, while nutrient starvation in presence of light leads to increased accumulation of starch and less biomass growth. These results are very logical, as starch is the energy storage, so under the stress, such as lack of nutrients, the alga accelerates its energy storage functions to prepare for the harsh period. Conversely, when conditions allow fast growth, the algae focus on biomass production. Low light leads to lower concentration of starch and as well as lesser biomass growth compared to the presence of nutrients and no light restriction because both starch production and biomass growth require much energy input.

To test the effect of variation in natural daylight and temperature on starch content in *U. ohnoi*, the biomass was subjected to nutrient starvation for 2 weeks separately in August, November and December 2017. The concentration of starch in *U. ohnoi* was analyzed before starvation and after starvation. In August, starch concentration before starvation was 3.51% DW and after two-week starvation, it increased to 7.33% DW. In November, starch concentration before and after starvation was 11.78 and 16.04% respectively. Maximum starch concentration was observed in December, as 19.9% and 21.44% starch before starvation and after two weeks of starvation respectively (Fig. 2b). This could be due to the lower temperatures and shorter days in December compared to those in August (Table 2), which apparently induce investment in energy storage. Further research is needed to substantiate these initial findings. The difference in starch content in *U. ohnoi* before and after starvation was 3.81% in August, 4.68% in



**Fig. 1.** Microscopic observation of *U. ohnoi* thallus. **a**, light microscope. **b**, light microscope after treatment with Iodine solution. **c**, Confocal microscope without autofluorescence. **d**, Confocal microscope with auto fluorescence. **e**, phase contrast microscope. **f**, Transmission electron microscope (All images were observed under 40 × magnification, except TEM image (5000 ×, bar = 10 μM). Starch granules are indicated by arrow.

**Table 1**  
*U. ohnoi* cultivation in ASW under nutrients and light stress.

Reactor no.	1st week	2nd week
1	Without nutrients	Without nutrients
2	Nutrients	Nutrients
3	Nutrients	Nutrients + 50% less light
4	Nutrients	2 × Nutrients + 50% less light
5	Nutrients + 50% light	Nutrients + 50% less light

Note: Nutrients = 6.4 g m<sup>-3</sup> of nitrogen and 0.97 g m<sup>-3</sup> of phosphorus. Details on light data are given in Table 2.

November and 1.2% in December. Student's *t*-test analysis showed that there were significant differences in starch content in August, November and December months (*P*-values = 0.007, 0.029 and 0.019 respectively). The increase in starch content from the initial concentration corresponds to 108.93%, 41.3%, 6.31% in August November and

December respectively. ANOVA test on the results obtained for all three months yielded a *P*-value of 0.02. These results indicate that there was a significant increase in the starch content upon starvation in all three months. Light and Nutrients (N, P or S) stress are known to induce accumulation of storage polysaccharides in microalgae species [55], whereas, in macroalgae such as *Ulva*, nutrient limitation (mainly nitrogen) induce sporulation process which is marked by the accumulation of starch reserves inside its cells [29,31–35]. Our results of nutrient starvation with *U. ohnoi* also confirm the phenomena of starch accumulation. These results show that the starch productivity in *U. ohnoi* can be significantly increased by growing them initially in complete nutrient condition for fast biomass production followed by nutrient starvation for fast starch production. Moreover, winter conditions seem to be ideal for starch production.

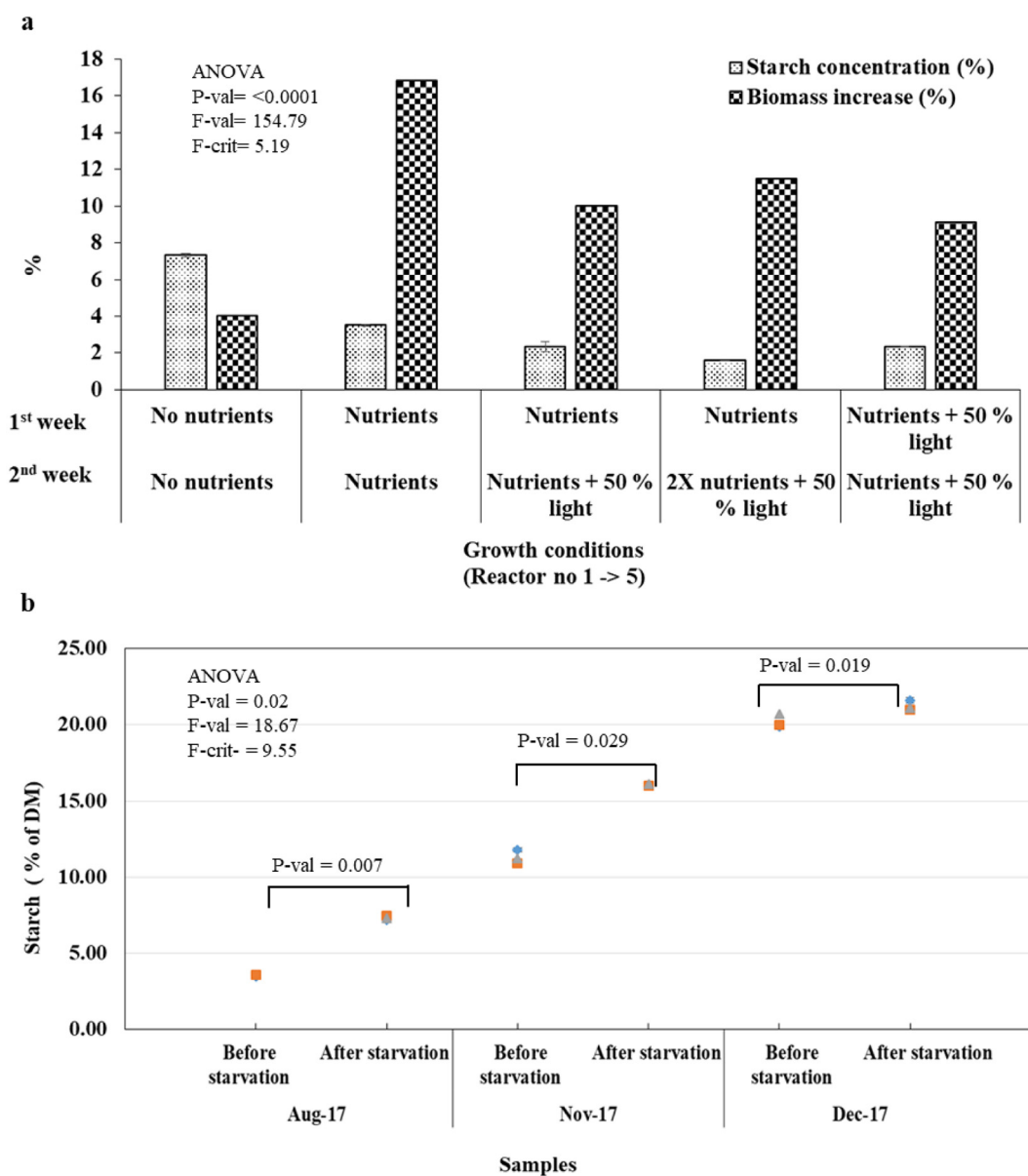


Fig. 2. Effect of nutrient and light on starch concentration in *U. ohnoi*. a, Effect of nutrients [6.4 g m<sup>-3</sup> of N (NH<sub>4</sub>NO<sub>3</sub>) and 0.97 g m<sup>-3</sup> of P (H<sub>3</sub>PO<sub>4</sub>)] and 50% less light on the starch content in *U. ohnoi*. b, Starch content in *U. ohnoi* before and after starvation during different months.

Table 2  
Temperature and light data during the experiment. Effect of nutrients, light, and temperature on starch content in *U. ohnoi* in August 2017.

	Temperature (°C)				Day light (W/m <sup>2</sup> )			
	Inside reactor		Outside reactor		Inside reactor	Outside reactor	50%, for 2 weeks	
	Day	Night	Day	Night				
August	Min	24.3	24.4	24.2	24.3	0.04	0.13	0.04
	Max	34.0	28.9	41.0	28.4	111.64	223.28	111.64
	Ave	30.5	26.7	31.9	26.5	46.82	54.78	46.82
2nd week	Min	25.6	26.0	25.6	25.8	0.09	0.04	0.04161
	Max	34.6	29.7	39.7	28.7	117.22	346.09	53.03
	Ave	31.6	27.5	32.1	27.1	33.65	65.94	14.89
November	Min	13.4	13.3	nd	nd	nd	0.04	NA
	Max	39.7	24.3	nd	nd	nd	893.14	
	Ave	23.3	17.6	nd	nd	nd	169.52	
December	Min	11.4	12.1	9.6	9.3	0.04	0.04	NA
	Max	21.4	21.2	39.5	23.0	50.24	848.48	
	Ave	17.4	17.4	22.0	16.2	11.86	175.48	

Note: nd = not determined, NA = not applicable.

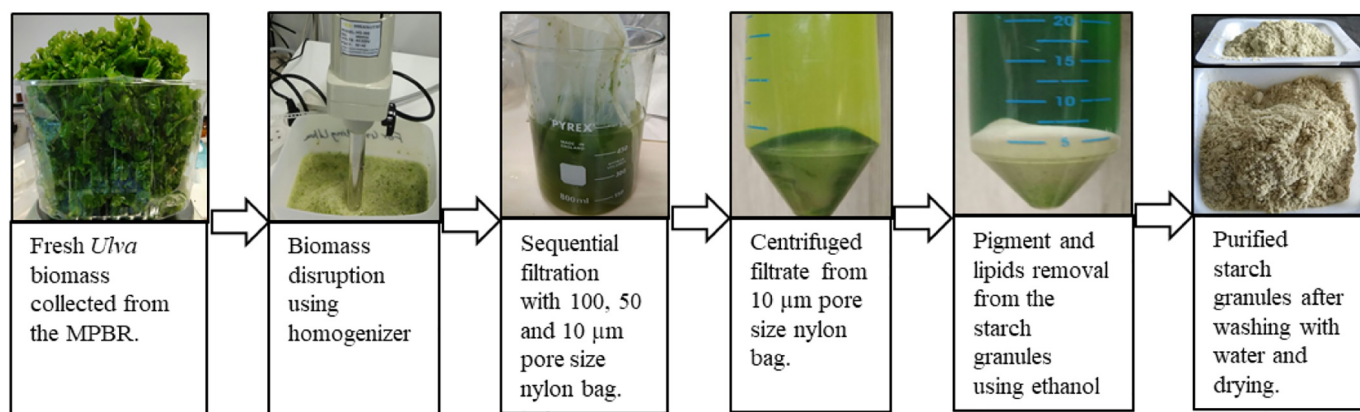


Fig. 3. Flow chart of starch extraction process from *U. ohnoi*.

### 3.3. Starch granules extraction, morphology, size distribution, and purity

Generally, extraction of starch from macroalgae in its native granular form is challenging due to its small granule size (5–7 µm) and low-temperature stability. *U. ohnoi* grown in December with 2 week starvation (having 21.44% starch of the DW) was used for starch extraction. The process followed for native starch granules extraction from *U. ohnoi* is shown in Fig. 3. This procedure can be scaled up for integration in a biorefinery process for the commercial recovery of the starch, similarly to what is done with wheat [56], and other products from plant biomass.

Spherical starch granules were visible inside *U. ohnoi* cells, as seen

under SEM, TEM and AFM (Fig. 4a, c & d). Although it resembled a sphere, other shapes such as ovoid, pear-shaped, and some irregularly shaped granules were also observed. The granule surface seems to be relatively smooth compared to the PS granules in SEM (Fig. 4a & b). Similar smooth surface morphology was seen in the AFM image (Fig. 4d).

TEM of *U. ohnoi* thallus (grown in December) showed the size of granules and majority of them were in the range of 5–7 µm (Fig. 4c). This was further confirmed by size analysis of extracted starch granules by Mastersizer. Similar observations about the size of US granules were made by SEM, TEM, and AFM. As can be seen in Fig. 4e, the average size and standard deviation of the extracted US granules were

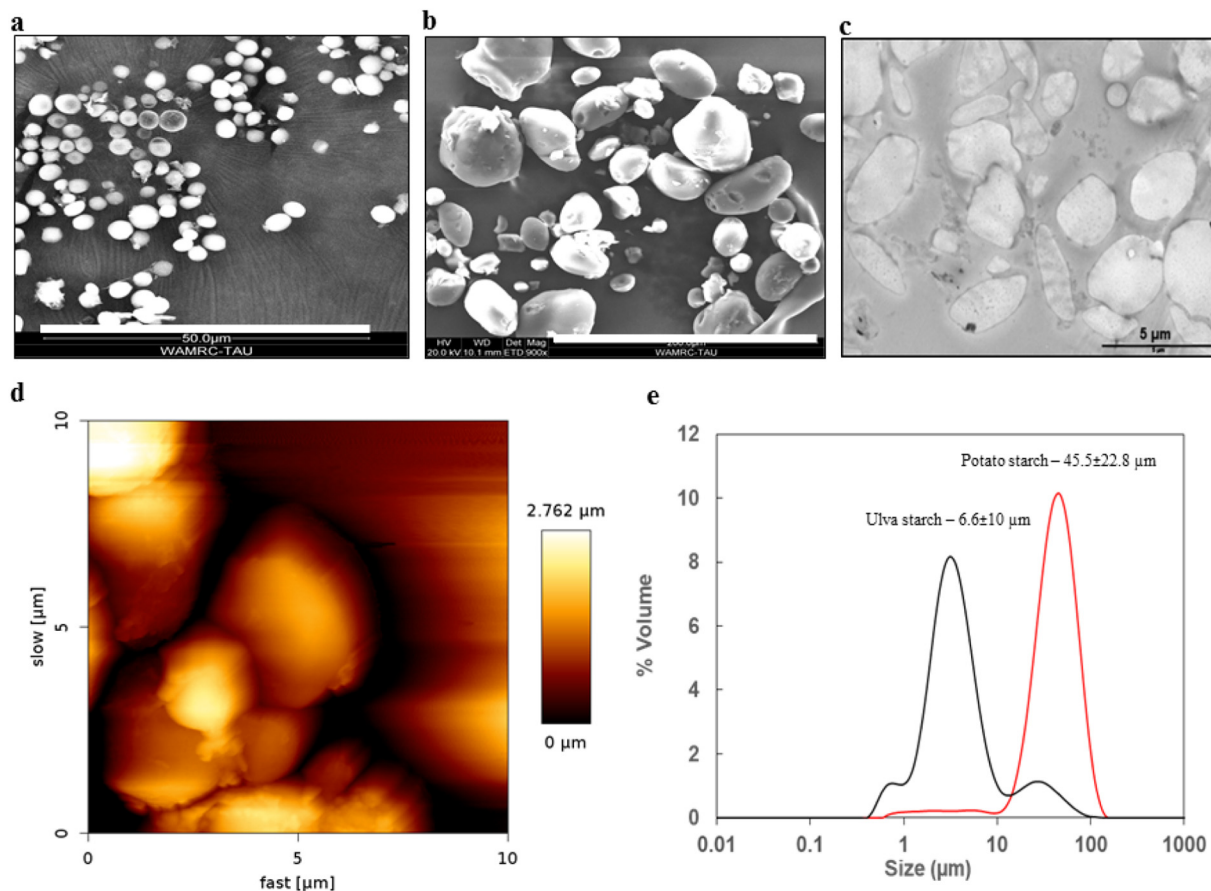
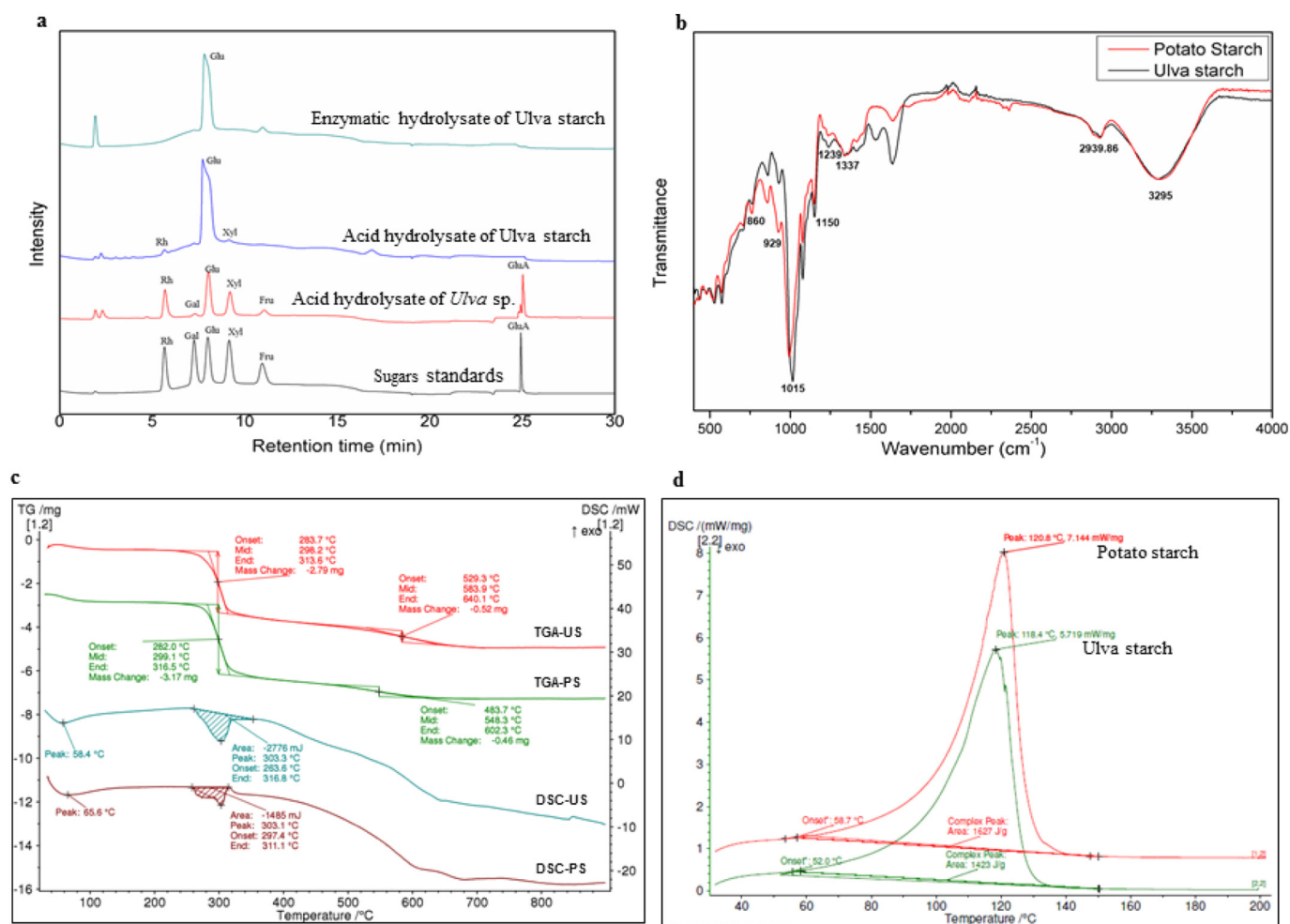


Fig. 4. Microscopic observation and size determination of US. a) SEM image of US granule (bar = 200 µm). b) SEM image of PS granules (bar = 200 µm). c) TEM image US granule. d) AFM image of US granule (scan size 10 × 10 µm, height difference 3.136 µm). e) Size distribution of extracted US and PS granules, determined by laser diffraction.



**Fig. 5.** Structural characterization of US. **a**, HPLC chromatogram of the enzymatic hydrolysate and acid hydrolysate of US, compared with acid hydrolysate of dry *U. ohnoi* biomass and standards sugars, peaks in chromatogram corresponds to Rh-Rhamnose, Gal-Galactose, Glu-Glucose, Xyl-Xylose, Fru-Fructose and GluA-Glucuronic acid. **b**, FTIR spectra of US granules compared with PS granules. **c**, TGA/DSC curve of US and PS granules. **d**, Thermograms displayed by US and PS granules in DSC.

$6.6 \pm 10 \mu\text{m}$  (the large standard deviation is due to the fact that the size distribution was trimodal, with sub-populations peaking at  $0.7 \mu\text{m}$ ,  $3.1$  and  $27.4 \mu\text{m}$ ). This is slightly larger than the typical size of red macroalgae starch granules ( $\sim 2\text{--}5 \mu\text{m}$ ) [57], and is quite similar to the size of rice starch granules ( $2\text{--}7 \mu\text{m}$ ) [58]. However, PS granules had a much larger average size and standard deviation of  $45.5 \pm 22.8 \mu\text{m}$ . The US granules we extracted were somewhat larger in size compared to starch granules from *U. ohnoi* ( $1\text{--}3 \mu\text{m}$ ) observed (qualitatively using TEM) in another study [59]. Electron microscopy and AFM analysis of these extracted granules also showed variability in shape (Fig. 4a, c & d). Starch granules vary enormously in size and shape depending on its botanical origin [58,60].

In plant leaf the size ranges from  $1$  to  $2 \mu\text{m}$ , in storage organs such as in potato tubers and rhizomes, it ranges from  $1$  to over  $100 \mu\text{m}$  [61] (in line with our observation, Fig. 4e). This variation in starch granule size and shape in plants is not clearly understood and may depend upon the timing of granule initiation during its synthesis and also reflects differences in a number of granules inside the cell. The smaller the granule, the “younger” it is, the more spherical its shape, and the higher its amylose content [60,62]. The same probably applies to the granule size and shape in *U. ohnoi*.

Various properties of starch and its final products are affected by its granules size distribution and find different industrial applications [63]. A recent study on rice starch granules has shown that starch with different granules sizes showed different gelatinization temperature,

pasting properties, crystallinity, and rate of hydrolysis [58,64]. US can find different potential industrial applications and further characterization in this regard is underway.

Upon extraction of starch from  $100 \text{ g}$  fresh *U. ohnoi* biomass (DM of 15%), all of the fractions obtained at the end were dried at  $105 \text{ }^\circ\text{C}$  and gravimetrically weighed for quantification and mass balance. The total mass of the residue remaining in the 3 nylon filters combined together was  $5.27 \text{ g}$  (35.17% of the DM used); the starch pellet obtained after centrifugation, purification and drying was  $2.14 \text{ g}$  (14.26% of DM); and the supernatant from centrifugation was also dried and found to be  $6.18 \text{ g}$  (41.2% of DM). The ethanolic fraction left from the starch purification was concentrated and dried using a rotary evaporator and was found to be  $0.8 \text{ g}$  (5.33% of the DM). The extracted fraction of US granules showed 75.45% starch content when analyzed using Megazyme total starch assay kit. This corresponds to 50.37% starch extraction yield (out of the potentially extractable starch content). The purity of US is close to the purity of various other starches such as from rice (73–86.8%), wheat (63–72%), potato (78.6%), cassava (80%), sweet potato (83%) [64–67]. Other components present in the starch fraction were cellulose 8.34%, protein 0.08%, lipid 2.43%, ash 1.51%, rhamnose 0.28% and xylose 0.77% (the latter two are ulvan components).



### 3.4. High-performance ion chromatography (HPIC)

Starch is mainly made up of glucose monomers comprising amylose and amylopectin polymers organized in multiple scales to form semi-crystalline granule [58,60,62]. The native granules are quite resistant to hydrolysis, but following gelatinization by heating in aqueous dispersion, the starch is susceptible to acidic and enzymatic degradation [68]. Acid treatment hydrolyzes all glycosidic bonds in the carbohydrate polymers (including ulvan and cellulose, a cell wall polysaccharides of in *Ulva* [69]), giving rise to their respective monosaccharides. Ulvan is a water-soluble fiber and was extracted using cold water as previously reported [70,71]. It is possible that some ulvan can get co-extracted during the starch extraction process followed in this study. Acid hydrolysis of starch followed by HPIC analysis was thus carried out to detect the presence of monosaccharides components of ulvan [20]. The enzymes amylase and amyloglucosidase specifically cleave glycosidic bonds in starch, breaking it down to its glucose monomers. The single major peak corresponding to glucose was observed in HPIC chromatogram of the hydrolysate obtained by enzymatic hydrolysis as well as by acid hydrolysis of the extracted fraction following its gelatinization (Fig. 5a). Small peaks of rhamnose (0.28%) and xylose (0.77%) observed in the HPIC analysis of acid hydrolysate indicated the presence of a small quantity of ulvan in the extracted starch fraction. HPIC results indicate that the extracted fraction mainly comprised of starch. Glucose detection in HPIC analysis suggests the gelatinized US is susceptible to hydrolysis by the digestive enzymes, amylase and amyloglucosidase. This provides an important piece of information about susceptibility of starch for enzymatic digestion, an important characteristic of starch used in the food industry. Further investigations on US digestibility need to be carried out so as to indicate its use as human or animal food supplement.

### 3.5. Attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectroscopy

Attenuated Total Reflectance-FTIR spectra of US extract and PS showed remarkable similarity in absorption pattern (Fig. 5b, Table 3). The peaks are characteristics to starch indicating the extracted fraction comprised starch [72]. The spectrum obtained was similar to the FTIR spectra reported for starch extracted from red algae *Furcellaria lumbicalis* [57], PS [73] and other native starches from various sources such as wheat and corn etc. [74]. The FTIR spectroscopy patterns can be used to understand the chemical bonding and short-range molecular order of starches. The O–H stretching vibration in US and PS samples is found to be a broad-band with peak position at 3295 and 3292 cm<sup>-1</sup> respectively. Such broad nature of the peak indicates that the starch is

**Table 3**  
Absorption peaks of *Ulva* starch (US) and potato starch (PS) in ATR-FTIR spectrum.

cm <sup>-1</sup>		Type of vibration of the functional group
US	PS	
3295	3292	O–H stretching
2930	2928	C–H stretching
1639	1645	H–O–H bending
1337	1353	(–C–H bending), (–CH <sub>2</sub> twist)
1242	1247	CH <sub>2</sub> OH side chain related mode (Weak)
1147.58,	1147.58	C–O–C Vibration, C–O–C, C–C stretching and C–O–H bending
1014.5		Characteristics to amorphous region C–O–H bending
	993.3	Characteristics to crystalline region
937	931	Skeletal mode vibration of α-1,4 glycosidic linkage (C–O–C)
860.2	858.3	C(1)–H, CH <sub>2</sub> deformation
763.7	761.8	C–C (stretching)

having strong hydrogen bonding interaction among themselves as well as with the water molecules present in it. The water absorbed in the amorphous region is confirmed by the presence of O–H bending vibration peaks at 1639 and 1645 cm<sup>-1</sup> in US and PS respectively [75]. A small variation in the peak position of CH<sub>2</sub> stretching vibration observed at 2930 and 2928 cm<sup>-1</sup> in US and PS respectively suggests the starch obtained from different botanical source [76]. All the peaks in the fingerprint region of starches (between 1500 and 400 cm<sup>-1</sup> were observed for both starches. Further, peaks at 574.7–572.8, 528.5–522.7 cm<sup>-1</sup> were attributed to skeletal vibrations of the pyranose ring.

The absorption peaks in the region 1100–900 cm<sup>-1</sup> have been shown to be sensitive to variation in starch structure, in particular bands at 1000, 1022 and 1047 cm<sup>-1</sup>, and have been reported to provide an idea about the crystalline and the amorphous regions in starch [72]. The peaks at 1022 cm<sup>-1</sup> seems to increase in more amorphous samples, while the bands at 1000 and 1047 cm<sup>-1</sup> become more defined in more crystalline samples [72]. In this study, US showed highest intensity peak at 1014.5 cm<sup>-1</sup> (indicating more amorphous) and PS showed highest intensity peak at 993.3 cm<sup>-1</sup> (indicating more crystalline). Furthermore, higher ratios in the absorption peaks at 995:1016 cm<sup>-1</sup> and 1048:1016 cm<sup>-1</sup> have been reported to impart the amorphous and ordered crystalline regions respectively [72,76]. The absorbance ratio of 995:1016 cm<sup>-1</sup> was 1.08 for US and 0.96 for PS respectively. The higher absorbance ratio of 995:1016 cm<sup>-1</sup> in US indicated that US has the higher amount of amorphous region as compared with PS, as well as, higher ratio of 995:1016 cm<sup>-1</sup> in US was indicative of higher affinity to water content due to higher amorphous content [76].

### 3.6. Thermogravimetry and differential scanning calorimetry (TGA/DSC)

Thermogravimetric analysis monitors the thermally-induced changes of a compound during heating. Structural features of starch granules influence their thermal degradation during combustion [51]. The TGA curve (Fig. 5c) showed loss of mass from the starch sample at rising temperatures. Under non-oxidative degradation, mass loss of 4.93% from 30 °C to 100 °C was recorded. Between 100 °C and 313.6 °C, 55.8% mass loss was observed, and another 10.4% mass loss occurred between 313.6 °C and 640 °C. The initial weight loss observed in the starch TGA curve can be attributed to the loss of residual moisture at a temperature range of ~40 °C–100 °C. The actual starch decomposition started only at a temperature of 283.7 °C and most of the starch decomposed at 298.2 °C.

Structural features of starch granules influence their thermal-degradation during combustion. In the DCS curve (Fig. 5c), the endothermic peak of the thermal phase transition was observed at 58.4 °C. This peak temperature is the melting phase transition point of US from a solid crystalline state to an amorphous molten state [77]. Following vaporization of the moisture content at higher temperatures, a second endothermic peak of starch degradation started at 263.6 °C [This was lower by 33.8 °C compared to that of the potato starch (297.4 °C)], with a decomposition peak at 303.3 °C and end point at 316.8 °C. This pattern of degradation of US observed by TGA and DSC was similar to that of PS.

In excess water, increase in temperature results in an endothermic peak in DSC at about 50–70 °C which is attributed to the gelatinization (thermally induced hydration & swelling) of the starch granules [58]. For US, an endothermic peak was observed with T<sub>o</sub> = 52 °C, T<sub>p</sub> = 118.4 °C, T<sub>c</sub> = 126.7 °C and ΔH of 21.01 J/g (Fig. 5d). The results were compared with those of PS, which showed T<sub>o</sub> = 58.7 °C, T<sub>p</sub> = 120.8 °C, T<sub>c</sub> = 127.8 °C and ΔH of 22.45 J/g (Fig. 5d), suggesting higher initial crystallinity and amylose content of PS granules, and is in line with their larger size (Fig. 3f). The gelatinization temperature (peak endothermic temperature (T<sub>p</sub>)) of US (118.4 °C) was higher than that of the Floridean starch from red algae *Gracilariopsis lemaneiformis*

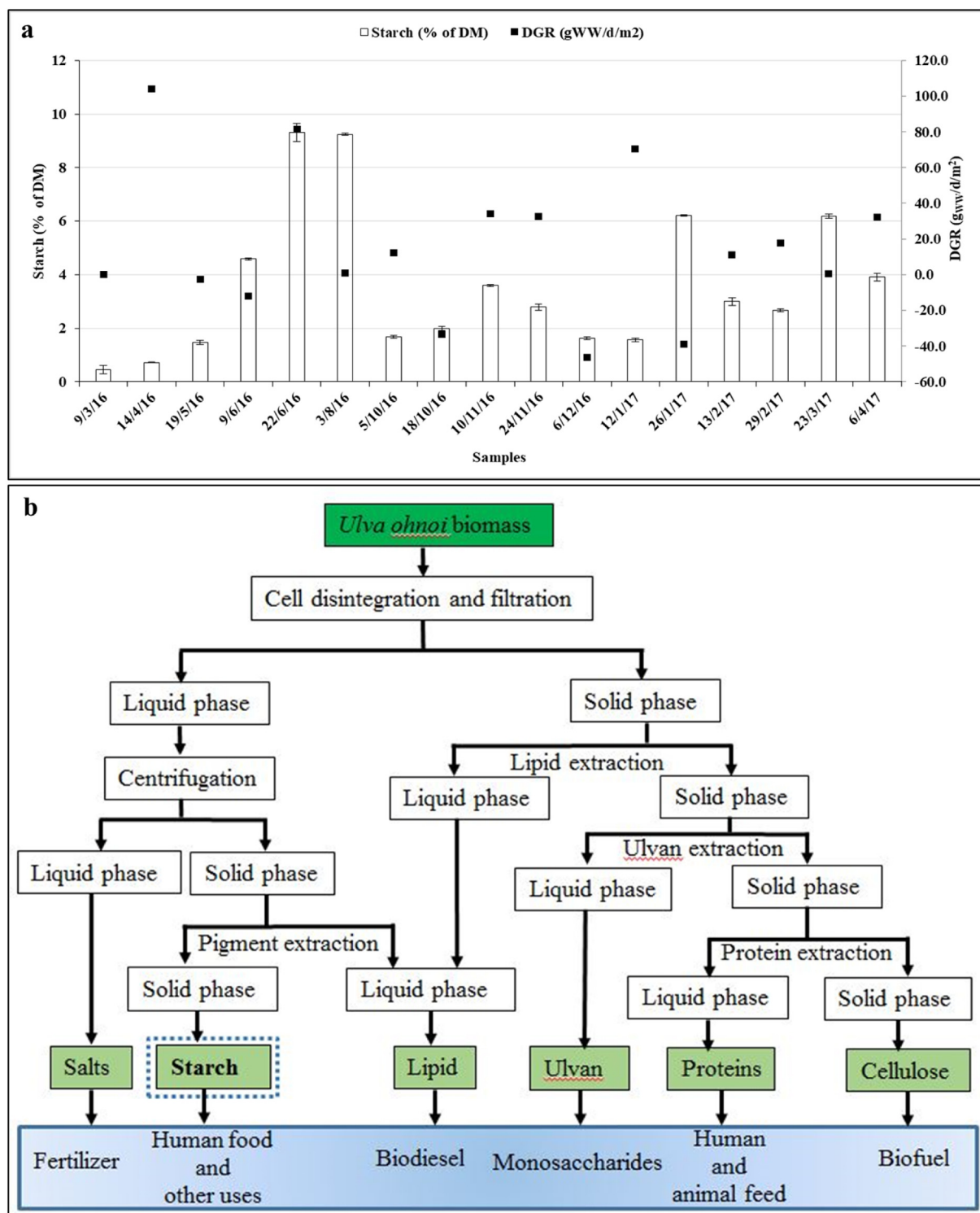


Fig. 6. Offshore *U. ohnoi* cultivation and biorefinery design. a, Starch content in *U. ohnoi* with daily growth rate (DGR) over a year cultivated in the offshore sea environment. b, Proposed *U. ohnoi* based integrated marine biorefinery process for co-production wide range of value products including starch.

(55.1 °C) and *G. chilensis* (52.7 °C) but lower than that of PS in our experiment (120.8 °C) and also reported by other authors (66.2 °C) [57]. Low gelatinization temperature of US can be attributed to smaller granule size or low amylose and high amylopectin content [64]. The peak gelatinization temperature and ΔH for PS observed were very similar to the values reported in the literature [78,79].

### 3.7. Starch monitoring in the offshore *Ulva ohnoi* cultivation and annual starch production

The starch concentration and growth of *U. ohnoi* in flat cultivation reactor placed at an offshore cultivation site was monitored for 13 months, from March 2016 to April 2017 (Fig. 6a). Highest average daily growth rate (DGR) of 183.5 g<sub>WW</sub>d<sup>-1</sup> m<sup>2</sup> was observed during the initial stages of the experiment from March 2016 to mid-April 2016.

This was followed by a significant decrease from May to August probably due to high temperatures are less favorable for *U. ohnoi* ( $\sim 30^\circ\text{C}$  seawater temperature in August) [52]. The average total DGR during the year was  $26.3\% \text{ WW d}^{-1} \text{ m}^2$  (or  $6.58 \text{ g d}^{-1} \text{ m}^2 \text{ DW}$ ). The variation in growth rate observed during this cultivation periods could be due to the degradation of *U. ohnoi* biomass during sporulation (as a part of reproduction event), or grazing by fish [80]. Many more general actual environmental parameters, such as temperature, salinity, pH, light intensity, or the presence of appropriate nutrients, may affect the DGR [52] and thus affect starch yield. Such loss of biomass could be avoided by cultivating a mutant sterile strain of *Ulva* (sterile strain without reproductive events) in controlled in-situ conditions [81]. Use of such sterile mutant strain of *Ulva* can be tested for starch accumulation which would enable on-going supply of biomass for continuous starch production.

Starch content was minimal (0.45–0.7%) during the initial growth period in March–May, and it increased gradually, reaching a maximum of 9.3% in June 2016. Although some biomass was lost in August, and the period was marked by minimal DGR, the concentration of starch in the remaining biomass was 9.23%. From October 2016 to mid-January 2017, the starch concentration was in the range of 1.5 to 3.6%, after which average moderate concentration of starch (4.5%) was observed until April 2017. Overall, the high starch concentration was linked to low growth rate. With an observed growth rate of  $-46 \text{ g WW d}^{-1} \text{ m}^2$  to  $183.5 \text{ g WW d}^{-1} \text{ m}^2$ , and DW/WW ratio of 0.15 in offshore cultivated *U. ohnoi*, a total biomass of 8.66 to  $31.32 \text{ t ha}^{-1} \text{ yr}^{-1}$  of DW could be obtained, with a total starch production (TSP) of 2.01 to  $3.38 \text{ t ha}^{-1} \text{ yr}^{-1}$  on the biomass dry weight basis. The details of annual biomass yield and starch concentration and their total yield can be found in Table S1 in the supplementary data.

As observed in this study as well as in our previous report [52], *U. ohnoi* biomass production yield was higher than some of the crops such as wheat, rice, and maize. With observed DW/WW ratio of 0.15 in offshore cultivation, a total of 8.66 to  $31.32 \text{ t ha}^{-1} \text{ yr}^{-1}$  of dry *U. ohnoi* biomass can be produced. This results in  $2.01\text{--}3.38 \text{ t ha}^{-1} \text{ yr}^{-1}$  of starch. In comparison, wheat gives 1.84 [82], rice gives 1.79 [82,83], maize gives 1.56 [82], potato gives 5.46 [84,85], sweet potato gives 4.52 [86], and cassava gives 10.39 [86,87],  $\text{t ha}^{-1} \text{ yr}^{-1}$  of starch. Intensive off-shore *U. ohnoi* farming can be carried out to further increase the biomass production, which would need less area for cultivation and also make the process economically viable [88]. As observed in Fig. 2a reactor 2, nutrient addition showed lower starch concentration, but biomass increase, which was almost four times that of the reactor with nil nutrients. This would lead to a proportionate increase in starch production too per hectare scale. The other advantages of higher biomass production are manifold- higher yields of other products too in a biorefinery model favoring economic feasibility of the process. One of several offshore biorefinery approaches could be followed for such macroalgae farming, to increase the diversity of products [54,89]. With several advantages, in terms of land, water and fertilizer, associated with the production of *Ulva* over many other terrestrial crops [54], starch production from *U. ohnoi* becomes very promising. Currently, various products are extracted from macroalgal biorefinery such as protein, carbohydrate, cellulose, fertilizer, animal feed or fuel, but starch is still not a part of macroalgal biorefinery [49,69,70,90,91]. Seaweed starch could be an important component of biomass that has not been reported so far in marine biorefinery. It indeed, could further strengthen the marine biorefinery concept as well as economic sustainability. Here we propose *U. ohnoi* based integrated marine biorefinery process for harvesting different added-value products with starch as an additional new product (Fig. 6b). Integrated extraction of this useful, high-added value product in a biorefinery, will confer additional efficiency and productivity gain, reduce waste (towards the zero-waste vision) and will further enhance the profitability of macroalgal biorefinery.

#### 4. Conclusion

We report on a new sustainable pathway to produce native starch from offshore grown *U. ohnoi* biomass. Nutrient starvation, particularly during winter, significantly increased the starch concentration in *U. ohnoi* grown in laboratory conditions. Characterization with HPIC, FTIR, and TGA/DSC results confirmed the extracted fraction contained starch with 75.4% purity. Enzymatic hydrolysis following gelatinization indicated the starch granules were susceptible to the digestion by amylase and amyloglucosidase. Offshore cultivation of *U. ohnoi* showed a total starch yield of 2.01 to  $3.38 \text{ t ha}^{-1} \text{ yr}^{-1}$  on biomass DW basis. Production of starch and other nutrients from *U. ohnoi* by off-shore farming and extraction in biorefineries, shows great promise to provide a novel and efficient way of improving future food security.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2018.11.007>.

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#### Competing interests

The authors declare no competing interests.

#### Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

#### Declaration of authors' agreement

All authors agreed to the authorship and submission of the manuscript to Algal Research for peer review.

#### Author contributions

M.P. – Conducted microscopy, starch estimation, design of starch extraction and characterization, data analysis and manuscript writing; A.C. – Conducted *U. ohnoi* cultivation experiment both in-situ and offshore, data analysis; R.G. – Conducted microscopy studies and discussion; M.K. – Conducted microscopy and size distribution; O.N. – Assisted in molecular characterization, microscopy, starch estimation and discussion; M.G. – Assisted in starch characterization; A.I., Y.L. and A.G. – Conceived the idea, discussion of objectives and critically reviewed the manuscript draft.

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