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A waste-free, microbial oil centered cyclic bio-refinery approach based on flexible macroalgae biomass

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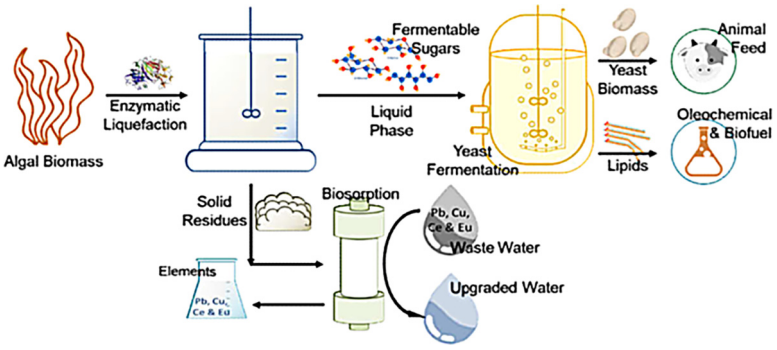
HIGHLIGHTS

- Holistic valorization of unexploited marine biomass.
- Integrated production of plant like yeast lipids, animal feed and biosorbents.
- Enzymatic macroalgae liquefaction to obtain yeast cultivation media.
- Utilization of hydrolysis residues as precious metal biosorbent.
- New, simple and rapid analysis tools for industrial lipid/ biosorption monitoring.

GRAPHICAL ABSTRACT



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ABSTRACT

Biofuels and the oleochemical industry are highly dependent on plant oils for the generation of renewable product lines. Consequently, production of plant lipids, such as palm and rapeseed oil, for industrial applications competes with agricultural activity and is associated with a negative environmental impact. Additionally, established chemical routes for upgrading bio-lipids to renewable products depend on metal-containing catalysts. Metal leaching during oil processing results in heavy metal contaminated process wastewater. This water is difficult to remediate and leads to the loss of precious metals. Therefore, the biofuels and chemical industry requires sustainable solutions for production and upgrading of bio-lipids. With regard to the former, a promising approach is the fermentative conversion of abundant, low-value biomass into microbial, particularly yeast-based lipids. This study describes the holistic, value-adding conversion of underexploited, macroalgae feedstocks into yeast oil, animal feed and biosorbents for metal-based detoxification of process wastewater. The initial step comprises a selective enzymatic liquefaction step that yields a supernatant containing 62.5% and 59.3% ($w/^{d}w_{biomass}$) fermentable sugars from *L. digitata* and *U. lactuca,* respectively. By dispensing with chemical pretreatment constraints, we achieved a 95% (w/w) glucose recovery. Therefore, the supernatant was qualified as a cultivation media without any detoxification step or nutrition addition. Additionally, the hydrolysis step provided 27–33% ($w/^{d}w_{biomass}$) of a solid residue, which was qualified as a metal biosorbent. Cultivation of the oleaginous yeast *C. oleaginosus* on the unprocessed hydrolysis supernatant provided 44.8 g L^{-1} yeast biomass containing 37.1% ($w/^{d}w_{biomass}$) lipids. The remaining yeast biomass after lipid extraction is targeted as a performance animal feed additive. Selectivity and capacity of solid macroalgae residues as biosorbents were assessed for removal and recycling of rare and heavy metals, such as Ce^{+3}, Pb^{+2}, Cu^{+2} and Ni^{+2} from model wastewater. The biosorption capacity of the macroalgae residues (sorption capacity ~ 0.7 mmol g^{-1}) exceeds

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that of relevant commercially available adsorption resins and biosorbents. To facilitate the integration of our technology in existing chemical and biotechnological production environments, we have devised simple, rapid and cost-efficient methods for monitoring both lipogenesis and metal sorption processes. The application of the new optical monitoring tools is essential to determine yeast cell harvesting times and biosorption capacities respectively. For the first time we report on a waste-free bioprocess that combines sustainable, microbial lipid production from low value marine biomass with in-process precious metal recycling options. Our data allowed for a preliminary economic analysis, which indicated that each product could be cost competitive with current market equivalents. Hence, the synaptic nature of the technology platform provides for the economic and ecologic viability of the overall process chain.

1. Introduction

The biofuels (i.e. biodiesel and biokerosene), oleochemicals (i.e. lubricants, surfactants and polymers) and the cosmetic sector are highly dependent on plant-derived triglyceride feedstocks to generate renewable product lines [1]. Due to their high areal yields, favorable chemical composition and low cost, palm and rapeseed oil are most desirable feedstocks for these processes [2]. However, the production of these plant-based lipids is associated with a negative ecological impact. More specifically, production of these chemical feedstocks competes with food production, accelerates land use change in sensitive ecosystems and thereby negatively impacts biodiversity [3]. By contrast, microbial oils generated from waste biomass streams within a bio-refinery setting potentially enable the generation of a diversified and sustainable product portfolio that is not associated with a negative environmental impact.

More recently, residual marine biomass, such as algae and seagrass, receives increasing attention as feedstock in bio-refinery processes to substitute the limited availability of residual terrestrial biomass in specific regions of the globe with dense populations and limited agricultural land (i.e. Germany, UK, Ireland, France, Japan, India, Malaysia, China) [4,5]. In this regard, macroalgae can expeditiously generate biomass from carbon dioxide, sunlight and inorganic nutrients by efficient photosynthesis [6,7]. With their high photon conversion efficiency [7], seaweeds like *Laminaria japonica* can reach a productivity of 1300 t ha^{-1} $year^{-1}$ biomass, which is 6.5 times higher than the productivity of sugarcane [8]. Furthermore, macroalgae are able to grow in a wide diversity of environments including fresh-, salt-, temperate- and municipal wastewater [9]. However, the use of macroalgae in biotechnological processes – much like terrestrial biomass feedstocks – requires the chemical or biological hydrolysis of polymeric carbohydrates that constitute the cellular matrix to release fermentable, monomeric carbohydrates. Previously, hydrolysis and liquefaction of macroalgae biomass has been demonstrated using chemical hydrolysis [10], chemo-enzymatic hydrolysis [11], biological degradation [12] and anaerobic digestion [13,14]. The resulting liquid hydrolysate has been reported to be enriched in fermentable sugars or volatile fatty acids [13], to be used in bioenergy generation, encompassing biogas

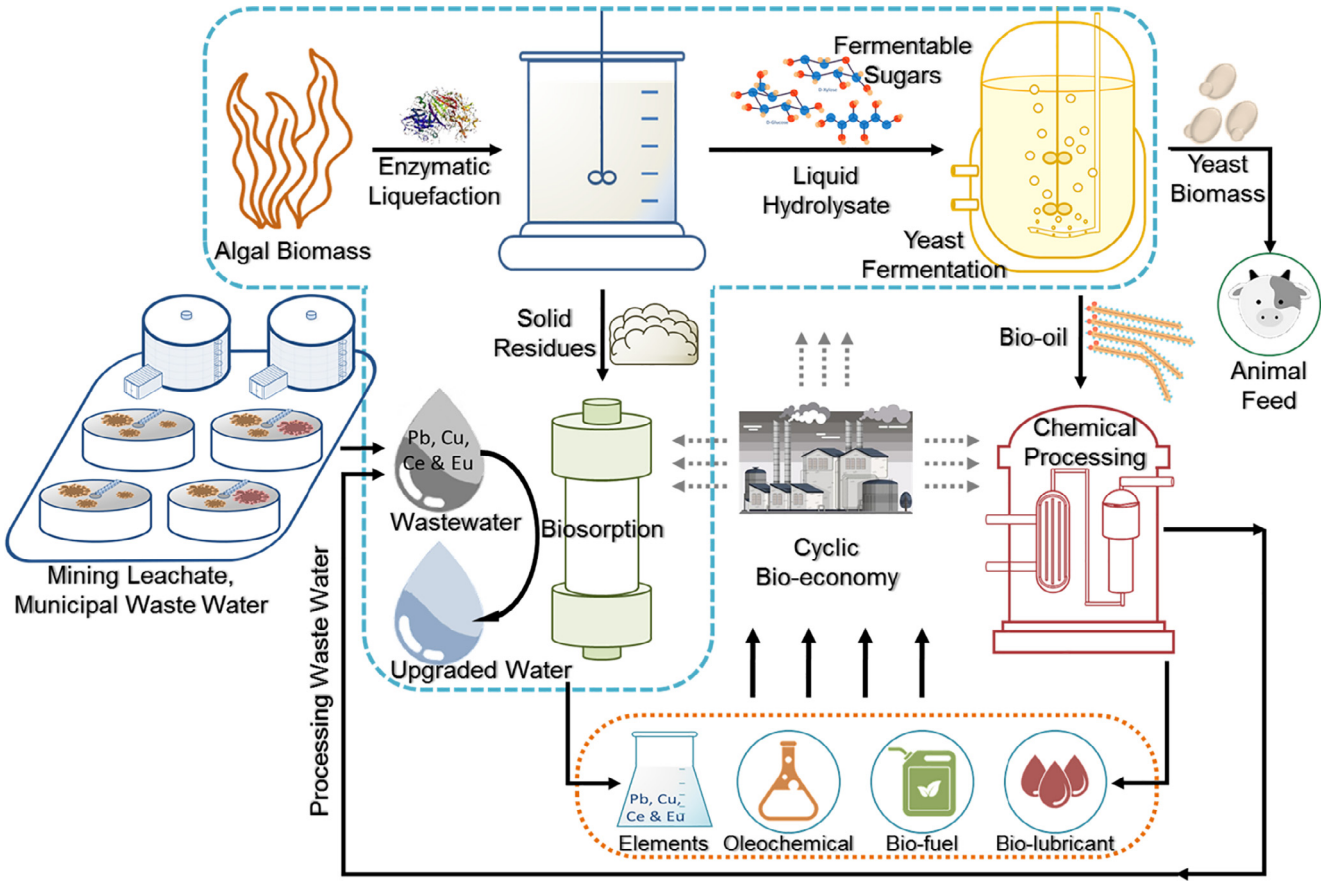


Fig. 1. The cyclic bio-refinery concept based on flexible macroalgae biomass feedstocks. The processes shown within the blue dashed line represent the focus of this study. All other processes are established at industrial scale and referenced throughout the text.

(methane) [14], bio-ethanol [15], acetone and bio-butanol generation [16]. In contrast, there are no value adding applications for the residual biomass fraction, which remains after biomass hydrolysis. At present, the most widespread application for this hydrolysis residue is application as fertilizer [9,17].

The utilization of the sugar rich liquid fraction as a fermentation base for generation of microbial oils has received increasing consideration [18,19]. In this application, designer bio-oils can be generated, that depending on the fatty acid profile could provide a sustainable resource for the production of high value pharmaceuticals, food additives, cosmetics, bio-lubricants or biofuels [20,21]. In that respect, microbial oils could directly substitute plant equivalents, such as palm oil, whose production is associated with land use change having a negative impact on biodiversity and food production [22]. Finally, biomass-hydrolysate of the marine microalgae *Scenedesmus* sp. [23] and beach-cast seagrass [5] have been demonstrated to be suitable fermentation substrates for yeast-based lipid production. The suitability of macroalgae hydrolysates for the fermentative generation of yeast oils has not been demonstrated.

This high value outlet of the liquid fraction contrasts the low value energetic use of the solid fraction remaining after enzymatic biomass hydrolysis. Therefore, the development of alternative high value applications for the solid residues that can be integrated in the bio-refinery work flow is highly desirable. One such application is biosorption, defined as passive uptake of molecules by biological matrices such as non-living cells as opposed to bioaccumulation involving active metabolism [24]. Marine macroalgae have been among the first biomass sources capable of binding high amounts of heavy metals, reaching capacities of 1.5 mmol g^{-1} [25]. Particularly, brown algae containing high amounts of alginate belong to the most studied biosorbing organisms [26]. Due to their growth performance and abundance (up to 16 million tons per year), brown algae are proposed to be a promising biosorbent [27]. Consequently, the development of biosorbents has to follow a “low-tech” and “low-price” philosophy, which can only be achieved by using inexpensive and abundant biomass residues. In a simplified model-process, desorption of biosorbents is not required since the loaded biomass can serve for energy generation as well.

In this context, process water containing heavy metals, found in the electronic, metal and chemical processing industries, poses a significant challenge for purification and recycling processes [28]. In general, heavy metal contaminants are difficult to remove by conventional technologies and potentially accumulate in the water column, where they are toxic to aquatic life [29]. Therefore, sustainable techniques for the removal and recycling of heavy metal contaminants that may contribute value to a bio-refinery set-up are in demand.

As chemo-catalytic processing of yeast triglycerides to end products, such as biolubricant and oleochemicals, potentially results in heavy metal (Ni, Cu, W, Pb, Pt, etc.[30]) contaminated wastewater streams, the solid residue was evaluated for its capacity to bind these metal toxins. Surprisingly, we could demonstrate that hydrolysis residues are excellent biosorbents, capable of opening avenues for in-process recycling of precious catalytic metals and simultaneous wastewater upcycling. Moreover, the residual solid fraction could be a separate value-adding product stream, which can be employed in the recovery and recycling of precious metals from mining waters or municipal waste leachates respectively (Fig. 1). An important group among these metals used in modern electronics are the lanthanides. Due to rising demand and unsustainable mining practices, they currently belong to the class of critical materials [31]. Rare earth metal recycling from waste streams is rather poor amounting to only 1% in 2011 [32]. However, there are some accessible resources, such as phosphogypsum leachate [33] and red mud [34] as the most prominent examples. As biosorbents are available at very low cost, it might be possible to process these residue streams. To quantify the specific sorption capacity, we use cerium, treated here as a model for the lanthanide group. Our simple and rapid luminescence-based approach also allows to measure kinetics and competitive biosorption in synthetic multicomponent solutions necessary for a reliable scale-up.

This study presents an entirely new cyclic bio-refinery concept based on flexible macroalgae biomass feedstocks. The assembly of value adding unit operations demonstrated herein are highly interconnected and interdependent, thereby eliminating any waste streams (Fig. 1). For the first time, we disclose optimized enzyme systems that enable selective, low energy hydrolysis of marine brown (*Laminaria digitata*) and green (*Ulva lactuca*) macroalgae biomass in the absence of physico-chemical pretreatment steps. The resulting liquid fraction, which is enriched in fermentable, monomeric sugars is applied as a fermentation base for the high yield production yeast oil triglycerides using the oleaginous yeast *Cutaneotrichosporon oleaginosus* (ATCC 20509). Fermentations carried out in aerated Erlenmeyer flasks (250 ml) and controlled stirred tank bioreactors (1L), demonstrated that *C. oleaginosus* can efficiently transform the chemically diverse macroalgae-derived sugar matrix into triglycerides, when a nutritional stressor was applied. In this context, a new staining-free flow cytometry-based method has been developed, which indicates the exact onset and extent of lipogenesis in *C. oleaginosus*. This method is generally applicable to time onset of lipid biosynthesis in oil-forming yeasts and allows for the exact determination of harvesting times, which are essential for process optimization in microbial oil-centered bio-refinery settings. While resulting yeast triglycerides are a “drop-in” feedstock for generation of bioactive agents, oleochemical building blocks and biofuels, the residual protein and carbohydrate rich yeast biomass can be used as animal feed. To close the mass balance of our cyclic bio-refinery concept we developed a new value-adding outlet for the solid residue fraction remaining after enzymatic macroalgae hydrolysis.

In summary, we present a completely new cyclic bio-refinery concept based on macroalgae biomass residues that has yeast oils as a primary outlet coupled with animal feed and biosorbent production. The bio-refinery process does not compete with agricultural activity, is not associated with land use change, is waste free and allows for in-process wastewater upgrading and metal recycling. Therefore, this set-up significantly contributes to the economic and ecologic efficiency of oleochemical generation.

2. Martials and methods

2.1. Macroalgae sample sourcing

Two brown algae (*Laminaria digitata*) and one green algae (*Ulva lactuca*) sample were harvested from the western cost of Ireland in March and June 2013 (North Seaweed Ltd – Netherlands). The samples were washed thoroughly (with distilled water) to eliminate salt, sand and contaminants. Subsequently, samples were dried and grind down to ≤0.5 mm grain size using a Planetary Ball Mill – (Fritsch, Germany).

2.2. Enzymatic liquefaction of brown algae biomass

Enzymatic liquefaction of each *L. digitata* and *U. lactuca* samples was conducted in acetate buffer (50.0 mM, *p*H 5.0) containing 7–8% (*w/v*) biomass. Hydrolysis parameters were maintained at: 50 °C, stirring at 400 rpm for 72 h. Enzymatic hydrolysis was conducted at 1 L for lab scale. For technical scale, *L. digitata* was hydrolyzed at 30 L scale.

In this study, an admixture of the commercial hydrolase enzyme preparations Cellic CTec 2®, Cellic HTec®, Novozymes 188®, (Novozymes, Denmark), *exo*-Laminarase (Megazyme-France) and *α*-Amylase® (Sigma, Germany) was used for biomass liquefaction. In the case of *U. lactuca* hydrolysis, the optimal enzyme admixture comprised a *w/w* ratio of 1.2: 0.3: 0.2: 0.0: 0.3 of the above enzyme preparations respectively. Analogously, the *L. digitata*, targeted hydrolase system was adjusted to a *w/w* admixture of 0.3:1.3:0.2:0.2:0.0, respectively. The respective enzyme admixture was dosed at total concentration of 2.0% ($w/^{d}w_{biomass}$) for brown and green algae respectively.

After biomass hydrolysis, the solid residue phase was separated by centrifugation at 8000*g* for 15 min. The liquid phase was purified by 10 kDa cross-flow. The filtrate was then sterilized with 0.2 μm filter and subjected to yeast fermentation. The retentate was pooled with the solid residue phase and subsequently used as a biosorbent for metal removal from dilute aqueous solutions.

2.3. Utilization of hydrolysis residues as a biosorption matrix

All biosorption experiments were conducted by incubating 20 mg air dried biosorbent in 2 ml of a defined metal solution for 3 h at room temperature. Subsequently, this suspension was centrifuged at 10,000*g* for 10 min and the cerium concentration was measured in supernatant. The cerium concentration ratio before and after the sorption process was used to calculate the binding capacity per gram biosorbent. Experiments were conducted as follows:

(1) Fresh biomass from March, fresh biomass from June, the mixture of both charges before and after hydrolysis. (2) Hydrolysis residue in solutions containing only cerium, and additionally nickel, copper and lead respectively as the second metal in the solution. The purpose was to determine the selectivity for cerium in presence of other metals. (3) Sorption kinetics with hydrolyzed biomass, for wet and dried samples.

2.4. Algae hydrolysates as cultivation media for yeast lipid production

2.4.1. Laboratory scale lipid production in aerated Erlenmeyer flasks

For yeast growth rate and lipid accumulation experiments, *Cutaneotrichosporon oleaginosus* (ATCC 20509) was cultivated in 1 L Erlenmeyer flasks containing 300 ml of the different enzymatic hydrolysates. The flasks were supplemented with an aeration system supplying the cultures with 0.2 L min^{-1} filtered air. Incubation was done at 28 °C, 120 rpm for 5 days with starting OD of 0.1. All experiments and analyses were conducted in triplicates.

2.4.2. Technical scale batch lipid production in controlled stirred tank bioreactors

C. oleaginosus was inoculated in the *L. digitata* hydrolysate. Batch cultivation of *C. oleaginosus* was performed in a 2 L bioreactor (INFORS HT system, Switzerland) with a working volume of 1.5 L in *L. digitata* hydrolysate with an approximately C/N ratio of 85. The temperature was kept constant at 28 °C, and the *p*H of the bioreactor was fixed at 6.5 $_{\pm 0.05}$ with 1 M NaOH by the system. Stirring (200–800 rpm) and aeration (air: 1.0–2.0 vvm) were regulated automatically to maintain dissolved oxygen above 50%. Foam formation was prevented by the addition of 0.01% (*v/v*) antifoam agent (Antifoam 204, Sigma Aldrich).

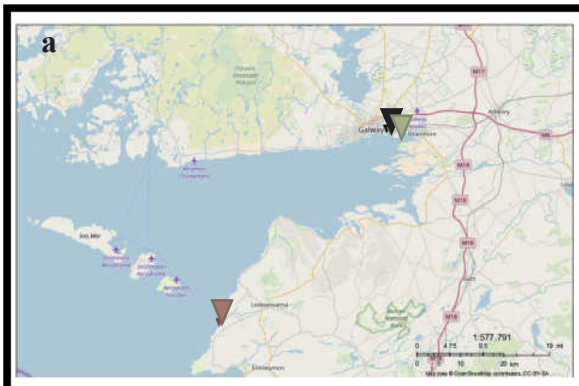
2.5. Analysis

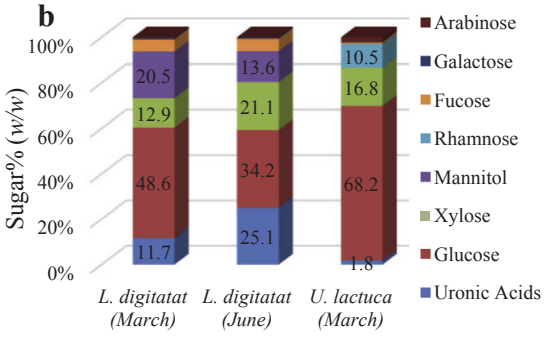
2.5.1. Biomass analysis

Sugar analysis was sequentially carried out by chemical hydrolysis and HPLC analysis as previously published [5]. Lipid analysis was performed via GC-FID [35]. Protein content was measured according to the Kjeldahl Standard operating procedure [36]. Finally, the ash content was determined gravimetrically after 1000 °C biomass incineration for 3 h [5].

2.5.2. Determination of total dissolved solids and elemental analysis

Total dissolved solids [TDS] and element analysis was carried by drying 100.0 ml of the hydrolysate at 105 °C (overnight). Resulting crystals were incinerated at 1500 °C for 3 h. Obtained ash material was subjected to scanning electron microscopy (SEM) with energy-dispersive X-ray (EDX) analysis using a JSM-7500F scanning electron microscope (JEOL, Japan). Crystals were mounted on carbon film and prepped for analysis. EDX analysis was performed on multiple areas (100 × 100 μm) in backscattered electron (BSE) mode for each ash sample. The average value was calculated to obtain the elemental composition for the ash of the hydrolysate.





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Sample	Location	% ($w/^{d}w_{biomass}$) Sugar	Protein	Ash	Lipid
***L. digitata* (March)**	Black	77.1 $_{\pm 0.52}$	10.3 $_{\pm 0.11}$	10.8 $_{\pm 0.42}$	1.8 $_{\pm 0.16}$
***L. digitata* (June)**	Red	75.5 $_{\pm 0.31}$	13.4 $_{\pm 0.14}$	9.9 $_{\pm 0.05}$	1.2 $_{\pm 0.59}$
***U. lactuca* (March)**	Green	62.5 $_{\pm 0.75}$	12.5 $_{\pm 0.15}$	22.8 $_{\pm 0.37}$	2.2 $_{\pm 0.09}$

Fig. 2. (a) An image of western cost of Ireland showed the location of selected samples; *L. digitata* [1] (black rectangle), *L. digitata* [2] (red rectangle) and *U. lactuca* (green rectangle). (b) Sugar profile of macroalgae samples; L. digitata [1], *L. digitata* [2] and *U. lactuca*, the relative standard deviation of all value is less that ± 4%. (c) The biomass balance of three samples; *L. digitata* [1] (harvested in March), *L. digitata* [2] (harvested in June) and *U. lactuca* (harvested in March). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

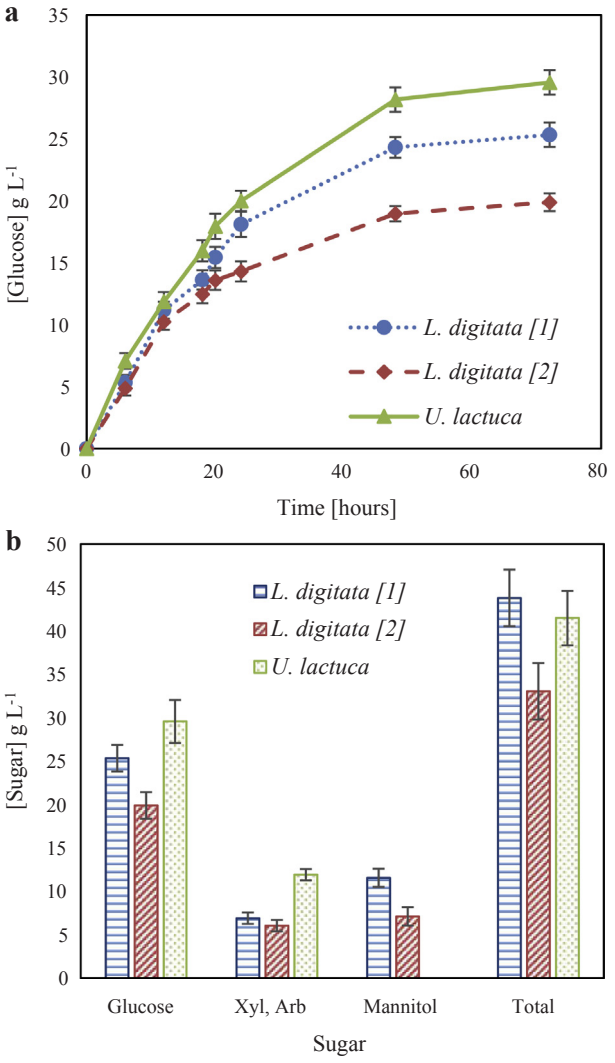

Fig. 3. (a) The proportional increase of glucose over the enzymatic hydrolysis time of *L. digitata* [1] (blue), *L. digitata* [1] (red) and *U. lactuca* (green). (b) The total sugar concentration at the final hydrolysate of *L. digitata* [1] (blue), *L. digitata* [1] (Red) and *U. lactuca* (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5.3. Gravimetric analysis of yeast biomass and lipids

Lyophilization was used to obtain dried yeast biomass. Lyophilization was carried out for 2 days at -80 °C and 0.04 mbar (Christ alpha 2–4 LD plus). For lipid extraction, cell destruction was performed with a high-pressure homogenizer (Mulsiflex C3, Avestine, Canada). After lyophilization, three times solvent extraction with Folch solution was sequentially carried out. Then gravimetric lipid quantification was carried out using the Bligh-Dyer method [37]. The lipid profile was assessed via GC-FID as described previously [5].

2.5.4. Flow cytometry-based cell counting

Cell counting via flow cytometry was carried out with a Bio-Rad S3 FACS (BioRad, Hercules, USA) equipped with 488 nm/ 100 mW laser beam. The counting was conducted using 100 µl of sample after 100 times dilution. The cell density diagram describes Side scatter [SSC] on "Y" axis and Forward scatter [FSC] on the "X"-axis.

2.5.5. Cerium concentration measurement

Samples were first diluted 1:99 by mixing 50 µl with 4.95 ml of deionized water. The measurement was then performed in a multi well plate with 100 µl diluted sample mixed with 100 µl buffer in each well. The buffer contained 100 mM sodium acetate, 10 mM copper (II), 10 mM nickel (II), and 10 mM lead (II). The *p*H was adjusted to 5.0 with glacial acetic acid. For every series of measurements, a calibration curve consisting of 4 points (cerium: 2.5 mM, 5 mM, 7.5 mM and 10 mM) was prepared the same way, as the samples. For measuring luminescence, a black quartz-glass 96-well microtiter plate manufactured by Hellma Analytics (Germany) and an EnSpire 2 Multimode Plate Reader (Perkin Elmer, USA) were used.

The reliability of the luminescence-based cerium detection method in presence of other metals has also been evaluated. To that end, triplicate samples with known copper concentration (10 mM, 20 mM and 40 mM) have been measured in the cerium concentration range 2.5 mM to 10 mM. All datasets can be described by a global linear regression curve with very good coefficients of determination (R-square) of 0.985 (Cu 40 mM) 0.987 (Cu 20 mM) and 0.992 (Cu 10 mM). The data plot with regression curve is depicted in the supplementary information (Supplementary Fig. 1S). The applicable limit for this method is about 1 mM cerium if interfering metals are present. For model samples containing only cerium the dilution factor can be decreased (until self-quenching occurs) leading to a limit of quantification (LOQ) at about 0.62 µM with signals at 308 RFU compared to 193 ± 12 RFU for the blank (Supplementary Table 1S). Notably, all metal chelating/binding agents should be eliminated from the assay solution. This was achieved, by repeated washing of the sample to eliminate any water-soluble components.

3. Results and discussion

3.1. Liquefaction of macroalgae biomass

Samples of brown macroalgae *Laminaria digitata* were harvested from the western coast of Ireland in March (black rectangle) and June 2013 (red rectangle), respectively (Fig. 2a). An additional sample of the green the macroalgae (*Ulva lactuca*) was harvested in March (green rectangle) from the same location. The overall chemical composition of the algal samples was analyzed using a detailed biomass analysis (Fig. 2c).

While the brown algae displayed a carbohydrate content of up to 75–77% ($w/^{d}w_{biomass}$), the green algae samples showed a 62% ($w/^{d}w_{biomass}$) carbohydrate content. Conversely, brown algae have less inorganic ash in relation to green algae. Protein and lipid content show convergent values in all samples. In spite of higher total sugar content in the brown algae, the glucose content was higher in the green algae (Fig. 2.b). It should be taken into consideration, that glucose content is important for fermentation since it is the most preferable sugar for microorganisms.

Seasonal sugar analysis of the *L. digitata* samples showed that the total sugar content is lower in June compared to the March batch. Moreover, the sugar profile showed considerable seasonal changes. In June the *L. digitata* batch contains less glucose and mannitol, which may be correlated with a decrease in the cellulosic fiber and laminarin. On the contrary, an increase in uronic acids was detected in the June batch, which could indicate an increase of alginate in the cell wall (Fig. 2b). Interestingly, the fucose concentration remained constant in both samples. In the case of *U. lactuca* glucose and xylose are the major sugars. In addition, a considerable amount of rhamnose was detected which could originate mainly from ulvan [38].

Independent of seasonal changes, glucose is the major carbohydrate monomer in the macroalgae hydrolysis supernatant. Therefore, glucose was used as indicator of hydrolysis efficiency. The glucose concentration was convergent in the first day of macroalgae hydrolysis (Fig. 3a). Thereafter, the difference in glucose release between samples is more pronounced. *U. lactuca* displays the highest glucose concentration followed by the March batch of *L. digitata* [1]. The synergistic effect of the optimized enzyme system facilitates cell wall lysis within the first 24 h,

which enhanced the sugar release over the remaining two days of hydrolysis. However, the total sugar composition showed different trends. The high amount of free mannitol in the brown algae samples, results in an overall higher concentration of total fermentable sugar in the *L. digitata* hydrolysate (Fig. 3b). The measured glucose and total fermentable sugar concentrations in the enzymatic hydrolysate were consistent with the biomass analysis. While, the glucose amount corresponds to almost 95% (*w/w*) of theoretical glucose content in the respective biomass sample, the total fermentable sugar concentrations indicate yields of 62.5, 47.1 and 59.3% ($w/^{d}w_{biomass}$) for dried *L. digitata* [1], *L. digitata* [2] and *U. lactuca* biomass respectively. The cumulative results indicate that with the exception of alginate and fucoidan, the synergistic enzyme activities result in an almost holistic lysis of the cellulosic and laminarin biomass components.

Our current data on recoverable fermentable sugars exceeds previous reports, where *Laminaria japonica* and *Ulva lactuca* were treated chemically prior to the enzymatic hydrolysis, resulting in relativity low glucose recovery compared to the theoretical yield [11]. Another report describes a single enzyme treatment that also lead to a low hydrolysis efficiencies and sugar yields with green and brown macroalgae biomass [39]. Most interestingly, our enzymatic hydrolysis process was so efficient for recovery of fermentable sugars, that no thermochemical pretreatment was required. To that end, the addition of laminarase, amylase and β-glucosidase significantly increased the sugar release. More generally, macroalgae are more amenable to enzymatic hydrolysis compared to terrestrial, lignocellulosic biomass resources (i.e. cereal straw or forestry waste), as they do not contain lignin and most sugar polymer are present in an amorphous and not a crystalline state [40]. The lack of lignin and crystalline polymeric carbohydrates circumvents the necessity of thermo-chemical pretreatments (i.e. steam explosion), which is associated with high energy expenditure and formation of fermentation inhibitors [41].

In addition to the fermentable supernatant, enzymatic hydrolysis also generates solid residue, which represent 27% and 32.5% ($w/^{d}w_{biomass}$) for *L. digitata* [1] and *U. lactuca* respectively. The analysis of the solid residue indicated, that it contained significant amounts of uronic acids, which are the main building blocks of alginate in addition to protein and inorganic components (i.e. $CaCO_3$) (Supplementary Fig. 2S).

Prior to using the liquid hydrolysis phase for fermentation, a 10 kDA cross-filtration step was conducted. The processed liquid phase was a light yellow clear liquid, which was used for downstream yeast fermentation without further processing. The highly viscous fraction that was retained after the cross-filtration step was subjected to component analysis. The resulting data indicated that this retentate was composed of glucose, mannitol and fucose respectively (Supplementary Fig. 2S). Based on the significant concentrations of fucose and glucose, the main sugar polymer remaining in the retentate was identified as fucoidan, which much like alginate is not accessible by commercial enzyme systems. Hence, alginate-rich solid hydrolysis residue and the fucoidan rich cross-filtration retentate was pooled and subsequently used as a biosorbent for metal removal from dilute aqueous solutions.

3.2. A cyclic biorefinery concept based on macroalgal biomass

3.2.1. Fermentative conversion of the liquid macroalgae hydrolysates to yeast oils

Sustainable bio-oil production from yeast is considered as the next generation source for triglycerides replacing plant-base lipids, in renewable chemical, cosmetic and pharmaceutical processes [21]. The application of oleaginous yeasts, such as *C. oleaginosus*, in a biorefinery setting has significant advantages over alternative microorganisms as they can rapidly grow to high cell density cultures with a biomass productivity of 2.18 g L^{-1} h^{-1} [42]. Moreover, oleaginous yeasts have the metabolic capacity to accumulate in excess of 75% ($w_{triglycerides}/^{d}w_{biomass}$) intracellularly, when nitrogen limiting conditions are applied [43]. The significant advantage of yeast oils over their plant equivalents is that they can be produced at the same yield and quality without seasonal variation. In contrast to industrial palm oil, microbial oil production does not negatively impact agricultural activity, food production or biodiversity as it does not induce land use change [22].

To test the suitability of macroalgae hydrolysates as feedstock, the oleaginous yeast *C. oleaginosus* was cultivated in each of the macroalgae hydrolysates as the sole fermentation medium. Results were compared to growth experiments conducted with artificial control media, whose chemical composition resembled the respective macroalgae hydrolysates.

The artificial control media were classified based on the nitrogen content into two categories; model complete hydrolysate and nitrogen-limited hydrolysate. The designed complete hydrolysates (model green algae and model brown algae hydrolysates) contained the C/N, C/S and C/P ratios as measured in the original brown or green macroalgae hydrolysates. By contrast, the nitrogen-limited hydrolysates (minimal green algae and minimal brown algae hydrolysates) featured a high C/N ratio (130–150) to induce lipogenesis. All components of nitrogen-limited hydrolysates were equivalent to earlier reports [44] with the exception of the sugar content. To ensure comparison of the artificial media to the original algae hydrolysates, the sugar profile was comparable to original algae hydrolysates.

During the first two days, the biomass formation and growth rate in all artificial media was comparable to typical cultivation in minimal media [5]. At the same time interval, the growth on real macroalgae hydrolysates was rather low. Unexpectedly by the third day, the growth rate in real hydrolysates showed a sharp increase, which exceeded that of all other media. At the end of the fermentation, the biomass yield of the real hydrolysate was considerably higher than in artificial media (Fig. 4a).

More specifically, *L. digitata* showed a growth inhibiting activity against four various bacterial or fungal species [45,46]. Interestingly, the antimicrobial natural product furaltadone was recently isolated from *U. lactuca*. To that end, changes in the yeast growth tendency over the first five day of fermentation using real and model hydrolysates could be attributed to an adaptive process of *C. oleaginosus* towards potentially cytotoxic hydrolysate compounds. The efficient adaptation of *C. oleaginosus* to these toxic components is then reflected in the rapid exponential growth phase observed from day three onwards. To that end, it is well documented that microorganism, such as oleaginous yeasts can adapt to toxic compounds contained in fermentation broth in a time dependent manner by switching on expression of detoxifying enzyme systems [47,48].

Yeast growth in model green algae hydrolysate has shown a slight superiority compared to model brown algae hydrolysate. This could be attributed to the high glucose content in model green algae hydrolysate, which is a most preferable sugar source for *C. oleaginosus*. By contrast, the real *L. digitata* hydrolysate (March batch, [sample 1]) showed a significantly higher biomass production (35.4 g L^{-1}) than the corresponding *U. lactuca* hydrolysate (28.9 g L^{-1}). In contrast, the *L. digitata* hydrolysate [2] (June batch [sample 2]) showed even lower biomass productivity (25.9 g L^{-1}).

Gravimetric lipid analysis showed 34 and 22% ($w/^{d}w_{biomass}$) produced biomass in *L. digitata* [1] and *U. lactuca* hydrolysates respectively (Fig. 4.b). It is noteworthy that this difference in lipid accumulation by yeast cells on different algae hydrolysates could additionally be confirmed by the microscopic imaging (Supplementary Fig. 3S). In that respect, intracellular lipids bodies could be detected in cells grown on brown algae hydrolysates. Specifically, yeast cells grown on original algae hydrolysate displayed an enlarged vacuole (Supplementary Fig. 3S). These results display the superiority of *L. digitata* [1] over *U. lactuca* hydrolysate as feedstock for oleaginous yeast *C. oleaginosus*.

With regard to the inhibited lipogenesis on *U. lactuca* hydrolysate, we suggest that the effect may be due to the presence of biologically

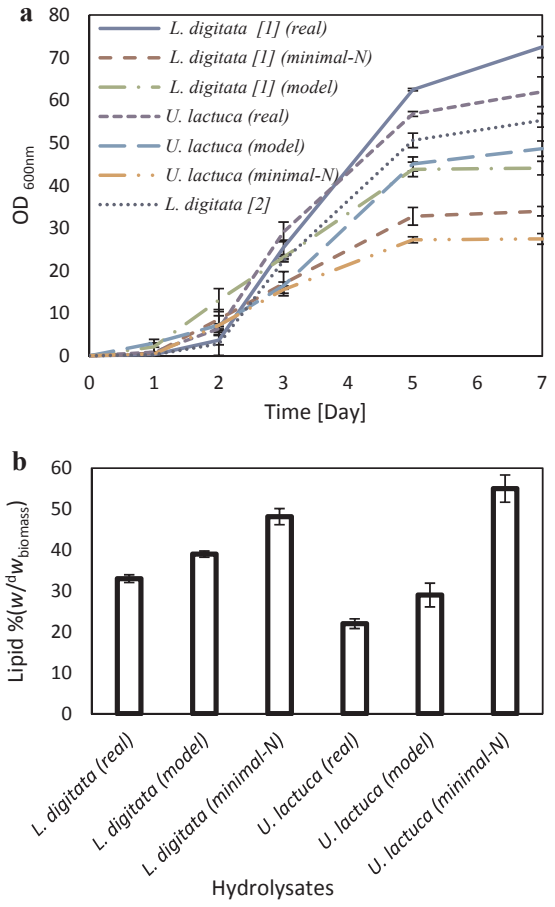

Fig. 4. (a) The increase of the optical density over the fermentation time of Real *L. digitata* [1], *L. digitata* [2] and *U. lactuca* and Model *L. digitata* [1] and *U. lactuca* and Minimal *L. digitata* [1] and *U. lactuca.* (b) The percentage of accumulated lipid by the dry biomass after 120 h of fermentation of real *L. digitata* [1] and *U. lactuca* and model *L. digitata* [1] and *U. lactuca* and minimal *L. digitata* [1] and *U. lactuca.*

active steroids in the green algae biomass such as 3-*O*-*β*-D glucopyranosyl-stigmasta-5,25-dien. This compound has been reported to have antimicrobial activity in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* [49].

In 2014, alginate from *Laminaria japonica* was fermented under anaerobic conditions to obtain volatile fatty acids [50]. The volatile fatty acids were consequently used as a carbon source for *C. oleaginosus,* for production of yeast triglycerides. In this set-up, a very low lipid productivity [Biomass: 4 g L^{-1}, contains lipid 48% (*w/w*)] was recorded [50]. Therefore, the data obtained in this study supersedes all previous data for biomass formation and total lipid yield with respect to oleaginous yeast fermentation. This can be attributed to the direct use of sugar rich liquid hydrolysates instead of volatile fatty acids as the yeast carbon source.

Based on the initial flask cultivations, *L. digitata* hydrolysate was deemed the best candidate for biomass and lipid production at 30 L scale. Hence, the brown algae hydrolysate was produced in scale of 30 L by enzymatic hydrolysis of *L. digitata*. The used biomass [2.4 kg–8.0% (*w/v*)] was a mixture of 1.9 kg from the March batch *L. digitata* [1] and 0.5 kg of June batch *L. digitata* [2]. After 72 h of incubation with the optimized enzyme solution, centrifugation followed by 10 KDa cross-flow filtration was performed in order to obtain a clarified liquid hydrolysate. The remaining solid residues were subjected to downstream biosorption assays. The resulting brown algae hydrolysate contained total 52 g L^{-1} fermentable sugars and 0.45 g L^{-1} total nitrogen resulting in a C/N ratio of 76.5 (Table 1). This C/N ratio is within the reported limits for lipid induction in oleaginous yeasts [21,51].

Without any nitrogen supplementation or external glucose feeding, the *L. digitata* hydrolysate was used in a 2 L scale fermentation of *C. oleaginosus*. As reported previously, respiration activity represented in pO_2 value was used as an indicator for the end of the fermentation. To that end, the fermentation was stopped at 120 h, when the pO_2 returned back to the saturation value. Biomass dry weight and cell count were used to monitor the growth. According to OD_{600} and dry weight, the exponential phase was terminated after 48 h of fermentation but flow cytometry measurements indicated that the cell saturation phase was already reached 20 h earlier (Fig. 5a). Lipid analysis showed an increase in the rate of lipid accumulation in the last two days of the fermentation (Fig. 5b). Under the applied conditions, 44.8 g L^{-1} yeast biomass containing 37.1% ($w/^{d}w_{biomass}$) lipids was obtained. Therefore, our total lipid of 16.5 g L^{-1} obtained with macroalgae hydrolysate is slightly higher than lipid yields obtained with *Cutaneotrichosporon* fermentations using corn cob (12. 3 g L^{-1} lipids) and rice straw (11.5 g L^{-1}) hydrolysate respectively [52,53]. Moreover, our previous work reported an equivalent C. oleaginosus fermentation using a crude hydrolysate of the seagrass P. oceanica as feedstock. In this context, we calculated a biomass-to-lipid conversion of 0.20 g g^{-1}, which is exactly the same as with brown algae in the current study [5].

The yeast triglycerides obtained in the current study have a similar composition to plant derived rape and palm oils, with oleic and palmitic acid being the dominant fatty acids (Supplementary Fig. 4S). Therefore, the yeast oil obtained in this study can be regarded as a direct substitute for plant oils in chemical processes [54]. In contrast to the plant equivalents, the yeast oils would have no impact negative on land use change, biodiversity or food production per-se. Moreover, they can be produced at consistent qualities, as their production process is not affected by environmental factors.

Detailed biomass analysis indicates that the residual yeast after lipid extraction contains about 15% (*w/w*) proteins and 85% (*w/w*) carbohydrates. Supplementary Fig. 5S depicts the sugar profile of the yeast residual biomass, mainly contains glucose and mannose. In addition, rhamnose and glucuronoxylomannan have been reported to be the main carbohydrate polymers of the *Cutaneotrichosporon* sp. [55]. Notably, previous studies confirm that mannan oligosaccharides contained biomass are excellent performance additives in weanling pigs feed formulations. In fact, MOS is considered as an alternative to an antimicrobial agent carbadox [56]. Therefore, we suggest that yeast cell residues obtained after oil extraction could be used as a performance additive in animal feed.

3.2.2. High through put monitoring of yeast lipogenesis by flow cytometry

Rapid measurements of the onset and extent of yeast lipogenesis is essential of in-process monitoring of yeast oil production and determination of harvesting times. Current methods either rely on gravimetric measurements or are depend on differential staining of lipid

Table 1
The chemical analysis of *L. digitata* hydrolysate.

Material	Concentration (g L^{-1})	Material	Concentration (g L^{-1})
Glucose	32.1	Nitrogen as NO_3/NO_2	0.15
Mannitol	12.2	Protein	1.56
Xylose	7.6	Phosphate (as PO_3^{-3})	0.6–2
Total Carbon	34.4	Total Sulfate (as S)	0.23
Total Nitrogen	0.45	Metal Ions	Na^+, K^+, Fe^{+2}, Ca^{+2}, Mg^{+2}, Mn^{+2}, Sr^{+2}
Nitrogen as NH_4	0.25		

[a]The relative standard deviation of all value is less that ± 5%.

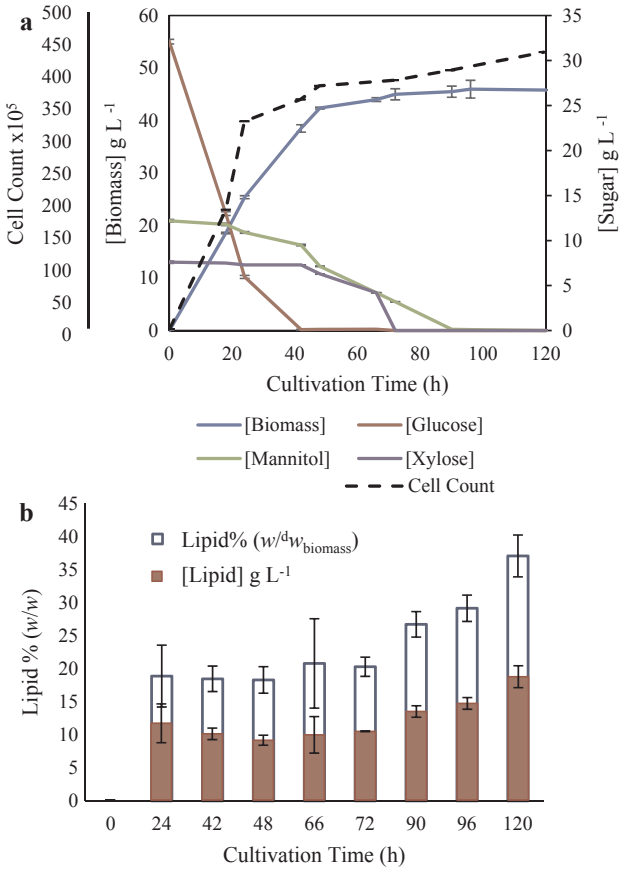

Fig. 5. Fermentation profile and lipid accumulation during 120 h of cultivation in 2 L bioreactor. (a) The cell count, dry biomass, optical density and consuming of fermentable sugar of the mixed *L. digitata* biomass. (b) The percentage of accumulated lipid and lipid productivity over the fermentation time in the mixed *L. digitata* hydrolysate.

bodies inside the yeast cells. Both methods are destructive, time consuming and they do not offer sufficient and information about cellular state of the culture. Additionally, many factors, such as cell size variation with phase of growth (lag, log, stationery), dead cell, shape of the cell, high cell density, chemicals and hydrophobic metabolites, can overlapped with obtained data causing under- or overestimation of biomass and lipids.

Therefore, rapid optical methods that could accurately determine these critical process parameters without destroying the sample would be a critical advantage when focusing on microbial oil centered biorefinery settings.

To that end, flow cytometry is an electro-optical-based technology able to analyze thousands of particles per second. Forward scatter [FSC] and side scatter [SSC] provides analysis of detailed cell features such as cell size and cell granularity respectively [57]. Previously, flow cytometry in combination with the Nile Red staining, has been used to quantify the intracellular lipid content of different microalgae [58,59] and the oil forming in some yeast like *Rhodotorula glutinis* [60]. Practically, we noted that lipid body staining in yeast cells by hydrophobic dyes such as Nile Red or Sudan black can be significantly affected by staining time, yeast type, lipid content and growth phase. Additionally, this effect significantly increased when the yeast cell has a rigid wall as in the case of *C. oleaginosus* [61]. Therefore, staining procedures do not offer reproducible assay results.

It is essential for any fermentative process to have an instantaneous assay method capable of monitoring the biomass, growth and lipid accumulation to gain instant feedback on the fermentation status.

During our microscopic assessments of the culture progress, we noted that both size and granularity of the yeast cells increased proportionally with the intracellular lipid content. Based these observations, flow cytometry-based parameters FSC and SSC *vs* lipid content were measured at same time intervals.

Fig. 6 shows the cell density plot integrated with the histogram of the FSC and SSC on X and Y axes respectively. At the starting point of the fermentation, the FSC histogram showed a wide cell distribution. At time point of 42 h, this distribution increasingly focused and moved towards a larger cell size. At the same time point, the cell count and biomass formation reached the saturation phase due to nitrogen depletion. Moreover, two populations could be distinguished in our flow cytometry experiments: A small population (P2) which newly appeared at the higher FSC and SSC in addition to the main plot (P1). With progressing fermentation, the FSC histogram of P1 was shifted towards higher size value. Later on, a third population (P3) was generated at the lower FSC and SSC.

With respect to the side scatter [SSC] values started sharp as the *C. oleaginosus* has an oval cell shape. With time; the SSC values became progressively boarder and moved to higher values, which indicated an increase in the cellular granularity. Most interestingly, this data indicated that cell granularity increases proportionally with increasing lipid content. This suggests, that the cell granularity is a direct indicator of the cellular lipid content.

According to the gravimetric assay, the changes in lipid content were about 19% from 18 to 37 ($w/^{d}w_{biomass}$)). These changes could remarkably be measured instantly by monitoring the FSC and SSC in our flow cytometry experiments. Additional experiments have demonstrated, that the FSC and SSC can exceed the value 10^{+3} of the logarithmic FSC, if the lipid content rose to about 75% ($w/^{d}w_{biomass}$). Moreover, microscopy confirmed that cells with 75% ($w/^{d}w_{biomass}$) lipid have remarkably larger sizes and almost a spherical shape (Supplementary Fig. 6S). In summary, flow cytometry-based on FSC and SSC data can be used for the rapid, non-invasive determination of the onset and extend of yeast lipogenesis. For the first time, this method provides for the rapid in-process monitoring of key parameters controlling oleaginous yeast cultivation progress and harvesting points. In addition, flow cytometry data provides information about possible culture contaminations and cell integrity.

In conclusion, flow cytometry presents on-time dynamic information for the vitality of the yeast. Nevertheless, gravimetric analysis and optical density are still necessary to quantitatively evaluate the overall fermentation but in conjunction with flow cytometry results and these data sets become more accurate and versatile. Moreover, the new flow cytometry-based method presented herein could enhance rapid and non-invasive lipid quantification. However, this process would require more monitoring for the yeast cultures at different conditions. Finally, this flow cytometry method allows fast evaluation in the case of media optimization or screen for best lipid producer from a mutation library. This high throughput methodology for monitoring in-process yeast lipogenesis is generally transferrable to other oleaginous organisms including lipid forming bacteria and other yeast species.

3.2.3. Solid residue as metal sorbent

To enable rapid screening of biosorbent metal binding capacities we developed a new luminescence-based method, which is reported in the context of this manuscript for the first time. The new screening methodology developed herein is based on the concentration dependent broadband cerium luminescence at approx. 365 nm when excited at 269 nm. The cerium luminescence is quenched when its concentration is in high concentrations (auto-quenching mechanism) or when some other metal ions are present in the solution. The latter quenching mechanism depends on the concentration as well as species of the respective metals. However, small changes of heavy metal concentrations do not have a measurable effect on the cerium luminescence. The method developed herein applies this observation. Therefore, diluted samples in a buffer containing high and defined concentrations of heavy

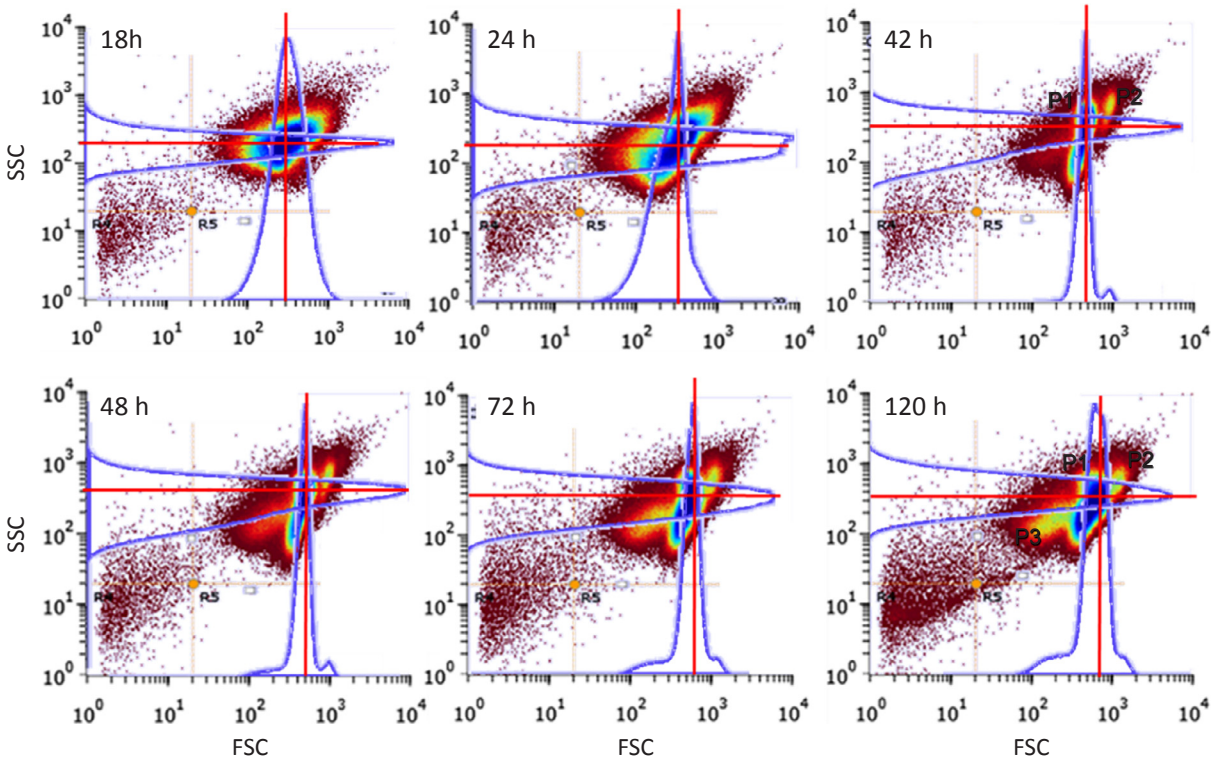

Fig. 6. Flow cytometry-based characterization of *C. oleaginosus* during 120 h of cultivation from samples of the 2 L bioreactor with mixed *L. digitata* hydrolysate. The cell density plot diagrams are showing intensity of the forward scatter [FSC] and side scatter [SSC].

metals are used to gain high fluorescence photo efficiency with controlled quenching. The molarities were chosen so, that a slight change of cerium concentration proportionally affects the luminescence, while a slight change in the alternative heavy metal concentration (if the sample contains metals other than cerium) only has marginal effects on the cerium luminescence. Furthermore, buffering with sodium acetate ensures a constant *p*H value for the measurement and stable complexation of heavy metal ions independent of the sample. The current methodology allows for the first time a rapid determination of the metal sorption capacity of any biological material.

The most important parameter of every sorbent is its loading (or sorption) capacity, indicating how much sorbate can be accumulated per gram of the sorbent. All four tested samples of *L. digitata* showed a very similar cerium sorption capacity at a fairly high level of about 0.60 mmol g^{-1} (84 mg g^{-1}). The raw biomass without hydrolysis performed only insignificantly better reaching 0.67 mmol g^{-1} (Table 2a). This suggests that the concept of residue valorization through biosorption is valid. In addition to a pooled and standardized brow algae residue, charges from different seasonal samples used for the hydrolysis were tested. In that respect, the batch harvested in March showed less cerium sorption capacity (sorption capacity: 0.58 mmol g^{-1}) than the second batch (sorption capacity: 0.67 mmol g^{-1}) harvested in June. As alginate is well characterized metal biosorbent [62] and the alginate was higher in the June than in the March batch (Fig. 2b), the higher cerium sorption capacity of the former may be related to the higher alginate content (Fig. 2b). This was supported by our control experiment were we determined the cerium sorption capacity of pure sodium alginate to be approximately 0.85 mmol g^{-1}. The data indicates that alginate may contribute the majority of sorption capacity in the macroalgae residue, while fucoidan has a minor role in this respect.

An ideally selective sorbent would bind its preferred metal up to its full sorption capacity regardless of the complexity of the solution it is used for. In reality, other ions generally hold influence on the sorption capacity of the metal in question – in this case cerium. A completely unselective sorbent (i.e. with binding spots suitable for all ions equally) would sequester metal ions proportionally to their concentration. Therefore, we measured the sorption capacity for cerium in the presence of potentially competing ions, including copper, lead and nickel (Table 2b). Compared to the control (only cerium in solution) a significant loss of cerium binding capacity was observed, when other metals were present in solution. Mechanistically, the competing ions are likely to occupy similar binding positions on the brown algae biosorbent thereby limiting the specific cerium sorption. With respect to lead the specific cerium binding capacity decreases to about one half. Interestingly, this situation is observed for the solid brown algae residue as well fresh unhydrolysed brown algae biomass.

However, the unhydrolysed biomass displays a higher selectivity, when nickel or copper instead of lead are present. By contrast, the solid hydrolysis residue appears to be less selective towards cerium sorption when competing metals are present in solution. Considering that equimolar amounts of both metals were present in each assay, we can conclude, that fresh biomass shows following selectivity: Pb = Ce > Cu > Ni. The hydrolysis residue is less selective having an

Table 2
(a) Sorption selectivity: comparison of raw *L. digitata* and residual biomass after hydrolysis. Shown is the sorption capacity for Ce when a second metal (Cu, Ni, Pb) is present in the solution (b) Sorption capacity of *L. digitata* raw biomass from both batches and of the hydrolysate.

(a) Sorption selectivity of [Ce^{+3}] as mmol g^{-1} for L. digitata [Mixed] in the present of:				
10 mmol	[Cu^{+2}]	[Ni^{+2}]	[Pb^{+2}]	No Addition
Original Biomass	0.36 ± 0.053	0.51 ± 0.019	0.27 ± 0.008	0.68 ± 0.009
Hydrolysis residues	0.25 ± 0.011	0.38 ± 0.063	0.26 ± 0.019	0.62 ± 0.041

(b) Total sorption capacity of [Ce^{+2}] of Hydrolysis residues:				
Hydrolysis residues	March Batch	June Batch	Mixed Biomass	Original Biomass
[Ce^{+3}] as mmol g^{-1}	0.59 ± 0.025	0.67 ± 0.005	0.62 ± 0.041	0.68 ± 0.009

apparent metal selectivity series of: Pb = Ce = Cu > Ni. Therefore, the hydrolysis residue is not usable for selective biosorption of cerium or other lanthanides from complex solutions. However, the hydrolysis residue represents a cost effective biosorbent for the general removal of metals from dilute solutions. A performance comparison between pure alginate and the brown algae hydrolysis residue (Supplementary Fig. 7S) indicates, that the loss of this component is probably responsible for lower selectivity towards cerium. In that respect, the sorption selectivity of the pure sodium alginate control has been determined as: Ce > Cu > Pb > Ni. In conclusion, the brown algae hydrolysis residue may serve as an effective sorption material for the general removal of heavy metals from aqueous solution and therefore could serve for process water upgrading in a cyclic biorefinery setting discussed here.

The second most important parameter to assess biosorption performance is the determination of sorption kinetics. This parameter provides a quantitation of the biomass contact time in metal containing solutions. From a technical perspective, the sorption kinetics is essential for the determination of required size and cost of respective biosorption reactors. To that end, we have tested both air dried (60 °C) and wet biomass samples originating directly from the hydrolysis step (Fig. 7). Slight differences in sorption kinetics were observed only within first three minutes. After that time the dried biomass completely resembled the wet samples, and no further points were measured.

The wet biomass bound the metals immediately after submersion and has a lag phase for the subsequent 10 min. Thereafter, a more stable binding period was observed, where the maximum loading capacity was reached between 30 and 100 min. By contrast, the dry brown algae hydrolysate residue required more time for the first phase, while it soaked with water, and began biosorption. In general the sorption rate (about 9.2 μmol g^{-1} min^{-1}) is fairly slow, as compared to green microalgae reaching 90% of maximum capacity already after few minutes [63]. Although, the maximum capacity of hydrolysis residue is significantly higher.

More generally, the cerium-based method for characterizing the metal sorption behavior of macroalgae residues is directly applicable to other bio-based metal sorption materials, such as microalgae biomass [64].

3.3. Economic evaluation of the L. digitata biorefinery system

Currently, the biofuels and oleochemical industry predominantly relies on the use of rapeseed and palm oil for biofuels and renewable chemical production. Particularly, production processes of palm oil have significant negative impact on the ecosystem, leading to deforestation and an associated reduction in biodiversity. As an alternative, the generation of microbial oils from terrestrial or marine waste biomass represents a scalable route for production of renewable oils targeted at the biofuels and chemicals industry without impacting the environment [65]. In that regard, the fermentative conversion of waste biomass hydrolysates via oleaginous yeasts, such as *C. oleaginosus* is economically most promising [5]. However, hydrolysis of chemically complex waste biomass often results in solid residues (i.e. lignin or unhydrolyzed sugar polymers) with limited technical applicability, which are either burned to generate process energy or used as simple fertilizer [9,17]. Therefore, these residues do not contribute to the economic viability of the bioprocess. Moreover, chemical yeast oil upgrading to fuels and chemicals may result in the environmental release of heavy metal contaminated process water, which negatively impacts the ecological footprint of the process [30]. To address the issue of economic and ecologic viability this study presents a new, waste-free, cyclic bio-refinery process focusing on the holistic conversion of marine macroalgae biomass for the generation of yeast based lipids and a biological metal sorbent that is capable to extract heavy metals from process water.

We have developed a selective liquefaction of macroalgae biomass using an optimized enzyme system, which provided a liquid phase that contained fermentable sugars. The liquid phase was used for cultivation of the oleaginous yeast *C. oleaginosus,* which achieved high biomass yields compared to artificial control media. The obtained microbial lipids can be converted to a variety of high value platform chemicals for the renewable manufacturing of oleochemicals, bio-lubricant or biofuels by established chemical procedures [20]. After lipid extraction, analysis showed that residues yeast biomass can be used as animal feed (Fig. 1).

Most recently, a techno-economic evaluation of a macroalgae-based biogas and bioethanol production facility has been reported [17]. In this process, the biogas digestate was applied for thermic electricity production or it can be used as animal feed. This study demonstrated that due to the low price for bioethanol and biogas, a scale of 680,000 t $year^{-1}$ of dry macroalgae biomass would be required to render the process economically viable [17]. However, we have applied our previous calculations that demonstrated that a fermentation medium based on marine biomass hydrolysates would cost about \$0.11 L^{-1} [5]. Whereas, the crude yeast oil product could reach costs \$5.5–7.3 kg^{-1} [5,66] based on the applied process. However, the crude yeast oil could be processed to high-value products, such as performance oleochemical ingredients targeting biolubricant, cosmetic and pharmaceutical industries[67]. These applications would justify the relatively high-cost of the fermentative processes. Moreover, this cost will be partially recovered, if the profits generated by utilizing the yeast biomass as animal feed and solid macroalgae residues as biosorbents are taken in consideration.

We have demonstrated that the solid macroalgae residue is able to recover about 0.6 mmol g^{-1} of heavy metals per dry biomass. For example, if lead is used as metal to be removed from wastewater, the binding capacity would be 124 mg g^{-1} dry biomass. This impressive capacity exceeds commercially available resins such as Amberlite® IRC-718 with a capacity of only 20 mg g^{-1} [68] at a cost of approx. \$92 kg^{-1} (http://en.chemmerce.com/chemicals/21312-54423/). Nevertheless, a direct comparison might be misleading, because synthetic resins offer greater long-term stability and no variability of binding capacities as opposed to products of biological origin. All this has been considered when marketing a similar algae based product, the AlgaSORB®, which is sold for approx. \$1.5 kg^{-1}. Although the sorption capacity of this product is not published, it is based on *Chlorella* sp. (eukaryotic green algae) and *Arthrospira* sp. (cyanobacteria) cells. To that end, it is reported, that the biosorption potential of brown algae is more than double of both green microalgae and cyanobacteria respectively. Therefore, brown macroalgae derived biosorbents could be marketed at an even higher price.

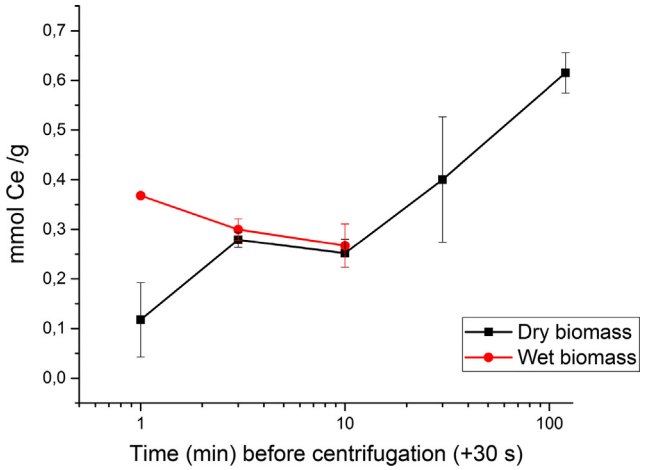

Fig. 7. Sorption kinetics of *L. digitata* residual biomass after hydrolysis for dry and wet biomass.

4. Conclusion

In this study we have selected marine green (*U. lactuca*) and brown (*L. digitata*) macroalgae biomass as process feedstocks, as their production has no negative impact on agricultural activity or the environment but provides higher biomass yields than the most favorable terrestrial crops. For the first time, we established a highly efficient enzymatic hydrolysis process that converts 62.5% (*L. digitata*) and 59.3% (*U. lactuca*) of the total biomass into soluble fermentable sugar monomers. In addition to the total sugar yield, we focused on the overall glucose yield as this sugar is most effectively metabolized by our oleaginous yeast strain. To that end, we actually obtained a glucose recovery of 95% (*w/w*) with respect to the dry macroalgae biomass feedstock. The high sugar recovery was essential for qualifying the macroalgae hydrolysis supernatant as a sole fermentation medium without further additives. In contrast to other biomass feedstocks, our enzymatic liquefaction of macroalgae feedstocks did not require any thermo-chemical pre-treatment, which generally is energy (cost) intensive and results in generation fermentation inhibitors, such as furfural. Processing of the hydrolysate resulted in a solid residue fraction containing both alginate and fucoidan polymers, which were successfully applied as an efficient metal biosorbent. By contrast, the supernatant served as the sole fermentation base for cultivation of the oleaginous yeast *C. oleaginosus*. The laboratory process was validated at technical scale of 30 L in a controlled bioreactor setting. Under these conditions, a biomass yield of 44.8 g L^{-1} yeast biomass containing 37.1% (w_{lipids} /$^{d}w_{biomass}$) was achieved. To enhance the economic viability of the process we suggest to use yeast biomass obtained in the process of lipid generation as a performance animal feed additive due to its favourable cell wall sugar composition. Moreover, the integrated production and use of a macroalgae-based biosorbent significantly enhances the ecological footprint of chemical process steps conventionally involved in renewable lipid upgrading to biofuels and oleochemicals. Furthermore, the application of biosorbents can be diversified to the recovery and recycling of precious metals (Loading capacity of 0.679 mmol g^{-1}; with selectivity to Pb = Ce = Cu > Ni), such as lanthanides, from industrial and mining wastewater sources. To our knowledge this is the first account of a bioprocess assembly that demonstrates the production of microbial oil in conjunction with process water remediation options. To facilitate integration of our technology platform in a conventional industrial production setting, we have devised simple, rapid and selective optical control methodologies for monitoring lipid productivity and metal sorption efficiencies with cost-efficient equipment. Specifically, we developed a new, flow cytometry-based method for determining the onset and extent of lipid biogenesis, which is essential to optimize harvest timeframes. Analogously, a spectrophotometric, cerium-based for metal sorption monitoring methodology was developed as a tool to control waste upcycling and remediation within the process chain.

In summary, we devised a zero waste bioprocess where every product outlet (microbial lipid, biosorbent and animal feed additive) is assigned a significant value in a diversified market scenario. A preliminary economic analysis of the reported process chain, indicated that each product is in the range to be cost competitive with current market equivalents. This synaptic nature of these biotechnological processes provides for the economic and ecologic viability of the overall process chain.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apenergy.2018.04.089.

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