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Study on Intracellular Extracts of *Chlorella* sp. KLSc61 to Promote Growth of *Lactobacillus plantarum* JCM 1149

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Abstract

Chlorella is one of the famous microalga genera used to study in various fields, including functional food. Several studies revealed that its dried biomass could be used as prebiotics to promote the growth of probiotics such as *Lactobacillus* sp. Using *Chlorella* dried cell mass might be ineffective while producing on an industrial scale. To find an alternative strategy, we tested if *Chlorella* crude extract comprised some bioactive compound that could promote the growth of *Lactobacillus* or not, this led to reduce the amount of *Chlorella* use. This study aimed to compare fresh and dried *Chlorella* crude extract to find out which one could be a better use, which was still unclear. We prepared fresh and dried intracellular ethanolic extracts of *Chlorella* sp. KLSC61, added them into the *Lactobacillus plantarum* JCM 1149 growth medium (MRS), and measured the growth of *L. plantarum* JCM 1149. The results showed that all dried crude extracts significantly showed better *L. plantarum* JCM 1149 growth promotion than fresh ones. The 0.1% (w/v) of dried-ethanolic crude extract could promote growth up to 1.1-1.2-fold compared to controls (MRS and glucose) at 14 and 24 h during the stationary phase. These preliminary results demonstrated that *Chlorella* sp. KLSC61 crude extracts contained some bioactive compounds that could enhance the growth of *L. plantarum* JCM 1149, and that compounds might be heat resistant. We propose analyzing the crude extract and finding out a specific compound that could benefit the growth of *L. plantarum* JCM 1149 for future study.

Keywords: *Chlorella* sp. KLSc61, Ethanol, Intracellular extract, *Lactobacillus plantarum*

1. Introduction

Microalgae have been used for several applications, e.g., aquaculture, pharmaceuticals, nutraceuticals, etc., because they consist of high-value compounds such as carotenoids, antimicrobial peptides, vitamins, and lipids (DHA and EPA), etc. Some microalgae, *Chlorella* sp. and *Spirulina* sp., can yield high biomass production and be suitable for an aquaculture-based economy (Abreu et al., 2023). *Chlamydomonas reinhardtii* was used to produce antimicrobial peptides such as taraxacam officinale antimicrobial peptide 4, bacteriocin LS2, and mytichitin-CB (Hadiatullah et al., 2020; Liu et al., 2020; Xue et al., 2020). In recent years, some microalgae, *Scenedesmus intermedius*, *Klebsormidium flaccidum*, and *Chlorella* spp., have offered a promising alternative for prebiotic production with high carbohydrate content (20-50% of dry weight), simpler structures, and additional nutrients (Babich et al., 2022; Bernaerts et al., 2018; Gao et al., 2024; Rizwan et al., 2018; Smith & Zeeman, 2020).

Chlorella sp. is a unicellular green microalga. *Chlorella* cells have a round shape, and half of their cells consist of a single chloroplast. *Chlorella* produces numerous nutrients, including vitamin D and B12, folate, and iron that are lacking in plant-derived food sources (Bito et al., 2020). In terms of biocontrol and functional food application, *Chlorella* biomass consists of polysaccharides that could increase the population of beneficial bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Akkermansia* as a bacterial prebiotic (Lv et al., 2022; Wan et al., 2023; Yu et al., 2019), For instance, *Chlorella vulgaris* biomass was revealed to enhance *Lactobacillus brevis* growth and its lactic acid (Ścieszka & Klewicka, 2020). Several studies frequently used *Chlorella* biomass to increase the population of probiotic bacteria, but this strategy could become inefficient for upscaling in industrial

application. There were some reports revealing that *Chlorella* extracts could be used as natural antimicrobial agents, extending the life of food products, including promoting the growth of *Lactobacillus plantarum* JCM 1149, a type strain of probiotic bacteria (Abdel-Raouf et al., 2012; Andrade et al., 2018; Zheng, 2012). *Chlorella* bioactive compounds such as phenolics and antimicrobial peptides have been found to inhibit the growth of pathogenic bacteria, including *Escherichia coli*, *Salmonella* sp., *Clostridium difficile*, and *Helicobacter pylori*, by interfering with bacterial enzymes and cell structures (Ilieva et al., 2024). From these properties, *Chlorella* extracts have become a valuable product for reducing the risk of gastrointestinal infections and promoting human health (Abdel-Raouf et al., 2012). However, there are still limitations in studying the strategy to use the extract because there have been no reports about whether fresh or dried extract is better to use, and it would be beneficial to explore the specific compounds in fresh or dried extracts that could affect probiotic growth.

This study focused on comparing fresh and dried *Chlorella* sp. KLSC61 (Laokua et al., 2022) crude extracts to see if the extract of *Chlorella* sp. KLSC61 could promote the growth of *L. plantarum* JCM 1149, a type of probiotic strain. The *Chlorella* extracts were performed by using 30% (v/v) and 70% (v/v) ethanolic extraction and subsequently tested on *Lactobacillus plantarum* JCM 1149 growth in MRS medium. This study could suggest that *Chlorella* sp. KLSC61 extract has some bioactive compounds that could promote probiotic growth. We proposed choosing the best *Lactobacillus* growth promoting extract and characterizing a specific bioactive compound that could be a potential application in the platform of dietary supplements production or other related fields.

2. Materials and Methods

2.1 *Chlorella* sp. KLSc61 cells preparation, cultivation and standard growth curve

Chlorella sp. KLSc61 was obtained from Cherdsak's laboratory (Laokua et al., 2022). Cells were grown and maintained on Tris Acetate Phosphate (TAP) agar (Gorman & Levine, 1965). To generate a standard growth curve of *Chlorella* sp. KLSc61, a starter culture was prepared by inoculating a single colony from the culture plate into 100 mL of TAP medium in a 250 mL Erlenmeyer flask and cultivated for 3 days. Subsequently, *Chlorella* cells were transferred into fresh 100 mL TAP medium in a 250 mL Erlenmeyer flask to achieve an initial cell concentration of 1×10^6 cells/mL. Cell counts were performed every 24 h for 10 days using a hemocytometer (Boeco, Germany), and a growth curve was generated thereafter by Microsoft Excel.

To prepare the *Chlorella* extract, the starter culture was prepared in the same way as for the growth curve. Then, algal cells were transferred into 1 L of TAP medium in a 2-liter Erlenmeyer flask. Algal cells were cultured for 5 days to reach the early stationary phase. *Chlorella* cells were harvested by centrifugation at 3,500×g for 5 min. The experiment was performed in triplicate to collect cell mass from a total culture volume of 3 L. All cells from the 3-liter culture were combined in the same 50-mL conical tube and stored at -20°C until use.

All *Chlorella* cultivation was conducted on a rotary shaker at 120 rpm with 24 hours of light exposure under a light intensity of 100 µmol/m²/s at 27°C in a culture room.

2.2 *Chlorella* sp. KLSc61 intracellular extract preparation

The cell disruption method was modified from Gerde et al. (2012). Briefly, *Chlorella* sp. KLSc61 cells (prepared from section 2.1) were resuspended in sterile distilled water in two separate beakers: 1) 10 g of cells were added to 70 mL sterile distilled water, and 2) 10 g of cells were added to 30 mL of sterile distilled water. In the next step, cell disruption was performed using a probe-type Ultrasonic Cell Disruptor (Sonics & Materials VCX500 Vibra Cell Ultrasonic Processor, USA). The process involved alternating cycles of 10 sec of sonication and 10 sec of rest, repeated 30 times, for a total sonication time of 5 min (the total processing time was 10 min).

After the sonication process, absolute ethanol (QRëc®, New Zealand) was added to the sonicated cell suspensions: 1) 30 mL of ethanol was added to 70 mL of sonicated cell suspension to generate a 30% ethanolic extract (v/v), and 2) 70 mL of ethanol was added to 30 mL of sonicated cell suspension to generate a 70% ethanolic extract (v/v). To extract *Chlorella* sp. KLSc61cells, both *Chlorella* cell mixtures were incubated in the dark at 4°C for 48 h. After that, both mixtures were centrifuged, and supernatants were transferred and collected in new 50-mL conical tubes as 30% and 70% *Chlorella* ethanolic extracts separately.

For fresh extract preparation, both supernatants (of 30%- and 70% ethanolic extract) were subjected to the ethanol removal by using a Rotary Evaporator at 40°C and 100 mbar for 3 h or until the solvent was completely

removed. Then, 30% ethanolic and 70% ethanolic extracts were resuspended in 20 mL of 1%(v/v) DMSO and stored at -20°C until use.

For dry extract preparation, both fresh extracts (30% and 70%) were poured into petri dishes and freezen in a -80°C freezer for overnight. It was then dried using a Freeze Dryer at 0.1 mbar for 6 h, or until completely dry. The dried *Chlorella* extract was ground into a fine powder by using a mortar and pestle and stored at -20°C until use.

2.3 *Lactobacillus plantarum* JCM 1149 cells preparation

Lactobacillus plantarum JCM 1149 was purchased from the Japan Collection of Microorganisms (JCM). The strain was maintained and stored on de Man, Rogosa, and Sharpe (MRS) agar (Difco™ & BBL™, USA) at -20°C until use. Before use, *L. plantarum* JCM 1149 was reactivated from the stock culture by streaking onto MRS agar and incubating at 37°C for 24 h. A single colony was then transferred into 5 mL of MRS broth in a new test tube and incubated at 37°C for 24 h.

After incubation, the culture was transferred into 5 mL of MRS broth in a new test tube to achieve an initial inoculum equivalent to a McFarland standard 0.5 and incubated at 37°C. Absorbance at 600 nm (A600) was measured using a microplate reader every 2 h from 0 to 12 h to determine the mid-logarithmic (mid-log) phase (A600 values are typically 0.3-0.6).

2.4 *In Vitro* study of *Lactobacillus plantarum* JCM 1149 growth after adding *Chlorella* sp. KLSc61 extracts

2.4.1 Fresh extract solution preparation

The fresh extract (prepared from section 2.2) was diluted to achieve final concentrations of 1%, 2.5%, 5%, and 10% (w/v) in MRS broth to make a total volume of 3.5 mL (Figure 1). The mixed solution was then divided, with 1.75 mL dispensed into two separate test tubes to serve as the sample solution and the blank solution. *L. plantarum* JCM 1149, with an initial cell count of 1×10^6 CFU/mL, was added to the sample solution tube. For the blank solution, MRS broth was added instead of the bacterial culture (Figure 1). Additionally, standard glucose solutions at concentrations of 1%, 2.5%, 5%, and 10% (w/v) in MRS broth were prepared similarly to serve as a comparison group (Figure 1).

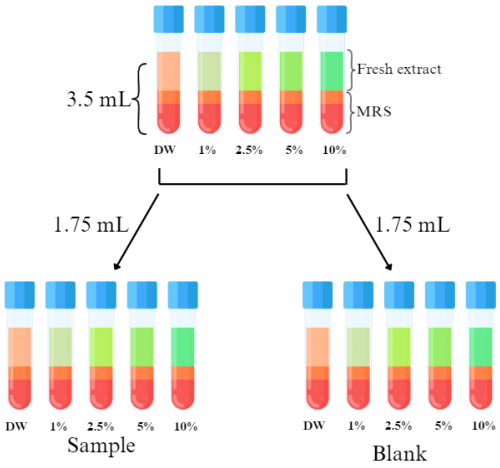


Figure 1. Fresh extract preparation.

2.4.2 Dried extract solution preparation

Stock solutions were prepared by dissolving the dried extract (from section 2.2) in 99.0% Dimethyl Sulfoxide (DMSO) (Loba, India) These stock solutions were then diluted in MRS broth to achieve final dried extract concentrations of 0.1% and 1.5% (w/v) (Figure 2). The dilution was calculated such that the final concentration of DMSO in the culture medium was 1% (v/v). Subsequently, the same procedure as for the preparation of the fresh extract solution was performed.

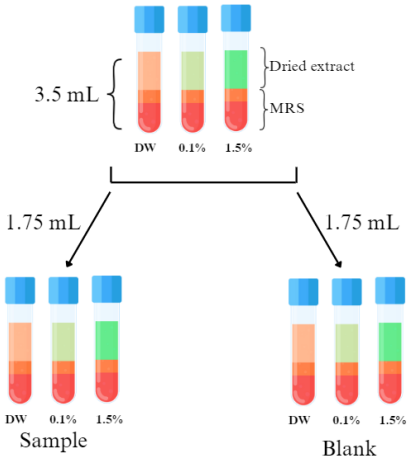


Figure 2. Dried extract preparation.

2.4.3 Growth measurement of *Lactobacillus plantarum* JCM 1149

The mixtures from 2.4.1 and 2.4.2 were added to a 96-well plate, 200 µL per well, in triplicate. The sample wells contained the solutions with *L. plantarum* JCM 1149, and the blank wells contained the respective solutions without the bacteria.

L. plantarum JCM 1149 was incubated at 37°C, and *L. Plantarum* JCM 1149 growth was measured using a microplate reader at a wavelength of 600 nm at 0, 2, 4, 6, 8, 10, 12, and 24 h. The data obtained were then used to generate a growth curve for *L. plantarum* JCM 1149 by using Microsoft Excel.

2.5 Statistical analysis

The experiment was conducted using a Completely Randomized Design (CRD). Data were analyzed for variance using One-way ANOVA, and mean differences were compared using Duncan's multiple range test with IBM SPSS Statistics version 29 (SPSS software, New York, USA).

3. Results and Discussion

3.1 The growth of *Chlorella* sp. KLSc61 and cell harvesting

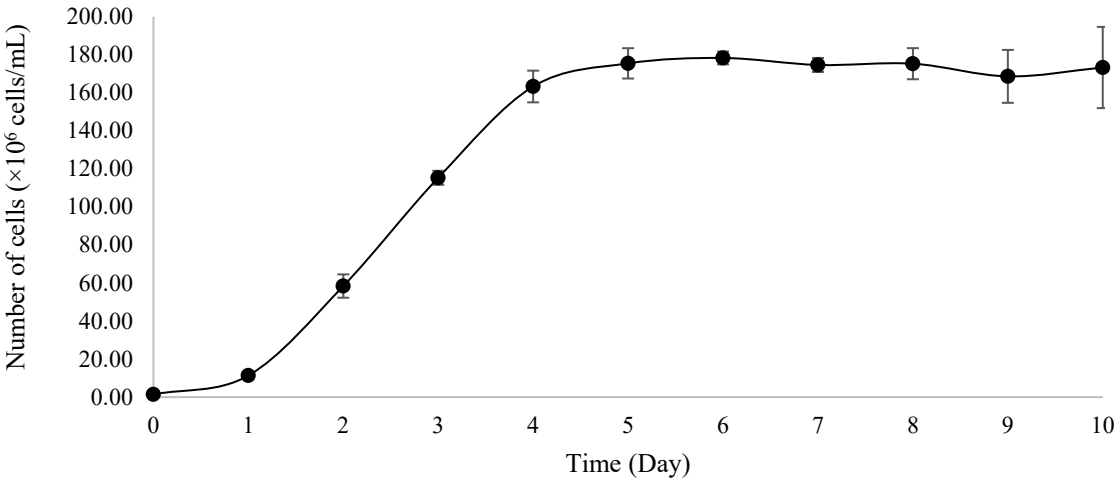


Figure 3. The standard growth curve of *Chlorella* sp. KLSc61 was measured using a hemocytometer over a period of 10 days. All experiments were done in triplicate and statistical analysis using ANOVA and DUNCAN at p value < 0.05.

To determine the harvesting stage, the growth curve of *Chlorella* sp. KLSc61 was generated. As shown in Figure 1, *Chlorella* cells remained in the lag phase until Day 1, after that they started entering the log phase until they reached the early stationary phase on Day 4 and the cells had the highest cell number accumulation of $1.76 \times 10^8 \pm 7.97$ cells/mL on Day 5. In this early stationary phase, cells began to accumulate secondary metabolites or bioactive compounds. According to the study of Tiwari and Dhakal, harvesting of *Chlorella* sp. cells at the beginning of the stationary phase resulted in an increase of the antioxidant activity of secondary metabolic compounds up to 20% compared with harvesting of *Chlorella* cells at other growth phases (Tiwari & Dhakal, 2023). *Chlorella* cells typically contain a rich source of pigments such as chlorophyll and carotenoids, phenolics, flavonoids including protein, polysaccharides as highly potent bioactive compounds (Georgiopoulou et al., 2021; Safi et al., 2014). We decided to harvest *Chlorella* cells by Day 5 because of the highest cell accumulation of *Chlorella* sp. KLSc61 and intracellular extract of *Chlorella* sp. KLSc61 was prepared and tested to see if the crude extract could promote *Lactobacillus*, a probiotic growth in the next experiment.

3.2 The growth of *Lactobacillus plantarum* JCM 1149

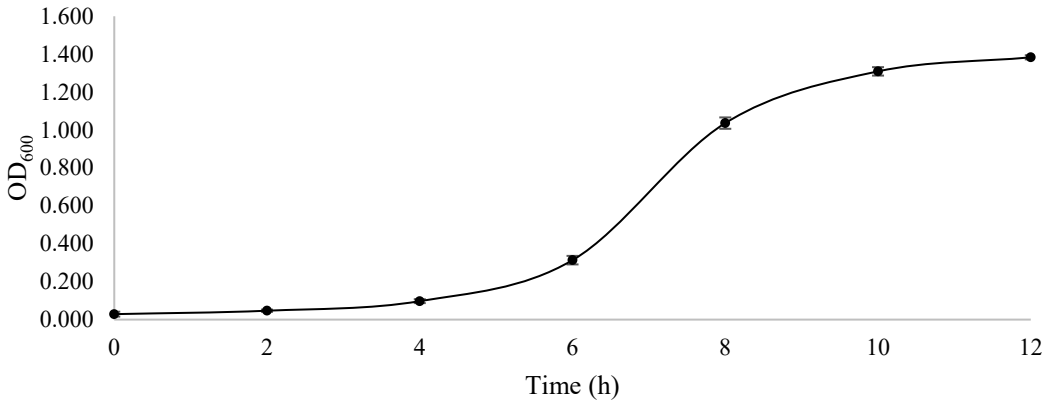


Figure 4. The standard growth curve of *Lactobacillus plantarum* JCM 1149 was measured using a microplate reader at a wavelength of 600 nm. All experiments were done in triplicate and statistical analysis using ANOVA and DUNCAN at p value < 0.05.

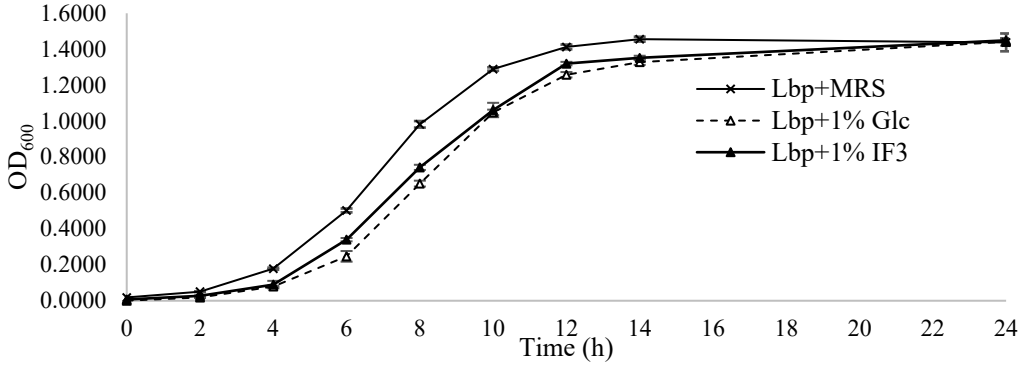
To determine the mid-log phase for cell harvesting, *Lactobacillus plantarum* JCM 1149 growth was observed for 12 h in MRS medium. As shown in Figure 2, *L. plantarum* JCM 1149 cells remained in the lag phase from 0 to 4 h, after that they started entering the log phase on 4 h until they reached the early stationary phase at 8 h. From the growth curve, the mid-log phase of *L. plantarum* JCM 1149 was 6 h, with an OD_{600} value was approximately 0.314 ± 0.023. Using bacterial cells in the mid-log phase of growth was commonly used in microbiological experiments because, during this period, bacteria divide rapidly and consistently. There is minimal interference from waste accumulation or nutrient depletion, which allows for a stable growth rate and easy control of experimental conditions. This phase provides a reliable, reproducible foundation for studying bacterial behavior or responses to environmental conditions or chemicals, making it ideal for obtaining accurate and repeatable results (Zavizion et al., 2010). Thus, *L. plantarum* JCM 1149 cells were grown and harvested at 6 h (approximately OD_{600} 0.3) and used for the next experiments.

3.3 Growth-promoting effects of *Lactobacillus plantarum* JCM 1149 by supplementing with *Chlorella* sp. KLSc61 intracellular extracts

3.3.1 Supplemented with fresh extracts of *Chlorella* sp. KLSc61

To find out if *Chlorella* sp. KLSc61 intracellular extract could help promote *Lactobacillus plantarum* JCM 1149 growth, we made fresh extracts using ethanol and added them to the growth medium (MRS) at amounts of 1%, 2.5%, 5%, and 10% (w/v). As shown in Figure 5, we found that by adding 30% of the fresh *Chlorella* ethanolic

extract at 1% (Lbp+1%IF3) (Figure 5A), 2.5% (Lbp+2.5%IF3) (Figure 5B), 5% (Lbp+5%IF3) (Figure 5C), and 10% (Lbp+10%IF3) (Figure 5D) (w/v), *L. plantarum* JCM 1149 cells had lower growth compared with the controls, adding MRS + *L. plantarum* (Lbp+MRS) and glucose (Lbp+Glc). Whereas 8 h of the *L. plantarum* growth, adding 70% (v/v) ethanolic extract at concentrations of 1%, 2.5%, 5%, and 10% (w/v) significantly increased the growth of *L. plantarum* JCM 1149 by up to 1-2-fold compared to the controls, MRS (Lbp+MRS) + *L. plantarum* and glucose (Lbp+Glc), respectively. During the stationary phase (after 10 h), adding fresh extract of 1% (w/v) of 70% ethanolic extract (Lbp+1%IF7) (Figure 5E) could promote better growth of *L. plantarum* JCM 1149 when compared it with the others. This finding indicated that 30% of *Chlorella* extract did not promote *L. plantarum* JCM 1149 growth, but 70% (v/v) ethanol showed an effect on *L. plantarum* growth promotion. From these results, 70% (v/v) ethanol, which is a solvent having less polarity than 30% (v/v) ethanol, could dissolve some moderately polar compounds, as described by the like dissolves like theory. These compounds have promising effects on *Lactobacillus* growth promotion. Based on previous studies, we hypothesized that the 1% (w/v) of 70% (v/v) ethanolic extract of *Chlorella* sp. KLSc61 was likely to contain some bioactive compounds such as phenolics, carotenoids, and simple or short-chain carbohydrates, which are moderate polarity substances due to solubility (Goiris et al., 2012; Safafar et al., 2015).



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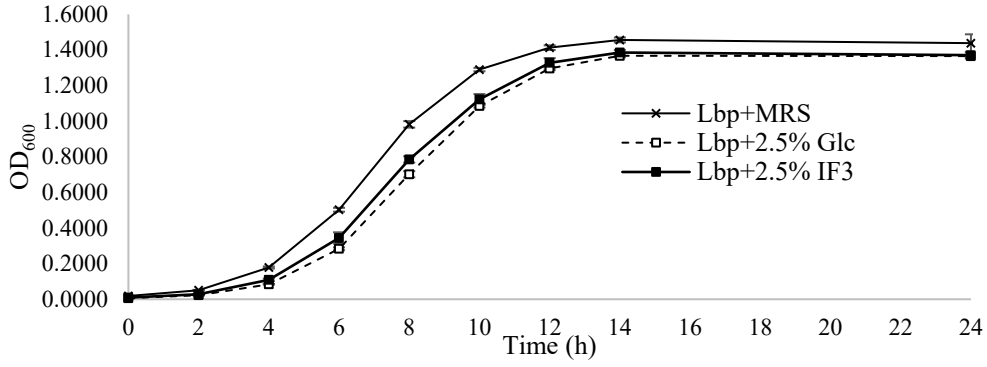

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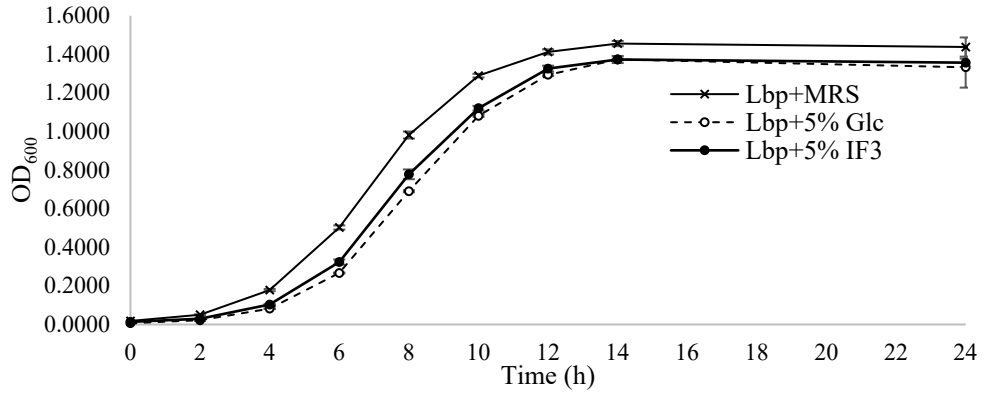

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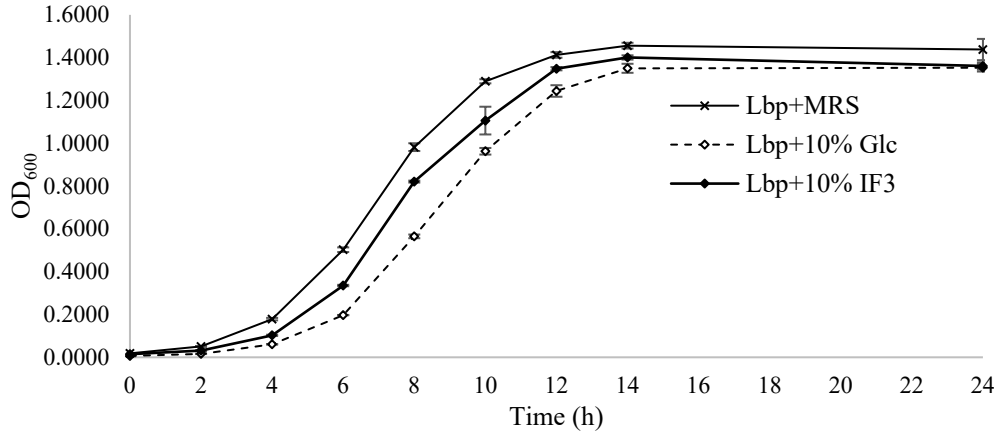
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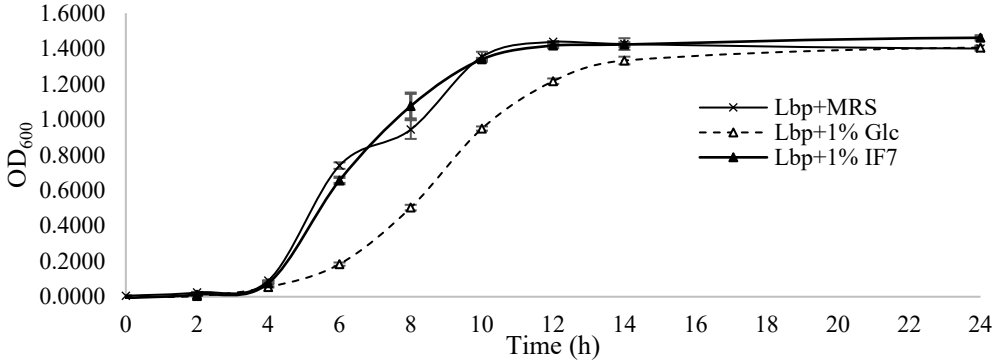
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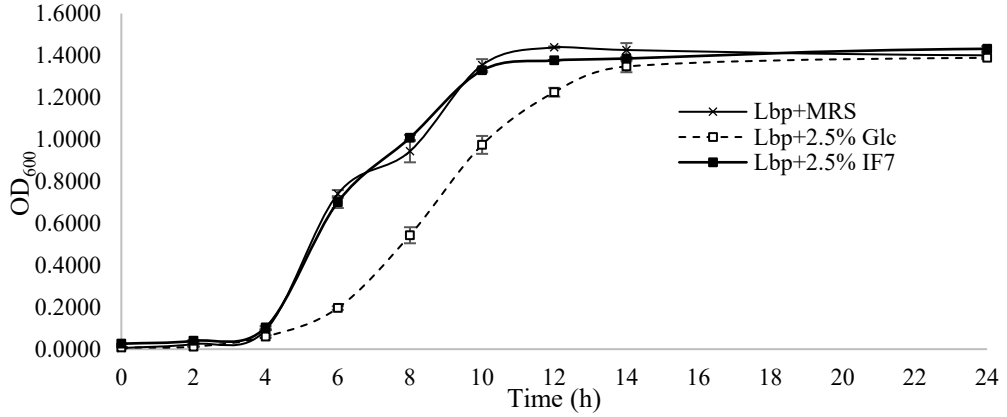
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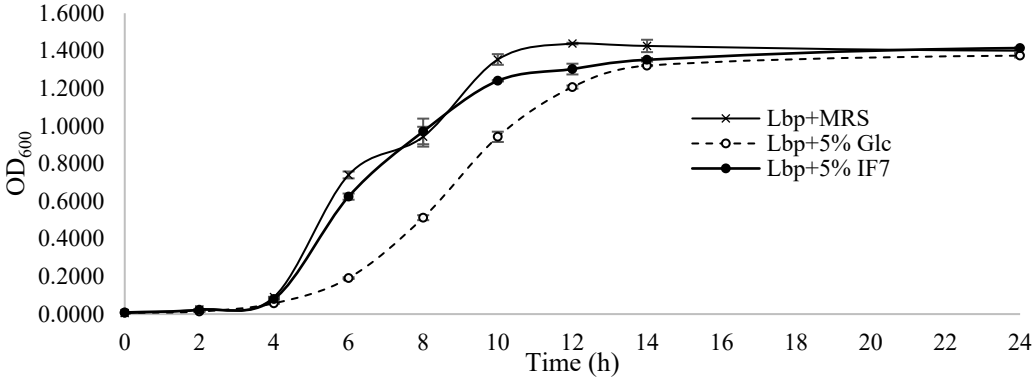
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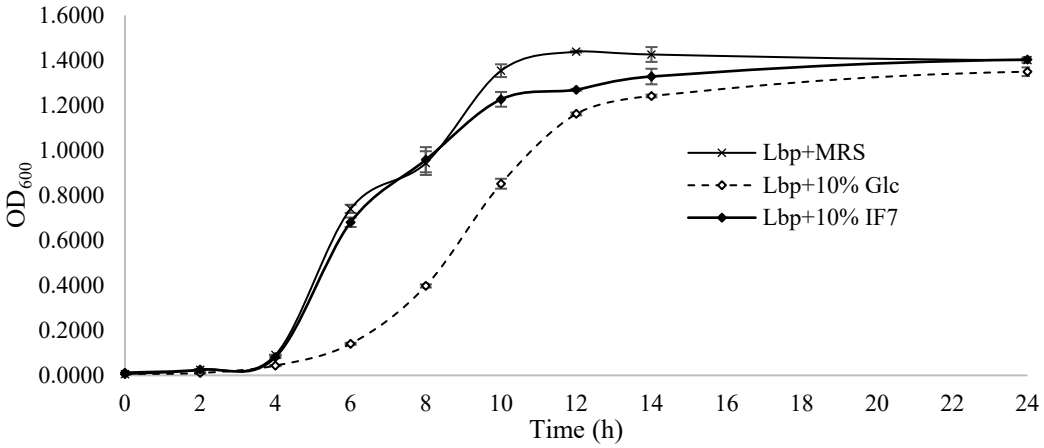
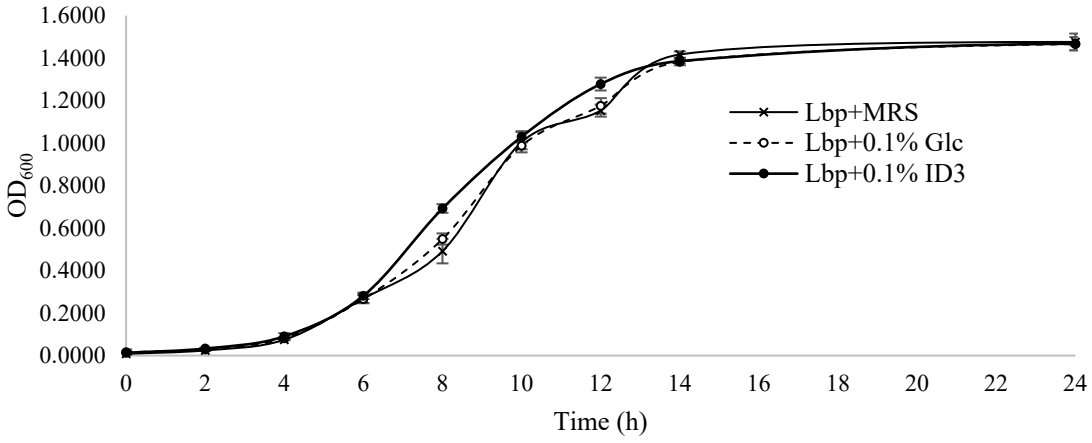


(H)

Figure 5. Growth measurement of *Lactobacillus plantarum* JCM 1149 supplemented with the 30% fresh ethanolic extract of *Chlorella* KLSc61 (A-D) and supplemented with the 70% fresh ethanolic extract of *Chlorella* KLSc61 (E-H); (A) supplemented with 1% (w/v); (B) supplemented with 2.5% (w/v); (C) supplemented with 5% (w/v); (D) supplemented with 10% (w/v); (E) supplemented with 1% (w/v); (F) supplemented with 2.5% (w/v); (G); (H) supplemented with 10% (w/v). All experiments were done in triplicate and statistical analysis using ANOVA and DUNCAN at p value < 0.05.

3.3.2 Dried extracts of *Chlorella* sp. KLSc61 obtained using ethanol 30% and 70%

To determine if dried intracellular extract could promote the growth of *Lactobacillus plantarum* JCM 1149, this experiment was performed by drying the intracellular extract using a freeze dryer. After that the *L. plantarum* JCM 1149 growth medium was supplemented with; 1) 0.1% (w/v) of 30% dried intracellular extract; 2) 0.1% (w/v) of 70% dried intracellular extract; 3) 1.5% (w/v) of 30% dried intracellular extract; 4) 1.5% (w/v) of 70% dried intracellular extract of *Chlorella* sp. KLSc61 (Figure 6).



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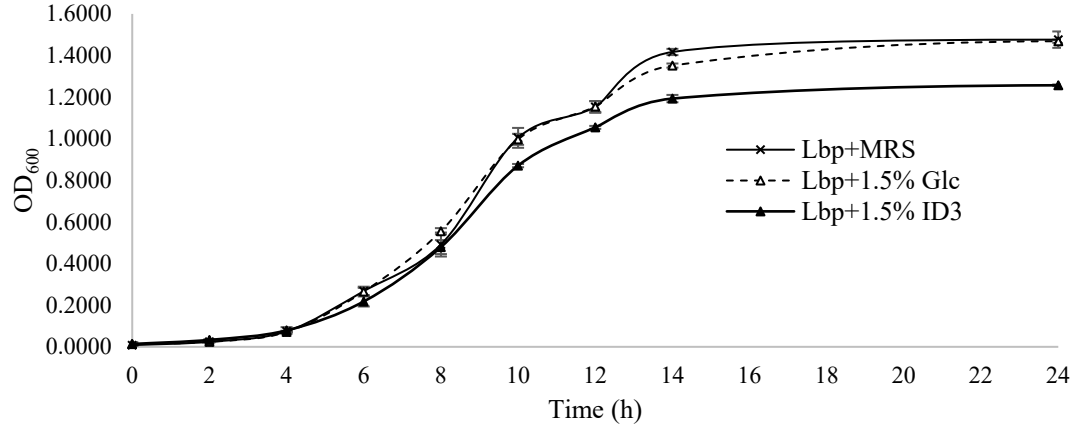

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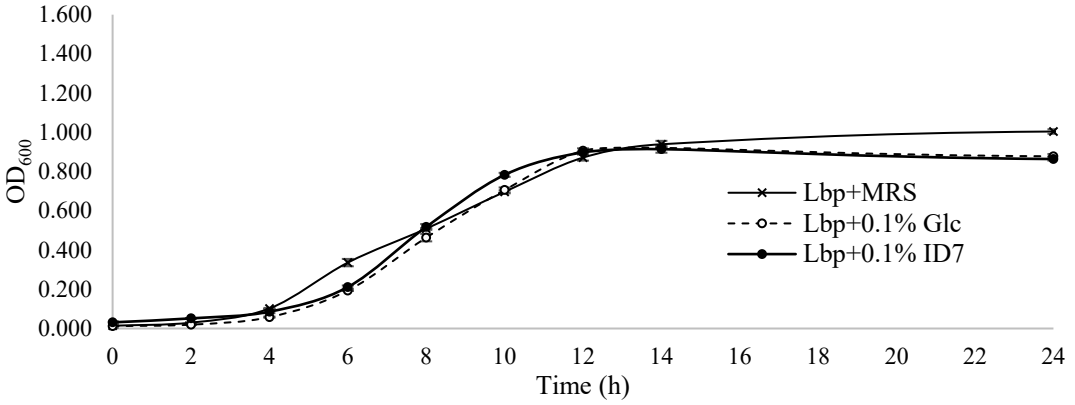

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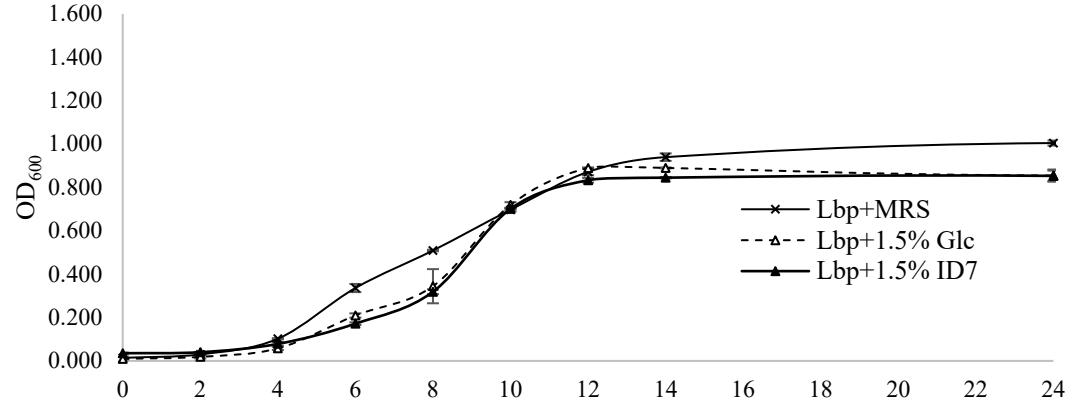

(D)

Figure 6. Growth measurement of *Lactobacillus plantarum* JCM 1149 supplemented with 30% dried intracellular ethanolic extract of *Chlorella* sp. KLSc61 (A-B) and 70% dried intracellular ethanolic extract of *Chlorella* sp. KLSc61 (C-D): (A) supplemented with 0.1% (w/v); (B) supplemented with 1.5% (w/v); (C) supplemented with 0.1% (w/v); (D) supplementation with 1.5% (w/v). All experiments were done in triplicate and statistical analysis using ANOVA and DUNCAN at p value < 0.05.

The experimental results showed that supplementing with 0.1% (w/v) of 30% (v/v) dried intracellular extract of *Chlorella* sp. KLSc61 (Lbp + 0.1% ID3) increased the growth of *Lactobacillus plantarum* JCM 1149 by approximately 1.1-fold compared to the control (Lbp + MRS), and yielded cell accumulation similar to that with 0.1% glucose supplementation (Lbp + 0.1% Glc) at the 6, 8, 10, and 12-h time points (Figure 6A). In contrast, increasing the concentration of the 30% (v/v) ethanolic extract from 0.1% to 1.5% (w/v) (Lbp + 1.5% ID3) (Figure

6B) did not promote the growth of *L. plantarum* JCM 1149. This result was consistent with that of the fresh ethanolic extract and was not surprising because 30% of dried intracellular extract was derived from 30% of fresh extract and the result confirmed that high polar compounds (soluble in 30% ethanol) were likely not to enhance *L. plantarum* JCM 1149 growth.

To compare with the 30% (v/v) dried intracellular extract of *Chlorella* sp. KLSc61 (Lbp + 0.1% ID3), adding both 0.1% (w/v) (Lbp + 0.1% ID7) and 1.5% (w/v) (Lbp + 1.5% ID7) of 70% (v/v) dried intracellular extract into the *L. plantarum* JCM 1149 medium, the *Lactobacillus* cells grew better than with the 30% ones (Figure 6A-B), but increasing the concentration of the 70% (v/v) ethanolic extract from 0.1% (Lbp + 0.1% ID7) to 1.5% (w/v) (Lbp + 1.5% ID7) did not promote the growth of *L. plantarum* JCM 1149 (Figure 6C-D). This result showed the same trend as the fresh extract because all 70% (v/v) of fresh and dried intracellular ethanolic extract increased *L. plantarum* growth compared with the 30% (v/v) ones. It seems likely that moderately polar substances could positively affect *Lactobacillus* growth.

In the stationary phase of *L. plantarum* growth between 14 and 24 h, adding 0.1% (w/v) (Lbp + 0.1% ID7) (Figure 6C) of 70% (v/v) dried intracellular extract resulted in *L. plantarum* JCM 1149 cell accumulation up to 1.1- and 1.2-fold compared with controls, adding glucose (Lbp+0.1%Glc) and MRS + *L. plantarum* (Lbp+MRS), respectively. This finding indicated that the addition of 70% (v/v) dried intracellular extract with 0.1% (w/v) is more effective than at 1.5% (w/v) in promoting the growth of *L. plantarum* JCM 1149. Interestingly, we found this contradicted with Sylwia and Elżbieta's study because they found that 1.5% of *Chlorella vulgaris* powder (dried biomass) could promote the growth of *Lactobacillus brevis* more than 0.1% of *Chlorella vulgaris* powder, especially in the log phase of *L. brevis* growth (Sylwia & Elżbieta, 2020). This does not allow us to conclude that *Chlorella* cell mass, which contained carbohydrates derived from cell walls including some bioactive compounds (heat-sensitive), is more effective than dried intracellular extract because the amount used was different and the dried intracellular extract was crude, therefore, it is necessary to purify it and retest for L. plantarum growth enhancement. From this study, we preliminarily hypothesized that *Chlorella* cells contain bioactive compounds that could be used as prebiotics to supplement *Lactobacillus* probiotic growing. This finding demonstrates the benefits of *Chlorella* sp. and explores the use of *Chlorella* cells as biomaterials in functional food applications. It also confirms that *Chlorella* sp. could be used as a promising prebiotic for future study.

4. Conclusions

In the study, we aimed to test both fresh and dried intracellular extracts from *Chlorella* sp. KLSc61 to promote *Lactobacillus plantarum* JCM 1149 growth. We found that the dried intracellular ethanolic extract resulted in greater *L. plantarum* JCM 1149 cell growth than the fresh extract. Moreover, using 70% dried intracellular ethanolic extract positively presented the best effect on *L. plantarum* JCM 1149 growth. These results can be explained that the dried extract, which was concentrated from the fresh crude, contained a higher number of bioactive substances, and the moderate polarity of those substances had an impact on *L. plantarum* JCM 1149 growth. Testing of purified bioactive compounds from those 70% of dried crude extracts was necessary to characterize what compounds could be used to promote *Lactobacillus* growth. Additionally, it was likely that lower polarity of ethanol could also be used to extract other promising bioactive compounds. These findings highlight the potential of *Chlorella* cells as an alternative prebiotic resource; this opens interesting opportunities for pharmaceutical, nutraceutical, and functional food applications.

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