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Original Paper

Cultivation of the green microalga *Coelastrella chongqingensis* in lactose-containing media

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Abstract

Currently, various agricultural by-products are being explored as cost-effective alternative media for microalgae cultivation, to enhance biomass productivity and reduce overall production costs. Among these substrates, dairy industry by-products remain particularly challenging to valorize. In this study, the biomass productivity of the axenic *Coelastrella chongqingensis* MSCL 1802 strain was evaluated under mixotrophic and heterotrophic conditions using cheese whey and whey permeate as alternative cultivation media, and compared to its productivity in a defined synthetic medium. The results demonstrated that the highest biomass productivity was achieved in whey permeate medium under mixotrophic conditions, reaching 0.32 ± 0.01 g L^{-1} day^{-1} (dry biomass). Furthermore, it was shown that *C. chongqingensis* can produce β-galactosidase and utilize lactose, i.e., the main sugar present in whey and permeate, under both mixotrophic and heterotrophic conditions.

Key words: β-galactosidase, lactose, galactose, whey, permeate, mixotrophic cultivation.
Abbreviations: BBM, Bold's basal medium; BOD, biological oxygen demand; COD, chemical oxygen demand; MSCL, Microbial Strain Collection of Latvia; OD, optical density; ONPG, ortho-nitrophenyl-β-galactoside.

Introduction

Currently, agriculture faces various challenges, including providing food for a rapidly growing human population, soil degradation, high rate of biodiversity loss, and climate change. Additionally, unsustainable practices within agriculture threaten soil fertility, water resources, and biodiversity, making it essential to adopt sustainable approaches (Koohafkan et al. 2011; Velten et al. 2015). By reducing agrochemical inputs, conserving ecosystems, improving efficiency of resource usage, and promoting interdisciplinary collaboration, sustainable agriculture can maintain productivity while ensuring long-term food security and mitigation of environmental problems (Velten et al. 2015). Furthermore, the circular economy model has emerged as an approach to enhance economic performance in agriculture while simultaneously achieving sustainability goals, by focusing on minimizing waste, maximizing resource efficiency, and keeping materials and nutrients in continuous use. Implementation of circular economy strategies in agriculture is increasing over time, but additional efforts are essential to achieve sustainability goals (Velasco-Muñoz et al. 2022).

Among agricultural sectors, the dairy industry is under growing pressure to align with circular economy and sustainability objectives while simultaneously addressing economic constraints, waste management challenges, environmental degradation, and rising public expectations. (Alrhmoun et al. 2025). The manufacturing of dairy products such as yogurt, cheese, and butter generates various side-streams, the volumes of which can range from 0.2 to 10 liters per liter of processed milk. Dairy industry by-products such as whey and permeate contain high concentrations of lactose, proteins, lipids, and minerals; however, their valorization remains unfeasible, and these by-products are often treated as waste (Carvalho et al. 2013; Kolev Slavov 2017). Subsequently, this waste requires complex and expensive purification procedures to prevent environmental damage upon discharge (Carvalho et al. 2013). Estimates indicate that approximately 50% of the world's whey production is disposed of without proper treatment, resulting in severe environmental damage. This is associated with the high biological oxygen demand (BOD) and chemical oxygen demand (COD) of the by-product, of which 70 to 90 % is attributed to lactose (Carvalho et al. 2013; Kolev Slavov 2017).

As highlighted in recent reviews, necessary steps must be taken to reduce the environmental impact of various dairy industry side-streams by applying circular economy principles (Schilling, Weiss 2021; Ozcelik et al. 2024). Therefore, the bioconversion of such side-streams by microalgae, i.e., a polyphyletic group of

microorganisms, has recently gained significant attention as a sustainable strategy for reducing dairy industry waste and promoting a zero-waste approach in this agricultural sector (Cheirsilp et al. 2023; Ozcelik et al. 2024). This is associated with enhanced ability of microalgae to grow in a wide range of substrates and to synthesize and accumulate valuable bioactive compounds with a plethora of potential commercial applications (Spolaore et al. 2006). Furthermore, the use of agricultural by-products can serve as a sustainable strategy for the commercialization of microalgae, as large-scale production often remains problematic due to high production costs and low biomass or target compound productivity (Rizwan et al. 2018).

Recent studies have demonstrated that selecting microalgae capable of growing on lactose-containing substrates can support the sustainable management of dairy industry by-products, while simultaneously enhancing microalgal biomass productivity and potentially reducing overall production costs (Bosso et al. 2020; Ozcelik et al. 2024). The successful growth in such substrates is directly connected with the ability of microalgae to produce β-galactosidase, the enzyme required for lactose hydrolysis (Husain 2010; Bosso et al. 2020). Recently, certain axenic microalgae, including *Tetradesmus obliquus* CPCC 5 (Bentahar, Deschênes 2021), *Graesiella emersonii* MSCL 1718 (Kolesovs et al. 2025a), and *Galdieria sulphuraria* 107.79 (Zimermann et al. 2020), have demonstrated the ability to utilize lactose present in various lactose-containing media and produce β-galactosidase enzyme. Additionally, mixotrophic cultivation (in the presence of light an organic carbon (C) source) and heterotrophic cultivation (in the dark with organic C) can be a more suitable approach for obtaining high-density cultures compared to photoautotrophic growth at high culture densities that limit illumination and decrease biomass productivity (Gim et al. 2014; Kumar et al. 2014).

Despite its high potential, the topic of microalgal lactose metabolism remains understudied to date, with a limited number of microalgal strains demonstrating the ability to utilize lactose (Ozcelik et al. 2024). As lactose is the main constituent for high BOD and COD, the microalgae's ability to remove this disaccharide is crucial for efficient dairy by-product treatment. For instance, a study by Gramegna et al. (2020) demonstrated that although cultivation of four microalgae, i.e., *Auxenochlorella protothecoides, Chlorella sorokiniana, Chlorella saccharophila*, and wild-type *Chlamydomonas reinhardtii* in various dairy industry side-streams reached considerable biomass productivity, the lactose remained unutilized, thus decreasing the polluting load only partially. Therefore, the selection of lactose-utilizing microalgae is necessary for efficient removal of pollutants (Gramegna et al. 2020; Kolesovs, Semjonovs 2023).

Among other understudied questions remains the lack of exact data on culture purity, lactose uptake, and the unspecified β-galactosidase activity of the selected cultures (Kolesovs, Semjonovs 2023). In some studies, a significant decrease of BOD and COD in the substrate served as an indirect indicator of a decrease in lactose concentrations. For instance, *Chlorella vulgaris* KMMCC-143 was cultivated under mixotrophic conditions for 10 days in dairy wastewater effluent, resulting in the removal of approximately 85.6% BOD, 80.62% COD, and 85.5% total nitrogen (N) from the effluent (Choi 2016). However, the purity of the culture was not specified, limiting the ability to draw definitive conclusions about the strain's lactose metabolism. Additionally, the ability to utilize lactose has never been tested for many microalgal species, significantly limiting the number of available candidates (Kolesovs, Semjonovs 2023). Therefore, the assessment of aspects such as lactose uptake under different growth conditions, β-galactosidase activity and localization, i.e., intra- or extracellular, lactose tolerance levels, is crucial for filling the fundamental gaps and achieving sustainable and cost-effective production of valuable compounds or biomass (Kolesovs, Semjonovs 2023; Ozcelik et al. 2024). Furthermore, the identification of microalgal candidates with a superior tolerance to various abiotic stress factors remains another limiting factor for large-scale microalgae cultivation (Yun et al. 2019).

The aim of this study is to evaluate suitability of the recently isolated green microalga *Coelastrella chongqingensis* MSCL 1802 for growth in lactose-containing substrates, which would expand our understanding of green microalgae performance in dairy industry by-products. Currently, there are no studies dedicated to this microalga species beyond its original morphological and taxonomic description in a study by Wang et al. (2021). In this study, we tested the ability of *C. chongqingensis* to utilize lactose, glucose, and galactose as external organic C, as well to assess its ability to produce β-galactosidase. Additionally, mixotrophic and heterotrophic cultivation modes were evaluated to identify a more suitable approach for achieving higher biomass productivity.

Materials and methods

Microalgal strain

Coelastrella chongqingensis MSCL 1802 is a freshwater green microalgal isolate obtained in Riga, Latvia. After isolation using the streak plate technique and identification, *C. chongqingensis* was deposited in the Microbial Strain Collection of Latvia (Institute of Microbiology and Biotechnology, University of Latvia). The identification procedure was performed at Ghent University (Belgium) by analyzing four markers, i.e., *SSU, ITS, LSU*, and *rbcL* genome sequences.

Experimental media

Modified synthetic Bold's basal medium (BBM) without

vitamins was used as a control medium for the trials and for the preparation of the inoculum in accordance with the Culture Collection of Algae and Protozoa instructions (Anonymous 2024). The following reagents were prepared in concentrated stocks and used at the following final concentrations: $NaNO_3$ 0.25 g L^{-1}, $CaCl_2 \times 2H_2O$ 0.0025 g L^{-1}, $MgSO_4 \times 7H_2O$ 0.0075 g L^{-1}, $K_2HPO_4 \times 3H_2O$, 0.0075 g L^{-1}, KH_2PO_4 0.0175 g L^{-1}, NaCl 0.0025 g L^{-1}, and by adding 3 mL of micronutrient solution prepared separately with following concentrations: Na_2EDTA 1.50 g L^{-1}, $FeCl_3 \times 6H_2O$ 0.194 g L^{-1}, $MnCl_2 \times 4H_2O$ 0.082 g L^{-1}, $ZnCl_2$ 0.010 g L^{-1}, $CoCl_2 \times 6H_2O$ 0.004 g L^{-1}, $Na_2MoO_4 \times 2H_2O$ 0.008 g L^{-1}. The vitamin solution was not added as provided in the recipe, as preliminary media assessment showed the *C. chongqingensis* growth was not affected by it (data not shown).

Additionally, BBM supplemented with lactose was used for mixotrophic and heterotrophic growth in the defined medium. The preparation of sterile media mixtures was described in previous work (Kolesovs et al. 2025a). Briefly, concentrated reagent mixtures were sterilized by filtering through a 0.22 µm syringe filter, and the final concentration was achieved by addition to autoclave-sterilized (121 °C for 15 min at 1.2 atm) deionized water or lactose solution. The pH was adjusted to 7.0 ± 0.1 using 0.1 M HCl or 0.1 M NaOH. For the assessment of culture purity after experiments, a version of BBM with agar was prepared by adding 15 g L^{-1} bacteriological agar and 5 g L^{-1} D-glucose.

Two types of dairy by-products, i.e., cheese (sweet) whey and cheese whey permeate, were obtained from local dairy product manufacturer JSC "Smiltenes Piens". Whey permeate was produced by removing proteins from whey through ultrafiltration. The same batches of whey and permeate were used for all trials and stored frozen in aliquots at –20 °C. Table 1 summarizes the approximate compositions along with the analytical methods used for their determination. The first two trials used whey as the cultivation medium, whereas the final trial employed whey permeate due to the excess proteins in whey, which complicated analytical procedures.

The whey and permeate media were prepared in different concentrations by diluting the sample with deionized water (*v*/*v*). Before the autoclavation, the pH was then increased to pH 7.0 ± 0.1, avoiding significant media composition changes during the process. The sterilization procedure usually resulted in a decrease in media pH to approximately 6.6 ± 0.5.

Experimental design

The trials were divided into two parts, i.e., preliminary screening of optimal media concentrations in 96-well microplates and two larger-scale experiments in flasks for the control of multiple parameters. The inoculum was prepared statically under photoautotrophic growth conditions at 22 °C in BBM, with photon flux density of 55 ± 2 µmol m^{-2} s^{-1} provided by cold white LED lamps.

Cultivation in 96-well microplates was carried out statically for 7 days at 25 °C using a Biosan ES-20 orbital incubator-shaker (Latvia). Each well contained 200 µL of the respective medium with 5% inoculum. The cultivation was conducted under both mixotrophic (12 h light and 12 h dark cycle at photon flux density of 55 ± 2 µmol m^{-2} s^{-1}, white LED lamps) and heterotrophic conditions to determine the optimal concentrations of lactose and whey. For heterotrophic growth conditions, the microplate was covered with aluminum foil. For the 96-well microplates, the other layer was filled with deionized water to minimize the evaporation of the experimental samples (Arumugam et al. 2020).

After the determination of optimal media concentrations, the trials were carried out in 50 mL flasks with 25 mL medium and 5% inoculum (~1 × 10^6 cells mL^{-1}) in a Brunswick Scientific I26 Refrigerated Incubator Shaker (USA) at 59 ± 2 µmol photons m^{-2} s^{-1} for 12 days. For heterotrophic cultivation, the flasks were covered with aluminum foil and placed alongside the mixotrophically cultivated flasks.

An additional assessment to verify ability of *C. chongqingensis* to utilize lactose was performed by adding ampicillin and kanamycin at 0.6 mg mL^{-1} concentration each to the 80% permeate media (lactose concentration 33.08 ± 0.29 g L^{-1}) to inhibit any potential bacterial growth.

All experimental groups were examined under a light microscope after the trials to verify culture purity. Additionally, samples were cultivated on BBM agar plates supplemented with glucose to monitor potential contaminant growth. The results presented in this article were obtained using axenic *C. chongqingensis* culture.

Table 1. Composition of dairy industry by-products. Approximate parameters of cheese whey and permeate used for the assessment of *C. chongqingensis* growth

Parameter	Cheese whey	Permeate	Method
Initial pH	6.2 ± 0.1	6.2 ± 0.1	pH electrode measurement
Lactose	39.82 ± 0.48 g L^{-1}	40.01 ± 0.31 g L^{-1}	Lactose/galactose enzymatic assay kit
Galactose	0.62 ± 0.09 g L^{-1}	0.67 ± 0.06 g L^{-1}	Lactose/galactose enzymatic assay kit
Glucose	0.08 ± 0.02 g L^{-1}	0.09 ± 0.02 g L^{-1}	Glucose enzymatic kit
Total proteins	6.68 ± 0.71 g L^{-1}	0.84 ± 0.03 g L^{-1}	Bredford reagent (Kruger 1994)
Total lipids	~0.5% (*w*/*v*)	~0.5% (*w*/*v*)	Folch method (Folch et al. 1956)

Assessment of biomass synthesis

For the 96-well microplate trial, optical density (OD) measurements of the BBM groups were performed at 750 nm (Hotos et al. 2020) using a Tecan Infinite M Nano spectrophotometer (Switzerland). The protein content in the whey groups significantly affected the OD measurements in the 96-well plates; therefore, cell counting using a haemocytometer was used for assessment of microalga growth. This approach has demonstrated its suitability for the estimation of optimal whey permeate media concentrations in our previous study (Kolesovs et al. 2025b).

The biomass dry weight (DW) measurements in flask-scale trials were performed following the methodology described in a recent study by Kolesovs et al. (2025a). Initially, the evaporated volume (usually 5 to 8 %) was adjusted by adding deionized water to the media. Then, a 10 mL sample was taken and washed three times by centrifugation and rinsed with water. An additional step was added for both whey and permeate media to remove excess proteins as described by Kolesovs et al. (2025b). Briefly, the pH of the sample was decreased to approximately pH 4 using 1 M HCl and put into a 36 °C water bath for 20 min. Then the sample was filtered through a cotton filter and rinsed with deionized water, which allowed the passing of microalgal cells but not the coagulated protein. Then the biomass was treated similarly to the BBM samples. Dry weight (g L^{-1}) was measured gravimetrically after drying samples at 105 °C, and dry biomass productivity (g L^{-1} day^{-1}) was calculated by dividing the dry weight by the cultivation period (in days).

Sugar uptake

For the flask-scale trials, the analyses of sugar uptake were conducted using a lactose/galactose assay kit (rapid) and a D-glucose assay kit (glucose oxidase/peroxidase format; Megazyme, Ireland). The 1 mL samples were taken under sterile conditions on day 0 and day 12 and stored frozen prior to the measurements. Prior to sampling, the culture volume was adjusted to the initial volume with deionized water to account for evaporation and prevent imprecise measurements. Additionally, the initial concentrations of lactose, glucose, and galactose of whey and permeate were evaluated using the same assay kits (Table 1).

β-galactosidase assay

The enzyme activity was determined to confirm the presence of β-galactosidase in the biomass (intracellular) and in the media (extracellular) by implementing the ortho-nitrophenyl-β-galactoside (ONPG) reaction at the final day of cultivation, as described in detail by Bentahar et al. (2019). For the intracellular enzyme activity, a 5 mL sample was centrifuged at 4 000 rpm for 2 min, rinsed once with deionized water, and repeatedly centrifuged. A 0.5 mL supernatant sample after the first centrifugation was used for the extracellular enzyme activity measurements. The intracellular enzyme activity was expressed in U g^{-1}, i.e., the normalized enzyme activity per gram of dry *C. chongqingensis* biomass, while the total extracellular β-galactosidase activity in the medium was calculated in U L^{-1}, representing the volumetric enzyme activity.

Data statistical analysis

All experiments were conducted with at least five biological replicates. For analytical procedures, i.e., sugar uptake, enzyme activity, samples from at least four biological replicates were used. The obtained data were analyzed using IBM SPSS Statistics for Windows (IBM Corp., 2023, Version 29.0.2.0 Armonk, NY: IBM Corp). Normality and homogeneity of variance were initially assessed, followed by one- or two-way analysis of variance, depending on the dataset. The changes in sugar concentrations between initial and final cultivation days were assessed by a paired sample t-test. The significance level of $p = 0.05$ was used for the analysis. The error bars in the figures represent the 95% confidence intervals.

Results

The initial screening of axenic *C. chongqingensis* isolate in 96-well microplates was conducted to estimate the optimal range of lactose and cheese whey concentrations (Fig. 1). For the assessment of *C. chongqingensis* growth in whey media, only the cell count was used (Fig. 1B), because the turbidity measurements were significantly compromised by the high protein content present in whey.

As shown in Fig. 1A, a significant increase in biomass turbidity in the model BBM groups at 750 nm was observed in both mixotrophically and heterotrophically cultivated *C. chongqingensis* compared to negative control groups, with the highest OD in the BBM with 10 g L^{-1} lactose. However, the 40 g L^{-1} lactose concentration in the mixotrophic group resulted in a significant decrease in biomass synthesis compared to the photoautotrophic control ($p < 0.01$). Although there was a statistically significant increase in OD at 750 nm (Fig. 1A), the cell count for the heterotrophic group did not change compared to the negative control (Fig. 1B). This can be explained by an increase in cell diameter and accumulation of reserves inside the cells. However, the precise assessment of the cell diameter was not performed in this study.

For the whey groups, the 10, 30, and 50% concentrations resulted in lower biomass synthesis, likely due to the low initial concentration of essential nutrients (Fig. 1B). There was no statistically significant difference between the mixotrophic 70 and 90% permeate groups ($p = 1$), which resulted in the highest cell count, although it remained lower compared to the BBM control.

Based on the screening data presented in Fig. 1, a clarified range of media concentrations was selected

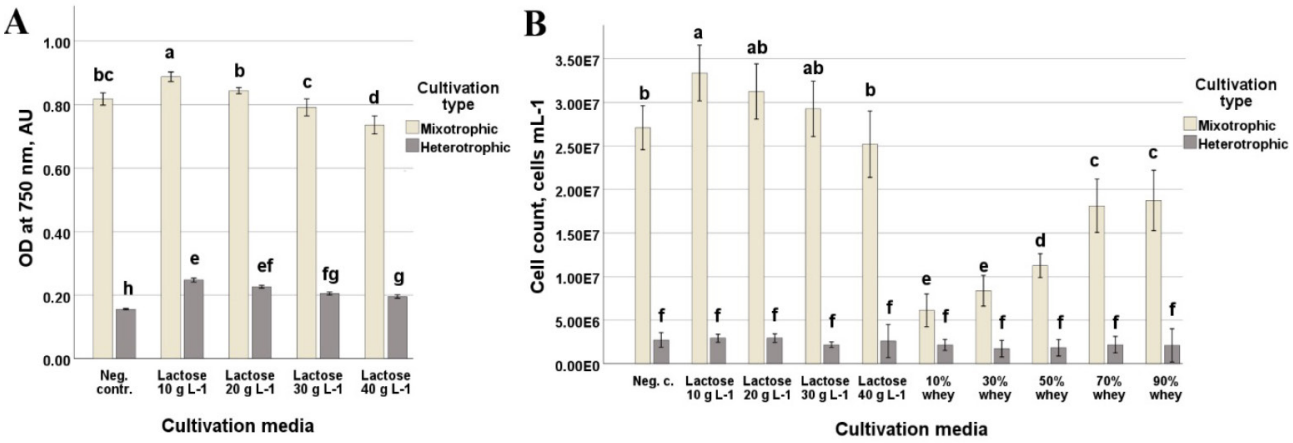

Fig. 1. Comparison of *C. chongqingensis* mixotrophic and heterotrophic growth. A, *C. chongqingensis* biomass turbidity at 750 nm after 7 days of cultivation in photoautotrophic (negative control), mixotrophic, and heterotrophic in model BBM with 10, 20, 30 or 40 g L^{-1} lactose concentrations. B, *C. chongqingensis* cell count after 7 days of cultivation in model BBM (without lactose – negative control; or supplemented with 10, 20, 30, or 40 g L^{-1} lactose) and cheese whey (10, 30, 50, 70, 90%).

for agitated mixotrophic cultivation in flasks to obtain additional information on *C. chongqingensis* performance in lactose-containing media. The assessment of biomass DW demonstrated that the *C. chongqingensis* biomass synthesis was significantly higher in all three whey media groups compared to the BBM control groups (Fig. 2). This can be associated with improved nutrient mixing and light availability under agitated cultivation conditions. Additionally, the pH changes during cultivation in whey media were minimal, reaching approximately 7.3 ± 0.2 on the final day. In contrast, the pH of BBM groups demonstrated significantly higher values at the end of cultivation, i.e., 8.6. ± 0.3 for the photoautotrophic and 9.1 ± 0.3 for the mixotrophic groups.

Among the tested concentrations, the 80% permeate resulted in a pronounced improvement in biomass synthesis, while the 95% whey medium resulted in a rapid decrease in biomass productivity, most likely due to an excessively high initial lactose concentration (37.33 ± 0.51 g L^{-1} in the 95% permeate group). Furthermore, a statistically significant increase in biomass synthesis compared to the photoautotrophic control was detected in the BBM group with 5 g L^{-1} lactose.

The high concentration of proteins in whey resulted in high variability of the DW results in the respective groups (Fig. 2) and significantly affected the intracellular enzyme activity measurements; therefore, based on the previous results the 80% whey permeate medium with significantly lower protein content (Table 1) was assessed in an additional trial under both mixotrophic and heterotrophic growth conditions. The permeate medium was prepared at 80% concentration to match the lactose content of 80% whey, as higher concentrations were found to inhibit the growth of *C. chongqingensis* (Table 1).

As shown in Fig. 3A, the biomass synthesis in the mixotrophic 80% permeate group resulted in a significant improvement of axenic *C. chongqingensis* biomass productivity up to 0.32 ± 0.01 g L^{-1} day^{-1}. The biomass synthesis in the heterotrophic permeate group, also resulted in a significantly higher biomass productivity compared to the photoautotrophic control group. Additionally, a minimal but statistically significant higher biomass synthesis compared to the negative control was detected in the heterotrophic BBM group with lactose ($p < 0.01$).

The assessment of β-galactosidase activity by the ONPG reaction demonstrated that the intracellular enzyme activity per gram of *C. chongqingensis* biomass on the 12th day was at similar levels in most experimental groups and remained below 6 U g^{-1} (Fig. 3B). The low intracellular enzyme activity

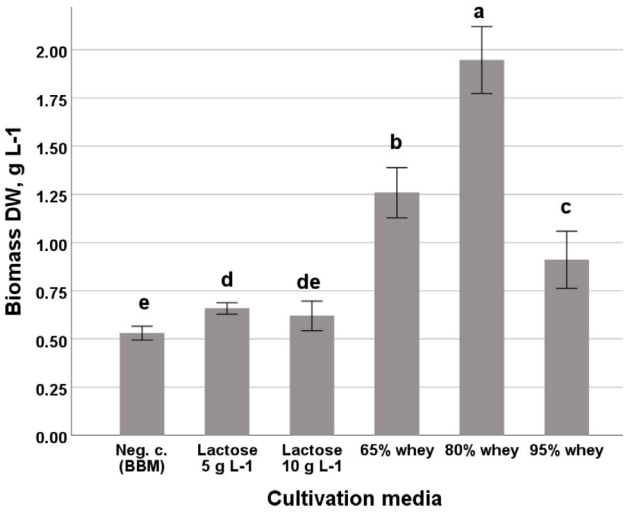

Fig. 2. Determination of optimal medium for *C. chongqingensis* biomass synthesis. *C. chongqingensis* dry biomass (DW) after 12 days of mixotrophic cultivation in BBM with lactose (5 and 10 g L^{-1}), cheese whey (65, 80, and 95%), and BBM without organic C (negative control; photoautotrophic cultivation).

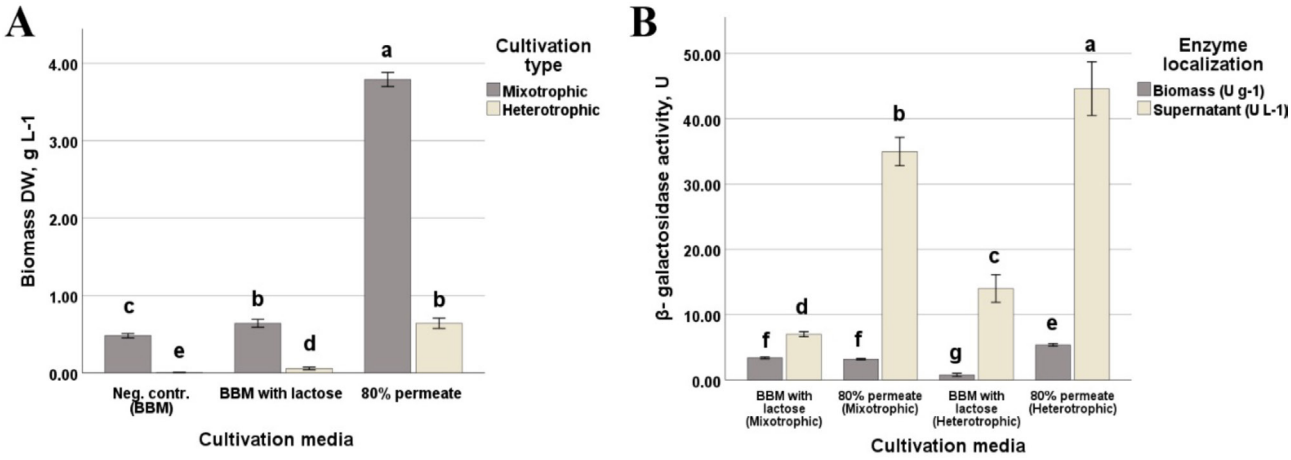

Fig. 3. Biomass synthesis and β-galactosidase activity of *C. chongqingensis* in optimized media. A, *C. chongqingensis* dry biomass (DW) after 12 days of mixotrophic or heterotrophic cultivation in 80% whey permeate medium, BBM with 5 g L^{-1} lactose and BBM without organic C (negative control). B, intracellular (U g^{-1}) and extracellular (U L^{-1}) β-galactosidase enzymatic activity in the *C. chongqingensis* biomass at the 12th cultivation day of mixotrophic or heterotrophic cultivation in 80% whey permeate medium, BBM with 5 g L^{-1} lactose.

measurements in the heterotrophic BBM group were probably compromised by the low biomass concentration of the sample. On the final day of cultivation, the permeate groups exhibited the highest extracellular enzyme activity, surpassing that of the BBM groups. This can be attributed to the previously mentioned pH stability of the permeate medium, maintaining the extracellular enzyme activity throughout the cultivation. Additionally, the heterotrophic cultivation , resulted in a significant increase in enzyme activity in the supernatant compared to the mixotrophic growth, since the microalga had to rely solely on, external organic C (Fig. 3B). However, considering the biomass productivity, mixotrophic cultivation is a more suitable option for *C. chongqingensis* growth (Fig. 3A).

The analysis of sugar concentrations indicated higher lactose reduction in the permeate groups compared to the BBM groups (Table 2). For the mixotrophic cultivation, a significant accumulation of galactose and glucose in the 80% permeate was observed, indicating that hydrolysis of the lactose occurred at a higher rate than the uptake of monosaccharides by the microalga. The final galactose concentrations indicated that this monosaccharide remained unmetabolized under mixotrophic cultivation conditions, with the microalga's growth sustained primarily by photosynthesis and glucose uptake. However, in the heterotrophic permeate group, statistically significant lactose hydrolysis was detected without the accumulation of galactose and glucose, highlighting the ability of *C. chongqingensis* to utilize all three sugars under heterotrophic growth conditions.

An additional clarification trial with galactose-supplemented BBM at 5 g L^{-1} was conducted to determine if *C. chongqingensis* can utilize galactose under mixotrophic growth conditions. Although the galactose uptake

Table 2. Changes in sugar concentration in the cultivation media. Comparison of changes in sugar concentrations in BBM with 5 g L^{-1} lactose and 80% permeate after 12 days of mixotrophic or heterotrophic cultivation of *C. chongqingensis*. * indicates a statistically significant ($p < 0.05$) increase in monosaccharide concentrations

Cultivation type	Experimental group	Sugar in the medium	Initial concentration (g L^{-1})	Final concentration (g L^{-1})
Mixotrophic	BBM with 5 g L^{-1} lactose	Lactose	4.96 ± 0.04	4.63 ± 0.02
		Galactose	0.03 ± 0.01	0.20 ± 0.01*
		Glucose	< 0.01	< 0.01
	80% permeate	Lactose	32.87 ± 0.59	28.73 ± 0.47
		Galactose	0.50 ± 0.01	1.76 ± 0.02*
		Glucose	0.06 ± 0.01	0.26 ± 0.02*
Heterotrophic	BBM with 5 g L^{-1} lactose	Lactose	4.99 ± 0.07	4.55 ± 0.09
		Galactose	0.03 ± 0.01	0.22 ± 0.07*
		Glucose	< 0.01	< 0.01
	80% permeate	Lactose	33.67 ± 0.18	30.72 ± 0.72
		Galactose	0.49 ± 0.02	0.53 ± 0.03
		Glucose	0.06 ± 0.02	0.02 ± 0.01

remained low, i.e., a decrease from 5.15 ± 0.03 to 4.86 ± 0.09 g L^{-1}, statistically significant changes in the concentrations were detected ($p < 0.05$), highlighting the ability of *C. chongqingensis* to utilize all three organic C sources under both mixotrophic and heterotrophic conditions.

The final trial was conducted with the *C. chongqingensis* in 80% permeate medium with antibiotics (kanamycin and ampicillin) to verify the ability of the axenic *C. chongqingensis* to hydrolyze lactose. The hydrolysis of 0.93 ± 0.05 g of lactose was detected, alongside the accumulation of galactose in the medium. However, the overall biomass productivity decreased significantly to 0.09 ± 0.01 g L^{-1} day^{-1}, which can be attributed to the inhibitory effect of kanamycin on the chloroplasts of the microalga (Bashir, Cho 2016). However, it should be noted that the effect of these antibiotics specifically on *C. chongqingensis* was previously unassessed.

Discussion

This is the first evaluation of axenic *C. chongqingensis* as a candidate for bioconversion of dairy industry by-products. The isolate can successfully utilize lactose in lactose-supplemented synthetic BBM and selected permeate medium (Table 2). Additionally, enzyme activity was assessed for the first time for the *C. chongqingensis* strain, expanding our knowledge on lactose utilization by microalgae. Based on the results, it can be hypothesized that under given cultivation conditions, the enzyme activity was more likely to be extracellular since the accumulation of the enzyme was observed in the supernatant and the enzymatic activity in the biomass was minimal (Fig. 3B). Additionally, the accumulation of both glucose and galactose, as summarized in Table 2, serves as an additional indication of the extracellular enzyme activity.

The biomass synthesis data highlight that mixotrophic cultivation in both lactose-supplemented BBM (5 g L^{-1}), as well as tested dairy by-products, resulted in a significant improvement in biomass productivity compared to the solely heterotrophic or photoautotrophic growth (Fig. 2; Fig. 3A). Therefore, the presence of light is required to maintain the higher biomass productivity rates for *C. chongqingensis* when cultivated in the permeate medium.

For certain microalgae, mixotrophic cultivation allows to overcome the limitations of ribulose-1,5-bisphosphate carboxylase/oxygenase, which is required for CO_2 fixation. As was demonstrated for *Chromochloris zofingiensis* ATCC30412 during mixotrophic cultivation, the glycolysis intermediates could directly enter the chloroplast, providing C for organic C metabolism while significantly decreasing chloroplast-fixed CO_2. This resulted in a more effective utilization of photosynthesis-derived energy, promoting growth of the microalga (Zhang et al. 2021). Although the transcriptome analysis of *C. chongqingensis* was not performed within this study, we can hypothesize that similar metabolic interactions may take place in the chloroplasts and cytosol during mixotrophic growth of the examined microalga.

Compared to other microalgal strains, *C. chongqingensis* has demonstrated considerable biomass productivity, reaching 0.32 ± 0.01 g L^{-1} day^{-1} in 80% permeate medium (Kolesovs, Semjonovs 2023; Ozcelik et al. 2024). For example, the cultivation of another axenic microalga, *Tetradesmus obliquus* CPCC 5, in cheese whey permeate medium resulted in the synthesis of approximately 2.19 g L^{-1} after 16 days of cultivation (Bentahar, Deschênes 2021). A similar biomass synthesis was demonstrated by *Dunaliella tertiolecta* (strain number not specified) in combined 20% (*v*/*v*) cheese whey and f/2 medium, resulting in 2.51 ± 0.01 g L^{-1} (DW) after 10 days of mixotrophic cultivation. However, in this specific study, the lactose content in 20% whey medium was approximately 6 g L^{-1}, and whey concentrations higher than 30% have demonstrated an inhibition of *D. tertiolecta* growth (Tsotsouli et al. 2025). Additionally, the *C. chongqingensis* demonstrated similar biomass productivity without any addition of synthetic medium to the permeate, while simultaneously tolerating higher lactose concentrations, i.e., up to 33.67 ± 0.18 g L^{-1} in 80% permeate medium, highlighting its potential suitability for growth in dairy industry side-streams.

Currently, the exact mechanisms involved in lactose metabolism in the microalgae remain unstudied and require detailed assessment in the future (Kolesovs, Semjonovs 2023). However, axenic *C. chongqingensis* has demonstrated the ability to utilize all three C sources under both mixotrophic and heterotrophic growth conditions. For instance, the previously mentioned *G. emersonii* MSCL 1718 did not utilize galactose under heterotrophic growth conditions in a semi-synthetic galactose-supplemented medium (Kolesovs et al. 2025a), while *Nannochloropsis oceanica* CCAP 849/10 microalga was unable to utilize lactose under heterotrophic growth conditions and required light availability for lactose utilization (Li et al. 2023). This highlights the versatile metabolism of *C. chongqingensis* as well as its potential for the removal of various organic C under different growth conditions.

The significant difference in biomass productivity between heterotrophic growth in the permeate medium and in lactose-supplemented BBM (Fig. 3A) can be attributed to the presence of multiple C sources in the permeate, primarily easily metabolizable glucose, resulting in mixed C source cultivation. In accordance with this cultivation mode, for many *Chlorophyta* microalgae, the utilization of galactose, lactose, or other less suitable C sources under heterotrophic growth conditions can be problematic, resulting in minimal or undetectable microalgae growth (Wu et al. 2024). Therefore, the addition of such C sources as glucose or acetate, which can be easily utilized by the microalgae, can stimulate the uptake of more problematic C, e.g., galactose, by providing energy or cofactors necessary

for the specific metabolic pathway (Hawkins 1999; Wu et al. 2024). It can be assumed that the glucose initially present in the permeate medium at minimal concentrations led to the initiation of *C. chongqingensis* heterotrophic growth and β-galactosidase production, enabling lactose hydrolysis and further glucose and galactose uptake throughout the cultivation (Table 2). Also, in contrast to other experimental groups, considerable galactose uptake, i.e., approximately 1.5 g as a result of lactose hydrolysis, was observed in the heterotrophic permeate group. However, considering that lactose and galactose metabolism and the respective pathways involved remain understudied for microalgae, further examination, including genome and transcriptome analyses, is required prior to making any specific conclusions.

The complex nutrient composition of whey and permeate and the improved stability of the pH provided a suitable environment for *C. chongqingensis* growth. Overall, the biomass synthesis in the tested by-products was significantly higher compared to synthetic BBM (Fig. 2; Fig. 3A). However, the permeate can be viewed as a more optimal medium for microalgae cultivation compared to whey, because the presence of proteins complicated handling of the whey media and resulted in lower biomass productivity (Fig. 2). Additionally, the shading effect of protein particles can significantly decrease light availability for the microalga. Moreover, from the industrial point of view, it would be advisable to recover proteins from whey for the production of other products prior to starting microalgae cultivation. We acknowledge that the transition from whey to permeate resulted in certain changes in the media composition besides the protein content, i.e., mainly in certain microelements, which concentrations were not evaluated in this study. Additionally, due to the heterogeneous composition of various dairy by-products, no standardized approach currently exists for microalgae cultivation on such substrates (Ozcelik et al. 2024; Tsotsouli et al. 2025).

Another significant obstacle remains for the proposed approach, which is associated with only partial lactose removal due to high initial levels of the disaccharide (Table 2) in the by-products (Ozcelik et al. 2024). Additionally, as was demonstrated during the initial screening, higher substrate dilutions significantly decrease the biomass synthesis by decreasing the availability of various critical nutrients (Fig. 1). To solve this issue, different strategies can be evaluated in future studies, for example, evaluation of other side-streams with lower lactose content, co-cultivation with other microalgae and/or lactose-utilizing microorganisms, as well as lactose enzymatic pre-treatment, which can potentially improve bioconversion of the substrate and reduce BOD and COD.

Changes in the various nutrients present in the dairy by-products were not evaluated, as the focus of this study was to assess the ability of *C. chongqingensis* to utilize lactose, produce β-galactosidase, and to conduct a preliminary evaluation of its growth on dairy by-products. However, further work must focus on the removal of other components, such as total N and P. Additionally, larger-scale trials are obligatory for assessing the feasibility of using dairy industry side-streams as a cultivation substrate for pilot- and industrial-scale microalgae production.

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