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Artículo de investigación

Biomass growth of water kefir with variations in sucrose and tibicos concentration

Crecimiento de la biomasa de kéfir de agua con variaciones en la concentración de sacarosa y tibicos

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Abstract

Water kefir is a slightly acidic fermented beverage made from sugar solutions. Its fermentation process involves a variety of microorganisms, mainly lactic acid bacteria and yeasts, which are embedded in a carbohydrate-based biopolymer known as dextran. In order to find the best conditions for obtaining this biopolymer with potential industrial applications and as a possible substitute for petroleum-derived polymers, this research evaluated the effect of the microbial consortium of commercial water kefir grains (tibicos) on the fermentation process. The best initial concentration of tibicos was 0.02 g/mL^{-1} in a culture medium composed of 100 g/L sucrose. The biomass production obtained at 48 hours was 23.9 g, standing for a 597% increase compared to the initial value of 4.0 g, with a final pH of 4.05 ± 0.1 and a pH reduction of 0.5 units at the end of fermentation. A total of 160 ± 0.06 g of dextran were recovered per kilogram of water kefir grains used. Dextran was isolated through acid purification and oven-drying. It was characterized and found using Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). The thermal properties of dextran were studied using Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA).

Keywords: culture medium, dextran, fermentation, tibicos, water kefir.

Resumen

El kéfir de agua es una bebida fermentada de sabor ligeramente ácido que se elabora a partir de soluciones azucaradas. En su proceso de fermentación intervienen una variedad de microorganismos, principalmente bacterias ácido lácticas y levaduras, que se encuentran embebidos en un biopolímero de naturaleza carbohidrato, conocido como dextrano. Con el fin de identificar las mejores condiciones para la obtención de este biopolímero con potenciales aplicaciones industriales y, como posible sustituto de los polímeros derivados del petróleo, esta investigación evaluó el efecto del consorcio microbiano de los tíbicos de kéfir de agua comercial en el proceso de fermentación. Se encontró que la concentración óptima de tíbicos fue de 0.02 g.mL^{1} en un medio de cultivo compuesto por 100 g.L^{1} de sacarosa. La producción de biomasa obtenida a las 48 horas fue de 23.9 g, lo que representó un incremento del 597 % respecto al valor inicial de 4.0 g, con un pH final de 4.05 ± 0.1 y una reducción de 0.5 unidades de pH al finalizar la fermentación. Se recuperaron 160 ± 0.06 g de dextrano por cada kilogramo de tíbicos de kéfir de agua utilizado. El dextrano fue aislado por purificación ácida y secado en horno. Se caracterizó e identificó mediante espectroscopia infrarroja por transformada de Fourier (FTIR) y difracción de rayos X (DRX). Las propiedades térmicas de dextrano se estudiaron por medio de Calorimetría diferencial de Barrido (DSC) y Análisis termogravimétrico (TGA).

Palabras Clave: dextrano, fermentación, kéfir de agua, medio de cultivo, tíbicos.

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1 Introduction

Water kefir grains, also known as tibicos, consist of a microbial consortium composed of lactic acid, acetic acid, and yeasts adhered to a polysaccharide matrix produced by bacteria. They are described as compact gelatinous granules of whitish or yellowish color, formed in a stable culture medium. They can be translucent or opalescent, with an irregular shape and an average diameter of 5 to 20 mm [1, 2].

The symbiosis among the microorganisms present in the tibicos allows the yeasts to benefit from the acidification of the culture medium, while the bacteria are favored by the vitamins and soluble nitrogen compounds produced by the yeasts [3, 4].

The main microorganisms responsible for biomass increase in tibicos are *Lactobacillus hilgardii, Lactobacillus paracasei, Lactobacillus nagelii*, and *Saccharomyces cerevisiae*. They contribute to the distinctive flavor of this beverage and are responsible for the production of antioxidants. This group of microbes can anaerobically ferment various sugary liquids, resulting mainly in the production of organic acids, ethanol, and carbon dioxide. As a result, the water becomes carbonated. It is important to note that the amount of alcohol produced does not exceed 2% (v/v) [1, 2, 5, 6].

During the sugar oxidation process, yeasts produce alcohol, while bacteria oxidize it to produce organic acids, as well as other secondary products such as mannitol. Other compounds, such as carbonyl groups, sulfur, and phenolic compounds, are byproducts of yeast fermentation that also contribute to the aroma of alcoholic beverages [7–9].

Carbonyl compounds of particular interest are aldehydes, which are intermediates in alcohol formation. Diacetyl and 2,3-pentanedione are formed during fermentation through the decarboxylation of α-acetolactic acid and α-acetohydroxybutyric acid. The phenolic compounds produced during alcoholic fermentation by the yeast consortium in tibicos are derived from the catalysis of p-coumaric acid, ferulic acid, and vanillin, and include compounds such as 4-ethylphenol, 4-ethylguaiacol, and 4-methylguaiacol [7, 9, 10].

The composition of the exopolysaccharide (EPS) or matrix of tibicos primarily consists of an insoluble glucose polymer with α-(1-6) linkages, known as dextran [1, 2]. This polysaccharide (Figure 1) has a high molecular weight, and its structure varies depending on the percentage, nature, and distribution of its linkages. It is typically produced in cultures of lactic acid bacteria such as *Streptococcus*, *Acetobacter*, or *Leuconostoc*, in media containing sucrose. The growing cell secretes an enzyme called dextransucrase that converts excess sucrose into dextran and releases fructose into the medium. *Lactobacillus hilgardii* is the main producer of dextran in water kefir grains, acting through the enzyme glucosyltransferase [1, 2].

Dextran has wide applications in the food industry and can be used as a thickening agent, stabilizer, emulsifier, fat substitute, or gelling agent. Moreover, it has been found to exhibit antitumor activity [11, 12].

This article presents the methodological aspects to determine the optimal growth conditions for water kefir grains through four experimental phases, including variations in inoculum quantity and sucrose concentration in the culture medium, in order to purify dextran for evaluation as a potential biopolymer with industrial applications and as a substitute for petroleum-derived polymers [13].

The biopolymer was characterized using Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD), and its thermal properties were studied using Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA).

2 Materials and Methods

Phase 1: Acclimatization of water kefir biomass

Preparation of the culture medium. A modified MRS medium, was prepared. This consisted of 100 g sucrose. L^{-1}, as the main carbon source, 2.25 g yeast extract L^{-1}, as the nitrogen source, and micronutrients including 2.0 g $K2HPO_4.L^{-1}$, 28 mg $MnSO_4$. L^{-1}, 200 mg $MgSO_4$. L^{-1} and 29 mg B complex.L^{-1} [11].

Acclimatization of water kefir biomass. In 500 mL Erlenmeyer flasks, 0.1 g of commercial water kefir grains were added per mL of culture medium, with a total liquid volume of 200 mL. Each assay was prepared by triplicate, including a negative control. The process was repeated until no further changes in the weight of produced kefir grains were observed. Fermentation was evaluated for 48 hours at a temperature of 25 °C in a Memmert UN75 universal oven. The cultured kefir grains were used for the assays in phases 2 and 3.

Phase 2: Evaluation of water kefir biomass growth at different kefir grain concentrations

After completing Phase 1, assays were prepared using the same culture medium and time-temperature conditions, but with variations in the initial kefir grain concentration ($g.mL^{-1}$): 0.02, 0.04, 0.06, 0.08, and 0.10.

Phase 3: Evaluation of water kefir grain growth at different sucrose concentrations

Once the inoculum concentration that showed the highest growth in biomass was identified, experiments were conducted under the same time-temperature conditions used in Phases 1 and 2, but with different sucrose concentrations ($g.L^{-1}$): 20, 40, 60, 80, and 100 [14].

Phase 4: Biomass growth under the best conditions identified in Phases 2 and 3

Finally, an assay was conducted under the best identified conditions, regarding kefir grain concentration, following a similar procedure as described in Phase 1 [14].

Physicochemical analysis of water kefir grains

pH determination. The pH was determined according to NTC 3651:1994. It was measured directly from the fermented liquid phase obtained every 48 hours, using an ORION 8107UWMMD ROSS Ultra pH/ATC triode electrode from Thermo Scientific. The ΔpH value was calculated by using Eq. (1).

$$\Delta pH = pH_f - pH_i \quad (1)$$

Determination of soluble solids (°Brix). °Brix was measured in the fermented liquid phase of each test, following the NTC 4624:1999 standard, using a portable refractometer (BRIXCO ATC 0-50%) to obtain the refractive index after 48 hours. The Δ°Brix value was calculated by using Eq. (2).

$$\Delta°\text{Brix} = °\text{Brix}_f - °\text{Brix}_i \quad (2)$$

Optical Density (OD). The absorbance values at 600 nm were measured for the liquid phase obtained from the fermentation process using an Iris HI 80 spectrophotometer. Eq. (3) was employed to determine the ΔOD value.

$$\Delta\text{DO} = \text{DO}_f - °\text{DO}_i \quad (3)$$

Cleaning and Drying. The water kefir grains were washed with distilled water and ethanol, followed by a drying process at 40 °C for 48 hours in a Memmert UN75 universal oven. They were then milled using a High-speed Multifunction Grinder HC-150 to achieve a particle size of 300 μm [15].

Purification of the Target Biopolymer. 200 mL of a 0.1 N H_2SO_4 solution was mixed with 100 g of clean tibicos. The mixture was continuously stirred and heated to 100 °C until complete dissolution of the grains. After cooling to room temperature, the solution was centrifuged at 7000 rpm for 15 minutes at 24 °C. The pH was adjusted to 7 using a NaOH solution, and then, three volumes of cold ethanol were added. The mixture was allowed to rest overnight at -20 °C. The supernatant was separated by decantation from the solid and moist mass, which was dried in an oven at 40 °C for 72 hours. It was then milled using an electric High-speed Multifunction Grinder HC-150 to obtain a uniform powder with a particle size of 300 μm [16].

Fourier Transform Infrared Spectroscopy (FTIR). The ATR infrared spectra of dextran from water kefir were obtained using a Nicolet IS50 analytical FTIR spectrometer. A total of 64 scans were collected with a resolution of 4, a scanning speed of 0.3165 cm^{-1}, using an aperture of 140, and a range of 500-4000 cm^{-1}.

X-ray Diffraction (XRD). The XRD patterns were obtained using a MiniFlex diffractometer with CuKα radiation (λ = 1.540 Å). Data were collected from 5° to 90° in 2θ with a step increment of 0.05°.

Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA). The DSC and TGA thermograms of dextran were obtained using a Setaram 1600 differential scanning calorimeter and thermogravimetric analyzer. The temperature conditions ranged from 25 to 1000 °C with a heating rate of 5 °C.min^{-1}, under a nitrogen flow of 50 mL.min^{-1}.

Statistical Analysis. All experiments were performed in triplicate and expressed as mean ± standard error. An ANOVA test was conducted, and the significance level for each test was set at p≤0.05. All statistical analyses were performed using the same software.

3 Results

3.1 Phase 1: Acclimation of water kefir biomass

Figure 1 shows that from the eighth to the sixteenth reseeding, the consortium of microorganisms reached stability in biomass growth, producing 7.0 g.L^{-1}, with a decrease of 35% compared to the initial seeded value. This indicates that the microorganisms present in the water kefir grains adapted to the amount of sucrose and other components in the culture medium.

Figure 2 shows that the variation in ΔpH during fermentation remained within a range of 4.3 to 4.8 units (the initial pH was 7.40 units), attributed to the growth of the kefir grains and the subsequent production of ethanol, favoring medium acidification [17, 18].

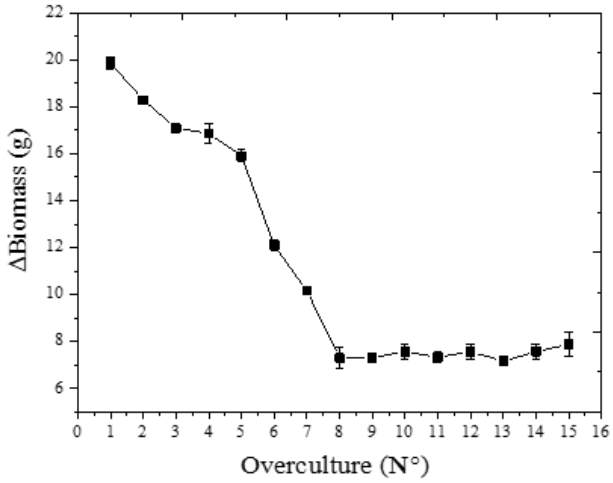

Figure 1: Biomass growth. Water kefir biomass recultivation.

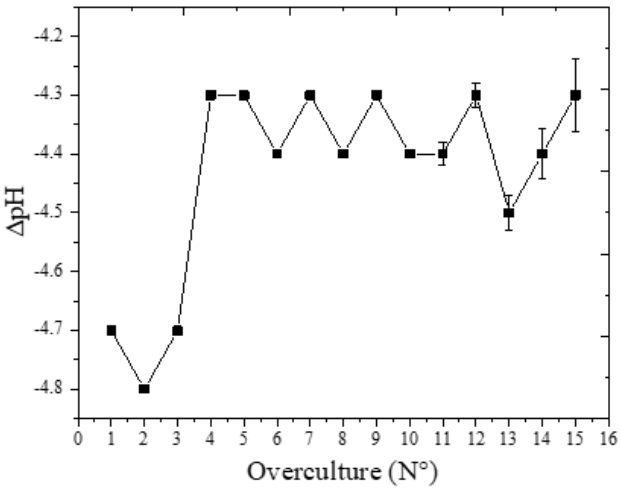

Figure 2: ΔpH. Water kefir biomass recultivation.

The optimal pH for the growth of water kefir grains, which

are the kefir granules, has been reported to be close to 4.0 [19]. At this specific pH, extracellular glucansaccharases enzymes produced by lactic acid bacteria (LAB) in water kefir exhibit their maximum activity. These enzymes are responsible for the growth and formation of water kefir grains.

It is important to note that the activity of glucansaccharases enzymes decreases at lower pH values. This means that if the culture medium's pH is more acidic or deviates from the optimal pH, enzymatic activity can be negatively affected. This, in turn, can influence the growth and development of water kefir grains, which may impact the °Brix and pH values of the culture medium. In other words, the decrease in water kefir biomass compared to the initial amount is mainly due to the adaptation of the microorganisms to the new environment, in particular the change in pH. When culture is initiated, there is a diversity of microorganisms present in the inoculum, as the pH decreases, some of these microorganisms adapt better to the acidic environment and consume the initial substrate to grow and multiply. During this adaptation process, not all microorganisms can survive or reproduce efficiently in the resulting acidic environment. Those that can thrive in these conditions become predominant, leading to a decline in microbial population diversity. That is, those microorganisms that cannot adapt to the new acidic environment decrease in number as others prevail. Once the predominant microorganisms consume the nutrients necessary for their growth and fully adapt to the acidic environment, the growth of the biomass stabilizes. This consortium of microorganisms forms a stable community that can survive and sustain itself in the acidic environment of water kefir, thus ensuring its subsistence.

These findings highlight the importance of maintaining appropriate pH conditions in water kefir culture to promote optimal enzymatic activity and healthy grain growth. Furthermore, they underscore the relationship between pH and enzymatic activity in the context of water kefir microorganisms and their impact on the physicochemical properties of the culture medium [19, 20].

Figure 3 shows the values of optical density (ΔOD), which is related to the cell concentration in the medium. Similar to biomass growth, ΔpH, and Δ°Brix, the ΔOD value stabilized from the eighth reseeding onwards, indicating a constant population of microorganisms in the medium from that point onwards [21].

The variation in soluble solids content (Δ°Brix) in each reseeding (Figure 4) shows that, in the in the first four reseedings, when there is a higher amount of water kefir grains in the substrates, the °Brix values decrease, indicating an inversely proportional relationship between the two variables. In this case, the °Brix value stabilizes at the eighth reseeding, coinciding with the stabilization of water kefir biomass growth. This suggests that the limit of substrate consumption by the microbial consortium is subject to the amount of sucrose in the medium, the number of reseedings, and the amount of inoculum used at the beginning of fermentation. As water kefir grains reproduce and increase in number, the availability of nutrients in the substrate may decrease. When there are more grains, competition for essential nutrients among microorganisms may be more intense. This could lead to a decrease in soluble solids content (Δ°Brix), as these nutrients could be being consumed more quickly by microbial growth. The microbial consortium may reach a point where it can no longer consume substrate due to its metabolic capacity. Despite the availability of nutrients, there is a limit to the rate and amount of substrate that microorganisms can metabolize in a given period. This limitation could cause the consortium's substrate consumption capacity to stabilize as the water kefir biomass increases. The fact that the °Brix value stabilizes at the eighth reseeding, coinciding with the stabilization of biomass growth, suggests a possible dynamic equilibrium reached between the available substrate and the consumption capacity of the microbial consortium. It may have reached a point where the amount of substrate and the rate of consumption are in equilibrium, resulting in a stabilization of both nutrient content and consortium growth [22].

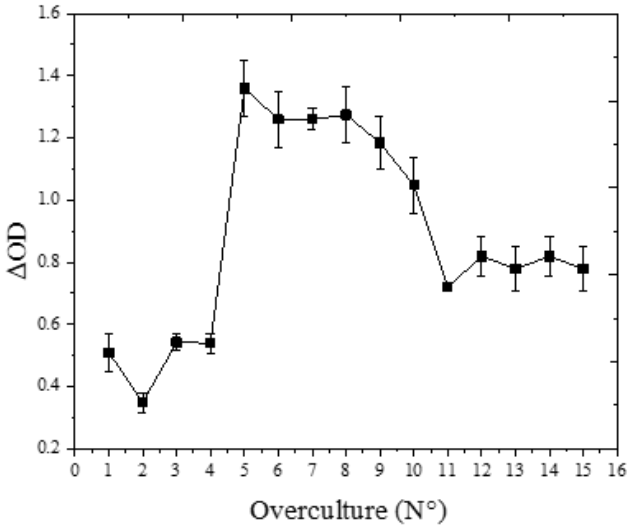

Figure 3: ΔOD. Water kefir biomass recultivation.

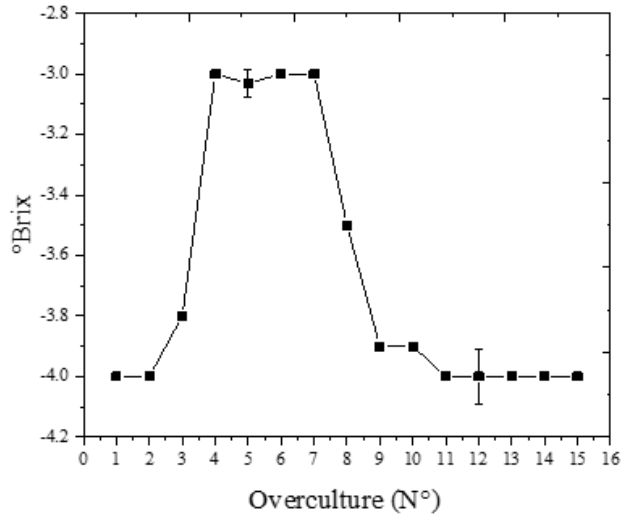

Figure 4: Δ°Brix. Water kefir biomass recultivation.

An increase in optical density generally indicates greater growth and proliferation of microorganisms in the culture medium. As microorganisms multiply, they consume nutrients present in the medium, including sugars. This can lead to a decrease in °Brix values, which indicate sugar concentration, as the available sugars are depleted. Additionally, water kefir microorganisms can produce various metabolic products during fermentation, such as organic acids. As optical density and microbial activity increase, acid production may also increase, resulting in a decrease in pH values [22].

This acclimation process of biomass promotes increased organism yield, facilitates the consumption of non-preferred substrates by bacteria, and can enhance tolerance to stress generated by fermentation by-products such as ethanol and acetic acid [23].

3.2 Phase 2. Evaluation of water kefir growth at different concentrations of tibicos

The highest biomass growth rate (12.0 $g.L^{-1}$) was achieved from 0.02 $g.mL^{-1}$ of kefir grains, representing a 300% increase compared to the initial seeded value (Figure 5(A)). A lower inoculum amount resulted in better adaptation to the culture medium. When using 0.1 $g.L^{-1}$, a growth of 7.0 $g.L^{-1}$ (32%) was observed.

There was non-significant difference observed between 0.06 $g.mL^{-1}$ and 0.08 $g.mL^{-1}$ of kefir grains, with growth rates of 50% and 41% respectively.

Figure 5(C) displays the optical density at 600 nm (OD600) for different amounts of kefir grains. It can be observed that the values stabilized at 0.14 units. This suggests that yeast growth may be favored under microaerobic conditions rather than anaerobic conditions [21].

Finally, Figures 5(D) and 5(B) show the final values of Δ°Brix and ΔpH obtained at the end of the fermentation process (2.03 and 4.0, respectively). This indicates that the microorganisms adapted to the components of the culture medium over the course of ten days (5 subcultures).

The information suggests that higher biomass growth can be obtained when using a lower inoculum amount. This may be due to the fact that a smaller inoculum minimizes natural substrate depletion, substrate inhibition, and acid stress in the medium.

The growth of water kefir grains occurs through the partial conversion of sucrose into an exopolysaccharide (EPS) called glucan. The synthesis of this EPS relies on the utilization of glucose by extracellular glucansucrase enzymes, which exhibit optimal activity at a pH range of 4.3 to 4.6. The activity of these enzymes dramatically decreases when the pH drops to 3.6 - 3.2, consequently inhibiting the production of glucansucrase rather than the enzymatic activity itself. As the growth of kefir grains decreases, less glucose is incorporated into their matrix, thereby increasing the availability of sugar for acid production by yeasts and acetic acid bacteria, leading to increased acid stress in the medium. Therefore, the low pH values can be associated with limited growth of kefir grains [6, 12], [24–26].

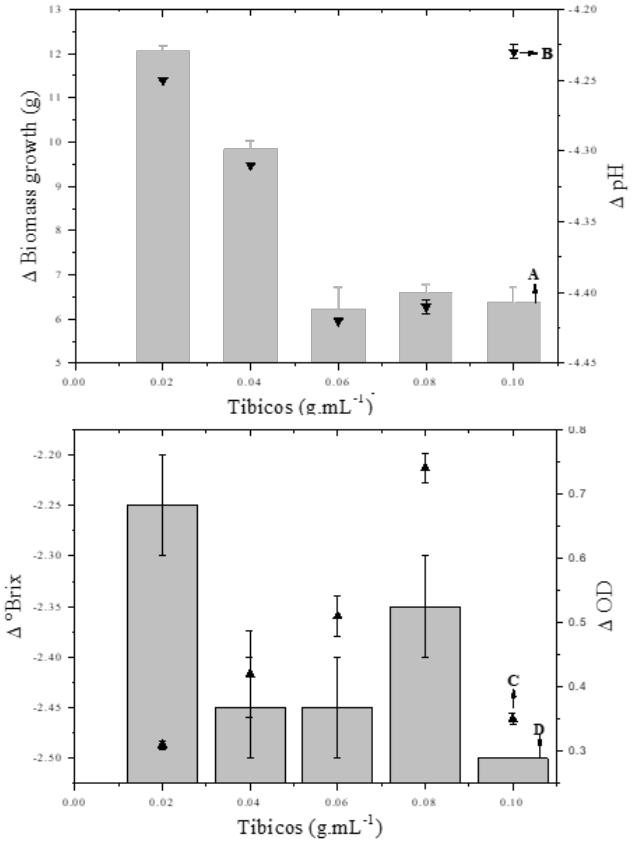

Figure 5: Evaluation of water kefir biomass growth at different concentrations of tibicos. (A) ΔGrowth; (B) ΔpH (C) ΔDO; (D) Δ°Brix.

The growth of water kefir grains occurs through the partial conversion of sucrose into an exopolysaccharide (EPS) called glucan. The synthesis of this EPS relies on the utilization of glucose by extracellular glucansucrase enzymes, which exhibit optimal activity at a pH range of 4.3 to 4.6. The activity of these enzymes dramatically decreases when the pH drops to 3.6 - 3.2, consequently inhibiting the production of glucansucrase rather than the enzymatic activity itself. As the growth of kefir grains decreases, less glucose is incorporated into their matrix, thereby increasing the availability of sugar for acid production by yeasts and acetic acid bacteria, leading to increased acid stress in the medium. Therefore, the low pH values can be associated with limited growth of kefir grains [6, 12, 24, 25, 26].

3.3 Phase 3. Evaluation of water kefir grain growth at different sucrose concentrations

Once 0.02 $g.mL^{-1}$ was identified as the inoculum amount that showed the highest biomass growth, the data represented in Figure 6A, corresponding to the variation of kefir grains with respect to sucrose concentration in the culture medium, were analyzed. It can be observed that when using 100 $g.mL^{-1}$ of sucrose, the kefir grains exhibited the highest increase in biomass, reaching 11.8 g (292.5% increase). With 80 $g.L^{-1}$ of sucrose, a final average weight of 7.5 g was obtained, corresponding to a 187% increase. Similarly, using 60 $g.L^{-1}$

and 40 g.L^{-1} of sucrose resulted in increases of 163.7% (6.5 g) and 171.2% (6.8 g), respectively. Finally, the lowest biomass increase was observed with 20 g.L^{-1} of sucrose, with a final average weight of 3.0 g (75

Based on the above, the following conclusions can be drawn: 1) The optimal sucrose concentration for the highest biomass increase in kefir grains is 100 g.L^{-1}, and 2) The sucrose concentration in the culture medium had a significant effect ($p<0.05$) on the growth of kefir grain biomass. The ΔDO values (from 0.73 to 0.83) and ΔpH values (from 4.3 to 4.4) in Figures 6C and 6B, respectively, remained stable during the fermentation process, indicating that the consortium of predominant microorganisms in water kefir tibics, which are characterized by their stability, remained constant despite different sucrose concentrations. [19, 20, 21].

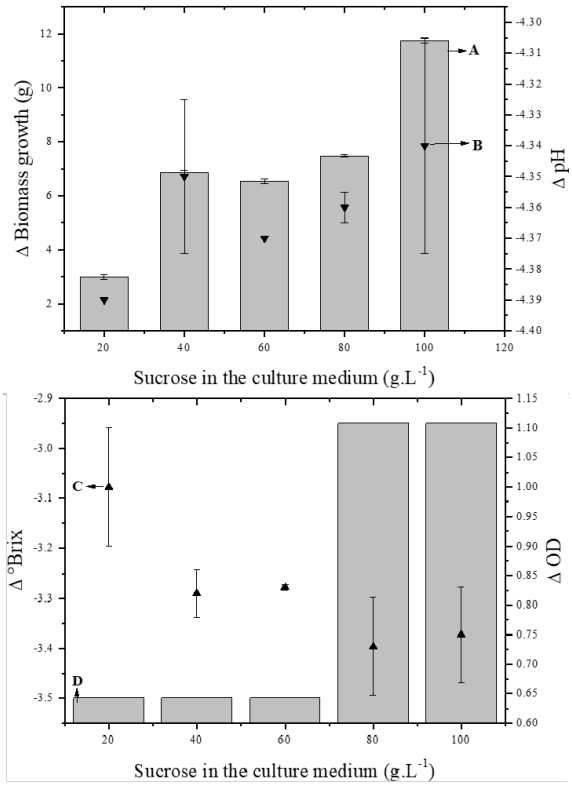

Figure 6: Evaluation of water kefir grain growth at different sucrose concentrations. (A) ΔGrowth; (B) ΔpH. (C) ΔDO; (D) Δ°Brix.

Lastly, the Δ°Brix value in Figure 6D indicated the consumption of sucrose by the microbial consortium. The consumption of sucrose by the microorganisms in the water kefir consortium directly impacts their growth. Sucrose is the main substrate used by the bacteria and yeasts present in water kefir for their metabolism and production of metabolites. When the microorganisms in water kefir consume sucrose, they break it down through fermentative processes. Lactic acid bacteria (LAB) convert sucrose into lactic acid, while yeasts ferment it to produce ethanol and carbon dioxide. These fermentation products contribute to the unique organoleptic characteristics of water kefir. The consumption of sucrose provides the necessary energy for the growth and reproduction of water kefir microorganisms.

As the microorganisms metabolize sucrose, they release energy that they utilize for their cellular processes. This allows them to increase their biomass and multiply in the culture medium. It is important to note that the consumption of sucrose by the microorganisms can also have an effect on the pH and °Brix values of the culture medium. Sucrose fermentation produces lactic acid, which can lower the pH of the medium. Additionally, the production of metabolites such as ethanol can affect the sugar concentration and, therefore, the °Brix values [25].

3.4 Phase 4. Biomass growth under the best conditions identified in phases 2 and 3

Figure 7 depicts the biomass growth rate, expressed in grams, of the strain's adaptation to the previously identified optimal fermentation conditions: 0.02 g.mL^{-1} of kefir grains and 100 g of sucrose per liter of culture medium. It can be observed that from the fourth to the fifteenth subculture, the biomass stabilizes its growth at 23 g.

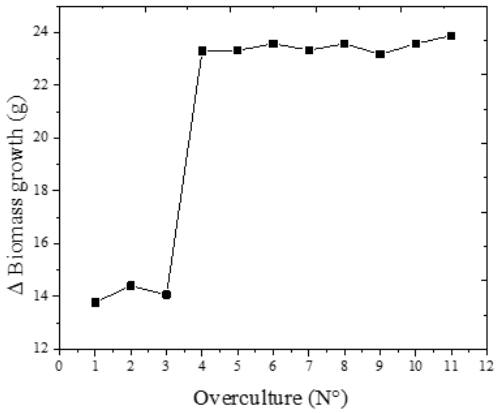

Figure 7: ΔGrowth. Biomass growth under the best conditions identified in phases 2 and 3.

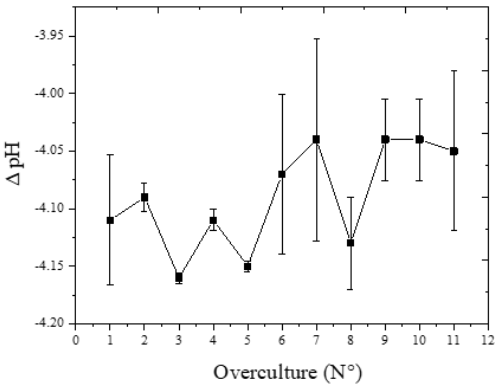

Figure 8: ΔpH. Biomass growth under the best conditions identified in phases 2 and 3.

Similarly, the ΔDO values stabilize at 0.14, Δ°Brix at -2.03,

and ΔpH at -4.0. This demonstrates that the microorganisms have adapted and stabilized to the components of the culture medium over the course of sixteen days (eight subcultures).

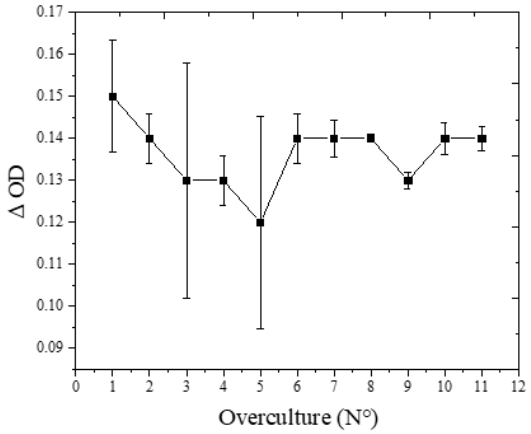

Figure 9: ΔDO. Biomass growth under the best conditions identified in phases 2 and 3.

Conducting the process of adapting the biomass to the identified optimal conditions is essential as it promotes the organism's performance and facilitates the utilization of non-preferred substrates by the bacteria. Additionally, this adaptation can contribute to improving tolerance to adverse effects generated by certain fermentation byproducts, such as ethanol and acetic acid.

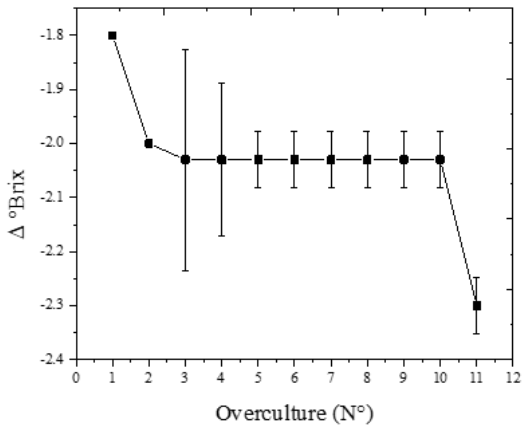

Figure 10: ΔBrix. Biomass growth under the best conditions identified in phases 2 and 3.

The aforementioned demonstrates that the microorganisms adapted to the components of the culture medium over the course of sixteen days (eight subcultures). In other words, the amount of sucrose and kefir grains in the culture medium had a significant effect ($p<0.05$) on the growth of the water kefir grain biomass.

The amount of dextran obtained was higher than that observed in other studies (15% or an average of 160 g of extract per kg of kefir grains, wet weight) that also utilized sucrose as a substrate. Previous studies have shown an average biomass growth of water kefir grains of 152.3 g of extract per kg of kefir grains, wet weight [16].

4 Physicochemical Characterization of Dextran from Water Kefir Tibicos

4.1 Fourier Transform Infrared Spectroscopy (FTIR)

Figure 11 displays the illustrated results of the FTIR spectra of dextran isolated using two extraction methods: (A) acid extraction and (B) oven drying, from water kefir Tibicos, allowing for the identification of its functional groups.

By comparing the bands of the obtained spectra with those identified by various authors, the data shown in Table 1 were obtained.

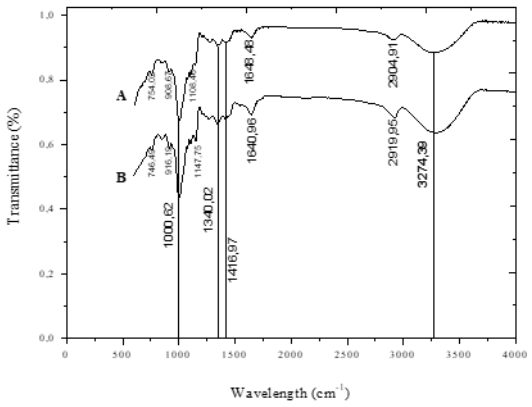

Figure 11: FTIR spectrum of dextran from water kefir Tibicos: A) Acid extraction; B) Oven drying.

According to the analysis of the data reported in Table 1, the obtained polysaccharide corresponds to dextran, as the values assigned to each functional group are characteristic of dextran, which coincides with the literature reports.

For example, peaks at 1640 cm^{-1} and 1648 cm^{-1} indicate the stretching vibration of the C=O bond [16]. Polysaccharides contain a large number of hydroxyl (O-H) groups, and the stretching vibrations of O-H usually appear as a strong and broad absorption peak between 3200 and 3600 cm^{-1} [18].

Therefore, the broad and intense band at 3274 cm^{-1} can be attributed to the stretching vibration of the O-H bond, which is characteristic of polysaccharides. Strong peaks at 2930.06 cm^{-1} are attributed to C-H stretching vibrations [27]. Thus, the bands at 2919 cm^{-1} and 2904 cm^{-1} are assigned to the stretching vibration of the C-H bond present in the isolated organic compound.

On the other hand, the bands between 1000 cm^{-1} and 1416 cm^{-1} represent the symmetric stretching vibration band of the free -COO- bond [28]. The C-O-C and C-O bonds are characteristic of carbohydrates, and thus, the infrared band at 1340.02 cm^{-1} is originated from the CH_2 bending modes [29].

The absorption peaks in the range of 800 cm^{-1} to 1200 cm^{-1} reflected the characteristics of a polysaccharide like dex-

tran [30]. The sharp peak at 1108.48 cm^{-1} and 1147.75 cm^{-1} indicates a highly flexible chain around α-(1→6) linkages [31].

The absorption peaks at 908.67 cm^{-1} and 916.58 cm^{-1} are characteristic of the asymmetric and telescopic vibrations of the glucopyranose ring present in dextran, and the band at 845.06 cm^{-1} indicates an α-glycosidic configuration [32].

Additional peaks at 754.02 cm^{-1} and 746.49 cm^{-1} are attributed to the stretching vibrations of the C-C bond in the molecule [29]. The profile of the functional groups indicates that the spectra correspond to dextran [28].

Finally, it can be deduced that the physicochemical characterization analysis (FTIR) was not affected by the techniques used for the isolation of the biopolymer, as no difference was observed between the two spectra in Figures 5A and 5B.

Table 1: Comparison of FTIR spectral bands of dextran.

Reference \ Functional group		O-H	C-H	C=O	C-O-C	C-O	Configuration α (abs)
		Band value (cm^{-1})					
This study	Oven	3274	2919	1640	1000	1416	916
	Acid		2904	1648			908
[33]		NA	NA	NA	1150	1010	912
[34]		3200-3500	2927	NA	1016-1020	1030-1040	825-850
[35]		3372	2935-2906	1649	1016	1420	NA
[36]		3438	2924	1637	NA	1018	NA
[33]		NA	NA	NA	1150	1010	912
[35]		3280	2800-3000	NA	NA	835	NA

4.2 X-Ray Diffraction (XRD)

The X-ray diffraction profile observed in Figure 12 was used to evaluate the crystallinity of the sample, indicating that the dextran extracted from water kefir tibicos exhibits an amorphous structure, as it lacks well-defined crystalline peaks. This characteristic is consistent with the polymeric nature of the exopolysaccharide, as observed in previous studies [37].

The diffractogram of the dextran sample isolated by acid extraction showed the formation of a broad peak at $2\theta°$ in the range of 15-21°, which is consistent with the crystallinity profiles observed in samples of commercial dextran and dextran obtained from other sources such as Lactobacillus mali CUPV271 and *Leuconostoc carnosum* CUPV411 strains [38, 39].

With regard to the comparison of crystallinity between the samples isolated using the two extraction methods of the *exopolysaccharide* mentioned, it is possible to infer that more well-defined crystalline peaks are observed in the diffractogram obtained from the acid extraction method.

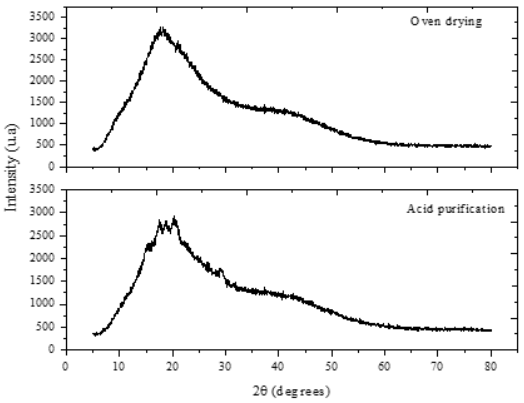

Figure 12: X-ray diffractogram of the dextran sample from water kefir grains.

4.3 Differential Scanning Calorimetry (DSC)

Figure 14A and 14B depict the differential scanning calorimetry (DSC) curves, showing endothermic peaks at 295 ºC and 288 ºC in both samples. The temperature at which this glass transition occurs (Tg) is high compared to other biopolymers, which can be attributed to the presence of strong hydrogen bonds between the dextran macromolecules in water kefir.

Previous studies have reported high Tg values for dextrans produced by lactic acid bacteria, including Leuconostoc pseudomesenteroides (278.4 °C), which is part of the microbial consortium of interest [38, 40, 41].

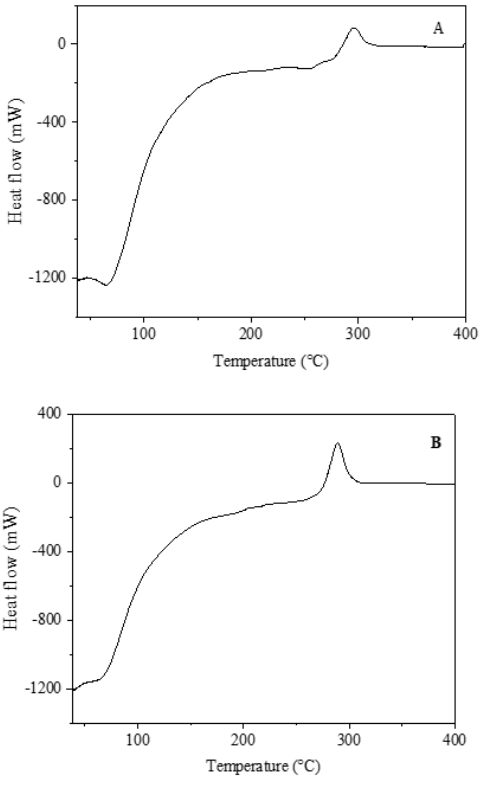

Figure 13: (A) DSC of the biopolymer obtained by acid extraction, (B) DSC of the biopolymer dried in an oven.

4.4 Thermogravimetric Analysis (TGA)

The thermal stability of the water kefir biopolymer was studied using thermogravimetric analysis (TGA). The thermogravimetric analysis curves and their respective derivatives are shown in Figures 9-A and 9-B. Both figures exhibit the same thermal behavior, with two mass loss events between 40 °C and 130 °C and a second loss in the range of 250 °C to 350 °C, with a peak at 295 °C for the biopolymer obtained by acid extraction and 288 °C for the one obtained by oven drying.

The initial mass loss is related to the reduction of free water bound to the material's structures and the condensation of hydroxyl groups on the surface. The second mass loss is associated with the degradation of the polysaccharide structure, resulting from depolymerization through the breaking of C-C and C-O bonds present in the sugar, and the release of small molecules during this process [28, 42].

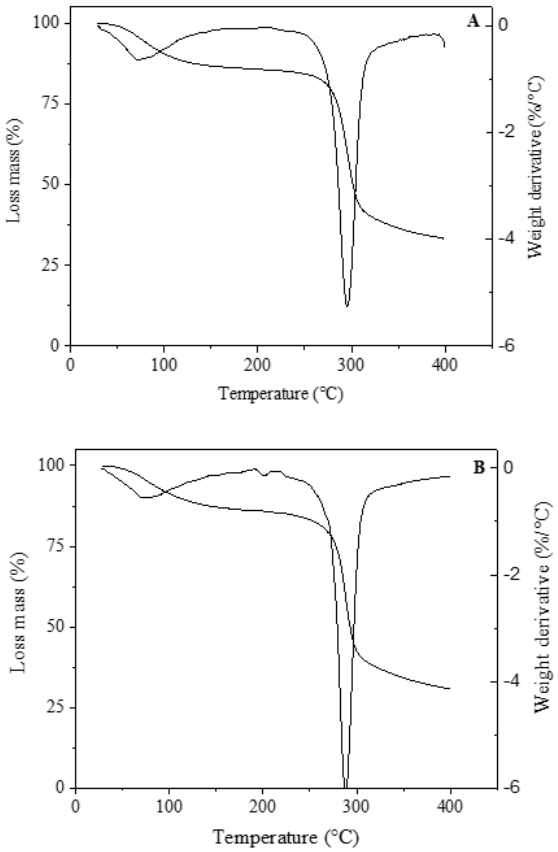

Figure 14: ((A) TGA and DSC derivative of the biopolymer obtained by acid extraction, (B) TGA and DSC derivative of the biopolymer dried in an oven.

In the oven drying process, it is possible that the biopolymer undergoes some structural modifications that provide it with a different molecular configuration and allow for a lower degradation temperature. Regardless of this, the TGA results suggest that the structure of the biopolymer is indeed dextran [35].

It is important to note the interesting thermal characteristics of the biopolymer obtained by both processes, considering their potential industrial applications where processing temperatures reach approximately 150 °C, such as autoclave sterilization in the pharmaceutical industry (15 min at 121 °C), spray drying (<200 °C), granulation, and fluidized bed drying (<200 °C), among others [43].

5 Conclusions

When bacteria in a culture medium reach the stationary phase, their growth stops because this growth equals cell death and therefore there is no increase in the number of cells. It is at this point that the relationship between constant growth and adaptation to the culture medium is proved. In this study, it was found that the microbial consortium of water kefir achieved its highest growth and stability by using 0.02 g/mL^{-1} of grains and 100 g/L of sucrose in the culture medium. This amount of sugar is the most significant carbon source for the incubation process. The stabilization of biomass growth values, ΔpH, ΔDO, and Δ°Brix, suggests that the strain took sixteen days (8 subcultures) to adapt to the medium. While °Brix is related to the substrate consumption rate and optical density to the number of cells in suspension, the pH value is particularly relevant in understanding biomass growth with a minimal inoculum. When there is little growth of the grains, the availability of glucose in the medium increases for acid production, which increases the medium's acid stress and directly affects grain growth. Therefore, continuous monitoring of pH is crucial for improving grain production processes.

It is important to highlight that in order to promote increased organism yield, ease the consumption of non-preferred substrates, and improve tolerance to stress caused by fermentation byproducts, it is necessary to conduct the biomass recultivation or adaptation process. It was found that from the eighth subculture, the biomass reached stability in its growth, showing that microorganisms adapted to the culture medium conditions.

Additionally, medium acidification was seen, possibly due to the production of organic acids by microorganisms, and it was found that the optimal activity of enzymes responsible for water kefir growth occurs at a pH close to 4.0. An inversely proportional relationship between the amount of grains in the substrate and the soluble solids content was also found. Overall, the study proved how water kefir grain microorganisms can adapt to different culture conditions and produce stable biomass.

The amount of sucrose in the culture medium has a significant effect on water kefir grain biomass growth, and the best amount for the greatest biomass increase under the study conditions is 100 g/L. It is also showed that Δ°Brix, ΔpH, and ΔDO values remained stable during the fermentation process, indicating that the amount of consumed substrate and medium acidity were constant and equivalent to biomass growth, and that the microorganisms present in the medium remained constant from the first inoculation. In summary, the amount of sucrose used in the culture medium is important for grain growth, and certain pH, substrate, and microorganism conditions must be kept achieving best biomass

production.

Starting from the fourth subculture, until the eleventh, the biomass growth rate stabilized at a constant value of 23.0 g, showing that the microbial consortium adapted to the culture medium conditions. Also, the amount of sucrose and grains in the culture medium has a significant effect on water kefir grain biomass growth.

Furthermore, it is noteworthy that the amount of dextran obtained was higher than that observed in other studies using sucrose as a substrate. In conclusion, these results prove that it is possible to control and enhance the growth of water kefir grains by manipulating fermentation conditions.

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Authors' Contribution

The authors confirm their contributions to the article as follows: All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interest Statement

The authors declare no conflicts of interest regarding the publication of this article.

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