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Mixotrophic cultivation of green microalga *Graesiella emersonii* on cheese whey permeate

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Abstract. Mixotrophic cultivation of microalgae using dairy industry by-products as a cheap alternative media offers a sustainable method for dairy industry waste reduction and stimulation of value-added biomass synthesis. Such integration can support the principles of circular economy and promotes environmentally friendly practices within the dairy industry sector. The current study focuses on the mixotrophic cultivation of a newly isolated green microalga *Graesiella emersonii* MSCL 1718 strain in concentrated cheese whey permeate. It was demonstrated that *G. emersonii* can successfully grow in whey permeate significantly surpassing the biomass synthesis of photoautotrophic control group cultivated in modified Bold's Basal medium. Different concentrations of permeate were assessed to determine an optimal media preparation strategy and *G. emersonii* lactose tolerance levels. It was shown that *G. emersonii* maintained significant biomass production rates with lactose exceeding 40 g L^{-1} concentrations in the experimental media. The highest biomass productivity was observed in 20% permeate medium, reaching 0.17 ± 0.02 g L^{-1} d^{-1} (dry weight). The results of this study highlight the suitability of *G. emersonii* for further bioconversion of dairy industry by-products into value-added biomass and commercial products. However, further assessment is required to optimise the biomass production and evaluate the bioconversion rates of various nutrients. Additionally, a pilot-scale assessment of biomass production is crucial for gaining a better understanding of the process and assessing the feasibility of the proposed approach.

Key words: *Graesiella emersonii, Desmodesmus subspicatus*, dairy industry by-products, β-galactosidase, lactose, bioconversion.

Introduction

According to recent data provided by OECD & FAO (2022), the global consumption of dairy products is projected to increase by approximately 1.4% per annum over the coming decade. This also leads to higher amounts of by-products generated, which require complex treatment procedures to prevent environmental damage. Currently, dairy industry by-products remain a problematic waste due to their high organic load, mainly from lactose (the primary organic carbon (C) in these side-streams), along with high nitrogen (N) and phosphorus (P) contents (Kolev Slavov, 2017). The valorisation of dairy industry by-products can help mitigate environmental pollution and reduce the high costs of proper waste treatment while aligning with the circular economy principles (Mehner *et al.*, 2024).

Over the past decade, microalgal bioconversion of lactose-containing substrates has attracted attention as a promising approach for dairy by-product valorisation. Different studies demonstrate that certain microalgal species exhibit high biomass productivity when cultivated in such substrates (Kolesovs & Semjonovs, 2023; Ozcelik *et al.*, 2024). Microalgae are a diverse group of primarily photosynthetic microorganisms that accumulate a plethora of valuable compounds, e.g., proteins, lipids, pigments, and vitamins, in their biomass (Jacob-Lopes *et al.*, 2020). To successfully grow on dairy industry by-products and reduce the organic C content microalgae must produce β-galactosidase enzyme for lactose hydrolysis. Recent studies have shown that certain strains, e.g., *Tetradesmus obliquus* CPCC5 (Bentahar & Deschênes, 2021), *Nannochloropsis limnetica* SAG

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18.99 (Li *et al.*, 2024) *Parachlorella kessleri* UTEX 3225 (Hidasi *et al.*, 2024), can successfully produce β-galactosidase and grow in various lactose-containing media. Additionally, the utilisation of cheap alternative cultivation substrates for enhanced microalgae growth can mitigate the problem of high biomass production costs, which still prevent the commercialisation of many microalgal species (Kolesovs & Semjonovs, 2023; Ozcelik *et al.*, 2024).

Despite the recent progress on this topic the mechanisms involved in microalgal lactose utilisation remain understudied. In many cases, studies assess the microalgal biomass productivity as well as N and P changes overlooking the lactose uptake in the media, β-galactosidase activity, and the purity of examined cultures (Kolesovs & Semjonovs, 2023). Additionally, the selection of strains capable of lactose hydrolysis is problematic with a recent study demonstrating that during preliminary screening only one out of 24 strains could utilise lactose (Hidasy *et al.*, 2024). Moreover, the performance of many lactose-utilising strains remains below optimal levels, for instance, *Chromochloris zofingiensis* ATCC 30412 (formerly *Chlorella zofingiensis*) strain showed the lowest biomass synthesis in lactose-supplemented medium among six tested C sources (Liu *et al.*, 2010). This emphasises the need to select microalgal strains capable of growing in dairy industry by-products, tolerate high lactose levels, and to study the mechanisms of lactose uptake. A better understanding of these fundamental processes would support further advancements in this field.

This study focuses on the cultivation of the green microalga *Graesiella emersonii* MSCL 1718 in concentrated cheese whey permeate. Preliminary studies have demonstrated that *G. emersonii* can utilise lactose and its monomers (glucose and galactose) for growth under mixotrophic conditions in a standard medium (Kolesovs *et al.*, 2025). The performance of *G. emersonii* was compared to another green microalga *Desmodesmus subspicatus* CCALA 467 which did not exhibit properties required for growth in lactose-containing substrates.

Materials and Methods

Microalgal strains

Green microalga *Graesiella emersonii* MSCL 1718 was selected for this study based on its ability to utilise lactose both mixotrophically and heterotrophically. This strain is an airborne freshwater isolate obtained during strain selection at the Institute of Microbiology and Biotechnology (Kolesovs *et al.*, 2025). After the identification procedure, it was placed in the Microbial Strain Collection of Latvia (University of Latvia). *Desmodesmus subspicatus* CCALA 467 was obtained from culture Collection of Autotrophic Organisms (Czech Republic) based on studies demonstrating that *Desmodesmus* sp. can grow on dairy industry by-products (Abril Bonett *et al.*, 2020). Axenic cultures were obtained by using streak plate method to eliminate any potential influence of other microorganisms.

Cultivation media

Bold's Basam medium with triple nitrogen and vitamins (3N-BBM-V) was used as a control medium for the trials (Bischoff & Bold, 1963). For the heterotrophic test a sterile lactose solution (final lactose concentration 10 g L^{-1}) was added to the 3N-BBM-V. Additionally, an agar medium with 3N-BBM-V and glucose (5 g L^{-1}) was used for verification of culture purity after each experiment.

Concentrated cheese whey permeate was obtained from JSC 'Smiltenes piens' (Smiltene, Latvia). The composition of permeate is provided in Table 1. Same batch of permeate was used for all trials and was stored frozen at –20 °C in aliquots to prevent significant changes in its composition.

Table 1

Composition of concentrated cheese whey permeate

Parameter	Method	Contents
Dry mater	GOST 3626-73	18%
Total carbohydrates	ISO 9622:2013	~17%
- Lactose	K-LACGAR kit	~160 g L^{-1}
- Glucose	K-GLUC kit	0.3 g L^{-1}
- Galactose	K-LACGAR kit	2.5 g L^{-1}
Total proteins	ISO 9622:2013	~1%
Total lipids	ISO 9622:2013	0.02%
pH	LVS ISO 10523:2008	6.3 ± 0.1

For the trials, the permeate media were prepared at final concentrations of 20, 40, 60, and 80 % for the first experiment and 5, 10, 20, 30% for the second cultivation (permeate diluted with distilled water). The pH was regulated to 7.0 ± 0.1 using 1 M NaOH prior to sterilisation to prevent significant media changes. Permeate media sterilisation was performed to exclude the growth of any potential microorganisms. Additionally, a 10% permeate medium sample was sterilised and kept at room temperature (22 °C) to assess any development of microorganisms. Subsequently, no microorganisms were detected at 10th day.

Experimental design

The inoculum for each experiment was prepared under photoautotrophic growth conditions for seven days (50 µmol photons m^{-2} s^{-1}, cold white LED light) in 3N-BBM-V. To establish the suitability of whey permeate for growth and to determine the approximate permeate concentrations that microalgae tolerate, *G. emersonii* and *D. subspicatus* cultures were cultivated mixotrophically in sterile 96-well microplates with 10% inoculum. Additionally, the heterotrophic growth test in 3N-BBM-V with lactose at 10 g L^{-1} or 3N-BBM-V without added organic C as negative control was also conducted in 96-well plates. The outer layer of each 96-well microplate was filled with distilled water to minimise evaporation effect of the samples as described by Arumugam *et al.* (2020). The microplates were cultivated statically at 25 °C in an incubator (Orbital incubator-shaker ES-20, Biosan, Latvia) for seven days.

Due to the suitability of *G. emersonii* for cultivation in permeate media, a larger-scale trial was conducted under agitated mixotrophic cultivation conditions in 100 mL Erlenmeyer flasks with 50 mL of culture volume, using an orbital incubator-shaker (Brunswick Scientific I26 Refrigerated Incubator Shaker, USA). The cultivation parameters were kept at 120 rpm and 12:12 daylight cycle maintained by cold white LED lamps with light intensity ~55 µmol photons m^{-2} s^{-1}, initial *G. emersonii* cell count 5 × 10^5 cells mL^{-1}, and the cultivation time extended to 10 days. The concentrated *G. emersonii* inoculum was added at 5% to prevent any significant changes in the permeate media composition (Kolesovs *et al.*, 2025).

Biomass productivity

Assessment of microalgal cell count was performed using the Goryaev chamber to evaluate the changes in the 96-well microplates. Cell count assessment was selected over optical density (OD) measurements because permeate medium introduces a significant error to the OD measurements.

Prior to measurements, the media volume was adjusted with distilled water to its initial level to account for evaporation. For the flask experiment a 20 mL biomass sample was taken from each flask and washed with centrifugation at 5,500 rpm for two minutes (Kolesovs *et al.*, 2025). To remove proteins from the permeate samples, an additional step was introduced after the first centrifugation. The supernatant was discarded, and 20 mL of water was added to resuspend the biomass with the remaining proteins. The pH was then lowered to 4.1 ± 0.1 using 1 M HCl, and the sample was placed in a water bath at 36 °C for 20 minutes to coagulate the remaining proteins. Following this, the suspension was filtered using a funnel and cotton wool filter and rinsed with water. This process resulted in the coagulated proteins attaching to the cotton wool, while the microalgal biomass passed through, significantly improving the precision of the DW measurements. Subsequently, the samples were rinsed two more times with distilled water to remove any residual media components. The biomass samples were dried in pre-weighted weighting bottles and calculated as DW g L^{-1} and biomass productivity g L^{-1} d^{-1} by dividing the DW with 10 cultivation days.

Changes in organic C and enzyme activity

Analysis of organic C was carried out using enzymatic kits K-LACGAR for lactose and galactose, as well as K-GLUC for glucose measurements in full accordance with manufacturer's guidelines (Megazyme, Ireland). Additionally, β-galactosidase activity analysis was performed on the final day of cultivation in accordance with the methodology described by Bentahar & Deschênes (2021).

Statistical analysis

Data collected from the trials were analysed IBM SPSS Statistics for Windows (IBM Corp., 2023, Version 29.0.2.0 Armonk, NY: IBM Corp), with one-way analysis of variance followed by Bonferroni post-hoc test for DW and β-galactosidase analysis and paired-sample T-test for changes of C concentrations at $p = 0.05$ significance level. Microalgal cultivation was performed with at least five biological replicates for each experimental group. Analytical procedures were conducted in at least four replicates.

Results and Discussion

The results of the microalgae screening are summarised in Table 2. For *G. emersonii* the 20% permeate medium resulted in higher biomass synthesis compared to photoautotrophic control medium. The screening demonstrated that permeate concentrations lower than 40% could potentially serve as a suitable medium for *G. emersonii* growth. On the other hand, *D. subspicatus* demonstrated the highest biomass synthesis under photoautotrophic cultivation conditions in the 3N-BBM-V negative control group (Table 2).

To clarify the strain's ability to grow in lactose-containing medium another trial was conducted with *D. subspicatus* under heterotrophic growth conditions using 3N-BBM-V without lactose (negative control) and 3N-BBM-V with 10 g L^{-1} lactose. The results indicated that no biomass synthesis was detected after 7 days of cultivation in both media with cell count remaining at initial levels of 1 × 10^6 cells mL^{-1}, while the *G. emersonii* cell count tripled from 1 × 10^5 to ~3 × 10^5 cells mL^{-1} under the same cultivation conditions. This allows to assume that *D. subspicatus* may be incapable of utilising lactose. Therefore,

Table 2

Changes in cell count of *G. emersonii* and *D. subspicatus* after 7 days of mixotrophic cultivation in whey permeate

Microalga	Group	Initial cell count, cells mL^{-1}	Average cell count, cells mL^{-1}
G. emersonii	3N-BBM-V (neg. control)	7.0×10^5	7.0×10^6
	20% permeate		**2.4×10^7**
	40% permeate		4.2×10^6
	60% permeate		$<1.0 \times 10^5$
	80% permeate		$<1.0 \times 10^5$
D. subspicatus	**3N-BBM-V (neg. control)**	1.3×10^6	**3.8×10^7**
	20% permeate		5.6×10^6
	40% permeate		5.1×10^6
	60% permeate		2.1×10^6
	80% permeate		$<1.0 \times 10^5$

the minimal growth of *D. subspicatus* in permeate may have been sustained by photosynthesis and mixotrophic utilisation of glucose present at 0.05 – 0.1 g L^{-1} concentrations in the media. Although it should be noted that the results for *D. subspicatus* were not fully conclusive, i.e., *D. subspicatus* was not tested in standard medium with lactose as main C under mixotrophic growth conditions and changes in lactose concentrations were not evaluated.

Based on the preliminary screening, *G. emersonii* was selected as a suitable candidate for growth in the permeate medium. Considering the limitations of 96-well microplates a larger-scale experiment was conducted for *G. emersonii* in 100 mL flasks with 50 mL of experimental media with 5, 10, 20, 30 % permeate. As shown in Figure 1, *G. emersonii* biomass synthesis was significantly higher in permeate media compared to the photoautotrophic 3N-BBM-V control. Among examined permeate groups the highest biomass DW was detected in the 20% permeate medium (1.69 ± 0.15 g L^{-1}; $p < 0.001$), with biomass productivity reaching 0.17 ± 0.02 g L^{-1} d^{-1}. Among tested concentrations the cultivation in 30% permeate medium has resulted in *G. emersonii* growth inhibition probably due to high initial lactose concentration (46.31 ± 0.46 g L^{-1}). However, biomass synthesis in the 30% permeate medium was still maintained at a higher rate compared to photoautotrophic growth reaching 0.07 ± 0.01 and 0.04 ± 0.01 g L^{-1} d^{-1}, respectively.

In literature the performance of microalgae varies greatly among different strains and lactose-containing substrates (Ozcelik *et al.*, 2024). In this study *G. emersonii* biomass synthesis was comparable

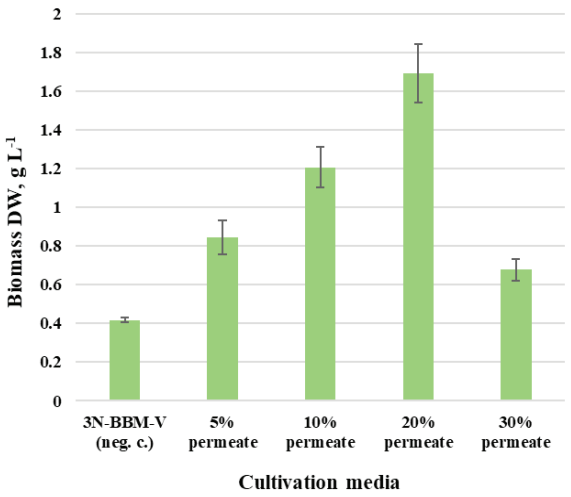

Figure 1. *G. emersonii* biomass DW after 10 days of agitated cultivation in the 3N-BBM-V negative control medium (photoautotrophic cultivation without organic C) and mixotrophic cultivation using 5, 10, 20, or 30 % whey permeate media.

to recently described lactose-utilising *N. limnetica* SAG 18.99 that produced 1.36 g L^{-1} (DW) after 8 days of mixotrophic cultivation in whey medium. It should be noted that a portion of this biomass was formed by native/phycospheric bacteria that was introduced together with *N. limnetica* inoculum (bacteria accounting to ~20% of total cells at the end of cultivation), which weren't dominant in the culture and probably formed symbiotic relationship with the microalga (Li *et al.*, 2024). Formation of similar phycosphere for *G. emersonii* could potentially

enhance the growth and increase the culture's tolerance to contamination by other microorganisms. However, another recently isolated microalga *P. kessleri* UTEX 3225 demonstrated 1.53 g L^{-1} d^{-1} biomass productivity under heterotrophic growth in modified BBM with 10 g L^{-1} lactose and 0.33 under mixotrophic growth in skimmed buttermilk wastewater (Hidasi *et al.*, 2024). This emphasises the need for further selection of best performing strains for improved performance in dairy by-products.

Changes in organic C concentrations in the experimental media are summarised in Table 3. The analysis of glucose concentrations demonstrated that the monosaccharide was detected at minimal levels (<0.1 g L^{-1}) in all experimental groups at both the start and the end of cultivation. Lactose was also hydrolysed with the highest uptake detected in the 20% permeate group (3.88 ± 0.52 g L^{-1}). This is also linked to β-galactosidase activity being significantly higher in the 10% and 20% permeate groups compared to the other groups (no statistically significant difference between 10% and 20% permeate media; $p = 0.77$). However, the enzyme activity was low in all groups at the 10th day and a more detailed assessment in further studies is required. Additionally, the lactose removal from permeate during the cultivation was only partial, i.e., 5 – 16 % based on the group, which is associated with the high initial lactose content in the media (Table 1).

A statistically significant increase in galactose concentration was observed in all experimental groups (Table 3) which is a consequence of lactose hydrolysis by *G. emersonii* and an indication that glucose is a preferable C source.

The accumulation of galactose in the supernatant can hypothetically be associated with intracellular lactose hydrolysis, when glucose is metabolised while some portion of galactose is excreted into the medium (Kolesovs *et al.*, 2025). Additionally, the formation of exopolysaccharides (EPS) can occur during cultivation of microalgae in dairy by-products. For example, *Porphyridium purpureum* (strain unspecified) produces EPS potentially as a mechanism for tolerating high lactose concentrations in the medium (Gălan *et al.*, 2022). This study did not investigate the potential formation of EPS by *G. emersonii* in whey permeate medium.

Further studies are required to improve *G. emersonii* growth in dairy side-streams. Modification of permeate, i.e., supplementation with trace elements or lactose pre-hydrolysis with commercial β-galactosidases, can potentially enhance biomass productivity. Additionally, other dairy industry side-streams should be evaluated, and the removal of N and P should also be assessed. Moreover, use of specialised equipment such as bioreactors can provide better cultivation condition stimulating the nutrient uptake. Furthermore, biomass production at a pilot-scale is essential for developing a better understanding of the process and evaluating the feasibility of the proposed approach.

The stability of pH values in permeate medium throughout the cultivation has been of particular interest to us. The changes observed in the permeate media, which remained close to the initial pH range of 6.5 – 7.0 throughout cultivation, indicate its buffering capacity. In comparison, cultivation in the 3N-BBM-V resulted in a gradual increase of pH (up to pH 8.5 ± 0.21). Another useful characteristic is the biomass flocculation effect observed in the permeate medium. After the agitation was stopped it took approximately 20 – 30 minutes for biomass to sediment compared to

Table 3

Changes in organic C concentrations after 10 days of *G. emersonii* cultivation in 5, 10, 20, 30 % permeate media. Glucose concentrations are not displayed due to insignificant concentrations in all experimental groups (below 0.1 g L^{-1}). An increase in galactose concentrations marked with*

Group	C in medium	Initial C, g L^{-1}	Final C, g L^{-1}	Δ C, g L^{-1}	β-galactosidase (10th day), U L^{-1}
5% permeate	Lactose	9.07 ± 0.02	7.61 ± 0.29	1.46 ± 0.26	3.66 ± 0.35
	Galactose	0.14 ± 0.01	0.71 ± 0.03	0.57 ± 0.03*	
10% permeate	Lactose	17.23 ± 0.23	15.02 ± 0.12	2.21 ± 0.24	6.73 ± 0.53
	Galactose	0.23 ± 0.02	0.79 ± 0.03	0.56 ± 0.02*	
20% permeate	Lactose	38.21 ± 0.39	34.33 ± 0.15	3.88 ± 0.52	7.67 ± 0.42
	Galactose	0.44 ± 0.02	0.98 ± 0.04	0.54 ± 0.05*	
30% permeate	Lactose	46.31 ± 0.46	44.24 ± 0.52	2.07 ± 0.83	2.33 ± 0.31
	Galactose	0.50 ± 0.05	0.78 ± 0.03	0.28 ± 0.04*	

significantly longer period for 3N-BBM-V medium. This can prove useful for larger-scale cultivation because efficient biomass harvesting technique (flocculation, concentration) is another factor that complicates the commercial production of microalgae (Deepa *et al.*, 2023).

The *G. emersonii* biomass can be potentially used in food industry as a sustainable source of proteins, lipids and pigments (Sawant Desai & Kelkar Mane, 2018). The effect of cultivation in whey permeate on the biomass composition was not assessed during this study. Future research should include a comprehensive biomass assessment, including fatty acid profiling and pigment composition analysis. Additionally, optimising media parameters are essential to enhance the production of bioactive compounds.

Conclusions

G. emersonii is capable of successfully growing in concentrated cheese whey permeate, with an optimal permeate concentration of 20%, resulting in a biomass productivity of 0.17 ± 0.02 g L^{-1} d^{-1}. The microalga exhibited tolerance to high lactose concentrations, achieving its highest biomass productivity at lactose levels of 17 – 38 g L^{-1}. However, lactose concentration above 40 g L^{-1} resulted in inhibition of *G. emersonii* biomass synthesis. Overall, *G. emersonii* can be viewed as a potential candidate for bioconversion of dairy industry by-products.

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