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Exploring the potential of microalgae in the recycling of dairy wastes

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ABSTRACT

Culturing microalgae using dairy-wastes offers the opportunity of producing valuable biomass for different industrial applications. The capability of four *Chlorella* species and a recombinant *Chlamydomonas reinhardtii* strain to mixotrophically grow in wastewaters from an Italian dairy factory was investigated. A robust algal growth could be efficiently sustained in these wastes, despite the abundance of D-Lactose (~4% w/v), that could not be metabolized by any microalgal species. Non axenic cocultivation of microalgae together with microbial communities from the dairy wastes resulted in a marked decrease of their pollution load, thus reducing the necessity of expensive treatments before their discharge in the municipal sewage system. Microalgae cultivated using dairy-wastes were characterized by a lipid content ranging from 12% to 21% (w/w), with *Auxenochlorella protothecoides* reaching the highest lipid productivity (~0.16 g/L/d) whereas the transplastomic *C. reinhardtii* strain expressing a thermostable β-glucosidase reached a recombinant enzyme productivity of 0.18 mg/L/d.

1. Introduction

Dairy industry is one of the major industries in the food sector. A steady increase in the demand of milk and milk-based products has led to an enormous growth of dairy industries with a consequent increase of dairy wastewater production. In 2018, the Food and Agriculture Organization of the United Nations (FAO) estimated a global milk output around 843 million tonnes, with an increase of 2.2% from 2017, whereas world trade in dairy products expanded to 75 million tonnes (in milk equivalents), with an increase of 2.9% from 2017 (Food and Agriculture Organization of the United Nations, 2019). Dairy industry generates large volumes of wastewaters, approximately 0.2–10 L waste per liter of processed milk (Ummalyma and Sukumaran, 2014). Dairy wastewaters, if not properly managed, represent a serious risk towards human beings, environment and aquatic life (Karadag et al., 2015). Despite their different compositions, mainly dependent on the technological operations employed for manufacturing dairy products, dairy effluents usually contain D-Lactose, soluble proteins, lipids and salts. Due to the high organic content, dairy wastewaters are characterized by high biochemical (BOD) and chemical oxygen demand (COD) values, varying from 0.1 to 100 g/L (Kolev Slavov, 2017). If disposed in the environment without appropriate treatments, dairy wastewater increase the risk of eutrophication, due to their high content in Phosphorous- and Nitrogen-based compounds (Kolev Slavov, 2017). Treatment of dairy wastewater includes the use of expensive physico-chemical methods requiring considerable amount of chemicals, energy and operating costs. Despite not still optimized enough, the biological treatment of wastewater is considered a more eco-friendly approach than physico-chemical methods (Kolev Slavov, 2017). In this regard, the use of microalgal cultivation in dairy wastewater has been proposed in recent years. Several algal species including *Chlorella pyrenoidosa*, *Anabaena ambigua*, *Scenedesmus abundans*, *Chlorella vulgaris*, *Chlamydomonas polypyrenoideum* and *Acutodesmus dimorphus* have been successfully cultivated in dairy wastewater (Brar et al., 2019; Choi, 2016; Chokshi et al., 2016; Kothari et al., 2013; Qin et al., 2014). The use of microalgae for the recycling of dairy wastewater represents an integrated system that

Abbreviations: Ap, *A. protothecoides*; BOD, biochemical oxygen demand; COD, chemical oxygen demand; Cr, *Chlamydomonas reinhardtii*; Csa, *C. saccharophila*; Cso, *C. sorokiniana*; Cv, *C. vulgaris*; D1, dairy waste 1; D2, dairy waste 2; PBRs, photobioreactors; Phi, phosphite; Pi, phosphate; *p*NPGal, *p*-nitrophenyl-β-galactopyranoside; *p*NPGlc, *p*-nitrophenyl-β-glucopyranoside; SM, salt mixture; WT, wild type; βG, β-glucosidase.

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reduces the consumption of freshwater by combining the treatment of wastewater with the production of valuable algal biomass. Microalgal biomass has attracted great interest for its potential use in several industrial applications, such as the production of high added-value products and metabolites with use in cosmetics, pharmaceuticals, health supplements (Barkia et al., 2019), animal feed (Yaakob et al., 2014) and biofuels (Brennan and Owende, 2010). Production of biodiesel from microalgae is considered a promising alternative to oil crops due to the high lipid productivity of certain microalgal species, whereas the use of closed growth systems, i.e., photobioreactors, offers the possibility of using waste land rather than arable land for microalgal cultivation, thus avoiding the competition with the agri-food sector (Chisti, 2007; Malcata, 2011; Menetrez, 2012; Rodolfi et al., 2009; Stephens et al., 2010). In this regard, mixotrophic cultivation of microalgae provides higher biomass and lipid productivity than cultivation under photoautotrophic conditions; however, the cost of the organic substrate accounts for 80% of the total cost of cultivation medium (Bhatnagar et al., 2011). Moreover, the use of organic sources in the culture medium increases the risk of contamination by microbes, thus requiring expensive sterilization procedures that negatively impact the economic sustainability of the entire process. Recently, dairy effluents have been proposed as cheaper organic sources for biodiesel production from microalgae (Abreu et al., 2012; Brar et al., 2019; Choi, 2016; Chokshi et al., 2016; Hena et al., 2015; Kothari et al., 2013). Another promising application of microalgae is the production of recombinant proteins of high industrial relevance. Microalgae have been proposed as interesting expression host since they are characterized by a faster growth cycle compared to land-plants and their cultivation is less expensive than that of bacteria and yeasts

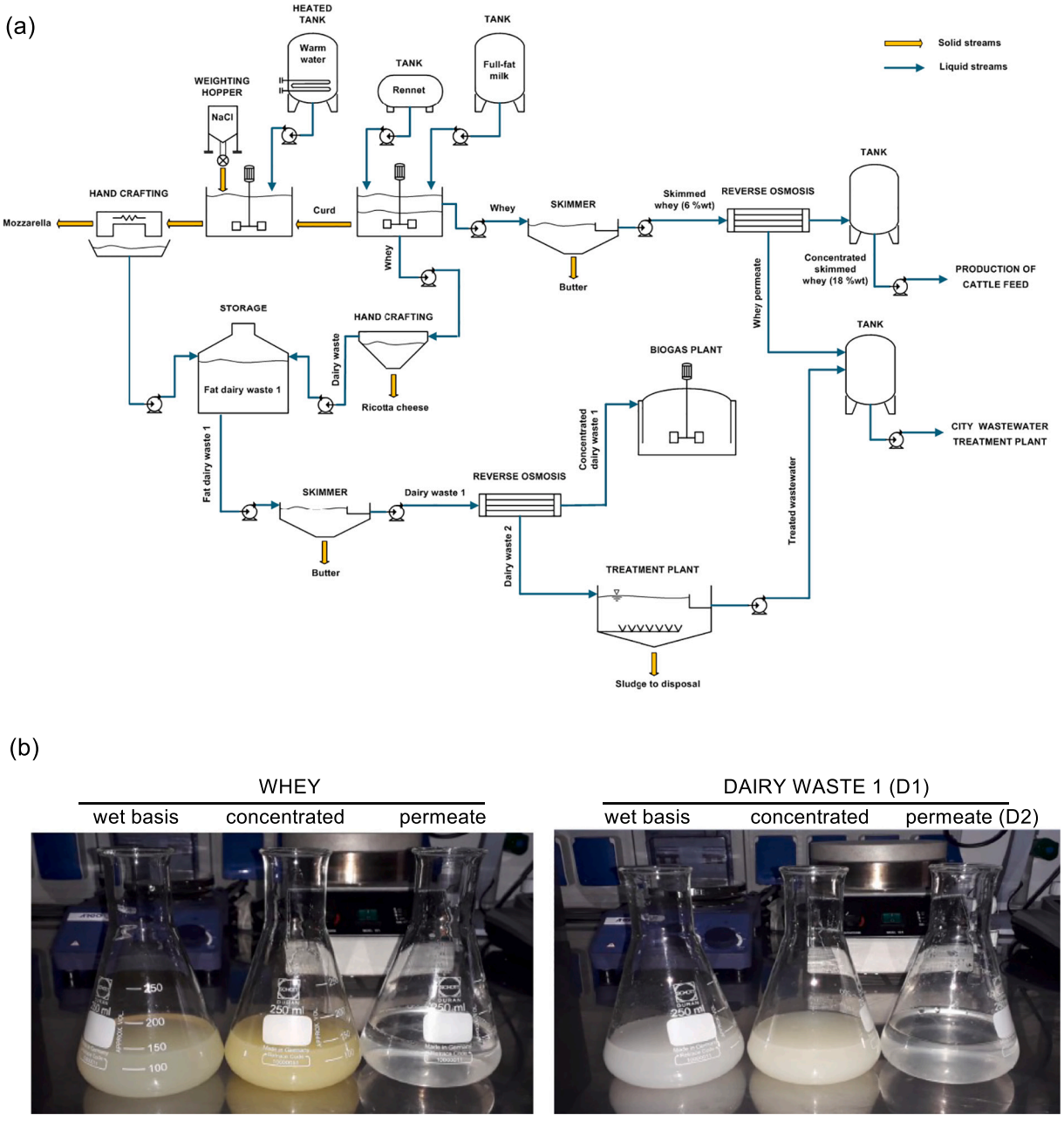

Fig. 1. Production scheme of the dairy factory. (a) The manufacturing pipeline of the three main dairy products (i.e., mozzarella PDO, ricotta cheese, butter) from Azienda Casearia Capurso is shown. The two major dairy waste effluxes (i.e., fat dairy waste 1 and skimmed whey) are indicated: skimmed dairy waste 1 (D1) is treated by reverse osmosis to obtain concentrated D1 and "permeate/dairy waste 2" (D2). The treatment of whey is also indicated. (b) Dairy waste fractions from skimmed whey (left) and D1 (right).

(Giovannoni et al., 2020). Moreover, microalgae are GRAS (Generally Recognized As Safe) organisms and are therefore suitable host for expressing proteins to be used in nutraceutical or biomedical fields (Benedetti et al., 2018). *Chlamydomonas reinhardtii* is the model microalga with the largest set of proven genetic tools, ranging from the heterologous expression of recombinant proteins to genome editing techniques (Baek et al., 2016; Scranton et al., 2015; Taunt et al., 2018). However, the use of dairy wastewater for the growth of recombinant microalgae has not been so far proposed.

1.1. The production scheme of the Italian dairy factory

In this study, the potential of the dairy effluents from an Italian dairy factory to produce biodiesel and recombinant enzymes from different microalgae species was investigated. The Italian dairy factory object of this study is Azienda Casearia Capurso (P. IVA 05982300724; Puglia, Italy). The dairy factory is involved in the manufacturing of various types of milk products, mainly mozzarella PDO (Protected Designation of Origin), ricotta cheese, and butter (Fig. 1a). The production pipeline generates two major effluents, namely "fat dairy waste" and skimmed whey. The production of mozzarella and ricotta cheese leads to the accumulation of fat dairy waste that, in turn, is skimmed to produce butter and skimmed dairy waste (D1) (Fig. 1a). Skimmed whey is the residual material from the whole whey, and it is the other main effluent of the dairy production pipeline (Fig. 1a). 39 million Liters of D1 and skimmed whey are produced annually, with an overall cost of 0.78 to 3.9 million € spent by the dairy factory for their disposal, plus an additional cost of 520,000 € for their transport. The dairy factory is equipped with a treatment plant for each effluent. Upon reverse osmosis, D1 is separated into (i) concentrated D1 (retentate), highly rich in organic carbon sources, and (ii) "permeate", here referred to as dairy waste 2 (D2) (Fig. 1b). For the concentrated D1, if not discharged by paying the municipal wastewater plant, an attempt of valorization was made by providing it as substrate for biogas production, however, the process was not satisfactory. Notably, an excessive administration of concentrated D1 over time in the biogas plant causes a reduction in the biogas yield due to the alteration of the anaerobic conditions of microbial communities inside the digester. D2, due to its biochemical composition, requires ad hoc treatment before its discharge into the city wastewater treatment plant. Skimmed whey with 6% wt of total solids (TS) content is concentrated by reverse osmosis as well, by generating the concentrated skimmed whey (18% wt TS) and "whey permeate" (Fig. 1b). Skimmed whey concentrate is valuable as cattle feed, thus representing a profit for the dairy company, while the whey permeate is disposed of directly in the municipal sewage system.

Therefore, our study focused on the management of dairy wastes derived from the production of mozzarella PDO and ricotta cheese, namely D1 and D2, by coupling the recycling of dairy wastes to the algal cultivation. An optimized combination of D1 + D2 enhanced a robust growth of five different microalgae species, i.e., *C. vulgaris*, *Chlorella sorokiniana*, *Chlorella saccharophila*, *Auxenochlorella protothecoides* and *C. reinhardtii*. At first, the algal biomass was analyzed in terms of lipid accumulation to determine its potential for biodiesel production. Subsequently, the enzyme yield of the recombinant β-glucosidase from *Pyrococcus furiosus* expressed in the chloroplast of *C. reinhardtii* (Benedetti et al., 2020) was evaluated after growing the transplastomic strain in the dairy waste-based media. It is worth noting that β-glucosidase from *P. furiosus* is also characterized by β-galactosidase activity (Kengen et al., 1993), i.e., the capability of degrading D-Lactose, making this enzyme highly valuable for the dairy factory itself since it can be used to produce lactose-free products. Finally, the levels of contaminants and chemicals were analyzed in the exhausted growth medium by providing further insights in the algal-based remediation of dairy wastes.

2. Materials and methods

2.1. Growth media

The dairy wastewater samples were collected from "Capurso Azienda Casearia srl", Gioia del Colle (BA, Italy).

Preliminary tests for the pre-treatment of dairy wastewater were performed in 6-well plates. The D1 waste was treated at 85 °C for 6 min, and then added in different concentrations (0.5, 1.5, 4.5 and 13% *v*/v) to 5 mL of salt-mixture (SM) medium (Table 1). The different mixtures were incubated for 144 h at 50 μmol photon m^{-2} s^{-1} and 25 °C in a rotary shaker (180 rpm) with a 16/8 h light/dark photoperiod. Growth experiments were performed using different growth media (Table 1), in particular:

- SM medium: a modified version of TAP medium (Kropat et al., 2011) containing 0.48 g/ L Tris and devoid of acetate; it was used for *C. reinhardtii* growth.
- SM^{ChlOpt} medium: SM medium in which Na_2SeO_3 is replaced by H_3BO_3; it was used for *Chlorella* growth.
- D1SM: 13% D1 (v/v) in SM medium.
- DWSM: 13% D1 (v/v) diluted in D2 (87%) supplemented with SM or SM^{ChlOpt} salts.
- DW: 13% D1 (v/v) diluted in D2 without salts supplementation.
- Phi-supplemented D1SM and DWSM: D1SM and DWSM media in which 1.2 mM PO_4^{3-} (Phosphate) was replaced by 4 mM KH_2PO_3 (Monopotassium Phosphite).

Monopotassium Phosphite was purchased from Wanjie Int., China (CAS No. 13977–65-6). All the media were prepared fresh, pH-adjusted (6.8) and heat-treated. Growth experiments in lab-scale photobioreactors were carried out using differently pretreated DWSM media. Pretreatment of DWSM medium was performed by autoclave sterilization (120 °C for 20 min), UV-radiation (overnight exposure) and heat (85 °C for 20 min), hereafter referred as mild heat treatment.

2.2. Microalgal strains and growth conditions

C. reinhardtii wild type 1a + (mt+) was obtained from the Chlamydomonas Resource Center (University of Minnesota). The recombinant βG-PTXD strain was obtained by transforming *C. reinhardtii* 1a + according to (Benedetti et al., 2020). *C. vulgaris* wild-type strain 211-11p was obtained from the Culture Collection of Algae (Göttingen University, Germany). *C. sorokiniana, C. saccharophila* and *A. protothecoides* were obtained from the UTEX Culture Collection (University of Texas, Austin, TX [http://web.biosci.utexas.edu/utex/]) as strains UTEX1230, UTEX2911 and UTEX25, respectively. Microalgal strains were maintained at 25 °C, with a 16/8 h light/dark photoperiod, light intensity of 50 μmol m^{-2} s^{-1}, in solid or liquid TAP medium (Table 1). Growth experiments were performed in 6-well culture plates at 50 μmol photon m^{-2} s^{-1}, 25 °C and 150 rpm, or in photobioreactor system under continuous light (PSI MC-1000, Photon Systems Instruments) by using a light intensity of 50 to 200 μmol photon m^{-2} s^{-1} and air bubbling supplementation. For growth experiments, microalgal cells were inoculated at a concentration of 2.5 × 10^5 cell/mL unless otherwise indicated. Growth was followed by measuring cell density using an automated cell counter (Countess II FL Cell Counter, ThermoFisher) or by recording OD at 720 nm.

2.3. Biomass production

The algal biomass was harvested by centrifugation at 1500*g* for 5 min and dried by freeze-drying lyophilization. All the biomass weights reported in the text are referred to the dry weight.

Table 1
Media composition and cost of TAP, TAP $^{Chl\ Opt}$ and SM. Reagent costs here reported were obtained from https://m.made-in-china.com/

g/L	TAP	TAP $^{Chl\ Opt}$	SM	SM $^{Chl\ Opt}$	SM cost €/L	SM $^{Chl\ Opt}$ cost €/L
Tris base	2.42	2.42	0.48	0.48	4.32E-04	4.32E-04
NH_4Cl	0.40	0.40	0.40	0.40	6.50E-05	6.50E-05
$MgSO_4$	0.05	0.05	0.05	0.05	3.90E-06	3.90E-06
$CaCl_2$	0.04	0.04	0.04	0.04	3.60E-06	3.60E-06
K_2HPO_4	0.09	0.09	0.09	0.09	1.14E-04	1.14E-04
KH_2PO_4	0.06	0.06	0.06	0.06	5.05E-05	5.05E-05
Na_2EDTA	0.02	0.02	0.02	0.02	3.48E-05	3.48E-05
$(NH_4)_6Mo_7O_{24}(4H2O)$	3.52E-05	3.52E-05	3.52E-05	3.52E-05	3.80E-07	3.80E-07
Na_2SeO_3	1.73E-05	–	1.73E-05	–	9.34E-07	–
$ZnSO_4(7H2O)$	7.19E-04	7.19E-04	7.19E-04	7.19E-04	4.53E-07	4.53E-07
$MnCl_2(4H2O)$	1.19E-03	1.19E-03	1.19E-03	1.19E-03	2.14E-06	2.14E-06
$FeCl_3(6H2O)$	5.41E-03	5.41E-03	5.41E-03	5.41E-03	1.70E-05	1.70E-05
Na_2CO_3	2.33E-03	2.33E-03	2.33E-03	2.33E-03	4.62E-07	4.62E-07
$CuCl_2(2H2O)$	3.41E-04	3.41E-04	3.41E-04	3.41E-04	9.21E-07	9.21E-07
H_3BO_3	–	4.95E-03	–	4.95E-03	–	1.56E-05
Acetate	1 mL	1 mL	–	–	–	–
Total cost					7.26E-04	7.41E-04

2.4. Lipid quantification

Total lipids were quantified using the sulfo-phospho-vanillin (SPV) assay according to Mishra et al., 2014.

2.5. Enzymes extraction, immunoblot analysis and activity assays

Protein extraction was performed by resuspending the dry algal biomass with extraction buffer [1 mL extraction buffer: 6 mg microalgal powder]. Extraction buffer was composed of 20 mM Na-Acetate buffer pH 5.5 supplemented with 0.3% (v/v) Tween 20. The resuspension was incubated for 50 min at 70 °C and, at the end of the procedure, the sample was centrifuged (14,000*g* × 10 min) and the supernatant used for downstream applications. Proteins extracted from 60 μg dry weight of biomass were separated in 10% of SDS-PAGE gel then transferred to nitrocellulose membrane and immuno-detected with a monoclonal AbHA (HA7 clone, Sigma-Aldrich). Enzymatic activity was assayed by incubating the algal extracts (10% v/v, 100 μL total volume) in 50 mM Na-Acetate buffer pH 5.5 at 75 °C by using the substrates 5 mM *p*-nitrophenyl-β-glucopyranoside (*p*NPGlc) and *p*-nitrophenyl-β-galactopyranoside (*p*NPGal) to determine β-glucosidase and β-galactosidase activity, respectively. The substrates were purchased from Sigma-Aldrich. Enzyme activity was expressed as Enzyme Units (μmol of *p*-nitrophenol released per min) per g (dry weight) of microalga.

2.6. Evaluation of daily productivity of algal biomass

Maximum biomass productivity (g/L/d) during the culture period was calculated in accordance with Abreu et al., 2012 by using the following equation:

$$P_{max} = (X_t - X_0)/(T_x - T_0)$$

where X_t was the biomass concentration (g/L) at the end of the exponential growth phase (T_x) and X_0 the initial biomass concentration (g/L) at t_0 (day). The different growth phases were determined by counting the microalgal cells at different time-points or, alternatively, by following the variations in absorbance (OD_{720}) during the microalgal growth.

2.7. Glucose and lactose quantification

D-Glucose and D-Lactose contents in dairy wastewater samples were quantified by the commercial GOPOD assay kit (Megazyme) and Lactose Assay Kit-Sequential/High Sensitivity (Megazyme), respectively.

2.8. Chemical analysis

At the end of the growth experiment, cultures were centrifugated at 1500*g* for 5 min, and the supernatants were collected and analyzed. Total Nitrogen and total Phosphorous were determined according to the APAT CNR IRSA 4060 Man 29 2003 method; COD was analyzed by the SO 15705:2002 method; Chloride was analyzed by APAT CNR IRSA 4020 Man 29 2003 method; protein Nitrogen was calculated by multiplying the total Nitrogen using the conversion factor 6.38. D-Lactose was analyzed by ionic chromatography (114 ed. 0 rev 2 2015 method). All parameters were analyzed in triplicate.

Removal efficiency (%) of the strains used was calculated by the equation:

$$RE\ (\%) = [(\text{Initial value} - \text{Final value})/\text{Initial value}] \times 100.$$

2.9. Design of the dairy factory

Microsoft VisioTM software has been used to draw the flowsheets of the processes shown in Figs. 1 and 6.

3. Results and discussion

3.1. Growth of wild type and transgenic C. reinhardtii in dairy wastes

The mixotrophic growth of *C. reinhardtii* was analyzed by supplementing D1 to SM medium, i.e., TAP medium devoid of acetate and with a reduced TRIS-base content (*see* Section 2.1). A pretreatment procedure was employed in order to reduce the starting microbial load in dairy wastes since D1 was characterized by a high microbial growth that must be reduced in order to avoid deleterious competition with algal growth (Supplementary Material). The use of a mild heat treatment was effective in reducing the microbial load in dairy wastes at a safe level for algal growth, making D1 a suitable medium for algal growth at a concentration as high as 13% (v/v) of the total culture volume (Supplementary Material). Sterilization procedures may alter the nature of dairy wastes on one side, by concomitantly affecting the economic feasibility of the process on the other. Mild heat treatment was effective in limiting, but not totally abolishing, the contamination of dairy waste, and could be adopted to replace expensive sterilization procedures for best sustainability at large-scale. It is worth noting that the Italian dairy plant is already equipped with several heated tanks that are routinely used to pasteurize fresh milk before starting the cheese manufacturing process.

Over the past years, easy and efficient transformation systems have been developed for *C. reinhardtii*; this microalga is gaining attention as a potential bioreactor for the production of recombinant proteins (Baier

et al., 2018; Benedetti et al., 2020; Perozeni et al., 2020; Scranton et al., 2015). Under this perspective, *C. reinhardtii* represents a potential system to combine the recycling of dairy wastes with the production of recombinant enzymes. The mixotrophic growth of the wild-type and a double transgenic *C. reinhardtii* strain, here named as βG-PTXD, was carried out in dairy wastes-based media (Supplementary Material) (Benedetti et al., 2020). βG-PTXD is a double mutant accumulating the thermostable β-glucosidase celB from the hyperthermophilic archea *Pyrococcus furiosus* (Kengen et al., 1993) in the chloroplast and the Phosphite Dehydrogenase D from *Pseudomonas stutzeri* WM88 in the cytosol (Costas et al., 2001). The *PtxD* gene encodes a NAD-dependent phosphite oxidoreductase able to oxidase phosphite (Phi) into phosphate (Pi) by using NAD as a cofactor (Loera-Quezada et al., 2016). The expression of PTXD confers to the βG-PTXD strain the ability of metabolizing Phi as sole Phosphorous source (Loera-Quezada et al., 2016). Importantly, Phi cannot be metabolized by plants, fungi and most bacteria, representing an eco-friendly strategy to prevent microbial contamination without using expensive sterilization procedures, antibiotics or herbicides. For this purpose, D1 was diluted in SM medium (Table 1) to obtain the D1SM medium in which Pi (1.2 mM) was replaced with different amount of Phi (i.e., 1-2-4 mM). The SM composition was the same as TAP medium (Kropat et al., 2011), except for the lack of acetate and a 80% reduced amount of TRIS-base (Table 1). In general, TRIS-base is used to counterbalance high acetate concentration in TAP-based formulation and was not necessary since acetate was not added. The effect of different concentrations of Phi on microbial contamination was investigated during the βG-PTXD growth (Supplementary Material). Although the use of Phi could not provide an alternative and unique Phosphorous source since D1 already contains "endogenous" Pi, it acted nonetheless as antimicrobial agent (Guest and Bompeix, 1990). Indeed, a significant restriction of microbial growth was observed in large Phi excess (4 mM) whereas Loera-Quezada and coworkers obtained microbial containment in Pi-depleted/Phi-repleted medium at much lower Phi concentration (i.e., 0.3 to 1 mM) (Loera-Quezada et al., 2016). At Phi concentration lower than 4 mM, we observed a weaker algal growth accompanied by bleaching effects, thus indicating an unhealthy condition for the algal cultures (Supplementary Material).

As previously mentioned, D2 needs expensive treatments before its discharge in the municipal sewage treatment plant. Since D2 is characterized by a low content of organic sources and a high salt concentration (Table 2), the possibility of using D2 to dilute D1 at a ratio [13% (v/v) D1: 87% (v/v) D2] was evaluated, with the purpose to avoid the use of fresh water for algal cultivation. The D1 + D2 medium was either supplemented with SM to obtain the DWSM medium or used without SM supplementation in the attempt to render the whole procedure less laborious and expensive (*see* Section 3.2).

Mixotrophic growth of βG-PTXD strain was evaluated in both D1SM and DWSM media by replacing Pi in SM with 4 mM Phi (Fig. 2a). As control, wild type *C. reinhardtii* strain 1a + (WT) was grown in Pi-supplemented D1SM and DWSM media. Phi-supplemented D1SM medium promoted a higher algal biomass production than the corresponding Pi-supplemented version (Fig. 2a), with an algal biomass yield of 2.07 g/L and 1.14 g/L for βG-PTXD and WT strain, respectively (Fig. 2b). After 10-days of growth, supernatants from Phi-supplemented D1SM medium were characterized by a reduced turbidity compared to those from Pi-supplemented D1SM medium, suggesting a more effective containment of microbial growth (Supplementary Material). Interestingly, the final biomass production of WT and βG-PTXD in Pi-supplemented DWSM (1.86 and 1.79 g/L) was comparable to that of βG-PTXD strain in Phi-supplemented D1SM medium (2.07 g/L) (Fig. 2a-b), indicating that dilution in D2 resembled the same effect of Phi supplementation towards microbial containment. Probably, the high content of Chloride in D2 (Table 2) played a role in limiting the microbial contamination in D2-based media. Therefore, the use of D2 for the cultivation of microalgae is beneficial not only for minimizing the use of freshwater, but also for reducing the microbial contamination without the addition of other antimicrobial agents. The use of dairy waste media has already been demonstrated effective in sustaining the growth of *C. vulgaris* (Abreu et al., 2012; Choi, 2016), and in this work a robust growth of *C. reinhardtii* was also obtained in such media. However, the use of dairy waste strongly increased the opalescence of the culture medium, making challenging to monitor *in continuum* the cell density related to the algal growth by measuring OD_{720}. Moreover, as opalescence hinders a homogenous penetration of light inside the photobioreactor, the light intensity was increased compared to that usually employed for mixotrophic growth, i.e., from 50-100 (Abreu et al., 2012; de Melo et al., 2018) to 200 μmol photons m^{-2} s^{-1}.

Table 2
Composition of D1 and D2. The legal limits for the disposal in a sewage system are in accordance with the Italian D. Lgs 152/06. n/a: not applicable.

Parameter	D1	D2	Legal limit for disposal in municipal sewage system
COD (mg O_2/L)	19,607	622	≤ 500
BOD5 (mg O_2/L)	10,041	582	≤ 250
D-Lactose (%, w/w)	0.42	<0.01	n/a
Chloride (mg/L)	5817	1088	≤ 1200
Phosphorous (mg/L)	108	2	≤ 10
Total nitrogen (mg/L)	593	37	≤ 30
Protein nitrogen (mg/L)	3785	235	≤ 30

3.2. Growth of four different Chlorella species and wild type C. reinhardtii in dairy wastes

Because of the rapid growth, ease of cultivation, high lipid content and lipid productivity, microalgae of the genus *Chlorella* have been proposed as promising feedstock for biodiesel production (Liu and Chen, 2014). *C. vulgaris*, *C. sorokiniana*, *C. saccharophila* and *A. protothecoides* vigorously grew in DWSM medium (Fig. 2c) after 10 days of growth. Amongst the four species analyzed, *A. protothecoides* showed a higher biomass production (3.3 g/L, Fig. 2b) than *C. vulgaris* (1.54 g/L), *C. sorokiniana* (2.1 g/L) and *C. saccharophila* (1.5 g/L) (Fig. 2d). Thus, *A. protothecoides* was the algal species showing the highest biomass production in dairy waste-based media.

In order to make the entire process less laborious in perspective of a large-scale application, the growth of *C. reinhardtii* and *A. protothecoides* in DW, i.e., DWSM without SM supplementation, was investigated. The microalgae were grown in different medium combinations, namely DW (13% D1 + 87% D2), SM medium (i.e., only basal salt mixtures) and DWSM (13% D1 + 87% D2 + SM) as positive control. As shown in Fig. 3a-b, DWSM was the only medium capable of supporting a robust growth for both microalgal species, thus indicating a synergistic effect between SM salts and the nutrients from dairy wastes. After 10 days of growth, the biomass yield from the DWSM medium was 2.9 g/L and 1.7 g/L for *A. protothecoides* and *C. reinhardtii*, respectively (Fig. 3c), whereas the absence of SM led to a drastic reduction in biomass production in both species, i.e., 0.5 g/L and 0.6 g/L for *A. protothecoides* and *C. reinhardtii*, respectively. The biomass production was further reduced when both microalgae were grown in the SM medium; i.e., 0.07 g/L and 0.1 g/L for *A. protothecoides* and *C. reinhardtii*, respectively. The decreased growth was likely due to an increase of microbial contaminants at the expense of microalgae, since SM medium did not contain any carbon source except for the newly formed microalgal biomass. In fact, the microalgal cultivation in SM medium was carried on in a non-axenic photo-autotrophic condition, and even if the mild heat treatment was effective in limiting the microbial contamination, the media were not sterile. In this regard, monitoring of all cultivations was extended up to 10 days in order to exclude the possibility of algal bleaching due to the stress conditions from prolonged non-axenic culturing (Loera-Quezada et al., 2016) (Fig. 3a-b).

In order to evaluate if culturing microalgae in dairy-waste media

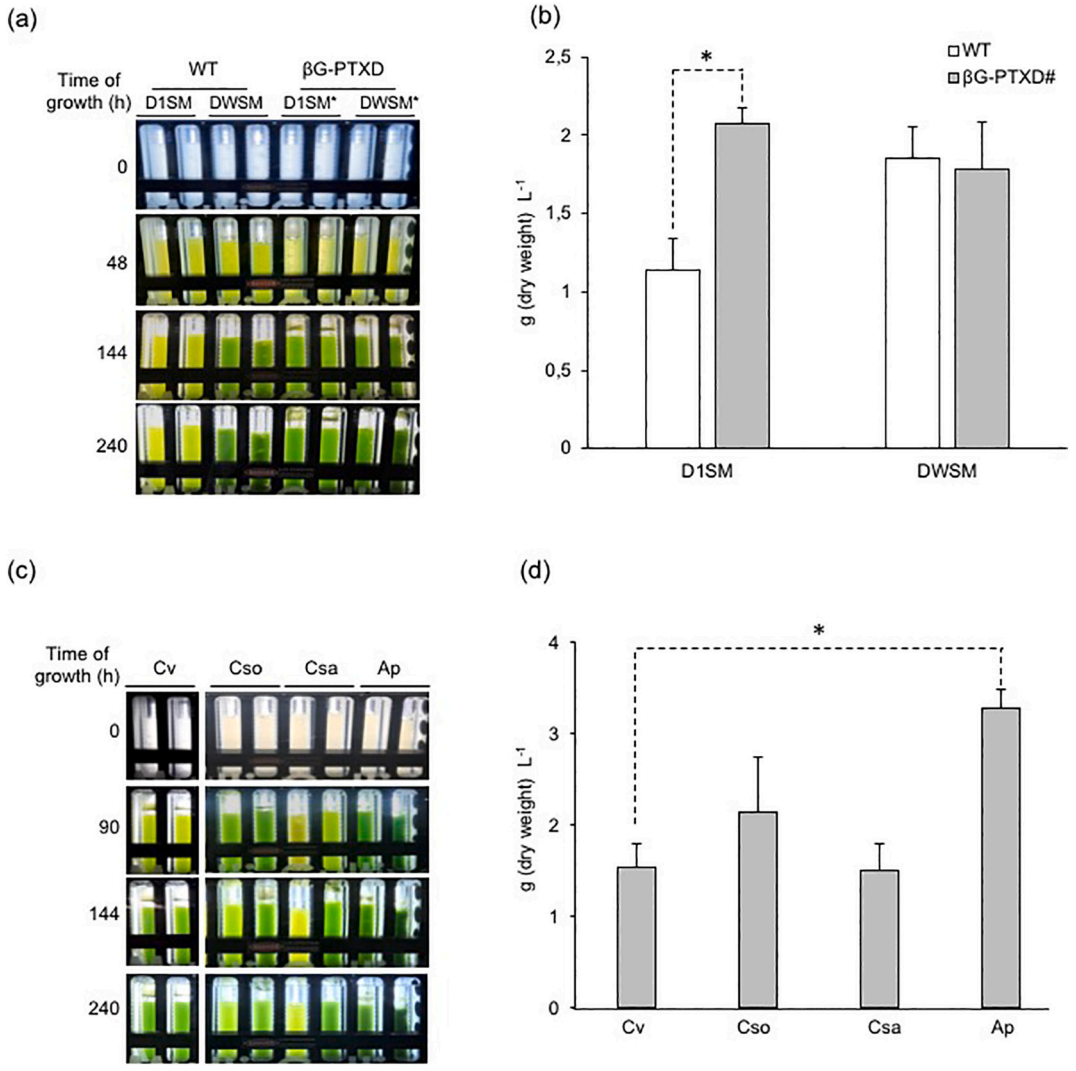

Fig. 2. Mixotrophic growth of *C. reinhardtii* and four different *Chlorella* species using dairy waste-based media. (a) Growth of WT *C. reinhardtii* and double transgenic βG-PTXD strains in D1SM (13% D1 + SM salts) and DWSM (13% D1 + 87% D2 + SM salts). #, βG-PTXD was grown in Phi-supplemented growth media (*see* Section 2.1 for details). Growth was performed at 200 μmol photons m^{-2} s^{-1} and air supplementation using 2.5 × 10^5 cell mL^{-1} as starting inoculum. (b) Biomass production from the same cultures described in (a). Values are means of 2 replicates ± SD. (c) *C. vulgaris*, *C. sorokiniana*, *C. saccharophila* and *A. protothecoides* were grown in DWSM at 200 μmol photons m^{-2} s^{-1} and air supplementation using 2.5 × 10^5 cell mL^{-1} as starting inoculum. (d) Biomass production from the same cultures described in (c). Ap, *A. protothecoides*; Csa, *C. saccharophila*; Cso, *C. sorokiniana*; Cr, *C. reinhardtii*; Cv, *C. vulgaris*. Values are means of 2 replicates ± SD. Asterisk indicates statistically significant difference according to Student's *t*-test (* $P < 0.05$).

under axenic condition could further improve the algal productivity, *A. protothecoides* was grown in sterilized DWSM (Supplementary Material). Sterilization of DWSM was carried out by using two different methods, i.e., UV-radiation and autoclave-sterilization. The cultivation of *A. protothecoides* in UV- and autoclave-treated DWSM showed more stunted and delayed growth, respectively, compared to that in the heat treated medium (Supplementary Material). The different growth may be ascribed to inefficient sterilization (UV-radiation) or to the alteration of certain compounds in the medium (autoclave-sterilization). Probably, UV-radiation could not efficiently sterilize dairy wastes due to the marked opalescence of such media that may hinder an optimal diffusion of UV rays in the medium. Moreover, it is worth noting that the use of autoclave to sterilize DWSM is not economically sustainable in a large-scale production process due to the high operating costs of the process. Therefore, the mild heat treatment represented the best compromise as it allowed a robust algal growth in a shorter cultivation time inside the photobioreactor.

3.3. Lipid accumulation in dairy waste grown microalgae

The determination of lipid yield in the algal biomass is a crucial parameter to determine their potential application for biodiesel production. *C. vulgaris*, *C. sorokiniana*, *C. saccharophila* and *A. protothecoides* showed a lipid content of 15.6%, 20.7%, 14.2% and 18.5% dry weight, respectively (Fig. 3d), with *A. protothecoides* displaying the highest lipid production (592.6 mg/L) when compared to that of the other algal species (238.5, 443.4 and 212.8 mg/L for *C. vulgaris*, *C. sorokiniana* and *C. saccharophila*, respectively). The maximum lipid content of *A. protothecoides* spanned from 5.1% to 10.6% to 18.5% of dry biomass in SM, DW and DWSM respectively, with a corresponding maximum lipid production of 3.6, 53.2 and 592.6 mg/L (Fig. 3d).

The lipid content and lipid production were 12% of dry biomass and 204.3 mg/L for DWSM-grown *C. reinhardtii*. In accordance with that observed for *A. protothecoides*, the lipid content of *C. reinhardtii* decreased to 9.5% and 4.4% in SM and DW medium, respectively, whereas the lipid production was 57.1 and 5 mg/L.

Considering the maximum biomass productivity (0.85 g/L/d) and

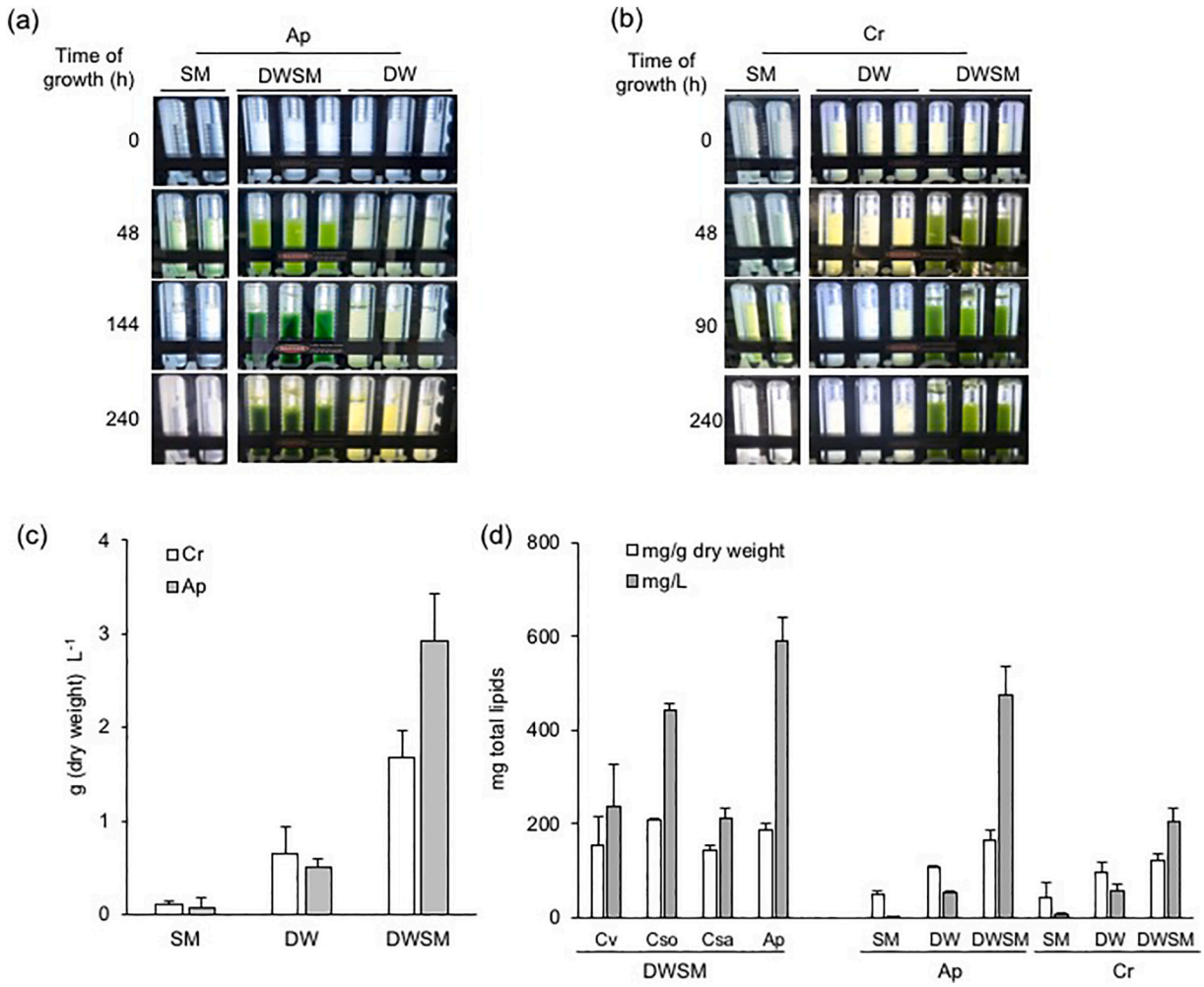

Fig. 3. Mixotrophic growth of *A. protothecoides* and *C. reinhardtii* in dairy waste-based media for the production of lipids. Growth of (a) *A. protothecoides* and (b) *C. reinhardtii* in only SM salts, DW (13% D1 + 87% D2) and DWSM (13% D1 + 87% D2 + SM salts) at 200 μmol photons m^{-2} s^{-1} and air supplementation using 2.5 × 10^5 cell mL^{-1} as starting inoculum. (c) Biomass production from the same cultures described in (a) and (b). (d) Total lipids from the microalgae cultures shown in (a), (b) and (Fig. 2c), as determined by SPV colorimetric assay. Ap, *A. protothecoides*; Csa, *C. saccharophila*; Cso, *C. sorokiniana*; Cr, *C. reinhardtii*; Cv, *C. vulgaris*. Values are means of 3 replicates ± SD.

lipid content (18.5%) obtained for *A. protothecoides*, a maximum lipid productivity of 0.16 g/L/d was achieved. Interestingly, the lipid content in the algal cells was positively related to the biomass production in each growth experiments (Figs. 2d, 3c-d), and lipid accumulation in *Chlorella* species was observed without the necessity of inducing specific stress conditions such as Nitrogen starvation (Dall'Osto et al., 2019), thus making the process less laborious and expensive. According to this experimental set-up, lipids were accumulated by microalgae in a straightforward manner, thus reducing the steps (and costs) required for their production.

3.4. Use of dairy wastes for the production of recombinant β-glucosidase

The expression of the thermostable β-glucosidase in the βG-PTXD strain upon growth in DWSM at 200 μmol m^{-2} s^{-1} was investigated by immuno-blot analysis and activity assay (Fig. 4a-b). In the algal extract of βG-PTXD strain, a protein band of the expected molecular weight (~58 kDa) was clearly detected (Fig. 4a). It is worth noting that the amount of β-glucosidase detected in the βG-PTXD extract was almost comparable to that obtained from a culture of βG-PTXD cells grown in TAP medium at 50 μmol m^{-2} s^{-1} under air supplementation and axenic condition (Fig. 4b). This fact was in part unexpected since the chloroplast expression of βG is regulated by the endogenous promoter PpsaA (i.e., the promoter of the PSI A subunit-encoding gene) and therefore is subjected to the host regulation. Light intensity as high as 200 μmol photons m^{-2} s^{-1} repressed the transcription of *psaA* and decreased the stability of the corresponding mRNA (Benedetti et al., 2020). In DWSM medium, the net light inside the culture was probably reduced due to the medium opalescence. Activity assays were then performed to evaluate β-glucosidase and β-galactosidase activity of recombinant βG. Extracts from βG-PTXD cultures grown in DWSM medium showed a specific activity towards the chromogenic substrates *p*NPGlc and *p*NPGal (25.5 and 22.6 U/g dry weight, respectively) almost comparable than that contained in the extract from TAP-grown cultures (32.9 and 28.4 U/g dry weight, respectively). These data demonstrate that DWSM can be used to efficiently grow recombinant *C. reinhardtii* βG-PTXD strain and that an active enzyme can be obtained from the harvested biomass. β-glucosidases are widely used in several industrial applications such as biofuel, food and feed processing, bakery, textile, cellulose pulping and paper industry as well as in the production of dietary supplements and nutraceuticals (Giovannoni et al., 2020). Due to the broad substrate specificity, β-glucosidases may be also used for removing D-Lactose from dairy products as well as to degrade cellobiose into D-Glucose for bioethanol production from lignocellulosic biomass. In order to evaluate a possible application in the same dairy industry, D-Lactose and D-Glucose were quantified in D1 medium upon treatment with extract from WT

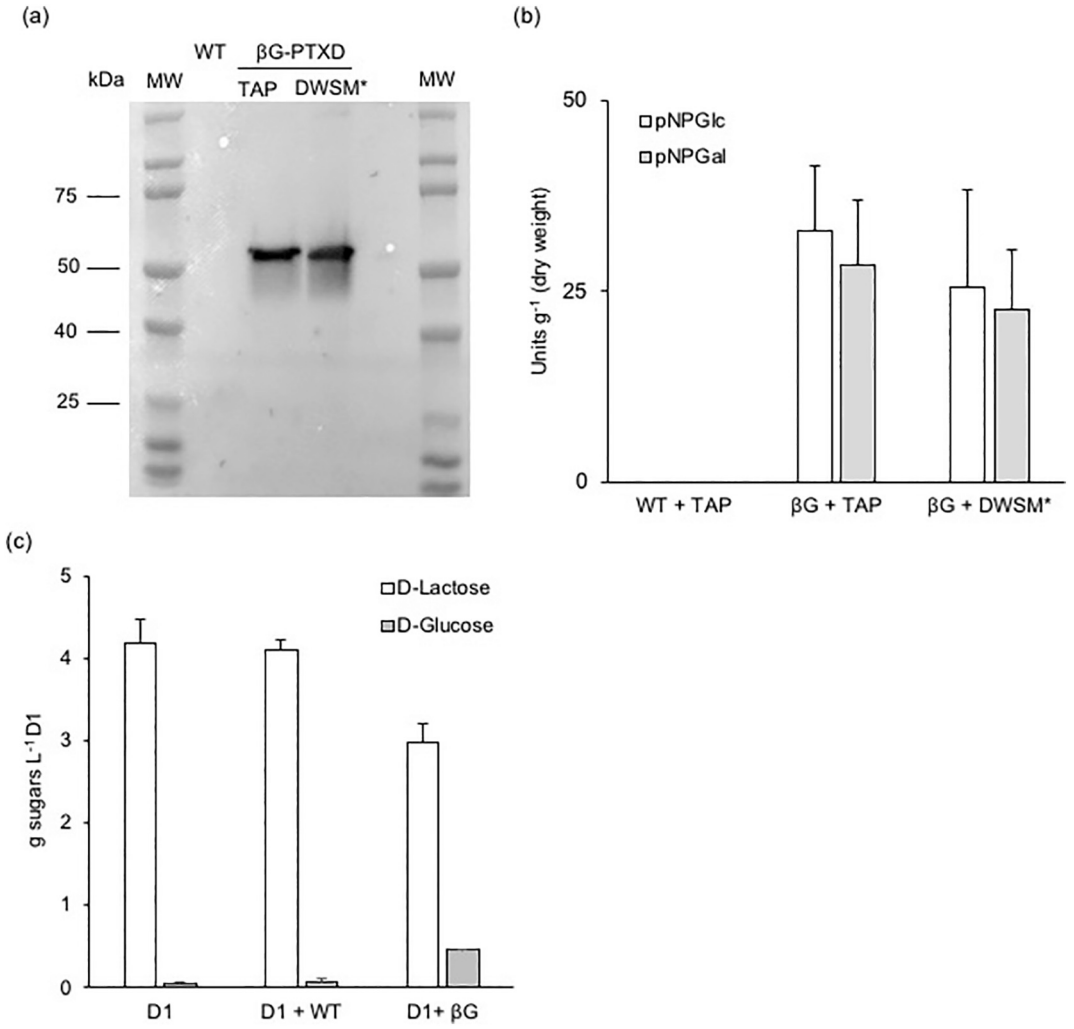

Fig. 4. Production of recombinant β-glucosidase from *C. reinhardtii* βG-PTXD using dairy waste-based media as growth medium. (a) Immunoblot and (b) activity assays of algal extracts from WT and βG-PTXD cultures grown in TAP or DWSM* medium. Growth was performed at 200 μmol photons m^{-2} s^{-1} under air supplementation. AbHA and *p*NPGlc/*p*NPGal were used for immunoblot and activity assays, respectively. Activity is expressed as Units per gram microalga biomass. 1 Units is defined as the amount of enzyme necessary to release 1 μmol of substrate per minute. *, 1.2 mM Pi was replaced with 4 mM Phi. 2.5×10^5 cells mL^{-1} was used as starting inoculum. (c) Determination of D-Lactose and D-glucose in 1 L of D1 upon 12 h-treatment with extracts obtained from 6 g (dry weight) of wild type *C. reinhardtii* (WT) and recombinant βG-PTXD (βG) biomass.

and βG-PTXD microalgae. As shown in Fig. 4c, 1 g of D-Lactose was hydrolyzed using the algal extract obtained from 6 g of βG-PTXD biomass upon 12 h-treatment; the conversion of D-Lactose into the constituting monosaccharides was also confirmed by the release of 0.46 g D-Glucose upon the enzymatic treatment, in accordance with the stoichiometry of the reaction (Fig. 4c).

The maximum biomass productivity of DWSM-grown *C. reinhardtii* was about 0.5 g/L/d. Considering that the production of βG from the βG-PTXD strain is about 0.35 mg/g dry weight (Benedetti et al., 2020), an enzyme productivity of about 0.18 mg/L/d was achieved. Further optimization is still required to improve the protein yield of βG from transplastomic microalgae (Benedetti et al., 2020). The algal-based biofactories here described has the advantage to combine the recycling of dairy wastes with the production of recombinant enzymes with potential use in other industrial fields. Moreover, the residual biomass after enzyme extraction can be further processed into livestock feed, organic fertilizer and bio-stimulants, or used for biogas production (Benedetti et al., 2018).

3.5. Remediation of dairy wastes by C. reinhardtii and A. protothecoides

The pollution load of untreated D1 and D2 largely exceeded the Italian legal limits for their direct discharge in the municipal sewage system (Table 2). The efficacy of the algal-based remediation in decreasing the amounts of the vary contaminating substances from dairy wastes was evaluated. The values of COD, D-Lactose, Chloride, Phosphorous, total Nitrogen and protein Nitrogen of both DWSM and DW were measured before and after the algal growth (Fig. 5).

The levels of COD, D-Lactose, Phosphorous, total Nitrogen and protein Nitrogen in DWSM were reduced upon *C. reinhardtii* growth by 76%, 84%, 87%, 65%, 65% respectively. Similarly, a significant reduction in COD (~65%), D-Lactose (~84%), Phosphorous (~77%), total Nitrogen (~43%) and protein Nitrogen (~43%) content was observed in the exhausted DWSM upon *A. protothecoides* growth while both algal species were unable to reduce the Chloride level since its concentration remained almost similar to that measured in the untreated DWSM medium (Fig. 5). Surprisingly, a similar (or even higher) extent of reduction was observed in the exhausted DW medium for both algal cultures despite the algal biomass production was significantly lower in DW than in DWSM medium. The reduction obtained after *A. protothecoides*

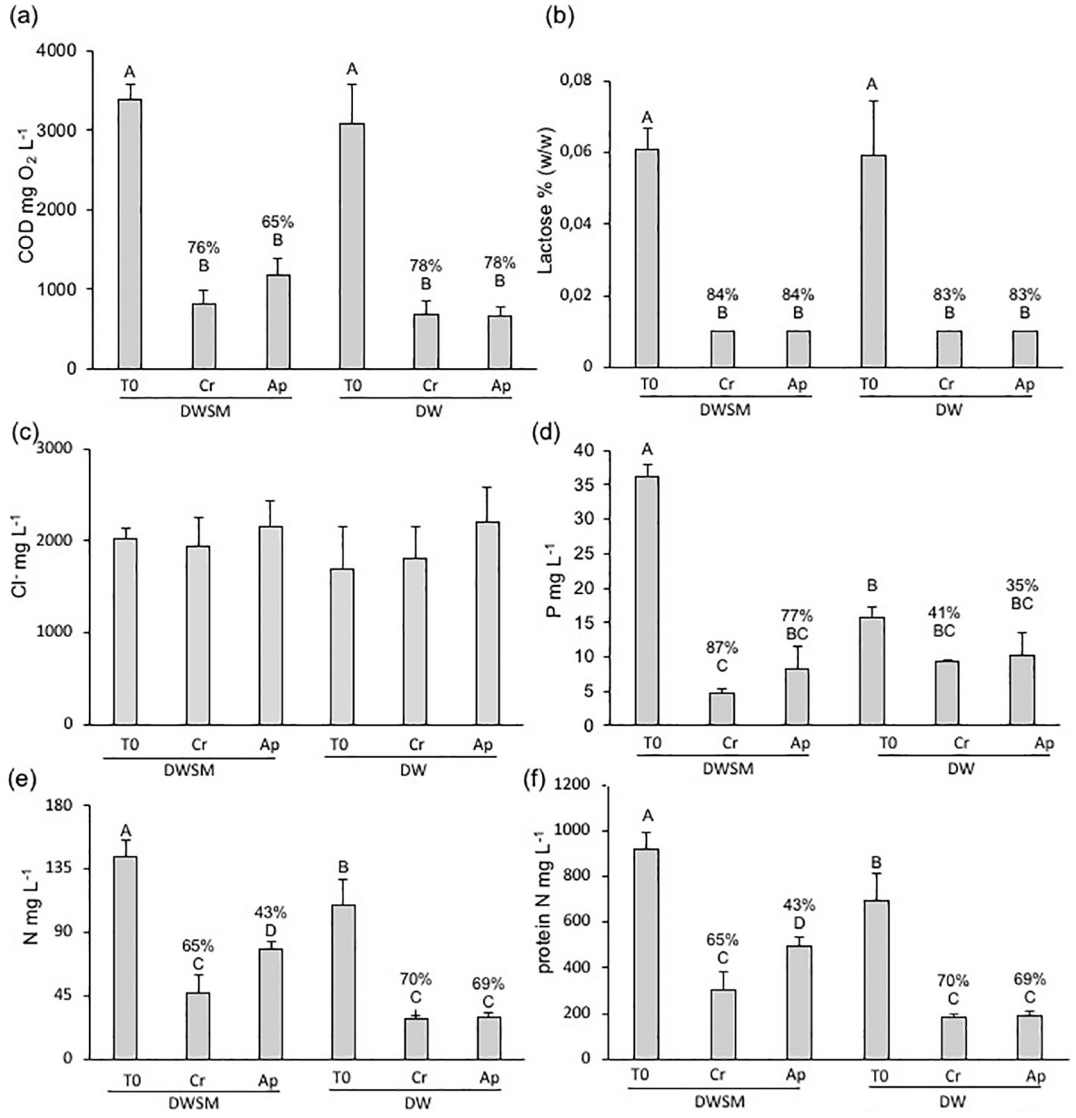

Fig. 5. Nutrient and salt removal from dairy waste-based growth medium by *C. reinhardtii* and *A. prototechoides*. Amounts of (a) COD, (b) D-Lactose, (c) Chloride, (d) Phosphorous, (e) total Nitrogen and (f) protein Nitrogen in the same cultures shown in (Fig. 3a-b) and in the corresponding untreated media (T0) expressed both as absolute values and percentage of reduction (top of the bar). Different letters above bars indicate statistically significant differences according to one-way ANOVA and Tukey test ($P < 0.05$). Ap, *A. protothecoides*; Cr, *C. reinhardtii*. Values are a mean of three biological replicates ± SD.

growth was significantly lower in DWSM compared to DW only in the case of total Nitrogen (43% vs 69%) and protein Nitrogen (43% vs 69%), suggesting that a robust growth of such microalga species may interfere with the removal of certain pollutants. These results, together with the observation that the biomass production of both microalgal species was not positively related to the reduction of pollution load (Fig. 3a-c), suggested that the "endogenous" microbial communities strongly contributed to the reduction of organic pollutants. Indeed, despite the mild heat treatment, the dairy waste-based media were not completely devoid of microbial contaminants. The disappearance of D-Lactose in such dairy wastes is a direct proof that contaminating microbes are the main responsible for the reduction of the pollution load; it is worth noting that D-Lactose concentration affects both COD and BOD values. Previous studies using dairy wastes for culturing different *Chlorella* species indicated their inability to assimilate D-Lactose (Abreu et al., 2012; Espinosa-Gonzalez et al., 2014). The ability of *C. vulgaris* to assimilate D-Lactose was also evaluated by carrying out a mixotrophic growth using both the monosaccharides constituting D-Lactose (i.e., D-Glucose and D-Galactose) and sole D-Lactose as carbon source (Supplementary Material). The growth curves suggested that *C. vulgaris* is unable to metabolize D-Lactose whereas it showed comparable assimilation rate of D-Glucose and D-Galactose (Supplementary Material). Accordingly, an increased yield in biomass of about 3-fold was observed in the presence of D-Glucose and D-Galactose compared to that observed with D-Lactose supplementation and pure photoautotrophic growth, clearly indicating that D-Lactose is not metabolized by *C. vulgaris* (Supplementary Material). In accordance with the previous results (Fig. 3d), the lipid content increased together with the biomass production in *C. vulgaris* (Supplementary Material). Notably, the maximum lipid production of *A. protothecoides* grown in DWSM (0.59 g/L) (Fig. 3d) was comparable to that obtained by *C. vulgaris* grown in 0.5% D-Glucose under axenic condition (0.59 g/L) (Supplementary Material), indicating that the lipid production in dairy waste-based medium was highly efficient. The beneficial role of DWSM in promoting algal growth may be ascribed to the presence of other nutrients rather than D-Lactose such as Phosphorous, Nitrogen, minerals, lipids and proteins (Table 2). The inability to assimilate D-Lactose was observed for all the algal species tested, i.e., *C. sorokiniana*, *C. saccharophila*, *A. protothecoides* and *C. reinhardtii* (Supplementary Material), the latter species being also unable to assimilate D-Glucose and D-Galactose (data not shown).

Nonetheless, the cocultivation between microalgae and microbes in DWSM medium allowed the production of valuable algal biomass and useful metabolites on one side, by concomitantly lowering the pollution load of the dairy wastes on the other (Fig. 5). To further confirm the role of co-cultivating microbes and microalgae using dairy wastes, the growth of *A. protothecoides* was evaluated under non-axenic condition by using an antibiotic to hinder the microbial growth. In accordance with the previous result, the disappearance of D-Lactose was not observed when the algal growth was performed in presence of ampicillin at 200 μg/mL (Supplementary Material). Several studies reported that bacteria and fungi are effective agents in bioremediation highlighting the advantages of using microalgal consortia over single-species cultures (González-Fernández et al., 2011; He et al., 2013; Muñoz and Guieysse, 2006). These results demonstrate that dairy wastes can be used for sustaining a robust algal growth and the production of algal-based metabolites, with a substantial reduction in their pollution load, the latter dependent on the cocultivation between microalgae and microbes rather than only microalgae.

3.6. Economic aspects of the recycling of dairy wastes using wild-type and transgenic microalgae

In this section, the economic evaluation of the microalgal-based dairy effluent treatment plant is summarized. Approximately 50.000 L D1 are produced daily by Azienda Casearia Capurso. By reverse osmosis treatment, "concentrate" D1 and "permeate" D2 are obtained from skimmed D1. The disposal of D1 is expensive and D2 requires additional treatments before its discharge in the municipal sewage system, therefore the management of these wastes is crucial for the economic and environmental sustainability of the dairy plant. In lab-scale experiment, the cultivation of *A. protothecoides* and *C. reinhardtii* in DWSM combined the dairy wastewater remediation with the production of valuable algal biomass and high added-value compounds. In order to test the effectiveness of the cultivation model here proposed, its scale up from pilot- to large-scale will be mandatory.

In the proposed model (Fig. 6), D1 is diluted using D2 in a ratio of 13:87 (*v*/v) with the addition of the salt mixture, i.e., SM, to obtain DWSM. The optimized medium is heated at 85 °C using a heated stirred reactor and then cooled to ambient temperature by refrigerated water. Once cooled, the medium is mixed in-line with the microalgal inoculum (2.5×10^5 cell/mL) and transferred to a tubular photobioreactor system

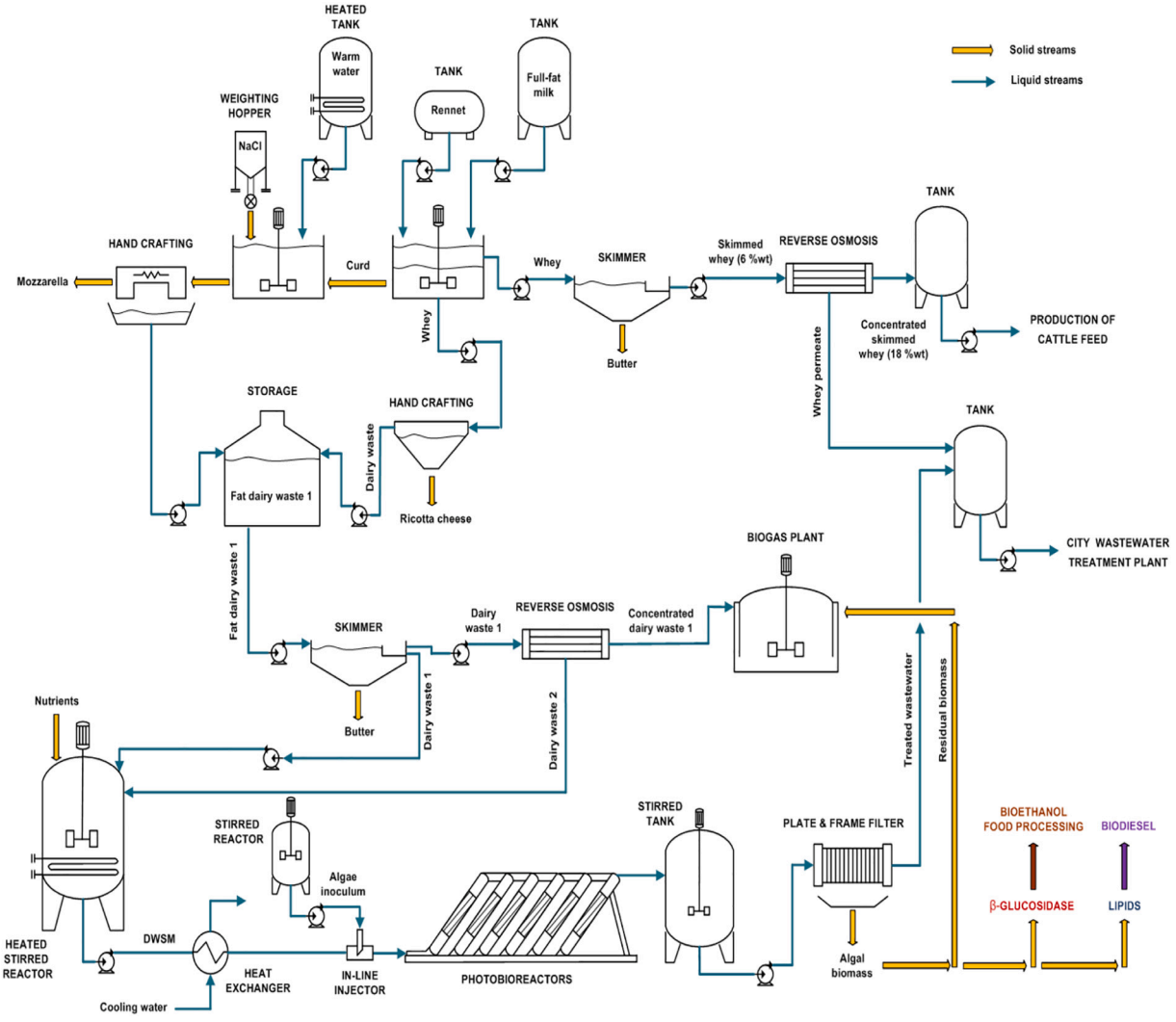

Fig. 6. Integrated model of the dairy factory coupled to the algal culture.
In the proposed model, D1 and D2, once supplemented with SM, are used for the cultivation of microalgae in order to combine the production of lipids (from *A. protothecoides*) and recombinant β-glucosidase (from transgenic *C. reinhardtii*) with that of valuable biomass. Then, the exhausted growth medium can be subjected to minimal wastewater treatment before effluent discharge.

(PBR) according to the PBR-design proposed in Tredici et al., 2016 (Green Wall Panel, GWP®). The algal cultivation in PBR required air supplementation and a light intensity of 200 μmol photons m^{-2} s^{-1}.

Every four days, half of the total culture volume is harvested and transferred into a storage tank, from which the algal biomass is separated from the liquid by a plate and frame filter. Fresh and heat-treated DWSM medium is pumped into the photobioreactors in order to restore the starting volume of the PBR-system. The exhausted DWSM medium, i.e., treated wastewater, will be discharged into the city wastewater treatment plant with minor treatment requirements. The algal biomass obtained from PBRs can be used for the extraction of biomolecules of commercial interest (i.e., lipids and β-glucosidase) while the residual biomass upon extraction can be sold for other purposes including the production of biogas (Jankowska et al., 2017). Alternatively, D1 (13% v/v) could be used for the cultivation of the recombinant strain βG-PTXD by exploiting Phi to restrict the microbial contamination, since Phi-supplemented media showed the same effect of D2-diluted media in limiting the microbial contamination. This may represent an additional economic saving for the company, overcoming the requirement of reverse osmosis process and the cost from concentrated D1 transport. Furthermore, using only D1, the starting Chloride level would be below the legal limits for the discharge in the municipal sewage system. However, the use of fresh-water is required to dilute D1 as well as further analyses are necessary to determine the polluting load in the exhausted Phi-supplemented media. An economic analysis on the biomolecules produced in accordance with the proposed model must take into account at least the cost of growth media, mainly represented by the cost of salts supplementation to DW (Table 1).

By scaling-up the culture to 100.000 L DWSM, a lipid and biomass productivity of 0.16 g/L/d and 0.85 g/L/d, respectively, may result in a daily production of 16 kg lipids and 85 kg algal biomass by *A. protothecoides*. Moreover, considering the best-case scenario in which (i) the conversion efficiency of lipids into biodiesel is higher than 99.5% (Topf et al., 2014) and (ii) the production cost of PBR-grown microalgae is 3.8 €/kg (Tredici et al., 2016), 1 t of biodiesel can be obtained from *A. protothecoides* in 62.5 days at a production cost of 20.187,5 €. Considering that the current selling price of biodiesel is 693 €/t (www.neste.com/corporate-info/investors/market-data/biodiesel-prices-sme-fame), lipid production alone cannot justify the economic benefit of culturing microalgae using dairy wastes. However, the main financial income is represented by the sale of algal biomass; considering a cheap market value of 5 to 10 €/kg algal biomass, the algal biomass accumulated over 62.5 days of growth (i.e., 5.312 kg) can be sold from 26.562 to 53.125 €, with a net profit of 6375 to 32.937,5 €.

On the other hand, a 100.000 L culture of βG-PTXD can produce about 18 g enzyme/d. A recombinant β-glucosidase from *E. coli* as obtained by high cell density fermentation has a production cost of ~280 €/kg whereas the enzyme productivity from *E.coli*-based biofactory is 2.41 g/L/d (da G. Ferreira et al., 2018). Considering (i) the enzyme productivity from the *C. reinhardtii*-based biofactory (0.18 mg/L/d), the biomass productivity (0.5 g/L/d) and the production cost of microalgae (i.e., 3.8 €/kg; Tredici et al., 2016), the same category of enzyme can be obtained from transplastomic *C. reinhardtii* in 55.5 days at a production cost of 10.545 €/kg. This result clearly shows the heterologous expression of recombinant proteins in *C. reinhardtii* urgently requires further optimization in order to be competitive with the current enzyme-biofactories. However, in the case of *C. reinhardtii* as a biofactory to produce recombinant enzymes, biocontainment strategies would be necessary to restrict its dispersion in the environment. Some technologies that could be combined with the recombinant Chlamydomonas is the "free-DNA" CRISPR-Cas9 technology, developed by Baek et al., 2016, that can be used to generate auxotrophic strains to ensure confinement in PBRs (Benedetti et al., 2020). Moreover, horizontal gene transfer of chloroplast transgenes to other microbes can be avoided by a codon reassignment-based strategy (Young and Purton, 2016). As observed for *A. protothecoides*, the algal biomass of *C. reinhardtii* accumulated over 55.5 days of growth (i.e., 2775 kg) to produce 1 kg of β-glucosidase can be sold from 13.875 to 27.750 €, with a net profit of 3330 to 17.205 €.

In summary, the results obtained provided a proof of concept of the whole supply chain, from the development of optimal medium for the algal growth and the selection of the algal species to their application for the production of high-value compounds. Advantages and limits of each step were highlighted, indicating the path to further improve the economic feasibility of microalgae-based production of biomolecules of industrial interest. Major profits for the dairy company could be represented by (i) the sale of algal biomass, that might be employed for various applications including the production of animal feed, biostimulants and dietary supplements (Benedetti et al., 2018) and (ii) the economic saving from the reduction of severe treatments necessary to clean the dairy wastes; however, the latter aspect would require a very high capacity algal cultivation system to be really effective.

4. Conclusions

The potential of different microalgae for dairy wastewater remediation and production of lipids and/or recombinant enzymes was explored. *A. protothecoides* proved to be a good candidate for biodiesel production, with highest biomass (3.3 g/L) and lipid (592 mg/L) yield. Cocultivation of microalgae and microbes reduced the pollution load in dairy wastes, decreasing Nitrogen and Phosphorous levels below wastewater discharge limits and significantly reducing COD and protein Nitrogen. Production of a thermostable β-glucosidase by transplastomic *C. reinhardtii* grown in dairy wastes revealed the potential for a sustainable biorefinery model combining waste remediation with the production of recombinant enzymes by microalgae.

CRediT authorship contribution statement

Giovanna Gramegna: Methodology, Investigation, Formal analysis, Writing- Original Draft.
Anna Scortica: Methodology, Investigation.
Valentina Scafati: Methodology, Investigation.
Moira Giovannoni: Methodology, Investigation.
Francesco Ferella: Methodology.
Libero Gurrieri: Methodology, Investigation, Formal analysis.
Francesca Sparla: Methodology, Investigation, Formal analysis.
Roberto Bassi: Formal analysis.
Benedetta Mattei: Conceptualization, Formal analysis, Supervision, Writing-Review and Editing, Project Administration, Funding acquisition.
Manuel Benedetti: Conceptualization, Methodology, Visualization, Formal analysis, Supervision, Writing-Review and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All relevant data are included in the article and/or its Supplementary Material files.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2020.100604.

References

Abreu, A.P., Fernandes, B., Vicente, A.A., Teixeira, J., Dragone, G., 2012. Mixotrophic cultivation of *Chlorella vulgaris* using industrial dairy waste as organic carbon source. Bioresour. Technol. 118, 61–66. https://doi.org/10.1016/j.biortech.2012.05.055.

Baek, K., Kim, D.H., Jeong, J., Sim, S.J., Melis, A., Kim, J.-S., Jin, E., Bae, S., 2016. DNA-free two-gene knockout in *Chlamydomonas reinhardtii* via CRISPR-Cas9 ribonucleoproteins. Sci. Rep. 6, 30620. https://doi.org/10.1038/srep30620.

Baier, T., Wichmann, J., Kruse, O., Lauersen, K.J., 2018. Intron-containing algal transgenes mediate efficient recombinant gene expression in the green microalga *Chlamydomonas reinhardtii*. Nucleic Acids Res. 46, 6909–6919. https://doi.org/10.1093/nar/gky532.

Barkia, I., Saari, N., Manning, S.R., 2019. Microalgae for high-value products towards human health and nutrition. Mar. Drugs 17, 304. https://doi.org/10.3390/md17050304.

Benedetti, M., Vecchi, V., Barera, S., Dall'Osto, L., 2018. Biomass from microalgae: the potential of domestication towards sustainable biofactories. Microb. Cell Factories 17, 173. https://doi.org/10.1186/s12934-018-1019-3.

Benedetti, M., Barera, S., Longoni, P., Guardini, Z., Herrero Garcia, N., Bolzonella, D., Lopez-Arredondo, D., Herrera-Estrella, L., Goldschmidt-Clermont, M., Bassi, R., Dall'Osto, L., 2020. A microalgal-based preparation with synergistic cellulolytic and detoxifying action towards chemical-treated lignocellulose. Plant Biotechnol. J. pbi.13447. doi:https://doi.org/10.1111/pbi.13447.

Bhatnagar, A., Chinnasamy, S., Singh, M., Das, K.C., 2011. Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. Appl. Energy 88, 3425–3431. https://doi.org/10.1016/j.apenergy.2010.12.064.

Brar, A., Kumar, M., Pareek, N., 2019. Comparative appraisal of biomass production, remediation and bioenergy generation potential of microalgae in dairy wastewater. Front. Microbiol. 10, 678. https://doi.org/10.3389/fmicb.2019.00678.

Brennan, L., Owende, P., 2010. Biofuels from microalgae-a review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sust. Energ. Rev. 14, 557–577. https://doi.org/10.1016/j.rser.2009.10.009.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306. https://doi.org/10.1016/j.biotechadv.2007.02.001.

Choi, H.-J., 2016. Dairy wastewater treatment using microalgae for potential biodiesel application. Environ. Eng. Res. 21, 393–400. https://doi.org/10.4491/eer.2015.151.

Chokshi, K., Pancha, I., Ghosh, A., Mishra, S., 2016. Microalgal biomass generation by phycoremediation of dairy industry wastewater: an integrated approach towards sustainable biofuel production. Bioresour. Technol. 221, 455–460. https://doi.org/10.1016/j.biortech.2016.09.070.

Costas, A.M.G., White, A.K., Metcalf, W.W., 2001. Purification and characterization of a novel phosphorus-oxidizing enzyme from *Pseudomonas stutzeri* WM88. J. Biol. Chem. 276, 17429–17436. https://doi.org/10.1074/jbc.M011764200.

da G. Ferreira, R., Azzoni, A.R., Freitas, S., 2018. Techno-economic analysis of the industrial production of a low-cost enzyme using *E. coli*: the case of recombinant β-glucosidase. Biotechnol. Biofuels 11, 81. https://doi.org/10.1186/s13068-018-1077-0.

Dall'Osto, L., Cazzaniga, S., Guardini, Z., Barera, S., Benedetti, M., Mannino, G., Maffei, M.E., Bassi, R., 2019. Combined resistance to oxidative stress and reduced antenna size enhance light-to-biomass conversion efficiency in *Chlorella vulgaris* cultures. Biotechnol. Biofuels 12, 221. https://doi.org/10.1186/s13068-019-1566-9.

de Melo, R.G., de Andrade, A.F., Bezerra, R.P., Correia, D.S., de Souza, V.C., Brasileiro-Vidal, A.C., de A. Viana Marques, D., Porto, A.L.F., 2018. Chlorella vulgaris mixotrophic growth enhanced biomass productivity and reduced toxicity from agro-industrial by-products. Chemosphere 204, 344–350. https://doi.org/10.1016/j.chemosphere.2018.04.039.

Espinosa-Gonzalez, I., Parashar, A., Bressler, D.C., 2014. Heterotrophic growth and lipid accumulation of *Chlorella protothecoides* in whey permeate, a dairy by-product stream, for biofuel production. Bioresour. Technol. 155, 170–176. https://doi.org/10.1016/j.biortech.2013.12.028.

Food and Agriculture Organization of the United Nations, 2019. Overview of Global Dairy Market Developments in 2018. Dairy Mark, Rev.

Giovannoni, M., Gramegna, G., Benedetti, M., Mattei, B., 2020. Industrial use of cell wall degrading enzymes: the fine line between production strategy and economic feasibility. Front. Bioeng. Biotechnol. 8, 356. https://doi.org/10.3389/fbioe.2020.00356.

González-Fernández, C., Molinuevo-Salces, B., García-González, M.C., 2011. Nitrogen transformations under different conditions in open ponds by means of microalgae–bacteria consortium treating pig slurry. Bioresour. Technol. 102, 960–966. https://doi.org/10.1016/j.biortech.2010.09.052.

Guest, D., Bompeix, G., 1990. The complex mode of action of phosphonates. Australas. Plant Pathol. 19, 113. https://doi.org/10.1071/APP9900113.

He, P.J., Mao, B., Lü, F., Shao, L.M., Lee, D.J., Chang, J.S., 2013. The combined effect of bacteria and *Chlorella vulgaris* on the treatment of municipal wastewaters. Bioresour. Technol. 146, 562–568. https://doi.org/10.1016/j.biortech.2013.07.111.

Hena, S., Fatimah, S., Tabassum, S., 2015. Cultivation of algae consortium in a dairy farm wastewater for biodiesel production. Water Resour. Ind. 10, 1–14. https://doi.org/10.1016/j.wri.2015.02.002.

Jankowska, E., Sahu, A.K., Oleskowicz-Popiel, P., 2017. Biogas from microalgae: review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion. Renew. Sust. Energ. Rev. 75, 692–709. https://doi.org/10.1016/j.rser.2016.11.045.

Karadag, D., Köroğlu, O.E., Ozkaya, B., Cakmakci, M., 2015. A review on anaerobic biofilm reactors for the treatment of dairy industry wastewater. Process Biochem. 50, 262–271. https://doi.org/10.1016/j.procbio.2014.11.005.

Kengen, S.W.M., Luesink, E.J., Stams, A.J.M., Zehnder, A.J.B., 1993. Purification and characterization of an extremely thermostable beta-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Eur. J. Biochem. 213, 305–312. https://doi.org/10.1111/j.1432-1033.1993.tb17763.x.

Kolev Slavov, A., 2017. Dairy wastewaters – general characteristics and treatment possibilities – a review. Food Technol. Biotechnol. 55. doi:10.17113/ftb.55.01.17.4520.

Kothari, R., Prasad, R., Kumar, V., Singh, D.P., 2013. Production of biodiesel from microalgae *Chlamydomonas polypyrenoideum* grown on dairy industry wastewater. Bioresour. Technol. 144, 499–503. https://doi.org/10.1016/j.biortech.2013.06.116.

Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent, P., Castruita, M., Pellegrini, M., Merchant, S.S., Malasarn, D., 2011. A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*: a revised mineral nutrient supplement for Chlamydomonas. Plant J. 66, 770–780. https://doi.org/10.1111/j.1365-313X.2011.04537.x.

Liu, J., Chen, F., 2014. Biology and industrial applications of *Chlorella*: advances and prospects. In: Posten, C., Feng Chen, S. (Eds.), Microalgae Biotechnology. Springer International Publishing, Cham, pp. 1–35. https://doi.org/10.1007/10_2014_286.

Loera-Quezada, M.M., Leyva-González, M.A., Velázquez-Juárez, G., Sanchez-Calderón, L., Do Nascimento, M., López-Arredondo, D., Herrera-Estrella, L., 2016. A novel genetic engineering platform for the effective management of biological contaminants for the production of microalgae. Plant Biotechnol. J. 14, 2066–2076. https://doi.org/10.1111/pbi.12564.

Malcata, F.X., 2011. Microalgae and biofuels: a promising partnership? Trends Biotechnol. 29, 542–549. https://doi.org/10.1016/j.tibtech.2011.05.005.

Menetrez, M.Y., 2012. An overview of algae biofuel production and potential environmental impact. Environ. Sci. Technol. 46, 7073–7085. https://doi.org/10.1021/es300917r.

Mishra, S.K., Suh, W.I., Farooq, W., Moon, M., Shrivastav, A., Park, M.S., Yang, J.-W., 2014. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. Bioresour. Technol. 155, 330–333. https://doi.org/10.1016/j.biortech.2013.12.077.

Muñoz, R., Guieysse, B., 2006. Algal–bacterial processes for the treatment of hazardous contaminants: a review. Water Res. 40, 2799–2815. https://doi.org/10.1016/j.watres.2006.06.011.

Perozeni, F., Cazzaniga, S., Baier, T., Zanoni, F., Zoccatelli, G., Lauersen, K.J., Wobbe, L., Ballottari, M., 2020. Turning a green alga red: engineering astaxanthin biosynthesis by intragenic pseudogene revival in *Chlamydomonas reinhardtii*. Plant Biotechnol. J. pbi.13364. doi:https://doi.org/10.1111/pbi.13364.

Qin, L., Shu, Q., Wang, Z., Shang, C., Zhu, S., Xu, J., Li, R., Zhu, L., Yuan, Z., 2014. Cultivation of *Chlorella vulgaris* in dairy wastewater pretreated by UV irradiation and sodium hypochlorite. Appl. Biochem. Biotechnol. 172, 1121–1130. https://doi.org/10.1007/s12010-013-0576-5.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100–112. https://doi.org/10.1002/bit.22033.

Scranton, M.A., Ostrand, J.T., Fields, F.J., Mayfield, S.P., 2015. *Chlamydomonas* as a model for biofuels and bio-products production. Plant J. 82, 523–531. https://doi.org/10.1111/tpj.12780.

Stephens, E., Ross, I.L., Mussgnug, J.H., Wagner, L.D., Borowitzka, M.A., Posten, C., Kruse, O., Hankamer, B., 2010. Future prospects of microalgal biofuel production systems. Trends Plant Sci. 15, 554–564. https://doi.org/10.1016/j.tplants.2010.06.003.

Taunt, H.N., Stoffels, L., Purton, S., 2018. Green biologics: the algal chloroplast as a platform for making biopharmaceuticals. Bioengineered 9, 48–54. https://doi.org/10.1080/21655979.2017.1377867.

Topf, M., Koberg, M., Kinel-Tahan, Y., Gedanken, A., Dubinsky, Z., Yehoshua, Y., 2014. Optimizing algal lipid production and its efficient conversion to biodiesel. Biofuels 5, 405–413. https://doi.org/10.1080/17597269.2014.985488.

Tredici, M.R., Rodolfi, L., Biondi, N., Bassi, N., Sampietro, G., 2016. Techno-economic analysis of microalgal biomass production in a 1-ha Green Wall Panel (GWP®) plant. Algal Res. 19, 253–263. https://doi.org/10.1016/j.algal.2016.09.005.

Ummalyma, S.B., Sukumaran, R.K., 2014. Cultivation of microalgae in dairy effluent for oil production and removal of organic pollution load. Bioresour. Technol. 165, 295–301. https://doi.org/10.1016/j.biortech.2014.03.028.

Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., Takriff, M., 2014. An overview: biomolecules from microalgae for animal feed and aquaculture. J. Biol. Res.-Thessalon. 21, 6. https://doi.org/10.1186/2241-5793-21-6.

Young, R.E.B., Purton, S., 2016. Codon reassignment to facilitate genetic engineering and biocontainment in the chloroplast of *Chlamydomonas reinhardtii*. Plant Biotechnol. J. 14, 1251–1260. https://doi.org/10.1111/pbi.12490.