

Production of Exopolysaccharides Through Fermentation of Secondary Whey with Kefir Grains [†]

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Abstract: The cheese industry produces millions of tons of lactose-rich whey yearly, of which 50% is discharged into water and soil, leading to significant environmental challenges. In Mexico, cheese whey is repurposed for traditional cheese production. However, another by-product named secondary whey (SW) remains. This study focused on the production of exopolysaccharides (EPSs) via SW fermentation of kefir grains, yielding 632.6 ± 30.8 mg/L of a freeze-dried solid, and the carbohydrate-to-protein ratio stood at 2.89, corresponding to the bound EPS protein content. Through the analysis of Fourier Transform Infrared Spectroscopy (FTIR) spectra, it was determined that EPSs were successfully produced, as a signal was observed between 1200 and 1000 cm^{-1} , characteristic of the glycosidic bonds in polysaccharides.

Keywords: exopolysaccharide; kefir grains; whey; lactic acid bacteria; fermentation



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1. Introduction

Exopolysaccharides (EPSs) are long-chain polymers comprising repeating units of sugars produced and excreted into their surrounding environment by microorganisms such as fungi and bacteria. They provide microbes with a barrier against desiccation, extreme temperatures, osmotic pressure, and salinity [1]. EPSs can be attached to proteins, lipids, ions, and other compounds [2]. They have broad physicochemical and rheological properties and applications in numerous industries, mainly as emulsifiers [3] and gelling agents [4]. They also present antioxidant [5], antimicrobial [6], and hypotensive activities [7], and more recently, they have been found to serve as bio-decontamination agents due to their capacity to bind to toxic compounds like aflatoxins, along with their GRAS (generally recognized as safe) status [8].

Production of EPSs has been achieved by lactic acid bacteria (LAB) fermentation in different mediums [9]. More recently, kefir grains (KGs) have been used as a source of EPSs. KGs comprise a symbiotic community of LAB, acetic acid bacteria, and yeasts that are embedded in a complex matrix of proteins, lipids, and an EPS named kefiran. Kefiran is produced mainly by *Lactobacillus kefiranofaciens*, *Lactobacillus kefir*, *Lactobacillus parakefir*, and other species of *Lactobacillus* during cooperative biosynthesis with the different microorganisms present in the grains [10].

The cheese industry generates approximately 190 million tons of whey annually worldwide. Although treated as a by-product, whey contains 55% of the milk nutrients. It is often

discarded in aqueous effluents, leading to serious environmental issues due to its high biological oxygen demand (>30,000 ppm) and chemical oxygen demand (>60,000 ppm). A sustainable alternative is to process whey into value-added products such as whey concentrates. Another approach is to fractionate whey into lactose, proteins, and minerals. However, only 50% of this waste material is processed [11,12]. According to Sutherland [13], a high carbon-to-nitrogen ratio in the fermentation medium favors EPS production by various microorganisms, making whey a suitable substrate for achieving this goal. Some studies have focused on KG fermentation in whey to obtain beverages, and some have focused on producing exopolysaccharides [14–16]. In Mexico, the manufacture of traditional whey cheese (*Requesón*) generates a lactose-rich by-product named secondary whey (SW), which still represents an environmental hazard. Therefore, this study proposes an alternative use of SW as a fermenting substrate for lactic acid bacteria (LAB) present in kefir grains (KGs) to produce functional bioproducts such as exopolysaccharides (EPSs). Unlike previous research, which has primarily focused on synthetic or dairy-based media, this work investigates the revalorization of an underutilized by-product in cheese production, offering an alternative approach to sustainable biopolymer production.

2. Materials and Methods

2.1. Kefir Grains and Maintenance

KGs were acquired from a household in Mexico City and maintained in a glass jar (sterilized at 90 °C for 15 min) containing 500 mL of UHT whole milk (Alpura®, Cuautitlán Izcalli, Mexico) at room temperature, replacing the milk every 24 h. The microbiota composition of the KGs was previously evaluated by microbiological methods, determining the presence of LAB (60%), acetic acid bacteria (5%), and yeasts (35%).

2.2. Secondary Whey Production

First, 1 L of pasteurized whole milk was added with 0.2 mL of rennet (CHR HANSEN A/S Denmark; 280 U/mL) and incubated at 35 °C for 1 h. The whey was separated from the curd by filtering using a cheesecloth [17]. The recovered whey was acidified to pH 4.5 with 50% (*w/v*) citric acid (J. T. Baker, Mexico State, Mexico) and brought to a boil for 30 min. Finally, SW was filtered off using Grade 4 Whatman® paper (diam. 125 mm), adjusted to pH 6.8 with NaOH (J.T. Baker, Mexico State, Mexico; 4 N), and pasteurized at 121 °C for 10 min.

2.3. Fermentation

The KGs (obtained as in Section 2.1) were washed with sterile distilled water and superficially dried with paper towels. Then, 1 L of medium (SW or semi-skimmed milk with 1.8% fat (Alpura®, Mexico), respectively) and KGs were added to a previously sterilized glass jar to reach a concentration of 10% *w/v*. Finally, the jar was covered with a cotton cloth. Fermentation was carried out in three batches at 30 °C for 24 h without agitation.

2.4. Extraction of EPS

KGs were separated from the fermentation media with a plastic strainer (previously washed with sterile water). Proteins and other solids were precipitated in a boiling water bath for 30 min and removed by centrifugation at 10,000 rpm for 20 min at 4 °C (Avanti J-E centrifugal, Beckman Coulter, Indianapolis, IN, USA). Two volumes of cold ethanol (J.T. Baker, Mexico State, Mexico) were added to the supernatant, previously adjusted to pH 7 with NaOH (4 N) solution, which was then maintained for 24 h at 4 °C. Then, the precipitate was separated by centrifugation at 10,000 rpm for 20 min at 4 °C, before being resuspended and dialyzed (dialysis tubing cut-off: 10 kDa; Sigma-Aldrich®, St. Louis, MO, USA) in deionized water (18.2 MΩ·cm) for 48 h at 4 °C. Finally, the dialysate was freeze-dried to obtain a solid from SW (FS-SW) and milk (FS-M) [16].

2.5. Secondary Whey and Freeze-Dried Solid Characterization

First, 1 mg of FS-SW or FS-M was dissolved in 1 mL of deionized water, while SW was diluted at a 1:100 ratio. Since EPSs are polysaccharides, the total amount of carbohydrates present in the solution was quantified by the Phenol–Sulfuric Acid Method (both from J. T. Baker, Mexico State, Mexico) [18] using a standard curve of glucose (Sigma-Aldrich®, St. Louis, MO, USA; 20–100 µg/mL). The Bradford micro-assay method (Sigma-Aldrich®, St. Louis, MO, USA) was used to estimate total soluble protein content [19], with a standard bovine serum albumin (BSA; Sigma-Aldrich®, St. Louis, MO, USA) curve from 20 to 100 µg/mL.

Fourier Transform Infrared Spectroscopy (FTIR) spectra for both the freeze-dried solid and Dextran 70 kDa (Sigma-Aldrich®, St. Louis, MO, USA), used as a reference for an isolated EPS, were recorded in the 4000–400 cm^{−1} range using a Frontier spectrometer (PerkinElmer, Shelton, CT, USA) with an ATR accessory.

2.6. Statical Analysis

All experiments were performed in triplicate, and the results were analyzed using the statistical software Minitab 17 (State College, PA, USA). The results were analyzed using an analysis of variance (ANOVA) with an analysis of means using the Tukey test with a value of $\alpha = 0.05$.

3. Results and Discussion

Freeze-Dried Solid Characterization

In this study, the SW obtained from whey cheese (*Requesón*) production contained 60.7 ± 0.4 g/L of lactose and 0.25 ± 0.01 g/L of protein. After fermentation and EPS extraction, the total sugar content in FS-SW and FS-M was similar; however, FS-M had a higher protein content, as shown in Table 1. Although the yield of freeze-dried solids was higher in milk fermentation, compositional analysis revealed that carbohydrate production was equivalent in both SW and milk fermentation. Meanwhile, FS-M exhibited a higher presence of associated proteins. This increase in protein content could be attributed to a higher carbon-to-nitrogen ratio in the fermentation medium, in accordance with Sutherland's observations [13].

Table 1. The product yields from fermentation and the composition of total sugars and soluble proteins of freeze-dried solids.

	FS-SW	FS-M
Freeze-dried solid yield (mg)	632.6 ± 30.8	6138.1 ± 493.2
Total sugars (mg _{eq} glucose/L)	75.33 ± 0.38	73.66 ± 1.68
Total soluble proteins (mg _{eq} BSA/L)	26.04 ± 2.63	86.18 ± 3.27

FS-SW: solids obtained from secondary whey; **FS-M:** solids obtained from milk; **BSA:** bovine serum albumin.

In previous studies where KGs were fermented in milk or whey (primary or secondary), the yields were reported as total sugar content values rather than in relation to solids. For example, in the study by Rimada and Abraham [20], milk fermentation resulted in an EPS production value of 0.645 mg/L. Additionally, in their earlier studies [16] it was reported the fermentation of “deproteinized whey”, which contained 53.4 ± 3.1 g/L of lactose and 3.19 ± 0.25 g/L of protein, yielded an EPS concentration of 51.1 ± 1.6 mg/L. As can be seen, the results obtained in this study are higher than those previously reported.

FTIR can help identify functional groups within the polysaccharides by identifying certain chemical bonds. According to Wang et al. [3], the most prominent absorption peak between 1200 and 1000 cm^{−1} corresponds to the fingerprint region of polysaccharides, due to the C-O-C glycosidic bond; also, hydroxyl groups (–OH), normally found in sugar

molecules, can be detected at around $3200\text{--}3400\text{ cm}^{-1}$. The FTIR spectra of FS-SW, FS-M, and Dextran are shown in Figure 1. As can be seen, FS-SW (blue line) and FS-M (green line) have well-defined peaks at $1000\text{--}1200\text{ cm}^{-1}$, confirming that they are polysaccharides; also, they have a shorter absorption band at 3300 cm^{-1} compared to Dextran 70 kDa. This difference could be attributed to proteins interacting with the polysaccharides' OH groups. To confirm the presence of proteins attached to the polysaccharide in Figure 1, we focused on the peaks at around 1643 and 550 cm^{-1} , which correspond to the C–N bending of an amide bond present in proteins and peptides, also being associated with the disulfide bridge. Therefore, it can be concluded that both FSs are composed of peptides/proteins and EPS.

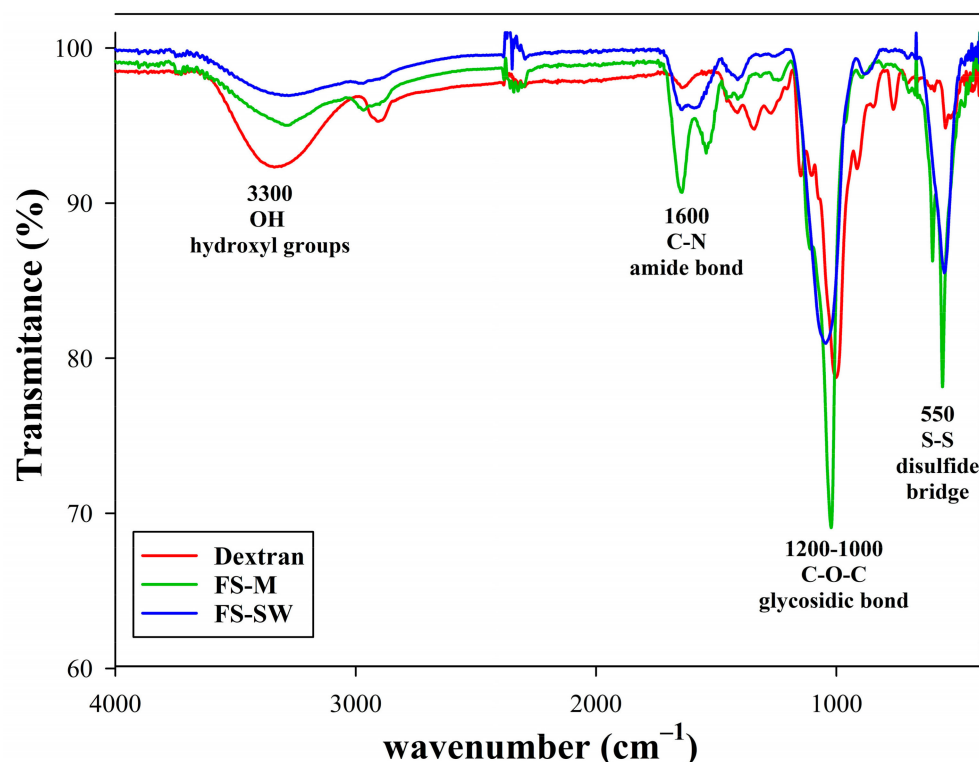


Figure 1. FTIR spectra of Dextran (70 kDa; red line), solids obtained from milk (FS-M; green line), and solids obtained from secondary whey (FS-SW; blue line) in the range of $4000\text{--}400\text{ cm}^{-1}$.

4. Conclusions

The use of secondary whey obtained from whey cheese (*Requesón*) production as a fermentation substrate for kefir grains is an effective way to utilize this by-product, enabling the production of significant amounts of value-added products such as exopolysaccharides. The freeze-dried solid obtained from SW exhibited a molecular fingerprint similar to that of a high-molecular-weight isolated exopolysaccharide (Dextran 70 kDa) and FS-M. However, the obtained product still contains bound proteins, which require an additional step of protein hydrolysis during the extraction process. Additionally, it is essential to determine the molecular weight (MW) of the produced EPS and carry out an analysis of their structure for better use of the solids. Finally, the possibility of scaling up this work is still pending, as the KG fermentation conditions need to be optimized in a continuous bioreactor to boost the growth of KGs' microbiota and EPS production.

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