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Cheese whey wastewater-derived Chlorella biomass promotes growth and β-galactosidase activity of Pediococcus sp. isolated from Gundurk

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Research Article

Keywords: Valorization, Microalgae, Lactic acid bacteria, Fermentation, Co-cultivation

Posted Date: August 31st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10465535/v1

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Additional Declarations: No competing interests reported.

Abstract

Background

Cheese whey wastewater (CWW), a nutrient-rich dairy waste, poses significant environmental concerns because of its high organic load. Valorizing such waste streams through microalgae cultivation provides a sustainable avenue for generating functional biomass for food biotechnology applications. Lactic acid bacteria (LAB) are used as starter cultures, probiotics, and microbial cell factories. Supplementing the LAB culture medium with microalgae may enhance both the growth and functional properties of these bacteria. In this study, we explored the effects of freshwater *Chlorella* sp. biomass on the growth and functional properties of *Pediococcus* sp., isolated from the traditional fermented food (*Gundruk*) of Nepal.

Results

Chlorella cultivated in standard Bold's basal medium (BBM) or 20% (v/v) CWW medium presented comparable growth rates, with cell densities of 1.59×10^7 and 1.73×10^7 cells/mL, respectively, on day 8. Fourier-transform infrared (FTIR) spectroscopy revealed an increase in lipid-associated peaks (2970 – 2850 cm^{-1}) in the biomass grown in CWW medium. Compared with 18.34 Miller units in the control de Man, Rogosa, Sharpe (MRS) medium, supplementation with *Chlorella* biomass significantly increased the growth of *Pediococcus* sp. and increased β-galactosidase activity to 49.33 and 38.82 Miller units when supplemented with BBM-cultured and CWW-cultured *Chlorella* biomass, respectively.

Conclusion

These findings demonstrate that microorganisms originating from distinct ecological niches can be used in a unified system for waste valorization and functional fermentation. CWW supports the cost-effective cultivation of *Chlorella* and provides functional biomass that enhances LAB performance. This finding highlights the potential of combining freshwater microalgae with beneficial bacteria from traditional fermented foods to support the bioremediation of dairy wastes and enhance the development of functional foods.

Background

Microalgae are unicellular photosynthetic microorganisms that produce highly valuable compounds, including proteins, lipids, carotenoids, vitamins, minerals, and polysaccharides, with significant applications in food, feed, pharmaceuticals, cosmetics, and biofuels [1] [2]. Among these, *Chlorella* is one of the most studied and largest microalgal genera used as a dietary supplement due to its high protein (55–65% of the cell dry weight), polysaccharides (25%–35%), chlorophyll (≤ 5.5%), and micronutrient content [3]. Additionally, bioactive compounds of *Chlorella* have demonstrated antimicrobial, antioxidant,

anti-inflammatory, and immunomodulatory effects, further supporting its role in health-promoting applications [2]. However, the biochemical composition varies depending on the source of isolation and cultivation conditions [4]. Yang et al. (2016) investigated three *Chlorella* strains from different geographical regions - arctic glacier, desert soil, and temperate lake and showed differences in lipid content, fatty acid profiles, and growth responses to temperature fluctuations, suggesting wild strains' biochemical composition differs based on the condition they grow in [5]. Thus, the potential of freshwater *Chlorella* sp. from diverse geographic regions should be further explored.

Cost-effective cultivation remains a barrier for large-scale microalgae production [6]. Cheese whey, a byproduct of the dairy industry rich in lactose, fat, protein, and minerals, may have a chemical oxygen demand (COD) of 50–80 g/L and a biological oxygen demand (BOD) of 40–60 g/L. When released into the environment as dairy wastewater, it may pose serious environmental and health risks. Proper management and treatment of this wastewater are crucial for reducing and addressing these problems [7] [8]. Nevertheless, microalgae cultivation in dairy wastewater offers a sustainable strategy for nutrient recovery, biomass production, and COD and BOD levels management [9]. The mixotrophic cultivation method, in which organisms utilize inorganic carbon through photosynthesis and organic carbon through assimilation, has emerged as a sustainable and effective approach for microalgae cultivation [10] [11]. Microalgae cultivation in dairy waste can offer dual benefits: nutrient recovery and cost-effective biomass cultivation. *Chlorella* sp. cultured in 40% (v/v) pretreated whey had biomass and lipid yields of 4.50 g L^{-1} and 1.80 g L^{-1}, respectively [12]. Furthermore, *Tetraselmis chuii* eliminated 98% of phosphorus and 93% of nitrogen from dairy waste, highlighting the role of light intensity and organic substrate in microalgal productivity [13]. When *Chlorella* sp. was cultured under non-sterile conditions, dairy wastewater produced 0.9 g L^{-1} of dry biomass in 6 days, with 84% COD removal [14], highlighting its potential beyond the controlled laboratory setting.

Lactic acid bacteria (LAB) are gram-positive, non-sporulating, facultative anaerobes that play a crucial role in food fermentation and human health [15]. Traditional fermented foods are considered reservoirs of diverse LAB that have been shaped by centuries of selective ecological pressure within microbial communities [16]. *Gundruk*, a non-salted, plant-based, fermented vegetable traditionally consumed by Nepalese communities in Nepal and in some parts of India, is prepared from *Brassica* species, including mustard, radish, and cauliflower leaves [17]. *Lactobacillus brevis*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici* are considered the dominant bacteria in these fermented vegetables, which are acid- and salt-tolerant and have diverse enzymatic repertoires that might lag in laboratory-maintained strains [18][19]. Among these, *Pediococcus* sp., a homofermentative LAB, contributes to fermentation through lactic acid production, pathogen inhibition, and enzymatic activity, including β-galactosidase, which catalyzes the hydrolysis of lactose into glucose and galactose[20][21]. These characteristics make this isolate relevant to functional dairy product development, as 65% of the adult population worldwide experiences lactose intolerance, making LAB strains with β-galactosidase activity a target of nutritional and industrial interest [22].

Microalgal biomass supplementation has been shown to improve bacterial growth, enzymatic activity, and the nutritional quality of fermented foods [23]. There is evidence showing *Chlorella vulgaris* at 0.1–1.5% (w/v) shortened log phase and increased lactic acid yield and enzyme activity (α-galactosidase, α-glucosidase and aminopeptidase) in the culture of *Lactobacillus brevis* [24]. Similarly, fermentation of *Arthrospira platensis* by *L. plantarum* resulted in higher LAB counts, a higher lactic acid concentration, and enhanced antioxidant activity and phenolic content [25]. Another study showed that the use of 0.25% and 0.5% of *Haematococcus pluvialis* biomass, along with pigment astaxanthin, increased the protein content and proliferated the growth of *Liquorilactobacillus nagelii*, *Lactobacillus hordei* and *Lactococcus lactis* by 5.8 times, 15 times and 13 times, respectively, highlighting its potential as an alternative probiotic source [26].

Despite advances in microalgae-based wastewater treatment and LAB supplementation for fermented foods, little is known about the functional impact of CWW-grown *Chlorella* biomass, as most studies have focused on general dairy wastewater. Additionally, most studies examining microalgae-LAB interactions focus on laboratory-maintained strains, thereby neglecting the potential of wild isolates. These wild *Chlorella* isolates from freshwater and LAB from fermented foods may exhibit functional versatility, as evidenced by differences in stress tolerance and metabolic composition. This study explores the use of wild strains (microalgae and LAB) isolated from freshwater ponds and traditional fermented foods, highlighting the potential of locally sourced microorganisms for sustainable bioprocessing. The current study addresses this gap by investigating (1) the growth performance of wild *Chlorella* sp. in CWW versus BBM; (2) the biochemical composition of the resulting biomass as characterized by FTIR spectroscopy; and (3) the effects of biomass supplementation on the growth and enzymatic activity of *Pediococcus* sp. The findings provide insights into the dual benefits of waste valorization and functional food enhancement, with implications for underexplored microbial ecosystems.

Materials and Methods

Screening, Isolation, and Identification of Chlorella sp.

Water samples were collected from a freshwater pond in Banepa, Nepal, in sterile 15 mL Falcon tubes. Each sample tube was washed with distilled water and BBM, respectively, then resuspended and observed under a microscope to confirm live microalgae cells. Petri plate cultures from the washed sample were done in 2% (w/v) bacteriological agar (Hi-Media) in BBM and incubated in a growth chamber at 25 ± 2°C for 2–3 weeks under cool daylight fluorescent tube lights (Bajaj Electricals) at an illumination of 3500–4000 Lux under an 18:6 h light-dark cycle. The isolated sample was identified based on its morphology and dimensions, as described by [27] [28] [29].

Cheese whey wastewater (CWW) preparation and Chlorella cultivation

Cheese whey wastewater (CWW) sample was obtained from Himal Farm (Satdobato, Lalitpur, Nepal) and transported to the laboratory in sterile vessels. An official letter from the Research Institute for

Bioscience and Biotechnology (RIBB) was submitted and approved by the farm management, following the written permission to collect and use the CWW sample for research purposes. CWW sample was centrifuged at 4000 rpm for 10 min to remove milk fat, and the supernatant obtained was autoclaved at 121°C for 15 min [30]. Residual precipitates in autoclaved CWW were removed by filtration. Autoclaved and filtered CWW diluted to 20% (v/v) using autoclaved distilled water and pH adjusted to 5.1–5.9 was used as a cultivation medium (determined with preliminary experiments, (Additional File 1: Figure. S1)), hereafter referred to as CWW medium. *Chlorella* sp. was cultivated in standard BBM and CWW medium using 250 mL (seeding culture) and 1 L conical flasks. The cultures were grown in a growth chamber at 25 ± 2°C under cool daylight fluorescent tube lights (Bajaj Electricals) at an illumination of 3500–4000 Lux under an 18:6 h light-dark cycle [31].

Growth monitoring and Biomass analyses

Growth of *Chlorella* sp. was monitored by measuring the Optical Density (OD) at 750 nm using a microplate reader (BioTek Epoch 2) and by performing cell counts on a Neubauer hemocytometer [32]. Cultures were simultaneously screened for microbial contamination (bacteria, fungi) and algal predators (e.g., rotifers). On day 8, culture biomass was harvested by centrifugation at 4000 rpm for 5 min, washed twice with sterile distilled water, and oven-dried at 60°C for 6–8 h [33]. The dried biomass was stored in airtight vials at -20°C for subsequent experiments.

FTIR analysis of Chlorella sp.

Dried biomass of *Chlorella* sp. obtained from BBM and CWW cultures was analyzed by Fourier-transform infrared (FTIR) spectroscopy. Approximately 1 mg of the dry biomass was mixed with 100 mg of dry Potassium bromide (KBr) powder and thoroughly triturated using a mortar and pestle to form a homogeneous mixture. This mixture was then compressed into a transparent disc using a hydraulic press. A background scan of a pure KBr disc was first recorded to perform baseline correction. The prepared sample discs were analyzed using a Shimadzu IR Tracer-100 spectrophotometer in the mid-infrared region (4000–400 cm^{-1}), and spectra were recorded [34].

Screening, Isolation and Characterization of Lactic Acid Bacteria (LAB)

LAB were isolated from traditionally fermented broadleaf mustard (*Brassica rapa*), locally known as *gundruk*, using the de Man, Rogosa, and Sharpe (MRS) medium (HiMedia, India). Approximately 40–50 g of *gundruk* from Pimbahal, Lalitpur (hereafter referred to as LpG) was collected in sterile zip-lock bags, transferred aseptically to the laboratory, and stored at -20°C until further use. Detailed metadata, including sample name, ethnic community, substrate type, fermentation duration, fermentation process, and date of manufacture, were recorded using a structured questionnaire (Additional File 2). 5 g of *gundruk* was homogenized in 45 mL of sterile 1X Phosphate-Buffered Saline (PBS) and shaken for 15 min. Serial dilutions were prepared, plated onto MRS agar, and incubated anaerobically at 37°C for 48 h

[35]. Isolates were subjected to standard morphological and biochemical characterization, including Gram staining, catalase, oxidase, and Sulphide Indole Motility (SIM) test [36]. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega), and the 16S rRNA gene was amplified using universal primers 27f (AGAGTTTGATCMTGGCTCA) and 1525r (AAGGAGGTGWTCCARCC). Amplicons were sequenced by Sanger sequencing (Strand Life Sciences, India), and the resulting sequences were analyzed using the isolateR and sangerseqR packages in RStudio (v4.4.3).

Growth characteristics of Pediococcus sp. supplemented with Chlorella dry biomass

The growth of *Pediococcus* sp. was evaluated in standard MRS medium and in modified MRS media supplemented with 1% (w/v) dry *Chlorella* biomass obtained from BBM and CWW cultures (Additional File 1: Figure. S2, Figure. S3, Table S1, Table S2). Serial dilutions were prepared by mixing 90 µL of PBS with 10 µL of *Pediococcus* broth culture, and dilutions up to 10^{-8} were spread onto sterile MRS agar plates (10 µL per plate). The plates were incubated at 37°C, and spot plate colony counting was conducted at 0, 6, 12, 18, 24, and 48 h. Colonies within countable spots were enumerated, and microbial load was calculated using Eq. (1).

$$\text{CFU/mL} = \frac{(Number\ of\ colonies \times\ Dilution\ factor)}{Volume\ (mL)} \quad (1)$$

β-galactosidase assay

The β-galactosidase activity of *Pediococcus* sp. was assessed in MRS and modified MRS media using Ortho-Nitrophenyl-β-D-Galactopyranoside (ONPG) as the substrate. Cell suspensions (0.4 mL) were washed twice with 1× PBS (pH 7.0) and resuspended in 0.6 mL Z-buffer (0.06 M $Na_2HPO_4{\cdot}7H_2O$, 0.04 M $NaH_2PO_4{\cdot}H_2O$, 0.01 M KCl, 0.001 M $MgSO_4{\cdot}7H_2O$, and 0.05 M β-mercaptoethanol, pH 7.0). Absorbance at 600 nm was recorded against the Z-buffer as a blank. Cells were permeabilized by adding 100 µL chloroform and 50 µL 0.1% SDS, vortexing for 60 s, and incubating at 37°C for 5 min. The reaction was initiated by adding 0.2 mL ONPG (4 mg/mL) and incubated at 37°C for 1 h. The reaction was terminated by 0.5 mL of 1 M Na_2CO_3, and samples were centrifuged to remove debris. Absorbances were recorded at 420 nm and 550 nm [21]. Enzyme activity was expressed as Miller units using the Eq. (2):

$$\text{Miller Units} = \frac{1000 \times\ [OD_{420} - (1.75 \times\ OD_{550})]}{t \times\ v \times OD_{600}} \quad (2)$$

Where OD_{420} and OD_{550} represent reaction absorbances, OD_{600} the cell density, t the incubation time, and v the reaction volume.

Statistical analysis

Experiments were done in triplicate, and results were presented as mean ± standard deviation. Two-way ANOVA (factors: medium and time interval) was applied to evaluate the growth response (n = 6), and

one-way ANOVA was applied for β-galactosidase activity (n = 3) of *Pediococcus* sp. in R software (RStudio v4.4.3). Following ANOVA results, post-hoc comparisons were conducted using Tukey's Honest Significant Difference (HSD) test ($p < 0.05$) (Microsoft Excel 2019).

Results

Screening, Isolation, and Identification of Chlorella sp.

After 2–3 weeks of incubation, green-pigmented colonies appeared on agar plates, indicating a viable microalgal culture. Morphological examination of the colony isolate under a compound microscope confirmed characteristic features consistent with the genus *Chlorella*. The identification was made with reference to the taxonomic keys and descriptions provided by [27]. Accordingly, the isolated microalga was identified as *Chlorella* sp.

Growth characteristics of Chlorella sp.

The growth kinetics of *Chlorella* sp. grown in BBM and CWW medium were assessed by OD_{750} and cell counts over the cultivation period (Figs. 1a and 1b). Comparable growth was observed in both BBM and 20% (v/v) CWW media, with final cell concentrations of 1.59×10^7 and 1.73×10^7 cells/mL, respectively, by day 8.

Despite the comparable growth performance based on cell counting, *Chlorella* grown in CWW produced a notably higher dry biomass yield (1.40 ± 0.09 g/L) than that cultivated in BBM (0.30 ± 0.05 g/L). No bacterial or fungal contamination was detected in the BBM and CWW cultures.

FTIR analysis of Chlorella sp.

The FTIR spectra of *Chlorella* sp. dry biomass cultivated in BBM and CWW revealed both similarities and differences in biochemical composition (Figure. 2). In both media, strong absorption bands were observed in the region of 3400 – 3300 cm^{-1}, corresponding to O-H stretching also contributed by N-H vibrations indicating the biomass is rich in polysaccharide and protein compounds [37].

Similarly, bands in the 2970 – 2850 cm^{-1} region represent CH_2 and CH_3 stretching vibrations characteristic of lipid aliphatic chains in biomass [38][39]. Protein-associated bands were observed in both samples, including the Amide I band (1680–1630 cm^{-1}) corresponding to C = O stretching and the Amide II band (1530–1560 cm^{-1}) corresponding to N–H bending and C–N stretching, with biomass from BBM cultures displaying pronounced peaks at 1543.05 cm^{-1}. CWW-grown biomass exhibited a strong peak at 1745 cm-1, assigned to the C = O stretching of lipids and ester groups[40]. The bands observed around the 1072–1099 cm^{-1} region suggested C-O-C stretching of polysaccharides and P = O stretching of phosphodiester groups, indicating the presence of carbohydrates and nucleic acid–related phosphate compounds [41]. Additionally, out-of-plane C = C vibrations near 669–671 cm^{-1}

corresponding to phosphodiester stretching confirmed the presence of unsaturated functional groups in both biomass types [42].

Screening, Isolation and Characterization of Lactic Acid Bacteria (LAB)

The colonies, exhibiting off-white to yellowish colors with distinct morphology, were initially isolated from the spread plate for subsequent analysis. Gram staining revealed that several isolates were Gram-positive cocci. Biochemical testing showed these isolates were negative for catalase, oxidase, sulphide, and indole, and were non-motile, consistent with LAB characteristics. Among the five isolates from LpG, one isolate (LpG3) was selected for further molecular characterization. DNA extraction from LpG3 was followed by PCR amplification, which yielded a ~ 1500 bp band observed via gel electrophoresis. Sanger sequencing of the 16S rRNA region and analysis of the raw data (.ab1 file) using isolateR and SangerseqR packages in R identified the isolate as *Pediococcus* sp. This *Pediococcus* sp. (LpG3) has been preserved at the RIBB Culture Collection Centre (RiCCO) in Nepal.

Effect of Chlorella biomass supplementation on the growth of Pediococcus sp.

Incorporating 1% (w/v) *Chlorella* biomass from BBM and CWW media influenced the growth of *Pediococcus* sp. in comparison to standard MRS-only media over different time points (Fig. 3). The growth of *Pediococcus* sp. increased over time in all treatments. However, treatment alone did not have a significant impact ($p = 0.584$). The interaction between treatment and time ($p < 0.001$) was significant, indicating that the effect of *Chlorella* supplementation varied across time points. At 0 h, all treatments exhibited comparable colony counts (6 log CFU/mL), with no significant differences among them. Between 6 and 12 h, bacterial populations increased rapidly across all treatments; however, no significant differences were observed. However, by 18 h, the formulated media supported significantly higher counts in both MRS + 1% *Chlorella* (BBM) and MRS + 1% *Chlorella* (CWW) than MRS alone ($p < 0.05$ and $p < 0.01$, respectively), indicating that *Chlorella* supplementation had a stimulatory effect during exponential growth. At 24 h, the CWW-supplemented medium continued to exhibit higher counts than MRS ($p < 0.01$), while BBM-supplemented media showed no significant differences. By 48 h, the trend reversed; MRS alone showed greater viability than formulated media, suggesting *Chlorella* biomass supplementation may not support long-term growth.

β-galactosidase activity of Pediococcus sp.

The production of β-galactosidase from *Pediococcus* sp. with MRS, 1% *Chlorella* from BBM, and 1% *Chlorella* from CWW was 18.34, 49.33 and 38.82 Miller Units (MU), respectively (Additional File 1: Figure. S4). This activity was significantly higher in BBM compared to CWW and MRS ($p < 0.01$). The ONPG test confirmed positive β-galactosidase activity, as indicated by the development of a yellow color, although the color change required a longer incubation period. The conversion of the yellow color took longer than the protocol suggested. To account for potential interference from microalgal biomass with OD

measurements, 1% *Chlorella* biomass without *Pediococcus* sp. was included as a control in the ONPG test.

Discussion

Growth characteristics of Chlorella sp.

The results indicate that CWW provides a nutrient-rich environment that enhances the growth of *Chlorella* sp., resulting in greater biomass accumulation than BBM. The higher biomass in CWW results may be attributed to the CWW content, including lactose, proteins, and other organic compounds. These findings are consistent with a previous study reporting that, over 12 days in BBM, the maximum biomass concentration reached 1.57 g/L with CO_2 supplementation and 0.98 g/L without CO_2 [43]. An earlier study demonstrated that *Chlorella vulgaris* improved growth in nutrient-supplemented wastewater, with cell count reaching 6.0×10^7 cells/mL after 8 days [44].

FTIR analysis of Chlorella sp.

The FTIR peaks analysis demonstrated the effect of the culture medium on the biochemical composition of *Chlorella* biomass. Overall, the spectral profiles suggest that BBM supports more pronounced protein synthesis, whereas the CWW shows greater prominence toward lipid synthesis. Under the mixotrophic condition, the availability of organic carbon, including lactose, nitrogen, and phosphorus in cheese whey [45], may have shifted the available carbon toward enhanced lipid biosynthesis [46][47]. These findings are consistent with previous studies linking wastewater-derived substrates to metabolic modulation in microalgae [48].

Screening, Isolation and Characterization of Lactic Acid Bacteria (LAB)

The LAB were successfully isolated from vegetable-based broadleaf mustard (*Brassica rapa*), locally known as *gundruk*, using MRS medium, a selective medium for lactobacilli [49]. The growth of *Pediococcus* sp. suggests these bacteria flourish in a selective medium. *Gundruk* is considered a reservoir for homofermentative and heterofermentative LAB, with *Lactobacillus plantarum* and *Pediococcus pentosaceus* reported as the dominant microorganisms during this spontaneous fermentation [19]. The morphological and biochemical screening employed in this study preliminarily confirmed that the isolates belonged to typical non-motile LAB genera [50]. Molecular identification through 16S rRNA gene amplification with universal primers and Sanger sequencing represents the most comprehensive and reliable strategy for accurate identification of LAB from traditional fermented foods [51], providing a validated strain library from a culturally and nutritionally significant fermented product of Nepal.

Effect of Chlorella biomass supplementation on the growth of Pediococcus sp.

The results indicate that *Chlorella* biomass enhanced the growth of *Pediococcus* sp. during the late log phase, with significant growth at 18 h and 24 h, followed by a decline thereafter. Previous studies have similarly demonstrated that *Chlorella* sp. supplementation enhanced LAB growth kinetics, shortened the log phase, increased the l-lactic acid production, and increased the enzymatic activity of valine arylamidase, α-galactosidase, and α-glucosidase [21]. In the present study, the enhanced growth of *Pediococcus* sp. supplemented with *Chlorella* biomass cultivated in CWW may be linked to the stronger lipid- and ester-associated peaks observed in the FTIR spectra, likely reflecting the presence of fatty acid components. Similarly, improved growth of *L. johnsonii* has been reported compared with cultivation in minimal *Lactobacillus* medium upon supplementation with fatty acids [52]. Consistent with these reports, our findings indicate that *Chlorella* biomass exerts its primary benefits during the late log phase, with limited impact on viability beyond 24 h of incubation.

β-galactosidase activity of Pediococcus sp.

The increased β-galactosidase activity in media supplemented with *Chlorella* indicates that microalgal biomass positively affects the metabolic activity of *Pediococcus* sp. A previous study with *Pediococcus acidilactici* NCDC 252 reported β-galactosidase activity of 65.08 ± 0.9 Miller units, nearly 22-fold higher than that of uninduced lac$^+$ strains (1.5–3.0 Miller units), highlighting the potential of LAB strains to express high levels of this enzyme under suitable conditions [21]. Similarly, supplementation of a LAB culture with 1.5% (w/v) *Chlorella vulgaris* resulted in either an increase or a decrease in enzymatic activity, depending on the strain. *Lactobacillus brevis* ŁOCK 0980 demonstrated higher enzymatic activity for valine arylamidase, α-galactosidase, and α-glucosidase when cultured in the presence of *Chlorella vulgaris* [24]. Likewise, in our study, supplementation with *Chlorella* demonstrated a clear functional benefit for *Pediococcus* sp., with a 2-fold increase in β-galactosidase activity compared to the standard medium. While CWW-derived *Chlorella* showed better bacterial growth, BBM-derived *Chlorella* resulted in higher β-galactosidase activity, indicating that different biomass components influenced the growth and enzymatic activity.

Conclusion

This study demonstrated that Cheese Whey Wastewater (CWW) is a viable substrate for the cultivation of freshwater *Chlorella* sp., producing biomass with a modified biochemical composition and enhanced lipid profiles compared to conventional BBM. When dry biomass was incorporated into *Pediococcus* sp., it improved growth during the exponential phase and elevated β-galactosidase activity. These findings establish CWW's dual role in supporting cost-effective algal cultivation and generating functional biomass for LAB applications. Beyond waste reduction, this method shows promising potential for use in the functional food and biotechnology industries. Future research should focus on optimizing supplementation levels, characterizing metabolic shifts, and scaling the process for industrial implementation.

Abbreviations

CWW
Cheese Whey Wastewater
BBM
Bold’s Basal Media
LAB
Lactic Acid Bacteria
MRS
de Man, Rogosa, Sharpe
OD
Optical Density
FTIR
Fourier-Transform Infrared Spectroscopy
PBS
Phosphate-Buffered Saline
ONPG
Ortho-Nitrophenyl-β-D-Galactopyranoside
COD
Chemical Oxygen Demand
BOD
Biological Oxygen Demand
DNA
Deoxyribonucleic Acid
RNA
Ribonucleic Acid
PCR
Polymerase Chain Reaction
rRNA
Ribosomal Ribonucleic Acid
KBr
Potassium Bromide
SD
Standard Deviation
ANOVA
Analysis of Variance
v/v
volume per volume
w/v
Weight Volume
µL
microliter

mL
milliliter
g/L
gram per Liter
RPM
Revolutions per Minute
°C
Degree Celsius

Declarations

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

Funding

The research was funded by a Thesis Support Grant from Kantipur Valley College (KVC), and SANN International College. Funding was also partially supported by Chr. Hansen (Seed Research Funding).

Author Contribution

M.K. and U.D. contributed equally to the protocol design, experimental work, data collection, data analysis, and manuscript preparation. S.K., B.C., B.M., L.P., K.D. designed and optimized the protocol, did the laboratory experiments and data acquisition. S.B. and P.R. contributed to manuscript revision and supervised the microbiological part of the study. S.L. conceived and supervised the study, contributed to experimental design, data interpretation, manuscript revision, and overall project coordination. All authors read and approved the final manuscript.

Acknowledgement

The authors would like to acknowledge and express sincere gratitude to Program Coordinator Mr. Sagar Thapa, Department of Biotechnology, Kantipur Valley College, and Himalaya Cheese Farm for providing us with the CWW, local people from Pimbahal, Lalitpur for fermented vegetable sample, Nepal Academy of Science and Technology (NAST) for FTIR Spectroscopy, all the thesis groups (SAAN, KVC) for their scientific contributions, Strand Life Sciences (India) for raw Sanger sequencing data, Dr. Raunak Shrestha, Mr. Gaurav Adhikari and Mr. Sagar Kurmi for their guidance.

Data Availability

The dataset generated during the current study is available in the GenBank repository, PZ770280.1 (https://www.ncbi.nlm.nih.gov/nuccore/3370611344).

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Figures

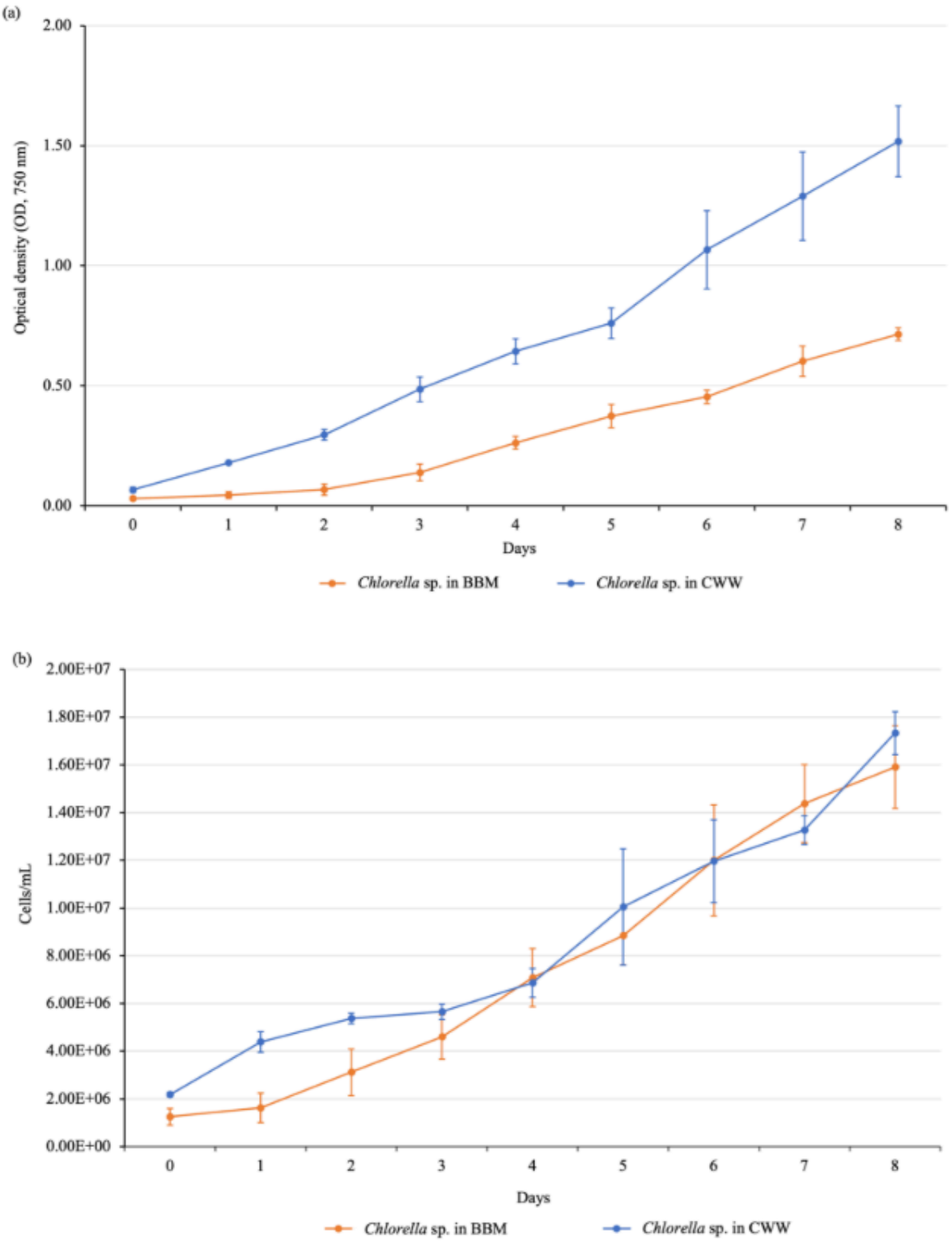


Figure 1

Growth curves of *Chlorella* sp. in BBM and CWW media based on (a) OD_{750} and (b) cell counting.

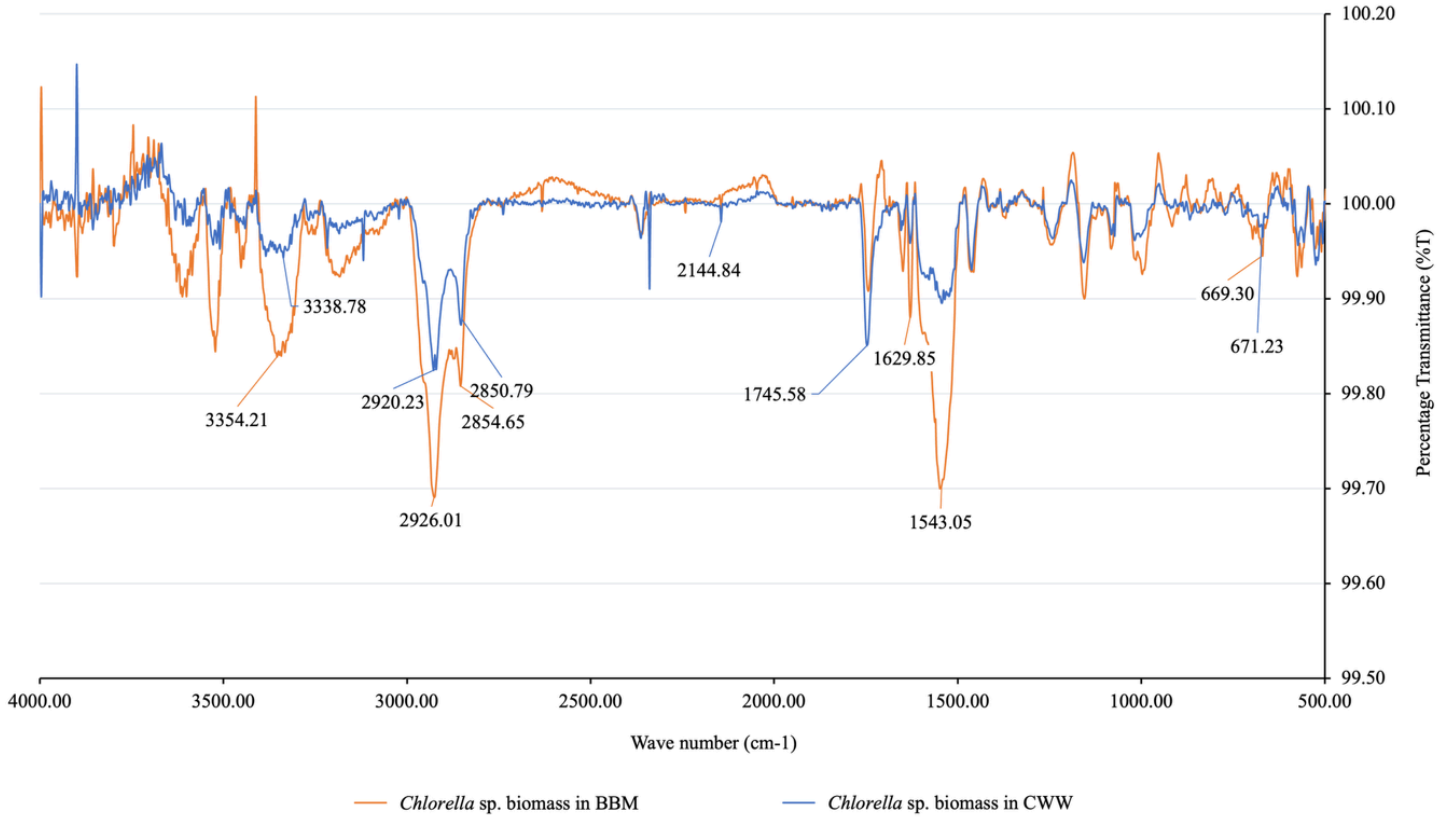


Figure 2

FTIR spectra of *Chlorella* sp. biomass cultivated in BBM and CWW media.

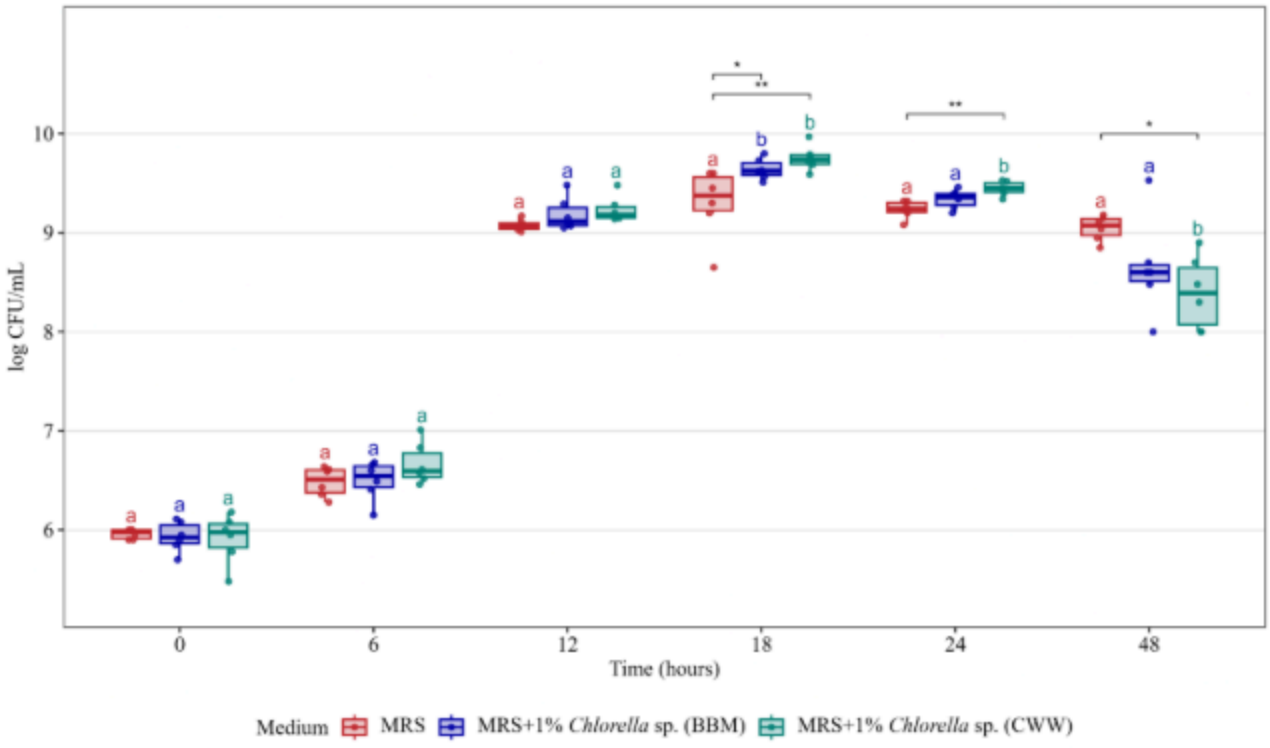


Figure 3

Boxplot showing growth of *Pediococcus* sp. (log CFU/mL) in three media (MRS and MRS supplemented with 1% (w/v) *Chlorella* biomass from BBM and CWW over 48 h (n = 6). Explanatory notes: the boxes represent the interquartile range (IQR), with horizontal lines indicating the median as individual data points are overlaid. Letters a and b indicate statistically significant differences between treatments at each time point, where groups sharing the same letter do not differ significantly. Brackets with asterisks denote significant pairwise differences (* $p < 0.05$, ** $p < 0.01$).

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