Bioactive Carbohydrates and Dietary Fibre 16 (2018) 14–21

Contents lists available at ScienceDirect

Bioactive Carbohydrates and Dietary Fibre

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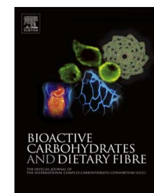

Optimization of culture conditions for kefiran production in whey: The structural and biocidal properties of the resulting polysaccharide

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A R T I C L E I N F O

Keywords:
Exopolysaccharide
Medium optimization
Biocidal properties
HPSEC
NMR

A B S T R A C T

In this study, the culture conditions necessary for the effective production of kefiran, using whey as a substrate, were evaluated through the one-factor-at-a-time and central-composite design methods. Using the optimal factors (whey supplemented with 15% (w/v) glucose fermented at 30 °C for 10 h without shaking), 0.20 g L^{-1} h^{-1} kefir grains composed of 18.57% (w/w) kefiran was obtained. The resulting polysaccharide was analyzed by high-pressure size-exclusion chromatography, gas chromatography-mass spectrometry, nuclear magnetic resonance, Fourier-transform infrared and Raman spectroscopy. Altogether, the results indicated that the purified polymer was homogenous and composed of galactose and glucose. The biocidal properties of the resulting kefiran were assessed, for the first time, using Live/Dead BacLight® real-time analysis, which indicated antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* strains. Additionally, the kefiran, when applied to various *S. typhimurium* strains, showed diverse biocidal results when in the presence of antibiotic multiresistance markers. In summary, these results demonstrated that kefiran can be produced from whey-supplemented media with high productivity and without losing its structural or biocidal properties.

1. Introduction

Kefir is a beverage that is commonly manufactured by fermenting milk using kefir grains that consist of polysaccharides and microorganisms (Chen et al., 2015). The polysaccharide present within kefir grains, referred to as kefiran, is a water-soluble glucogalactan that is produced mainly by *Lactobacillus kefiranofaciens* (Ahmed et al., 2013; Ghasemlou, Khodaiyan, & Oromiehie, 2011; Zajšek, Kolar, & Goršek, 2011). Kefiran polysaccharide has been associated with several bioactive properties, including antitumor, antibacterial, antioxidant, and anti-inflammatory activities (Chen et al., 2015; Rodrigues, Carvalho, & Schneedorf, 2005; Wang, Ahmed, Feng, Li, & Song, 2008); healing properties (Rodrigues, Caputo, Carvalho, Evangelista, & Schneedorf, 2005); gut immune-system modulation (Medrano, Racedo, Rolny, Abraham, & Pérez, 2011; Serafini et al., 2014; Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006); protection of epithelial cells against microbial toxins (Medrano, Pérez, & Abraham, 2008); and reduction of hypertension-induced high blood pressure (Maeda, Zhu, Suzuki, Suzuki, & Kitamura, 2004a, 2004b).

Because kefir grains can metabolize lactose, they can be used to ferment cheese whey, a lactose-rich waste product of negligible cost (Freitas, Alves, & Reis, 2011). Cheese whey's lactose content and its other elements, which include essential nutrients for microbial growth, turn this dairy byproduct into a potential feedstock for the production of valuable compounds through fermentation processes (Huertas, 2009; Panesar, Kennedy, Gandhi, & Bunko, 2007). In this sense, whey has attracted increasing interest as a substrate for kefiran production; this process has been tested using various sources (e.g., kefir grains, pure cultures of L. *kefiranofaciens*, and mixed cultures), for various culture media, and under various physicochemical conditions (Cheirsilp & Radchabut, 2011; Cheirsilp, Shoji, Shimizu, & Shioya, 2003; Dailin et al., 2016; Ghasemlou, Khodaiyan, Jahanbin, Gharibzahedi, & Taheri, 2012; Maeda et al., 2004a, 2004b; Rimada & Abraham, 2001; Tada, Katakura, Ninomiya, & Shioya, 2007; Yeesang, Chanthachum, & Cheirsilp, 2008; Zajšek, Goršek, & Kolar, 2013a, 2013b).

To make the production of whey-based kefiran economically viable, process optimization is of vital importance. It is possible to influence

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https://doi.org/10.1016/j.bcdf.2018.02.001
Received 1 September 2017; Received in revised form 30 January 2018; Accepted 9 February 2018
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the production yield and the microbial polysaccharide properties by modifying culture conditions such as incubation temperature, carbon supplementation, and agitation. In this regard, the aims of this work were to optimize the medium composition and the physicochemical conditions for kefiran production from whey waste and to characterize the chemical structure and biocidal activity of the resulting polysaccharide.

2. Materials and methods

2.1. Kefir grains and inoculum preparation

Kefir grains that had been isolated from Tibetan milk kefir beverages were used in the experiments (Fiorda, de Melo Pereira, Thomaz-Soccol, Rakshit, & Soccol, 2016). The inoculum was prepared by cultivating kefir grains in pasteurized whole milk that was renewed daily for 7 days. After this time, the grains were washed with sterile distilled water and inoculated into the cheese-whey media.

Glucose, lactose, sucrose, galactose, ethanol, $NaNO_2$, NaN_3, TFA, and KBr were purchased from Sigma Aldrich (Sao Paulo, Brazil). The cheese whey was donated by Anila Industries (Paraná, Brazil).

2.2. Media and fermentation conditions

The cheese whey was supplemented with various carbon sources (e.g., galactose, glucose, lactose, and sucrose); the kefir grains were then inoculated into the whey and tested at 5%, 10%, 15%, and 20% (w/v). A whey medium without a sugar supplement was used as the control. Batch fermentations were carried out in 250 mL Erlenmeyer flasks at 30 °C for 36 h. The fermentation volume was 100 mL, and the initial humid weight of the kefir grains was 4.2 g. At the end of the process, the kefir grains were separated from the fermentation product through filtration; the grains were then washed with cold, distilled water and dried carefully with filter paper. Later, the kefiran was extracted from the grains, and two responses were calculated: the biomass increase (Zajšek et al., 2013a, 2013b), and the kefiran productivity (in $g\,L^{-1}\,h^{-1}$). This is the formula used to calculate the biomass increase:

$$Biomass\ \ increase = \frac{Wf - Wi}{Wi} x100 \tag{1}$$

where W_i and W_f are the initial and final weights, respectively. The experiments were performed in triplicate. Statistical treatments were carried out using a one-way analysis of variance with a significance level of $\alpha = 0.05$ in R (R version 3.2.3; December 10, 2015; R Foundation for Statistical Computing).

The medium composition with the best kefiran productivity was the whey supplemented with 15% glucose; this medium was optimized through the evaluation of three physicochemical properties (i.e., temperature, time, and agitation). A central-composite design with six axial points and four central points was developed (Table S1). The analysis of variance and the response surfaces were carried out using the rsm package in R (Lenth, 2009). At the end of each experiment, the kefir grains were separated and processed as mentioned above, and the biomass increase and productivity ($g\,L^{-1}\,h^{-1}$) were calculated.

2.3. Kefiran extraction

The kefiran content of each sample was extracted using the protocol previously described by Rimada and Abraham (2003). A weighted amount of kefir grains, which were maintained in whey supplemented with carbohydrates (based on the optimization design; see below) was suspended in boiling distilled water for 15 min with discontinuous stirring. After cooling, to eliminate biomass residues, the mixture was centrifuged at 10,000*g* for 20 min at 20 °C. The addition of three volumes of cold (− 20 °C) ethanol was used to precipitate the supernatant containing the soluble fraction (e.g., the polysaccharide and other components). The suspension was then centrifuged at 10,000*g* for 20 min at 4 °C; the resulting precipitate (containing the biopolymer) was then dissolved in hot, distilled water. The precipitation procedure was repeated twice to eliminate protein and biomass residues. Finally, the polysaccharide was freeze-dried.

2.4. Structural analysis

2.4.1. High-pressure size-exclusion chromatography (HPSEC)

Kefiran (1.0 $mg\,mL^{-1}$) was dissolved in 0.1 M $NaNO_2$ containing NaN_3 (0.2 $g\,L^{-1}$) at 25 °C. The samples were filtered using 0.2 mm cellulose acetate membranes. The biopolymer analysis was performed using a differential refractive index detector (Waters 2410) coupled with a multi-angle laser light scattering detector (Wyatt Technology Dawn DSP). The products were separated isocratically at 0.6 $mL\,min^{-1}$ (Waters 515 peristaltic pump), and four size-exclusion columns with exclusion limits of 7.10^6, 4.10^5, 8.10^4, and 5.10^3 $g\,mol^{-1}$ were placed in series (Waters, Massachusetts, USA). The HPSEC data were collected and analyzed with ASTRA (Wyatt Technology, Massachusetts, USA).

2.4.2. Monosaccharide composition

The kefiran was totally hydrolyzed to determine its monosaccharide composition. In this process, 2.0 mg of the polysaccharide was solubilized in 0.5 mL of 2 M TFA and maintained at 100 °C for 2 h; then, the solution was evaporated, purging with nitrogen gas. The hydrolyzed sample was solubilized in 0.5 mL of distilled water, and the monosaccharides were reduced with $NaBH_4$ for 12 h. The solution was acidified with acetic acid (reaching pH 5.0) and dried with nitrogen; the residual boric acid was then codistilled with methanol. The alditols were acetylated with 0.5 mL of acetic anhydride at 120 °C for 1 h and analyzed by gas chromatography-mass spectrometry.

2.4.3. Nuclear magnetic resonance (NMR) spectroscopy

Kefiran samples were dissolved in D_2O 40 $mg\,mL^{-1}$ for ^{13}C and in 15 $mg\,mL^{-1}$ for ^1H NMR, and the results were recorded at 50 °C using a spectrometer (Bruker Avance DRX400). The base frequencies were 400.13 and 100.61 MHz for the ^1H and ^{13}C nuclei, respectively. Chemical shifts were expressed relative to acetone (internal standard) at 30.59 for ^{13}C.

2.4.4. Fourier-transform infrared (FTIR) spectroscopy

The FTIR spectrum was obtained using KBr pellets; the kefiran sample was pressed into the KBr (0.1% w/w), and the FTIR spectrum was recorded (Bomem, Hartmann & Braun, MB-Series) with a resolution of 4 cm^{-1} for 32 scans min^{-1} using the transmittance technique. The wavenumbers from 400 to 4000 cm^{-1} were scanned. The data obtained were analyzed using the ACD NMR processor (academic version).

2.4.5. Raman spectroscopy

Raman spectroscopy was performed on an upgraded WITec UHTS300 confocal microscope system (WITec GmbH, Ulm, Germany) with a 532 nm frequency-doubled Nd: YAG laser and a Nikon 100× objective. The Raman spectra were measured with a 600 nm grating in the 350–3980 cm^{-1} range. An area of 50 × 50 mm^2 was scanned in 0.39 mm steps, resulting in a map with 1024 × 127 pixels. A survey scan was run with 100 s integration times to determine the quality of the sample in the chosen area, and then a full scan was conducted at 1 s $pixel^{-1}$ to obtain reasonable spectra.

2.5. Biocidal properties

The Live/Dead BacLight® kit was used to measure the antimicrobial activity of kefiran against *P. aeruginosa*, *E. coli*, *S. aureus*, *S. typhimurium* LT2 (the type strain of *S. typhimurium*, resistant to streptomycin, rifampicin, and nalidixic acid), *S. typhimurium* CQ27 (resistant to

amikacin and gentamicin), *S. typhimurium* CQ28 (resistant to amikacin, gentamicin, and nitrofurantoin), and *S. typhimurium* CQ29 (resistant to nitrofurantoin). Bacteria were grown in the late exponential phase and inoculated in a soft nutrient agar (1:10 dilution); a 20 µL drop was then placed on the surface of a glass slide, which was incubated for 24 h to allow biofilm formation. Subsequently, the biofilm was covered with 1.0% (wt) kefiran for 30 and 60 min. After treatment, the biofilms were carefully washed with deionized water. Biofilm staining was prepared by mixing both dyes in equal proportions (0.75 µL of each in 0.5 mL of sterile deionized water). The resulting dye solution was applied to the entire biofilm and held in darkness for 20 min. Then, the samples were washed using deionized water and observed using an epifluorescence microscope (Leica DM 2500, Germany) equipped with ultraviolet filters (495–505 nm) to determine the bacteria's viability.

The detection was set at excitation wavelengths between 510 and 550 nm and at an emission wavelength of 590 nm (U-MWG2 filter) for living bacteria (green), and at an excitation wavelength of 460 nm and emission wavelengths of 490–520 nm (U-MWB2 filter) for dead bacteria (red).

3. Results and discussion

3.1. Evaluation of medium parameters for kefiran production in whey

Some methods have been proposed for whey valorization through kefir fermentation, including functional beverage formulation and kefiran exopolysaccharide production (Golfinopoulos, Soupioni, Kopsahelis, Tsaousi, & Koutinas, 2012; Magalhães et al., 2010; Soupioni, Golfinopoulos, Kanellaki, & Koutinas, 2013). Nevertheless, important aspects such as medium composition and exopolysaccharide structure must first be clarified before kefir grains can be fully applied to whey fermentations. To evaluate the best conditions for kefiran production in whey, carbon supplementation (i.e., galactose, glucose, lactose, and sucrose) was analyzed at the 5%, 10%, 15%, and 20% (w/v) levels (Fig. 1). The best result was obtained with 15% glucose, which yielded $0.090 \pm 0.07\,\mathrm{g\,L^{-1}\,h^{-1}}$; for this reason, 15% glucose was chosen to evaluate the effects of temperature, time, and agitation through a central-composite design. The results for biomass increase and kefiran productivity across various conditions, are visualized in Figs. 2 and 3, and Table S1.

A model was fitted with a second-order polynomial to adequately describe the kefir grains' growth. Eq. (2) is the model for this growth:

$$Y_1 = -554.670^{***} + 24.145X_1^{***} + 6.456X_2^{***} + 4.476X_3^{**} - 0.079X_1X_3^{*} - 0.317X_1^{2***} - 0.109X_2^{2***} - 0.015X_3^{2**} \quad (2)$$

where Y_1 is the biomass increase, X_1 is the temperature, X_2 is the time, and X_3 is the agitation speed.

Eq. (2) and Fig. 2 show the significance of the three studied parameters in biomass production. Agitation has a particularly strong negative effect on biomass production, confirming data from Kourkoutas, Sipsas, Papavasiliou, and Koutinas (2007). Kefiran production, however, does not depend on agitation. Eq. (3) shows the model for kefiran productivity, and Fig. 3 shows the resulting response-surface plot.

$$Y_2 = 0.103X_1^{*} - 0.002X_1^{2**} + 4.393 * 10^{-4}X_2^{2*} \quad (3)$$

where Y_2 is the productivity, X_1 is the temperature, and X_2 is the time.

In summary, the best conditions for kefiran production in whey were 15% (w/v) glucose supplementation and fermentation at 30 °C for 10 h without shaking. Under these conditions, $0.20\,\mathrm{g\,L^{-1}\,h^{-1}}$ (kefir grains composed of 18.57% (w/w) kefiran) was obtained, which is higher than the levels obtained in previous, whey-based kefiran studies in the literature (Cheirsilp & Radchabut, 2011; Rimada & Abraham, 2001). Rimada & Abraham (2001) reported $0.0085\,\mathrm{g\,L^{-1}\,h^{-1}}$ kefiran productivity for kefir grains during whey fermentation. Using a mixed L. *kefiranofaciens* and *S. cerevisiae* culture, Cheirsilp and Radchabut (2011) reached a maximum productivity of $0.067\,\mathrm{g\,L^{-1}\,h^{-1}}$ in a fed-batch, yeast-extract-supplemented whey fermentation. More recently, Dailin et al. (2015) reached a kefiran productivity of $0.02\,\mathrm{g\,L^{-1}\,h^{-1}}$ using a pure culture of L. *kefiranofaciens* in whey supplemented with sucrose and yeast extract.

3.2. Structural analysis

An important feature associated with biopolymer production is the purity of the resulting polysaccharide. In this study, the biopolymer sample's elution profile in HPSEC (using a multi-angle laser light scattering and refractive index detector) showed a unique and symmetric peak, indicating that the polysaccharide had a homogeneous molecular mass distribution (Fig. 4). The basic structural units of kefiran, according to the gas chromatography-mass spectrometry results, were glucose and galactose. This monosaccharide composition was also reported for the exopolysaccharide produced by both kefir grains of other origins and single-strain cultures isolated from kefir grains (Maeda et al., 2004a, 2004b; Wang et al., 2008; Zajšek et al., 2013a, 2013b).

Fig. 5 shows the ^{1}H- and ^{13}C NMR biopolymer analyses. The ^{1}H NMR spectrum shows, in the anomeric region (i.e., δ 5.3–4.4), five intense and well-defined signals (Fig. 5A). There are two overlapped signals: at 4.49, attributed to [→6)-β-D-Glcp-(1→], and at 4.53, overlapping between [→3)-β-D-Galp-(1→] and [→4)-β-D-Glcp-(1→. The signal at δ 4.65 was attributed to [→2,6)-β-D-Galp-(1→], while a duplet at δ 4.82 was attributed to [β-D-Glcp-(1→]. Finally, a singlet attributed to [→4)-α-D-Galp-(1→] was observed at δ 5.14.

The ^{13}C NMR spectrum (Fig. 5B) shows two main signals in the anomeric region (δ 110–90). The resonance at δ 98.5 was assigned to C-1 of [→4)-α-D-Gal*p*-(1→] residues (C-4 at δ 82.6). The intense anomeric signal at δ 105.7 corresponds to the C-1 overlapping of β-hexopyranosyl residues. The DEPT NMR experiment (Fig. S1) produced inverted signals at δ 63.7 and 63.1, corresponding to unsubstituted C-6 of hexopyranosyl residues, respectively; and at δ 72.5 and 71.6, attributed to substituted C-6 of [→2,6)-β-D-Gal*p*-(1→] and [→6)-β-D-Glcp-(1→] residues, respectively. In this way, the ^{1}H- and ^{13}C NMR patterns of kefiran obtained in a simple, inexpensive medium are identical to those previously reported for kefiran produced using a PYG medium with glucose as the carbon source (Maeda et al., 2004a, 2004b). This indicates that the exopolysaccharides have the same chemical structure when produced under different conditions.

The biopolymer sample was also analyzed using FTIR and Raman spectroscopy to identify the functional groups that were present in the exopolysaccharide's structure. The broad band around $3480\,\mathrm{cm^{-1}}$ in the FTIR spectrum was assigned to the O–H stretching of the hydroxyl groups, which is typically associated with polysaccharide structures (Aminov & Mackie, 2007; Wang et al., 2008). The peaks around 1400 and $1270\,\mathrm{cm^{-1}}$ in the Raman spectrum suggested -CH_2 or -CH_3 bending vibrations, a -CH_2OH related mode, and -CH_2 deformation (Xing et al., 2016). The peaks around $1038\,\mathrm{cm^{-1}}$ in the FTIR spectrum and $1080\,\mathrm{cm^{-1}}$ in the Raman spectrum could also be assigned to the ring vibrations that overlap with the stretching vibrations of the C–OH side groups and with the C–O–C glycosidic band vibration, each of which is characteristic a polysaccharide (Cael, Koenig, & Blackwell, 1973; Semeniuc, 2013). Finally, in the Raman spectrum, the vibration bands at $895\,\mathrm{cm^{-1}}$ indicated the presence of β-glycosidic linkages (Chen et al., 2015), and those around $736\,\mathrm{cm^{-1}}$ indicated C–H bending (Ambrose, Lohumi, Lee, & Cho, 2016).

The chemical and spectroscopic results imply that the isolated exopolysaccharide had the same chemical structure as kefiran produced in nutrient-rich substrates (Maeda et al., 2004a, 2004b).

3.3. Biocidal properties

The global problem of antibiotic resistance is increasing, and about 70% of known pathogenic bacteria are resistant to at least one

Fig. 1. Effect of supplementing whey with different carbon sources on the biomass increase and the kefiran production (Errors: SD ≤ 10%, n = 3).

antibiotic (Yılmaz & Özcengiz, 2017). Some studies have suggested the presence and evolution of resistance genes, which could be associated with a predisposition among these organisms to obtain resistance or to a lateral transfer of genes due to DNA contaminants in commercial antibiotics (Aminov & Mackie, 2007). It is crucial to find antibiotic molecules that have alternative mechanisms such as cell membrane damage. Recent studies have shown that kefiran has the ability to disrupt cell membranes, inducing pores (Barbosa, Santos, Lucho, & Schneedorf, 2011a, 2011b).

The present work, for the first time, shows kefiran's antimicrobial activity against seven pathogenic bacteria; this effect was investigated in real time using the Live/Dead BacLight® kit, thus establishing the

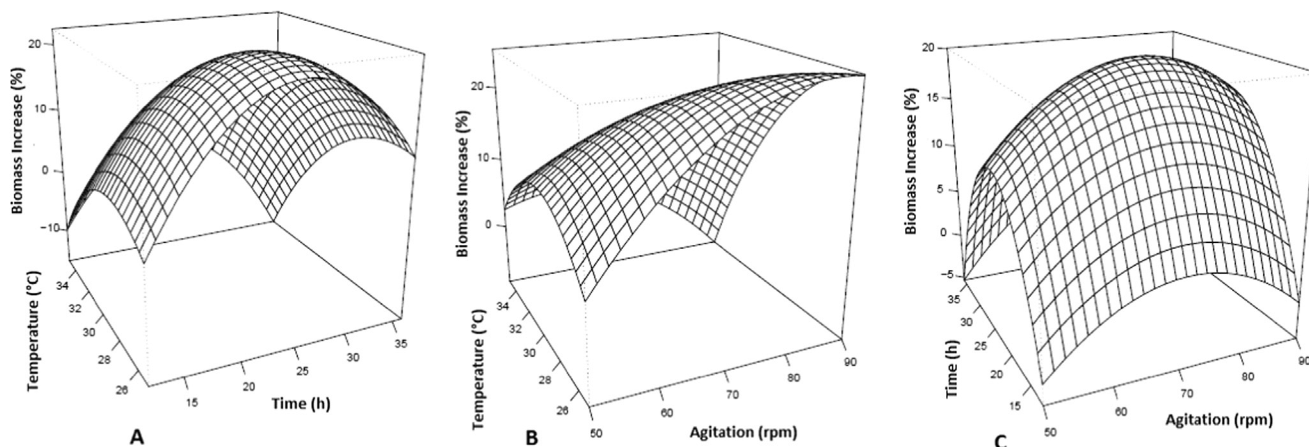


Fig. 2. Response surface plots for the biomass increase. A. Temperature and time, B. Temperature and agitation C. Time and agitation.

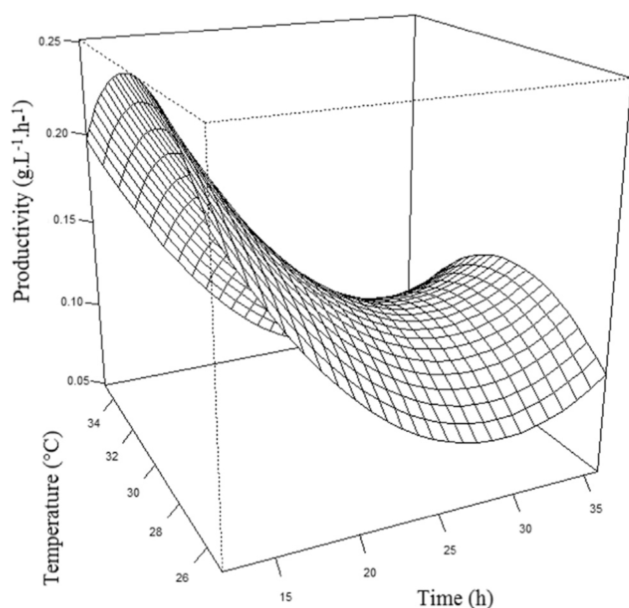


Fig. 3. Response surface plot for kefiran productivity.

effectiveness of the polymer's biocidal activity. The Live/Dead BacLight® was developed to distinguish between viable and nonviable bacteria according to the damage in their cell membranes (Hu, Murata, & Zhang, 2016). Among the Gram-negative strains tested, *P. aeruginosa* seemed to be the most sensitive to kefiran treatment (1.0% for 30 min) because it showed a considerable red stain under fluorescent microscopy (Fig. 6). Red staining indicates a compromised bacterial membrane resulting from either cell damage or loss of membrane integrity due to contact between the biopolymer and the cell surface. In *E. coli*, 50% of the population was affected after just 30 min of incubation with kefiran, but most of the cells were dead after 1 h of polymer treatment. On the other hand, after 30 min of incubation, Gram-positive *S. aureus* exhibited a lower kefiran bactericidal effect than did Gram-negative bacteria (as the former exhibited some green spots). This difference could be attributed to the presence in the Gram-positive strain of a bacterial cell wall composed of peptidoglycan, which may have reduced kefiran diffusion to the cell membrane. However, deteriorated bacteria were noticed in the Gram-positive images after 1 h of polymer incubation under the same experimental conditions.

Given the potential applications of kefiran as a biocontrol in the food industry, its bioactivity was tested against several *S. typhimurium* strains that had various antibiotic-resistant markers (Fig. 6). The *S. typhimurium* LT2 strain was less susceptible to kefiran treatment, but after 1 h, a small population of dead bacteria began to appear, probably because of the strain's reported antibiotic multiresistance to streptomycin, rifampicin, and nalidixic acid. Among the clinical isolates, *S. typhimurium* CQ27 (resistant to amikacin and gentamicin) was the most susceptible; *S. typhimurium* CQ28 (resistant to amikacin, gentamicin, and nitrofurantoin) seemed to be resistant to the kefiran treatment. Finally, the clinical isolate *S. typhimurium* CQ29 (resistant to nitrofurantoin) showed an intermediate behavior, with resistance during a 30 min exposure to kefiran but with visible red spots indicating compromised bacteria after 1 h.

4. Conclusions

The present study demonstrated the viability of high-productivity kefiran generation under minimal control conditions and using a simple purification method. Kefiran production is achieved in whey supplemented with 15% (w/v) glucose after fermentation at 30 °C for 10 h without shaking; this method produced 0.20 $g\,L^{-1}\,h^{-1}$ of kefiran from 4.2 $g\,L^{-1}$ of starter kefir grains. Additionally, the structural analysis indicated that the obtained biopolymer's structure does not change when produced in nutrient-rich substrates.

The kinetic analysis of kefiran's antimicrobial activity against selected microbes used fluorescent dyes and showed high effectiveness against *P. aeruginosa*, followed by *E. coli* and *S. aureus*; this could suggest higher biocidal activity against Gram-negative microorganisms than against Gram-positive bacteria that affect the cell membrane. However, the antimicrobial results suggest that the mechanism of action is related to damage in the cell membrane.

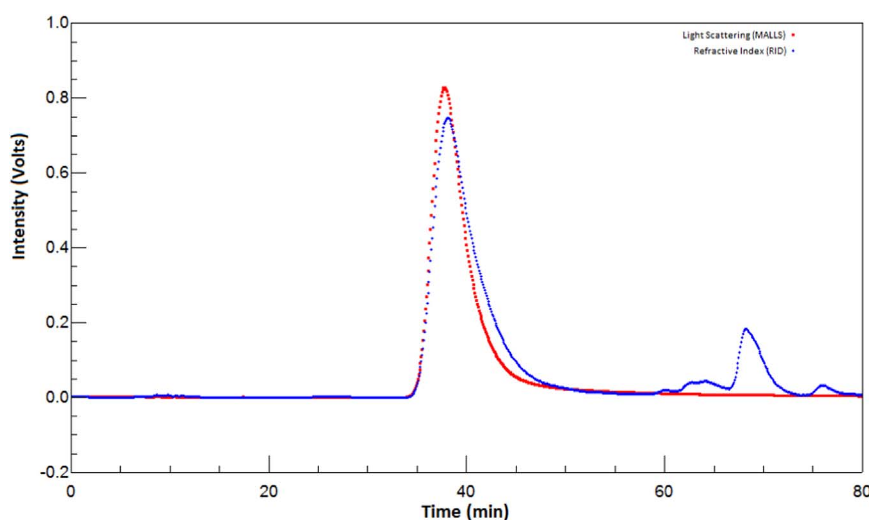
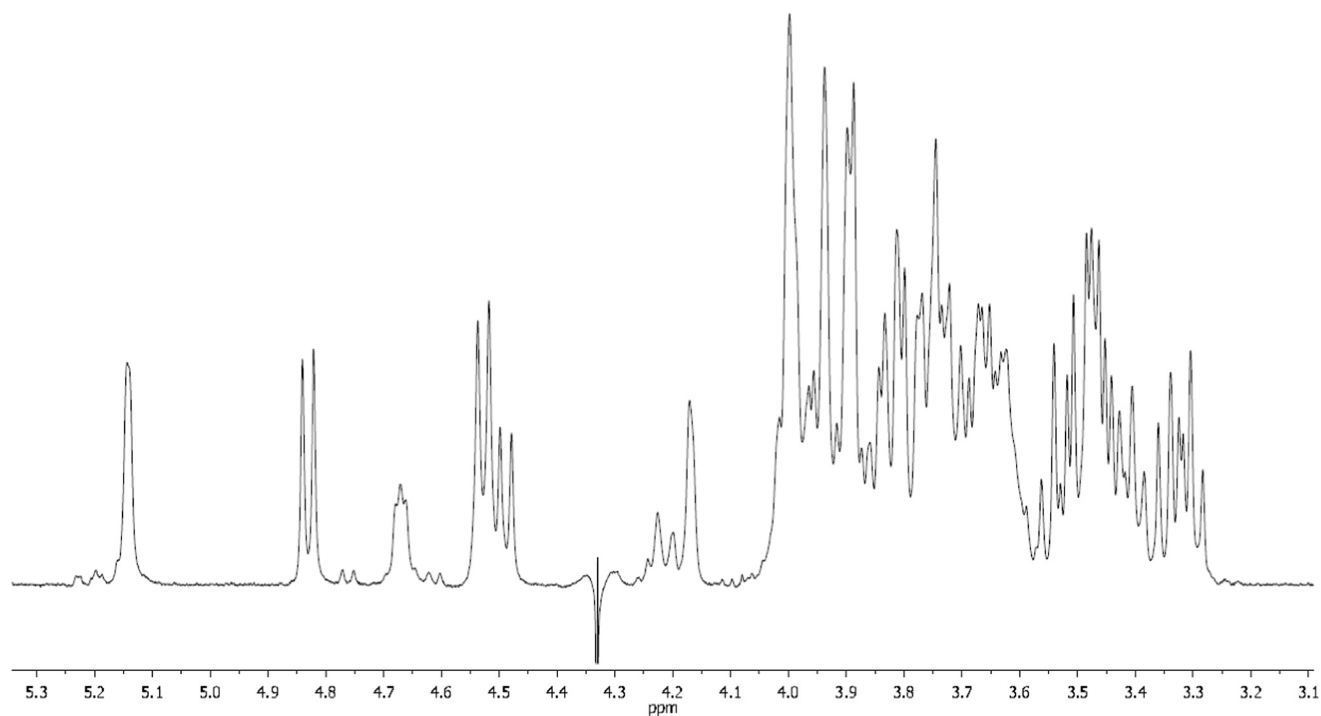


Fig. 4. HPSEC-MALLS-RID elution profile of the biopolymer obtained using the optimized conditions.



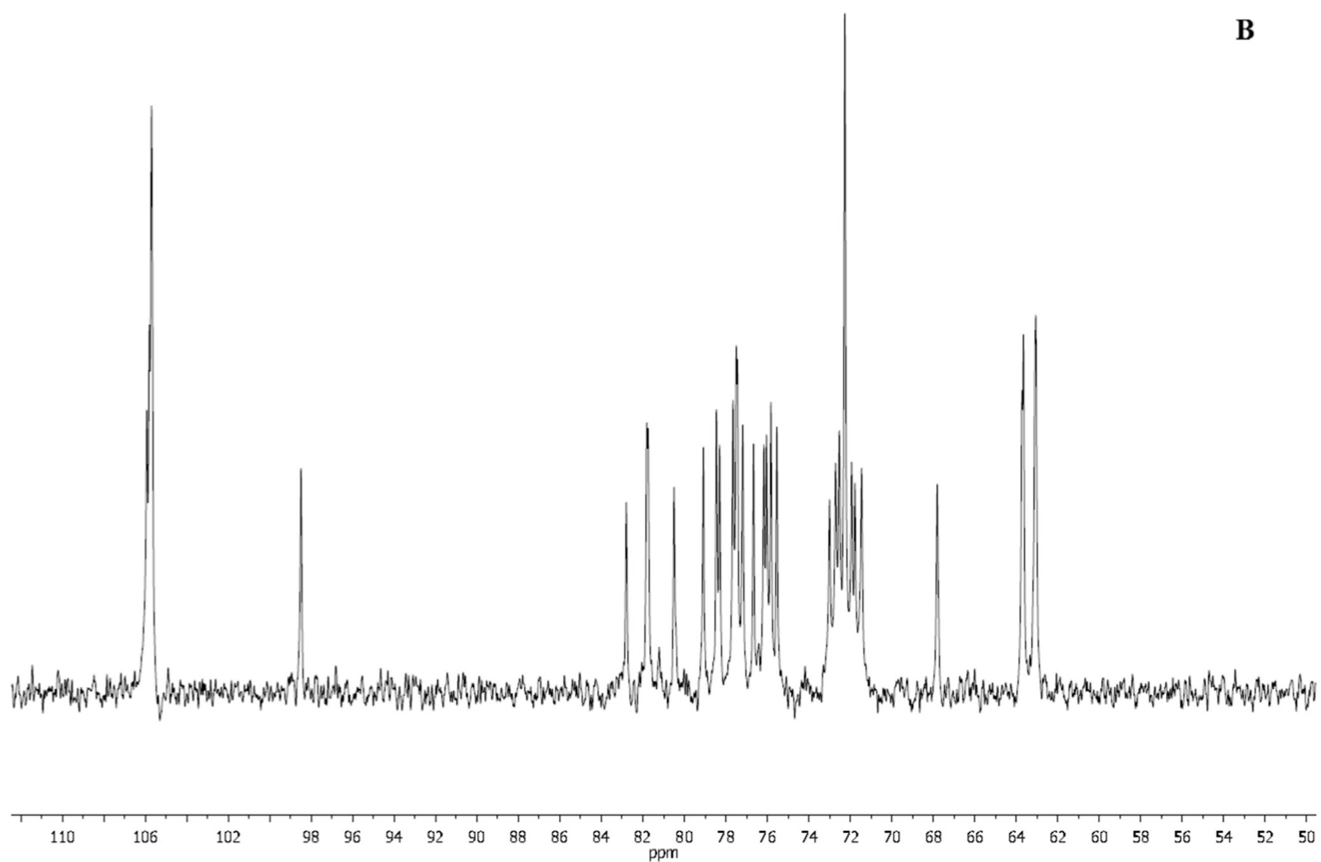


Fig. 5. ^{1}H NMR spectrum of the biopolymer obtained using the optimized conditions A. ^{13}C NMR spectrum of the biopolymer obtained using the optimized conditions B.

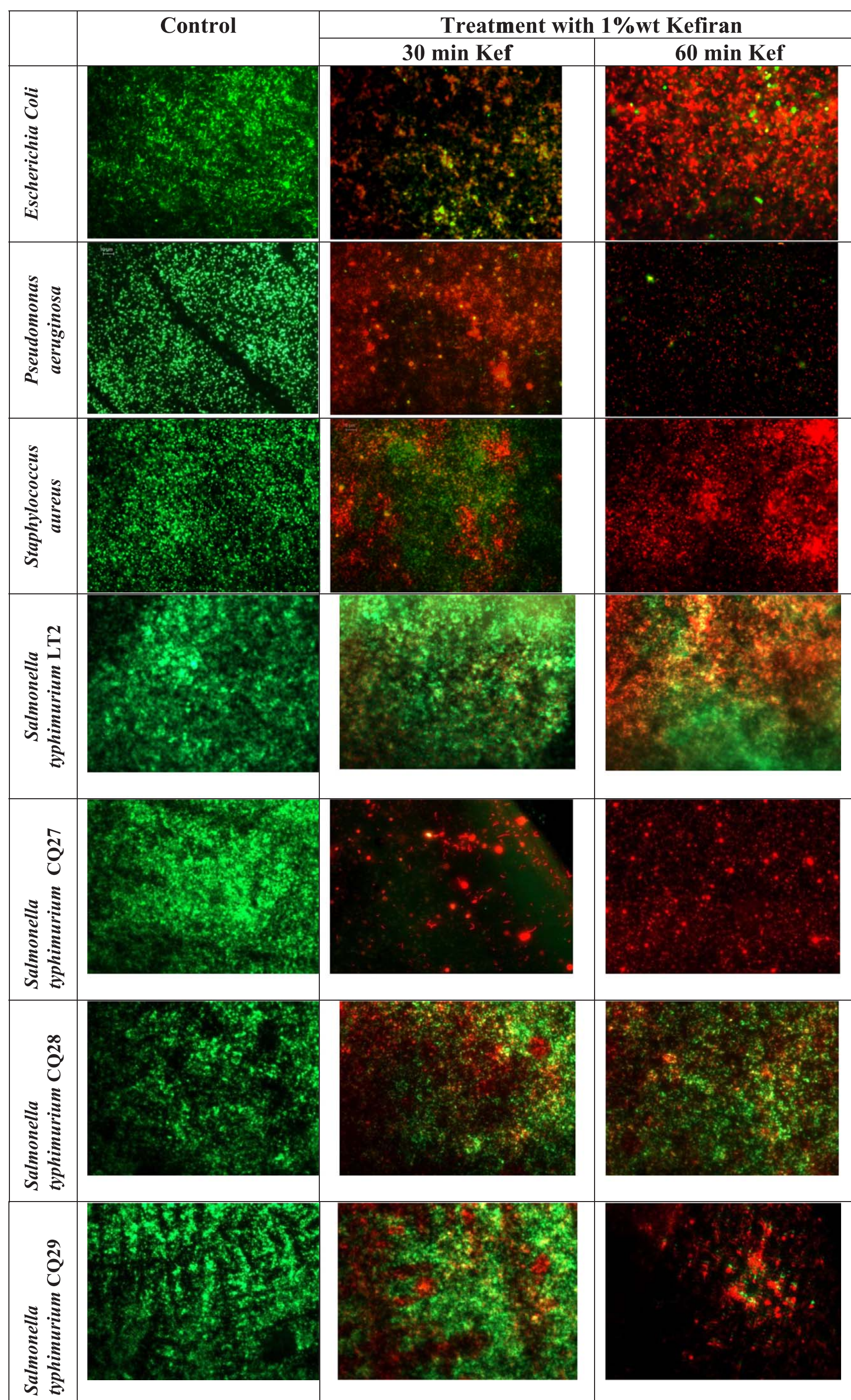


Fig. 6. Kefiran (1 wt%) effect on bacterial on viability after 30 and 60 min treatment, tested by Live/Dead BacLight® kit. Green color indicates live bacteria while red are dead ones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

The Argentinian support from CONICET, Universidad Nacional de La Plata, and ANPCyT (PIP-0214, X/545, and PICT2011-2116, respectively) is gratefully acknowledged. The authors also want to thank CNPq and CAPES Brazilian agencies.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bcdf.2018.02.001.

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