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Fingerprint profiling of polysaccharide kefiran extracted from kefir grains biomass

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Abstract

Kefiran is a water-soluble polysaccharide extracted from kefir grains biomass. The identification and quantification of kefiran monosaccharides were carried out by HPTLC after the complete acid hydrolysis of kefiran solutions. The mobile phase was a mixture of n-propanol: acetic acid: water (70:20:10, v/v). For derivatization was used *p*-aminobenzoic acid and *o*-phosphoric acid in methanol. The identified HPTLC fractions were glucose (Rf 0.71) and galactose (Rf 0.66), which indicates a high purity of the extracted kefiran. The relative concentrations of each monosaccharide identified in samples are dependent on the initial molecular weight of the polymer chain. The kefiran isolated from kefir grains grown in milk is a heteropolysaccharide which contains D-glucose and D-galactose units in a ratio of 0.94:1.1.

Keywords: kefiran, monosaccharide, HPTLC, kefir grains

1. Introduction

Bacterial growth is often followed by the production of exopolysaccharides (EPS), which have significant ecological and physiological functions. To date, EPS produced by lactic acid bacteria (LAB) have received increasing interest, mainly because of their GRAS (generally recognised as safe) status and their rheological properties in food, which improve the textures of fermented dairy products, such as yoghurt, cheese, kefir or fermented milks [1].

Kefiran is considered to be a metabolite of capsular bacteria such as *Lactobacillus kefir* [2], *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae* [3,4].

Kefiran is a slimy-gel polysaccharide, whose viscosity and adhesion properties are due to its protein-amino acid-fat complex, as a stable biological mass.

On a scientific basis, the molecular structure of kefiran is not well understood, but it was proposed to be a branched hexa - or hepta - saccharide repeating units (D-glucose and D-galactose in almost equal proportion) [5, 1]. Each unit is composed of a regular pentasaccharide, to which one or two sugar residues are randomly linked. For this reason, kefiran is resistant to enzymes attack [6].

Kefiran has an antibacterial and antitumor activity, modulates gut immune system and can also be used as a food grade additive for the fermented product since it enhances the rheological properties of chemically acidified skim milk [1].

Numerous applications in the industrial segments of microbial exopolysaccharide polymers were reported

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mainly due to their rheological properties (formation of viscous solutions even at low concentrations, good stability in a wide pH and temperature ranges) [7]. The functionality, stable cost and abundant supply of polysaccharides as biopolymer membranes and films, recommend their use in different industries with tailored flow properties [8].

Thin-layer chromatography is without doubt one of the most versatile and widely used separation methods in chromatography. The concept of TLC is simple and samples require minimal pretreatment. The previous image of TLC regarding low sensitivity, poor resolution, and reproducibility made it stagnant and forgotten technique few years back. Now, it is the most used chromatographic technique and likely to remain so for times to come. The recent advancements made in TLC have revolutionized and transformed it into a modern instrumental technique, called HPTLC. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, enable it to be an analytical tool for chromatographic information of complex mixtures of inorganic, organic, and biomolecules [9].

The aim of this study was to evaluate the influence of extraction conditions (temperature and time) on the composition of kefiran solutions using HPTLC. The kefir grains, as by-products, were used to obtain the polysaccharide kefiran.

2. Material and methods

2.1. Isolation and purification of kefiran

Kefiran was extracted from kefir grains and frozen before use (for at least 24 hours). To the weighted amount of kefir grains was added water in a ratio 1:10 (w/w) in four different conditions: at 70°C/100 minutes (M2), at 80°C/30 minutes (M4), at 90°C/20 minutes (M3), and at 100°C/5 minutes (M1). The mixture was then cooled and centrifuged at 10000 *g* for 10 min. The polysaccharide dissolved in the supernatant was purified by freezing at -20°C overnight followed by a slow thawing to avoid the destruction of polysaccharide structure. After centrifugation at 5000 *g* for 10 minutes at 4°C, the kefiran pellets were dissolved in distilled water at 60°C. The precipitationwas repeated two times. The kefiran solutions obtained showed high purities [10, 11].

2.2. Chemical hydrolysis of polysaccharide kefiran

The analysis of kefiran polysaccharide consists in the determination of sugar residues obtained by chemical hydrolysis. The complete acid hydrolysis of kefiran was carried out using a heating block. The hydrolysis was performed based on the slightly modified protocol of Garna et al. (2004) [12]. Forty mg of kefiran solution was hydrolyzed with 2.5 mL 0.2M trifluoroacetic acid (TFA) at 80°C for 24 h. The reaction medium was neutralized with ammonium hydroxide (14 M), drop by drop until pH 7 and diluted to 25 mL with milli-Q water. The hydrolysates were stored at 4°C until further analysis. The neutralization was performed prior to analysis. Samples were filtered through 0.45-µm Millipore filters.

2.3. Monosaccharides identification and quantification by HPTLC

The composition of polysaccharides was investigated using the method of Piermaria et al. (2008) [13]. The mobile phase used was a mixture n-propanol: acetic acid: water (70:20:10). For derivatization, 7g/L p-aminobenzoic acid and 30 g/L o-phosphoric acid in methanol were used.

The stock solutions fo standards were prepared by dissolving 0.01 g of D-glucose and D-galactose (purchased from Sigma-Aldrich) in 100 mL distilled water. The final concentration of standards was 100 ng/µL. it was used the protocol described in Camag Application Notes.

Other studies [14,15,16,17] have shown that the kefiran polysaccharide contains approximately equal amounts of glucose and galactose. For this reason, glucose and galactose were used as standards in the current study.

Separation and identification of monosaccharides from hydrolyzed kefiran solutions were carried out by thin layer chromatography on silica gel TLC plates (60 F254, 20x10 cm, Merck, Germany). The protocol used was that described in the Camag Application Notes. The polynomial regression data for the calibration plots exhibited good linear relationships (r = 0.99087 for galactose standard and r=0.99703 for glucose).

The plates were pre-washed with methanol (pre-chromatography) and dried at 105°C for 60 min using an Memmert drying oven (Memmert GmbH & Co. KG, Frankfurt, Germany). Samples and standards were applied on a plate with an Automatic TLC sampler (Linomat V) in 8-mm bonds. Nitrogen was used as a spray gas. The distance between tracks was 11.6 mm, the distance from the lower edge of the plate was 10 mm, and the distance from the sides was 30 mm.

The absorbance was measured at 620 nm using the Camag TLC Scanner 3 with WINCATS software (Camag, Switzerland) The polynomial regression data for the calibration plots were indicative of a good linear relationship between peak area and concentration over the range 100-700 ng per spot. Between the slopes of the calibration plots, there was no significant difference.

Repeatability of standards and samples application and measurement of peak area were carried out using two replicates of the same spot.

2.4. Statistical analysis

ANOVA (an analysis of variance) was used to compare the means using the SPSS 19.0 statistical analysis system, and a Tukey HSD test with a confidence interval of 95 or 99%. Differences were considered significant at $P < 0.05$.

3. Results and Discussion

Figure 1 shows the TLC plate spotted with the hydrolyzed samples of kefiran and standard solutions. As a result of complete chemical analysis, the monosaccharides band shows two closely spaced spots (glucose - Rf 0.71 and galactose - Rf 0.66).

Zhang et al. (2006) [18] also used the thin layer technique for analysis of monosaccharides resulted from kefiran hydrolysis. They did not achieved the best results because the two monosaccharides were not separated from each other. The method used by Piermaria et al. (2008) [13] has good repeatability, the spots are well separated, and the colour development occur after the derivatization step.

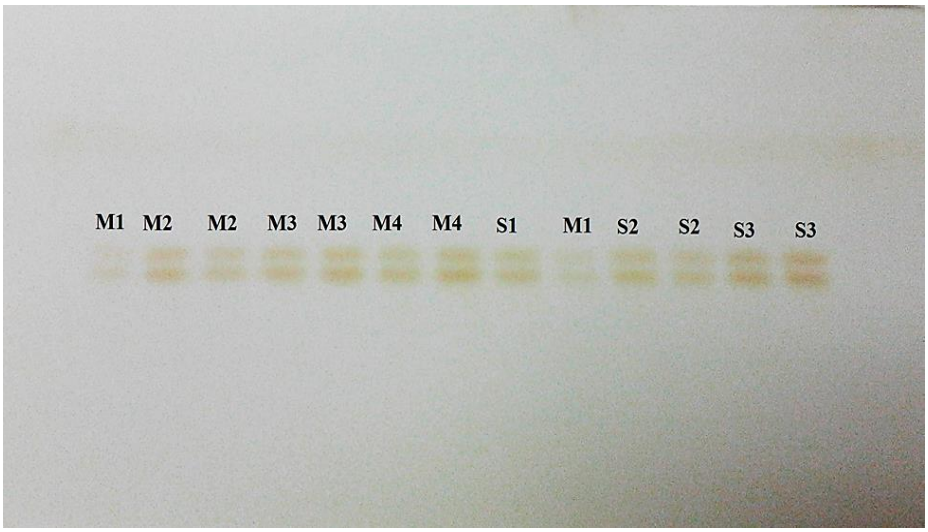

Figure 1. TLC plate with hydrolyzed samples of kefiran and standards. M1 – Kefiran extracted at 100ºC/5 minutes; M2 – Kefiran extracted at 70ºC/100 minutes; M 3 – Kefiran extracted at 90ºC/20 minutes; M4 – Kefiran extracted at 80ºC/30 minutes); S 1 – Standards (Glu:Gal, ratio of 1:1) 1600.00 ng; S 2 – Standards (Glu:Gal, ratio of 1:1) 2400.00 ng; S3 – Standards (Glu:Gal, ratio of 1:1) 3600.00 ng

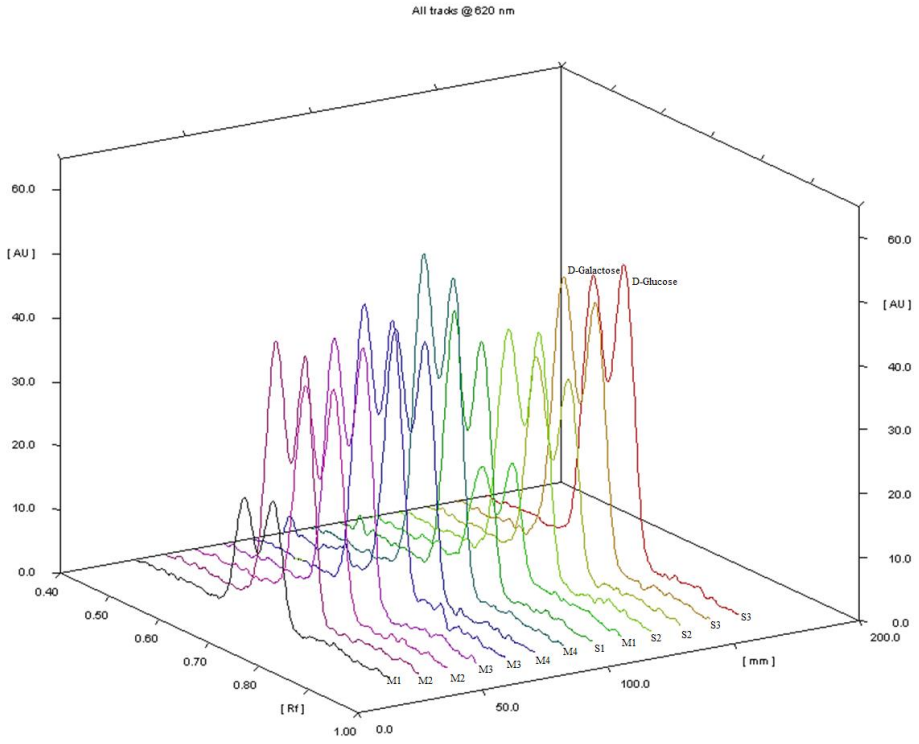

Figure 2. Three dimensional overlay of HPTLC desitograms of samples and standards (D-galatose and D-glucose)

Table 1.Glucose and galactose levels in hydrolysed kefiran solutions

Samples	Substance	Rf	Level(ng)	CV (%)	n	Recovery (%)
Sample M1	Galactose	0.65	669.93±0.1131	1.898	2	96.62
	Glucose	0.71	714.93±0.250	2.135	2	97.21
Sample M4	Galactose	0.66	2208.50±0.7071	4.830	2	95.12
	Glucose	0.71	2401.50±0.7071	4.426	2	96.33
Sample M2	Galactose	0.66	1441.50±0.7071	5.346	2	96.35
	Glucose	0.71	1509.00±1.4142	5.123	2	96.33
Sample M3	Galactose	0.66	1441.50±0.7071	2.145	2	94.89
	Glucose	0.71	1309.50±0.7071	2,564	2	95.32

*Values are the means of two applications on the same plate ± standard deviation

Figure 2 shows 3D overlay of HPTLC densitograms of the hydrolyzed samples of kefiran and standards (D-glucose and D-galactose), using 60 F254 TLC plates and n-propanol: acetic acid: water (70:20:10, v/v). The obtained fractions matched with the standards. Rf value of samples matches with those of standards. The monosaccharides identified in samples were glucose and galactose, which indicates a high purity of the kefiran.

The levels of glucose and galactose in hydrolysed kefiran solutions were calculated using the calibration curves of these monosaccharides.

The level of monosaccharides in tested samples are dependent on the kefiran extraction conditions. The highest level of monosaccharides was found in sample treated at 80ºC/30 minutes; followed by the one treated at 70ºC/100 minutes, at 90ºC/20 minutes, and at 100ºC/5 minutes (see Table 1).

4. Conclusions

The HPTLC method was validated using the HPLC determination of kefiran monosaccharides. The results demonstrated that the kefiran isolated from kefir grains grown in milk is a heteropolysaccharide which contains D-glucose and D-galactose units in a ratio of 0.94:1.1.

The physicochemical properties of the polysaccharide kefiran depend on extraction parameters (temperature and time).

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Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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