Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.105922

Evaluation of the biosynthesis, structural and rheological characterization of succinoglycan obtained from a formulation composed of whole and deproteinized whey

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Abstract

In this study, succinoglycan was produced from whey and its rheological and structural characteristics were elucidated. Eight culture media were prepared with different ions for bioconversion of whey into succinoglycan. The formulation composed of deproteinized whey, monobasic potassium phosphate and magnesium sulfate allowed the production of 13.7 ± 0.43 g/L of succinoglycan. The apparent molar mass of succinoglycan was estimated to be 9.033×10^5 g/mol and the polydispersity index was 1.044, representing the homogeneity of the sample. Monosaccharide composition of glucose and galactose for the succinoglycan produced was 6.6:1.0. The ^{1}H RMN analysis revealed the non-saccharide substituent content of 1.2%, 3.0% and 8.1% for acetyl, succinate and pyruvate, respectively. The rheological results showed that the apparent viscosity of the succinoglycan solutions was directly proportional to the concentration, and the solution showed pseudoplastic behavior. Dynamic frequency sweep tests identified that a concentration of 2.0% of succinoglycan is required for formation of the gel system. Temperature influenced the viscoelastic behavior of succinoglycan and revealed the melting point and reversibility of the gel. Whey was shown to be a promising carbon source for the production of succinoglycan with thickening potential and viscosity modifier.

Keywords: succinoglycan; whey powder; rheological properties; *Rhizobium radiobacter*.

Practical Application: This study demonstrates the effective application of the reuse of whole and deproteinized whey for succinoglycan biosynthesis.

1 Introduction

Succinoglycan is an acidic, water-soluble exopolysaccharide, composed of galactose and glucose residues, joined by β-links in a molar ratio of 1:7, has some non-saccharide substituents such as pyruvate, succinate, and acetate (Halder et al., 2017).

Unique characteristics give the succinoglycan molecule a high potential for industrial application, with chemical stability under drastic operating conditions, such as high temperature and pressure, extreme salinity and pH values, or high shear rates (Ruiz et al., 2015). Your rheological properties are determined by the chemical composition, molecule size, and mainly by the amount and type of non-saccharide substituents (Delani et al., 2022; Zhou et al., 2014). They have a high thickening capacity in aqueous solutions due to the high molar mass and the presence of different substituents in the chemical structure of the molecule (Gao et al., 2021).

Some technological properties of succinoglycan enable their practical application in the food industries and food development: thickening or viscosifying activity and emulsification properties (Delani et al., 2022). These properties make it possible for succinoglycan to be incorporated into beverages such as fermented milk, yogurt, dairy drinks, and sauces. An interesting possibility is to use succinoglycan as a fat substitute in ice cream and mayonnaise. Recently, research has shown that succinoglycan in the form of oligosaccharides was incorporated into fermented soy and rice-based beverages, bringing food alternatives to groups of individuals who have dietary restrictions (Nascimento et al., 2022). Due to the potential applicability of exopolysaccharides (Moyib et al., 2019), especially succinoglycan, in recent years several renewable carbon sources have been investigated for the production of bacterial biopolymers, aiming to produce biomaterials with lower environmental impact, higher performance, better applicability and lower production cost (Andhare et al., 2017b; Moosavi-Nasab et al., 2012).

Aiming at the use of industrial residues and the reduction of production costs of exopolysaccharides, the whey, resulting from the manufacture of cheese, could be a promising carbon source. Corresponding to 90% of the milk volume and, due to the high carbohydrate content, it can be considered a rich

Received 15 Sept., 2022
Accepted 21 Nov., 2022
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and easy to obtain culture medium (Li et al., 2020). World whey production is estimated at more than 108 tons per year (Húngaro et al., 2013). According to Embrapa, in 2020, the cheese industry in Brazil absorbed approximately 8.7 billion liters of milk (Empresa Brasileira de Pesquisa Agropecuária, 2021). However, only 15% of the available serum is reused by the industries, and a large part is still discarded as wastewater into the environment (Húngaro et al., 2013; Trindade et al., 2019). In addition, Brazil produces approximately 8 billion liters of whey per year, which can supply the production demand (Empresa Brasileira de Pesquisa Agropecuária, 2019).

To the best of the authors' knowledge, to date, there are no descriptions in the literature of the use of deproteinized whey powder for succinoglycan production and whether whey proteins interfere in the bioconversion process. Therefore, the purpose of this study was to evaluate the production of succinoglycan by *Rhizobium radiobacter* ATCC 4720 using deproteinized whey powder as carbon source, and the interference of nitrogen, potassium and magnesium ions on the bioconversion of this substrate. The structural characteristics and rheological properties of succinoglycan were also evaluated.

2 Materials and methods

2.1 Materials

Rhizobium radiobacter ATCC 4720 was acquired by Fundação André Tosello - Tropical Culture Collection (Campinas, SP). All chemical reagents used in the study were of analytical grade. The whey powder was donated by Alibra Ingredientes Ltda (Marechal Cândido Rondon, PR) and the commercial succinoglycan (Rheozan®) was donated by Rhodia Solvay Group (São Paulo, Brazil).

2.2 Microorganism and cultivation conditions

Rhizobium radiobacter ATCC 4720 was reactivated in growth medium according to the supplier's specifications: meat extract 3 g/L; polypeptone 5 g/L; pH 7.0 and incubated at 30 °C for 24 h. The methodology was conducted according to Ruiz et al. (2015).

2.3 Whey deproteinization treatment

The deproteinization of the whey powder solution was performed to avoid possible interactions of milk proteins during the bioconversion process. The methodology was conducted according to Húngaro et al. (2013).

2.4 Determination of lactose in deproteinized whey

The lactose concentration was determined using the DNS (3,5 dinitrosalicylic acid) method of Miller. Deproteinized whey presented 49.3 ± 1.2 g/L of lactose and was used as carbon source. This result was used to adjust the concentration in the succinoglycan production medium to 5% lactose.

2.5 Succinoglycan production

The methodology for succinoglycan production was developed according to Moosavi-Nasab et al. (2012) and Ruiz et al. (2015). According to Table 1, eight formulations were developed to verify the capacity of succinoglycan production by the microorganism, with whole milk whey and deproteinized whey as carbon source, and their concentrations adjusted to a 5% lactose solution. During the entire production process, the pH values of the solutions were corrected with sterile HCl or NaOH and kept at pH 7.0.

2.6 Extraction and evaluation of succinoglycan production

The methodology for succinoglycan production was developed according to Ruiz et al. (2015).

2.7 Chemical characterization

FT-IR analysis

Fourier transform infrared spectroscopy was conducted to elucidate the presence of major structural groups of biopolymer in a spectrometer with ATR accessory (Bruker, Model Vertex 70V). The material was packed in a diamond sample holder and the final spectrum was an average of 256 scans, in the region between 400 and 4000 cm^{-1} with a resolution of 4 cm^{-1}.

Determination of monosaccharide composition by Gas Chromatography (GC)

Monosaccharide composition analyses were performed as previously described (Reichembach & Petkowicz, 2020). The analysis was performed on a THERMO Trace GC Ultra chromatograph equipped with a flame ionization detector, Ross injector and DB-225 capillary column [30 m × 0.25 mm (i.d.)], with a film thickness of 0.25 μm.

Determination of the molecular weight of succinoglycan

For this analysis, it was used the technique of steric exclusion chromatography coupled with a multi-angle laser light scattering detector and a differential refractive index detector (HPSEC-MALLS/RI). The analysis were performed on an equipment consisting of an HPLC pump (Waters 515), injector, 4 Ultrahydrogel columns - 120, 250, 500 and 2000 - with exclusion limits of 5 × 103, 8 × 104, 4 × 105 and 7 × 106 g/mol respectively, DAWN DSP Light Scattering (Wyatt Technology) and a differential refractive index detector model 2410 (Waters). The molecular weight was calculated from light scattering data as previously described (Reichembach & Petkowicz, 2020).

Table 1. Composition of media for succinoglycan production by *R. radiobacter* ATCC 4720.

Compounds	F1	F2	F3	F4
Lactose carbon source (g/L)	50.0	50.0	50.0	50.0
Monobasic potassium phosphate (g/L)	1.0	1.0	1.0	1.0
Magnesium sulfate (g/L)	0.25	0.25	0.25	-
Ammonium phosphate dibasic (g/L)	1.0	-	-	-
Trace elements solution (mL)	10.0	10.0	-	-

NMR analysis

Proton NMR (^{1}H) and single heteronuclear quantum coherence (HSQC) spectra of the succinoglycan solution (40 mg/mL in D_2O) were conducted at 70 °C in a Bruker DRX 400 Avance spectrometer (Bruker, Germany). Acetone was used as internal standard (δ = 30.2 for ^{13}C and δ = 2.22 for ^{1}H). TopSpin software version 4.0.9 (Bruker, Germany) was used for data analysis. Analysis of the substituents was performed by quantifying the acetyl groups of succinoglycan by the methodology described by Hestrin (1949). The reaction of the acetyl functional group with hydroxylamine was measured at 540 nm and penta-O-acetyl-β-D-galactopyranose was used as a standard. The other substituents were estimated by comparing the areas of the ^{1}H NMR peaks of acetyl versus succinate and pyruvate.

2.8 Rheological properties

Rheological studies were performed in an advanced rheometer HAAKE MARS II with stress and strain control (Thermo Fisher Scientific, Waltham, USA), by using rotational and oscillatory tests. The geometry used was cone and plate (diameter 35 mm and cone angle 2°). In this configuration the minimum spacing between cone and plate is 100 μm.

In the rotational tests concerning the rheological behavior, the apparent viscosity of the samples (η_{ap}) was determined by progressively increasing the shear rate $(\dot{\gamma})$ in the range between $0.01 s^{-1}$ and $1000 s^{-1}$ at $25.0℃$. All viscosity curves were modeled using the Williamson equation (Equation 1),

$$\eta = \frac{\eta_o}{\left(1+\left(k\dot{\gamma}\right)^n\right)} \quad (1)$$

Where k is the consistency index and η is the flow index, and η_o is the zero shear viscosity.

Before each dynamic oscillatory experiment, the linear viscoelastic region was evaluated at $1 Hz$ by strain sweep experiments, with 8% strain within the linear region for all samples. The viscoelastic properties, elastic recovery modulus (G') and viscous dissipation modulus (G'') were determined by using small amplitude oscillatory sweeps with frequency control, in the range between $0.01 Hz$ and $10 Hz$ at $25.0℃$. All rheological tests were conducted in triplicate. Dynamic temperature sweeps were performed at 0.1 Hz between 5.0 °C and 75.0 °C in the heating and subsequent cooling cycle, at a rate of ± 2.0 °C. A thin layer of low-viscosity mineral oil was used to cover the sample and prevent evaporation during the thermal tests. All rheological tests were conducted in triplicate.

2.9 Statistical analysis

All tests were performed in triplicate and the results were submitted to analysis of variance (ANOVA) and Scott-knott test at 5% significance using the SISVAR 5.6 program. For rheological and structural analysis, the Origin Pro 8 software was used.

3 Results and discussion

3.1 Evaluation of culture medium for succinoglycan production

Since whey is rich in nutrients and already has proteins in its composition (Smithers, 2008; Trindade et al., 2019), in this study we investigated whether whey proteins could prevent or decrease the bioconversion of this substrate into succinoglycan. In addition, it was also evaluated whether the presence of nitrogen, potassium, magnesium ions could influence the production of succinoglycan in the formulations with whole and deproteinized whey. Thus, to evaluate the production of succinoglycan, eight formulations of culture medium were prepared using whole whey and deproteinized whey as carbon sources. The results of succinoglycan production from these formulations are shown in Figure 1.

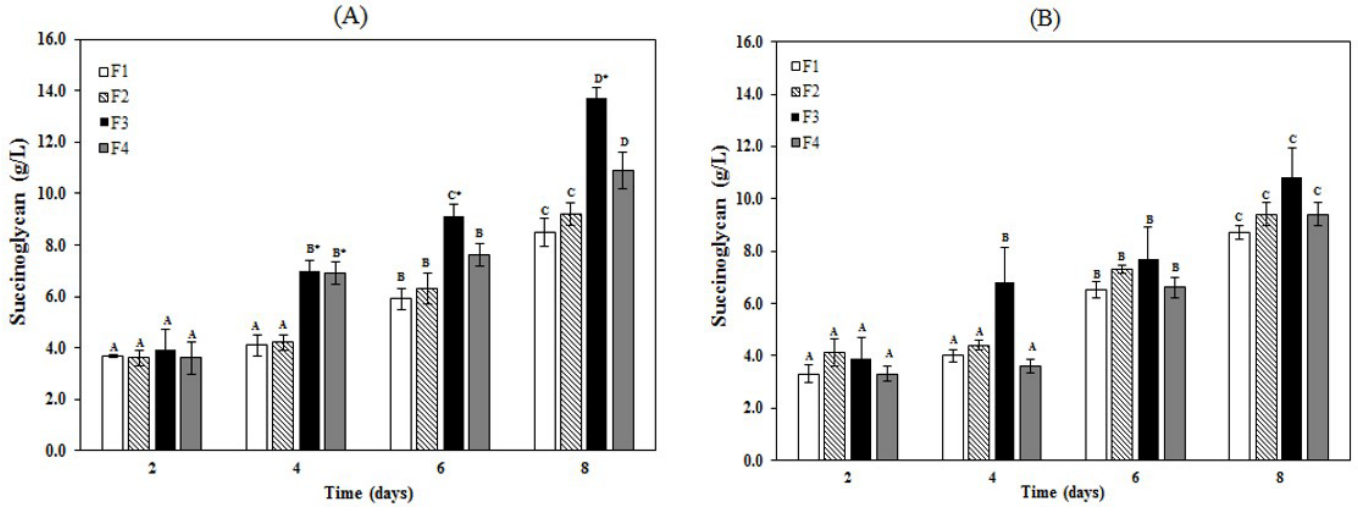

Figure 1. Succinoglycan production (g/L) by *Rhizobium radiobacter* ATCC 4720 for 8 consecutive days at pH 7 from deproteinized whey (A) and whole whey (B) substrate in different media formulations F1; F2; F3 and F4. The letters and letters * represent statistical comparisons between the eight formulations on the different production days with statistical difference ($p < 0.05$) by ANOVA test followed by the Scott-Knott test.

Among the different culture media studied, the highest bioconversion was found in the deproteinized whey substrate in formulation F3, which produced 13.7 g/L of succinoglycan on the eighth day, with statistically significant difference ($p < 0.05$) from the other formulations (Figure 1A). Formulation F4 had a succinoglycan yield of 10.9 g/L, followed by formulations F1 and F2, which showed no statistical differences ($p < 0.05$).

For whole whey powder (Figure 1B), formulations F1, F2, F4 did not show statistical differences. Formulation F3, for whole whey, was also the one that showed the best production, with biosynthesis of 10.8 g/L of succinoglycan, when compared with the other formulations using whole whey.

According to the results obtained with the eight formulations it was possible to observe that whey proteins interfere in the production of succinoglycan, since the best production occurred in the fermentative medium composed only of deproteinized whey, magnesium and potassium (F3) followed by the formulation of deproteinized whey and potassium ion (F4).

Whey proteins are direct sources of nitrogen, an essential physiological supplement for the multiplication of bacteria. However, to obtain a good microbial growth curve it is essential that the culture medium provides carbon as a source of energy and nitrogen for cell multiplication and protein synthesis. Still, the production of exopolysaccharides only occurs when there is exhaustion of the nitrogen source (Li et al., 2020; Liang et al., 2017). Since in this study a reactivation of the bacteria in growth medium is first performed for 24 h, the second step consisting of the carbon source, demonstrated that whey proteins interfere with production and are not necessary for bioconversion into succinoglycan.

Nitschke et al. (2001) using the microorganism *Xanthomonas campestris* C_7L, demonstrated that the capacity of whey bioconversion into xanthan gum was also dependent on the carbon/nitrogen ratio in the culture medium, and thus proposed a two-step combined fermentative system, using whole whey in the first step and filtered (deproteinized) whey in the second. In the combined fermentative system, the whole whey presented 0.35% protein and the filtered whey (deproteinized) showed 0.18% protein. This strategy increased the yield and final concentration of xanthan, with 13 g/kg of xanthan being obtained in the first phase and 28 g/kg of xanthan after 30 hours of production in the second phase.

In formulation F3, composed of deproteinized whey, potassium and magnesium, an improved succinoglycan production was obtained when compared to the absence of magnesium in the fermentation medium. Thus, in addition to the influence of carbon and nitrogen supplementation on the bioconversion of whey into succinoglycan, it is suggested that the production yield is affected when there are changes in the supply of some ions.

Pedroso et al. (2019) evaluated the ability of *R. radiobacter* ATCC 4720 to use sugars from rice husk hydrolysis as carbon source in the synthesis of exopolysaccharide. From the experimental design with different formulations, they found that besides the carbon source, only supplementation with yeast extract and KH_2PO_4 were necessary.

Important changes in the structure and chemical composition of an exopolysaccharide can also be caused by cultivation conditions. Therefore, knowing the physiological needs of the microorganism used can ensure the successful production of an exopolysaccharide, resulting in good yield and attractive rheological characteristics for industrial application (Delani et al., 2022).

Reducing production cost by replacing sucrose from the conventional fermentation process with agroindustrial residues is a viable alternative to stimulate the synthesis of new exopolysaccharides and increase their applicability. Furthermore, the use of reusable sources contributes to a better destination of this waste, reflecting in care for the environment (Angelin & Kavitha, 2020; Li et al., 2020). Evaluating the results already obtained by other research groups, it can be emphasized that, according to the carbon source and the strain used, there is a variation in the yield of the exopolysaccharide produced. However, in the present research, using deproteinized whey powder containing 5% of lactose in the composition as carbon source, it was possible to obtain a yield of 13.7 g/L of succinoglycan on the eighth day of production. Thus, the exopolysaccharide obtained from the formulation with the best yield was selected to perform the chemical and rheological characterization studies.

3.2 Chemical characterization

In order to confirm the presence of succinoglycan, your chemical structure has been investigated. The polysaccharide extracted was analysed by FT-IR spectroscopy (Figure 2A).

The spectra show that the commercial sample and the succinoglycan produced with deproteinized whey powder substrate are similar, both showed the bands related to the identification of the exopolysaccharide succinoglycan in the bonding regions of the OH groups, to the bands referring to the vibrations and deformations of the CH structures of the carbohydrates, as reported in studies by Andhare et al. (2017a) and Bakhtiyari et al. (2015). As observed in the spectrum, some characteristic bands were assigned. The band at 3286 cm^{-1} was clearly attributed to the hydroxyl group (O-H) stretching vibration of polysaccharides. The bands near 2900 cm^{-1} are assigned to the axial deformation of the CH bond in the carbohydrate structure. The bands near 1400 cm^{-1} can be associated with symmetric COO^- stretching (Najbjerg et al., 2011; Ruiz et al., 2015; Wiercigroch et al., 2017). whereas the band at 1035 cm^{-1}, present in the standard sample, can be related to C-O-C bonding of the glucose ring (Mangolim et al., 2017). The band at 1018 and 1022 cm^{-1} indicate the presence of ester (C-O) bands (Ruiz et al., 2015). The bands at 894 and 892 cm^{-1} for standard and succinoglycan, respectively, may indicate the presence of β-type glycosidic bonds in both samples (Monteiro et al., 2012).

The determination of the monosaccharide composition was performed by gas chromatography analysis after total acid hydrolysis followed by derivatization to alditol acetates. The polysaccharide had glucose and galactose in an average ratio of 6.6:1.0. Similar values were found by Gao et al. (2021) for succinoglycans produced by a high-yielding mutant strain (glucose:galactose molar ratio of 6.65:1.00 and 6.86:1.00). Thus,

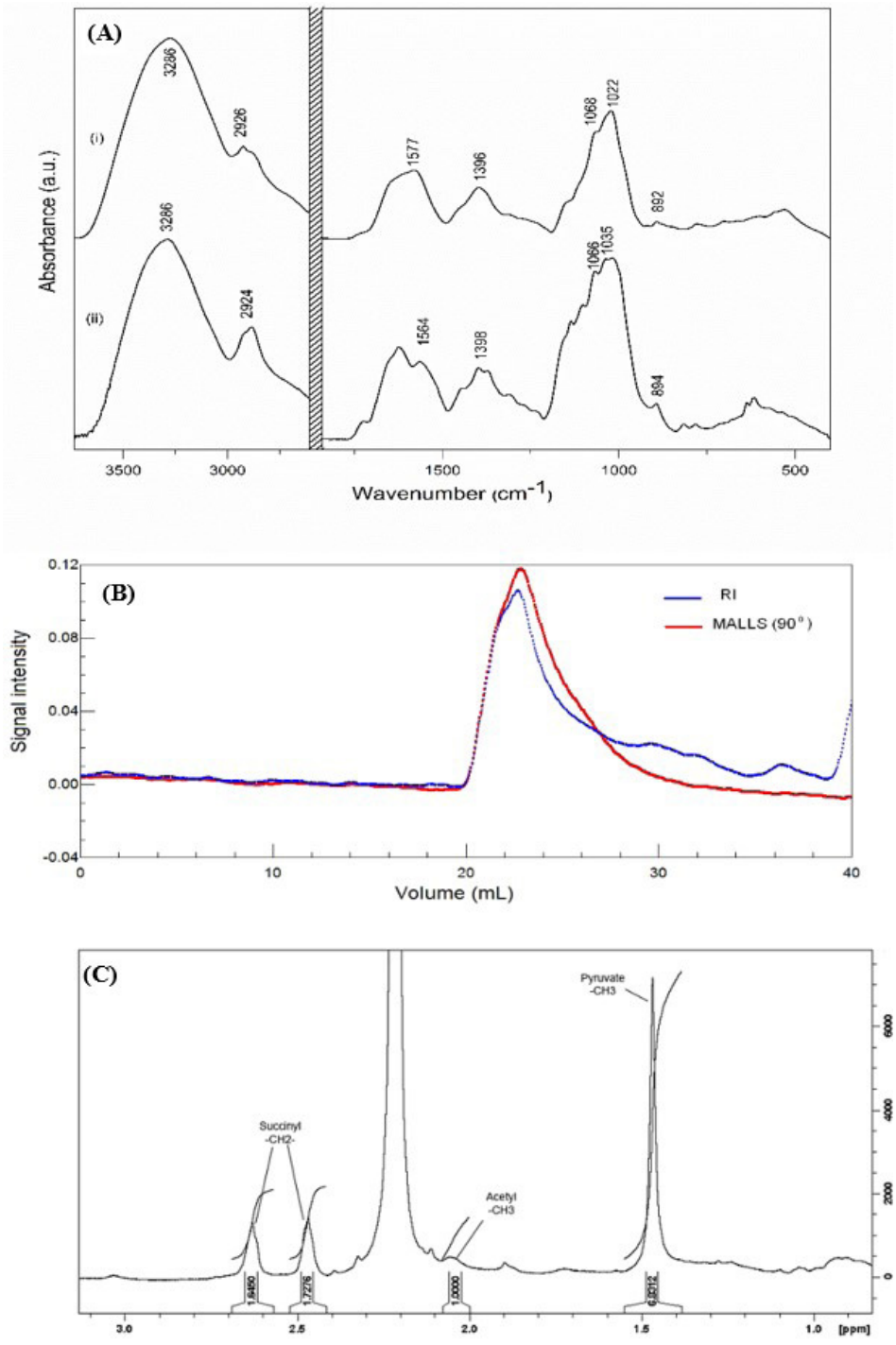

Figure 2. FT-IR/ATR spectra of succinoglycan samples. (i) Succinoglycan produced from deproteinized whey and (ii) commercial succinoglycan (A). Elution profile of the succinoglycan sample by (HPSEC-MALLS) (B). Spectrogram obtained by ^{1}H NMR with identification of succinate, acetyl and pyruvate substituents of succinoglycan obtained with deproteinated whey (C).

the results confirmed that exopolysaccharide isolated in the present study is a succinoglycan.

The elution profile of the succinoglycan sample obtained by HPSEC using multi angle laser light scattering (MALLS) and refractive index (RI) detectors are shown in Figure 2B. The polysaccharide eluted in a single peak, detected by both the light scattering detector and the refractive index detector, indicating the presence of a high molar mass polymer. The molar mass was calculated to be 9.033×10^{5} g/mol. The polydispersity index, which is related with the homogeneity of the sample, was low, similar to the value (1.06) reported by Kavitake et al. (2019).

The polysaccharide was qualitatively analysed by ^{1}H NMR spectroscopies have shown specific carbohydrate signals (Figure 2C). The ^{1}H NMR spectrum revealed the presence of

main characteristic signals from succinate and acetate group by the methyl protons resonances with chemical shifts of 2.5 ppm and 2.0 ppm respectively. The acetyl content of succinoglycan obtained by the colorimetric method was 1.2% ± 0.1%. By integration of the succinate and pyruvate peaks obtained by ^{1}H NMR and in comparison with the acetyl peak area, it was possible to estimate the total amount of succinate and pyruvate substituents in succinoglycan, being respectively 3.0% and 8.1%.

3.3 Rheological analysis

Figure 3 illustrates the effect of shear rate on apparent viscosity at the different concentrations of succinoglycan solutions studied at 25 °C. The results showed that the apparent viscosity was directly proportional to the concentration of succinoglycan, and the solution showed pseudoplastic behavior with increasing shear rate. Ruiz et al. (2015) and Moosavi-Nasab et al. (2012) also observed the same behavior. Despite the lower molar mass, at the same concentration the succinoglycan isolated in the present study had much higher viscosity than those obtained by Gao et al. (2021).

The apparent viscosity of all solutions evaluated decreased significantly with increasing shear rate (up to $1000 s^{-1}$). This shear thinning is caused by the stretching of succinoglycan molecules during the shear. It is an important rheological characteristic, and is related to several applications involving industrial processing.

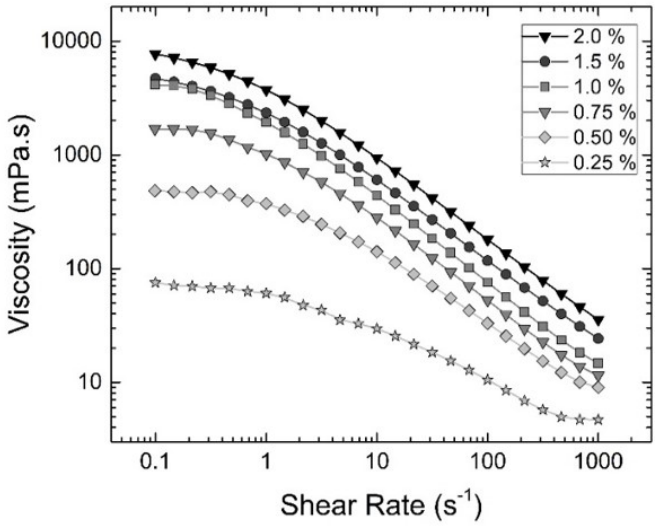

Figure 3. Viscosity curves of succinoglycan solutions at 25.0 °C. The symbols represent the concentrations (w/v).

The results indicate that solutions containing succinoglycan will flow easily when poured from a container or during various operations, such as pumping, spray drying, and agitation, despite their high initial viscosity (zero shear viscosity - η_o) (Andhare et al., 2017a). Table 2 shows the rheological parameters related to the concentration of the succinoglycan solutions obtained by numerical fitting of the data in Figure 3 and from Equation 1. The flow behavior (n) was lower than 1.00 for all succinoglycan concentrations, ranging from 0.603 to 0.858. This result confirms the pseudoplastic flow behavior shown by the solutions.

The results of the dynamic frequency sweep tests for the different concentrations of succinoglycan solutions (w/v) are illustrated in Figure 4.

At low concentrations of succinoglycan (0.25%) the rheological behavior of the solutions exhibited properties typical of a viscous fluid with the viscous dissipation modulus ($G"$) greater than the elastic recovery modulus (G') throughout the frequency spectrum explored (Figure 4). For higher concentrations (0.50%, 0.75%, 1.00% and 1.50% w/v), both the $G"$ and G'modulus grew with increasing frequency (Figure 4a-4e), but the G' modulus grew faster than the $G"$ modulus. As a result, the G' modulus curve intersects the $G"$ modulus curve at the crossover point ($crossover\ point - f_c$). From this frequency on, the values of the G' modulus are predominantly larger than those of the modulus $G"$. Succinoglycan solutions transitioned from a fluid-like to a gel-like structure. For concentrations of $2.0\% w/v$ (Figure 4f), the G' values exceeded the $G"$ values throughout the explored frequency spectrum, and a frequency dependence was observed, which indicated the presence of an apparent gel network in the system. The heating/cooling curves of moduli G' and G" of the 2.0% w/v aqueous solution of succinoglycan are illustrated in Figure 5. They were obtained at a heating/cooling rate of ± 2.0 °C/min at a constant frequency of 0.1 Hz.

At the beginning of the heating cycle, in the range from 5.0 °C to 60.0 °C, moduli G' and G" exhibited little dependence with temperature (Figure 5a). In this domain, the prevalence of G' over G" was observed (Figure 5a). Between 64.0 °C and 66.0 °C both moduli decreased dramatically, and the two curves intersected, indicating that the gel system began to melt. Therefore, the melting point of the gel was reached at 65.0 °C. The moduli remained practically constant between 66.0 °C and 75.0 °C, with G" prevailing over G'. In the cooling cycle (Figure 5b), the G' and G" curves were approximately reversible compared to the heating curves, with the gelling point occurring at approximately 60.0 °C. This result indicates a thermal hysteresis regarding the melting point around 5.0 °C. At the end of the procedure,

Table 2. Rheological parameters (η_o, k e n) of succinoglycan solutions as a function of concentration in distilled water at a room temperature of 25 °C.

Concentration (%)	$\eta_o\left(mPa.s\right)$	$k\left(Pa.s^n\right)$	n	R^2
0.25	83.5	0.266	0.603	0.996
0.50	530.9	0.359	0.794	0.998
0.75	1977.0	0.905	0.845	0.997
1.00	5246.4	1.779	0.858	0.998
1.50	5966.7	1.78	0.757	0.999
2.00	9913.9	1.95	0.759	0.999

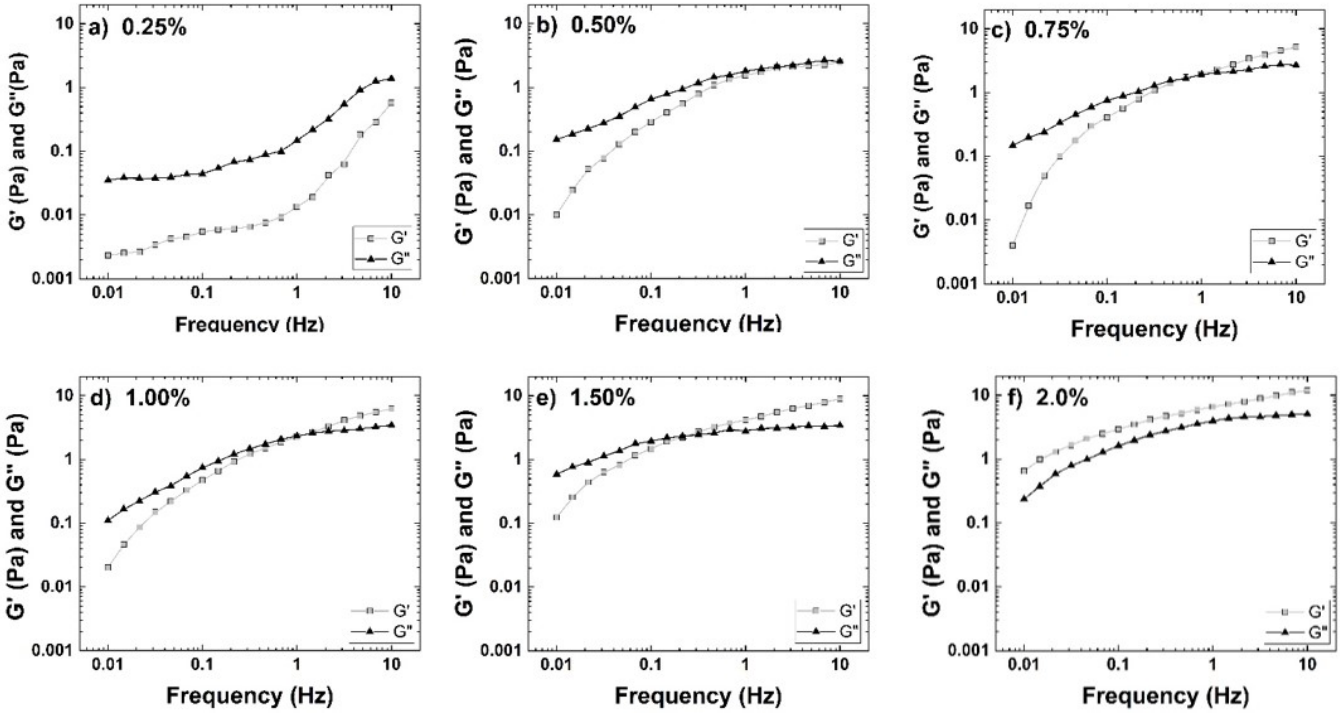

Figure 4. Mechanical spectrum of succinoglycan solutions at different concentrations at 25.0°*C*: (a) 0.25%, (b) 0.50%, (c) 0.75%, (d) 1.00%, (e) 1.50% and (f) 2.00%(w/v).

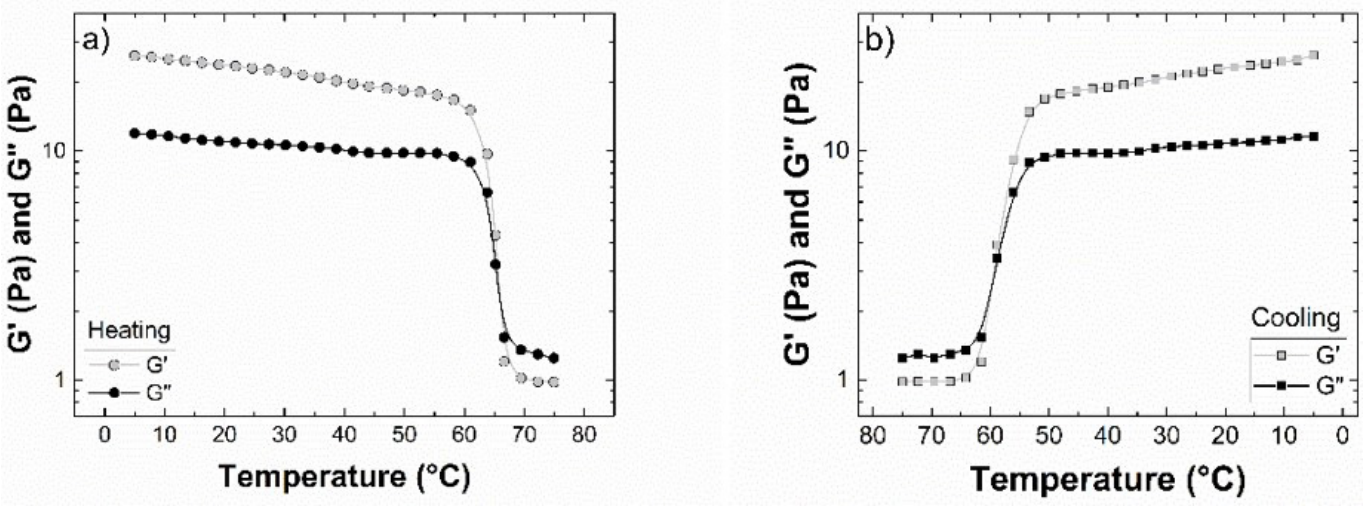

Figure 5. Changes in elastic recovery modulus G' and viscous loss modulus G "during the heating cycle (a) if subsequent cooling cycle (b) in the range from 5.0 °C to 75.0 °C at a rate of ±2.0 °C / min exhibited by the 2.0 %(w/v) succinoglycan solution.

both moduli almost returned to the original levels, indicating that heating and cooling did not influence the gelling ability of succinoglycan, and that the gel formed is thermally reversible.

4 Conclusion

Agroindustrial whey waste was bioconverted into succinoglycan by *Rhizobium radiobacter* ATCC 4720. A better production of succinoglycan occurred in the absence of whey proteins and in the presence of magnesium and potassium ions. The chemical characterization tests showed that the monosaccharide composition of glucose and galactose for the produced succinoglycan was 6.6:1.0 and revealed the content of non-saccharide substituents for acetate, succinate and pyruvate of 1.2%, 3.0% and 8.1%. The advanced rheological studies of the succinoglycan solutions revealed non-Newtonian and pseudoplastic behavior. The apparent viscosity of the evaluated solutions decreased significantly with increasing shear rate, an important rheological characteristic, as it demonstrates that the obtained succinoglycan can be applied in industrial processing. Temperature influenced the viscoelastic

behavior of succinoglycan and revealed the melting point and reversibility of the gel. According to the results obtained, the succinoglycan produced has the potential to be used as a thickener and viscosity modifier in food and other products. Whey has shown to be a promising and viable carbon source in the fermentation process, which enables the correct management and disposal of this waste, positively impacting the preservation of the environment.

Acknowledgements

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Conselho de Desenvolvimento Científico e Tecnológico (CNPQ) and Fundação Araucária (FA) for financially supporting this study.

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