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Study of the Fermentation Potential of *Eichhornia crassipes* Hydrolysate with Water Kefir Tibicos

Estudio del potencial fermentativo del hidrolizado de *Eichhornia crassipes* con tíbicos de kéfir de agua

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ABSTRACT

Eichhornia crassipes is an invasive aquatic plant that reduces oxygen availability, posing a threat to aquatic ecosystems. This was evidenced in the cooling lagoons of the Paipa Thermoelectric Power Plant in Boyacá, Colombia. Due to its low lignin content (~15%), this plant does not require rigorous treatments for utilization, which allows exploring its potential as a precursor in obtaining value-added products while contributing to the conservation of affected aquatic ecosystems. The objective of this study was to evaluate the growth of kefir tibicos in a culture medium with hydrolysates of E. crassipes obtained from sequential and simultaneous pretreatments with cellulase and the inclusion of activated charcoal suspensions, using sucrose as a co-substrate. All processes were monitored with measurements of soluble solids, reducing sugars, acidity, and pH. Finally, the kefir tibicos were characterized using Fourier-transform infrared spectroscopy and X-ray diffraction, and the thermal properties of dextran were identified through differential scanning calorimetry and thermogravimetric analysis. According to the results, the difference in exopolysaccharide production between the sequential and simultaneous applications of cellulase was less than 10%. However, the inclusion of activated charcoal increased the difference to 22.8% and revealed that insoluble dextran could be applied as a matrix for the *in situ* immobilization of particles during the microorganism growth stage.

Keywords: cellulose hydrolysis, cellulase, dextran, kefir tibicos, lactic acid bacteria, yeast

RESUMEN

Eichhornia crassipes es una planta acuática invasora que genera disminución en la disponibilidad de oxígeno, representando una amenaza para los ecosistemas acuáticos. Esto se evidenció en las lagunas de enfriamiento de la Central Termoeléctrica de Paipa, en Boyacá, Colombia. Debido a su bajo contenido de lignina (~15 %), esta planta no requiere tratamientos rigurosos para su aprovechamiento, lo que permite explorar su potencial como precursora en la obtención de productos de valor agregado, al tiempo que se contribuye a la conservación de ecosistemas acuáticos afectados. El objetivo de este estudio fue evaluar el crecimiento de tíbicos de kéfir en un medio de cultivo con hidrolizados de *E. crassipes*, obtenidos a partir de pretratamientos secuenciales y simultáneos con celulasa y de la inclusión de suspensiones de carbón activado, utilizando sacarosa como cosustrato. Todos los procesos se monitorearon con mediciones de sólidos solubles, azúcares reductores, acidez y pH. Por último, los tíbicos fueron caracterizados mediante espectroscopia infrarroja por transformada de Fourier y difracción de rayos X, y se identificaron las propiedades térmicas del dextrano mediante calorimetría diferencial de Barrido y análisis termogravimétrico. De acuerdo con los resultados, la diferencia en la producción de exopolisacárido entre la aplicación secuencial y simultánea de celulasa fue inferior al 10 %. Sin embargo, la inclusión de carbón activado hizo que la diferencia se incrementase hasta 22.8 % y reveló que el dextrano insoluble podría aplicarse como matriz para la inmovilización de partículas *in situ* en la etapa de crecimiento de los microorganismos.

Palabras clave: hidrólisis de celulosa, celulasa, dextrano, granos de kéfir, bacterias ácido lácticas, levadura

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Introduction

Eichhornia crassipes is an aquatic plant native to the Amazon basin, commonly known as the *water hyacinth*. It thrives in tropical freshwater environments and is listed among the 100 most dangerous invasive species around the world. This classification stems from its high competitiveness against native species and its ability to cause a reduction in water flow and oxygen availability in nutrient-excessive aquatic systems [1], [2]. Hence, it poses a threat to aquatic ecosystems, as it blocks sunlight, hindering the proper development of fish and other present organisms [1]. This is due to alterations in factors such as temperature,

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radiation, water nutrient levels, and the decomposition of the plant itself [3], [4].

The content of lignin in the water hyacinth is between 7 and 11%, which means that it does not require rigorous treatments for utilization. This enables the exploration of its potential as a precursor in obtaining value-added products while contributing to the conservation of affected aquatic ecosystems [5], [6]. However, the cost and environmental impact of the processes and technologies necessary for the pretreatment of plant biomass, coupled with the Colombia's high dependence on the petrochemical industry, have hindered comprehensive research into new alternative substrate acquisition processes that could generate various products from existing ones, such as bioethanol or biodiesel.

There are various alternatives for purifying this raw material, wherein biotechnological options play a significant role in implementing processes with low energy consumption. Mainly, the use of enzymes such as cellulase is the basis for generating different platforms like sugars, furanic derivatives, phenolics, and acids, which are used in the production of value-added products with potential applications in the biomedical industry, 3D printing, drug delivery, and tissue engineering [7].

The aforementioned achievement is made possible by incorporating microorganisms that act as biofactories in the production of multiple value-added compounds. Among the most significant examples are consortia such as water kefir tibicos, composed of lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeasts, adhered to a polysaccharide matrix produced by the bacteria themselves. These are compact, gelatinous granules of a whitish or yellowish color, produced in a stable culture medium. These tibicos may appear translucent or opalescent and are irregularly shaped, with an average diameter ranging from 5 to 20 mm [8], [9].

The symbiosis between the microorganisms present in water kefir tibicos allows yeasts to benefit from the acidification of the culture medium, while the vitamins and soluble nitrogenous compounds produced by the yeasts favor the bacteria [10], [11].

The composition of the exopolysaccharide (EPS) matrix of water kefir tibicos consists mainly of a glucose polymer with α-(1-6) linkages, which is insoluble in water, known as *dextran* [8], [9]. This polysaccharide exhibits a high molecular weight, with a structure that varies based on the percentage, nature, and distribution of its linkages. Typically, it is produced in cultures of LAB such as *Streptococcus*, *Acetobacter*, or *Leuconostoc* in sucrose-based media. During cell growth, an enzyme called dextransucrase is secreted, converting the excess sucrose into dextran and releasing fructose into the medium. The primary source of dextran production in water kefir tibicos is *Lactobacillus hilgardii*, which acts through the enzyme glucosyltransferase [8], [9].

Dextran has extensive applications in the food industry as a stabilizing thickener, emulsifier, fat substitute, or gelling agent. Additionally, it exhibits antitumor activity [12], [13]. Therefore, this study aims to purify dextran in order to evaluate its potential as a biopolymer with industrial applications, aiming to assess its viability as a substitute for petroleum-derived polymers.

This article aims to address the following research question: *What are the conditions required for the water kefir microbial consortium to produce biopolymers such as dextran through fermentation processes, using substrates derived from the aquatic plant E. crassipes?* To this effect, the following null and alternative hypotheses have been proposed:

- **H_0:** The fermentation of a culture medium with *E. crassipes* hydrolysates obtained from pretreatments with cellulase—including activated charcoal suspensions and using sucrose as a cosubstrate both sequentially and simultaneously—has no effect on the formation of kefir tibicos.
- **H_1:** The fermentation of a culture medium with *E. crassipes* hydrolysates obtained from a pretreatments with cellulase—including activated charcoal suspensions and using sucrose as a cosubstrate both sequentially and simultaneously—has an effect on the formation of kefir tibicos.

The methodological aspects for determining the fermentation kinetic behavior of *E. crassipes* hydrolysates, using a commercial water kefir microbial consortium and sucrose as a cosubstrate, are presented in this manuscript. The experimental development is detailed in the methodology section, where the composition of the raw material (*E. crassipes*) is specified, as well as the alkaline-enzymatic pretreatment of the biomass; the hydrolysis of the cellulose at varying substrate ratios; the preparation of the culture medium; the acclimatization of water kefir biomass; the evaluation of biomass growth of water kefir with *E. crassipes* hydrolysates, encompassing four experiments involving the growth of kefir tibicos in a culture medium with *E. crassipes* hydrolysate, enzymatic hydrolysis, the simultaneous growth of kefir tibicos, and a treatment with activated charcoal; a chemical analysis that considers the pH, soluble solids, and acidity; and dextran purification, which was characterized using X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR). The thermal properties of dextran were studied through differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The subsequent section is dedicated to the results and analysis of the alkaline-enzymatic pretreatment processes; the hydrolysis of cellulose at different substrate proportions; the acclimatization of the water kefir biomass; the evaluation of the biomass growth of water kefir with *E. crassipes* hydrolysates; and the physicochemical characterization of dextran from water kefir tibicos. In the final section, the conclusions of this work are detailed.

Methodology

Preliminary sampling and analysis

The water hyacinth was collected from the northern cooling lagoon of the Paipa Thermoelectric Power Plant in Colombia, with the following coordinates: N °5 46′ 8.3′′ W 73° 08′ 40.3′. The sample was dehydrated at room temperature, after which the leaves and roots were separated. The leaves were taken, and their particle size was reduced by spraying. Table I shows the components of the pulverized plant's leaves.

Table I. Composition of the raw material – *Eichhornia crassipes*

Component (%)	**Leaves**
Cellulose	23.7
Lignin	14.3
Hemicellulose	44.4
Removable products	2.4
Ash	7.9

Source: Authors

Alkaline-enzymatic pretreatment

In a 500 mL Erlenmeyer flask, water hyacinth was mixed with a NaOH solution in different concentrations (%w/v): 1.0, 2.0, and a control (without the addition of a base), at a 1:20 ratio. The mixture underwent a reaction at 100 °C in an autoclave for 1 h. Upon completion of the process, the solid phase was filtered and washed to neutral pH. Subsequently, the material was returned to the Erlenmeyer flask, and 200 mL of Rouyangmoer commercial cellulase solution were added, with an equivalent of 50 FPU/g.

Every 24 h for five days, 2 mL of the supernatant were extracted for reducing sugars analysis [14]. The remaining available biomass was subjected to the best identified pretreatment conditions, in order to obtain the hydrolysate for use during the fermentation process.

Hydrolysis of cellulose at varying substrate ratios

Based on the previous experiments, biomass was pre-treated with distilled water at 100 °C for 1 h, then dried in an oven. From this sample, four quantities were taken (1.25, 2.50, 3.75, and 5.00 g) for enzymatic hydrolysis, separately dissolved in 100 mL of distilled water. After 24 h of reaction, the produced reducing sugars were quantified to determine the percentage of hydrolysis concerning the water hyacinth cellulose.

Preparing the culture medium

A modified MRS liquid culture medium was prepared, which consisted of 100 g/L of sucrose as the primary carbon source, 2.25 g/L of yeast extract as the nitrogen source, and micronutrients including 2 $g.L^{-1}$ of K_2HPO_4, 28 $mg.L^{-1}$ of $MnSO_4$, 200 $mg.L^{-1}$ of $MgSO_4$, and 29 $mg.L^{-1}$ of B-complex [13].

Acclimatizing the water kefir biomass

We added 0.02 g of commercial water kefir tibicos per mL of culture medium to 500 mL Erlenmeyer flasks, totaling 200 mL of liquid. Each test was prepared in triplicate, along with a negative control. The process was repeated until no changes in the weight of the produced kefir tibicos were observed. Fermentation was evaluated for 48 h at a temperature of 25°C using a Memmert UN75 universal incubator.

Evaluation of biomass growth of water kefir with water hyacinth hydrolysates

Tibicos growth after water hyacinth hydrolysis

Using distilled water, 2.5 g of pre-treated water hyacinth were hydrolyzed in 200 mL of cellulase solution (50 U/g). The mixture was incubated for 24 h at 25 °C, with constant agitation at 150 rpm in an orbital shaker. The reaction was stopped by subjecting it to ± 92 °C for 5 min. Afterwards, it was filtered, and the value of soluble solids (°Brix) in the liquid phase was determined. The sugar concentration was then adjusted to 20 g/L with sucrose, and the missing components of the culture medium were added as indicated in the acclimatization section. The experiments were conducted in triplicates, considering two controls: B1 - without the addition of sucrose but with tibicos, and B2 - with the addition of sucrose but without tibicos.

All experiments were sterilized in an autoclave at 121 °C for 20 minutes, cooled to room temperature (±25 °C), and then inoculated with water kefir tibicos (0.02 $g.mL^{-1}$). The mixtures were stored in an incubator at 25 °C.

Every 12 h (for a period of 60), we monitored the variation in soluble solids, pH, and acidity in the liquid phase. Upon completion of the process, the quantity of produced tibicos was evaluated through wet weighing.

Concurrently, similar experiments were conducted with an additional 2.5 g of activated charcoal in the culture medium.

Enzymatic hydrolysis and simultaneous tibicos growth

2.5 g of pre-treated water hyacinth were added to 200 mL of a buffer solution at pH 4.8, followed by the addition of 16 g of sucrose and the missing components of the culture medium, excluding the enzyme. The mixture was then sterilized in an autoclave for 20 min at 121 °C. After cooling to room temperature (±25 °C), the enzyme was added, and the water kefir tibicos were inoculated. The experiments were conducted in triplicate, using a culture medium with hydrolysate and sucrose. Two control samples were considered: B3, without the addition of sucrose or tibicos but with the enzyme; and B4, with the addition of sucrose but without tibicos or the enzyme.

Concurrently, similar experiments were performed, also with an additional 2.5 g of activated charcoal in the culture medium.

Following the inoculation, the cultivation proceeded similarly to the procedures outlined in the biomass growth section.

Chemical analysis

pH. Direct determination was conducted in the fermented liquid phase, using an ORION 8107UWMMD ROSS Ultra pH/ATC triode electrode by Thermo Scientific.

Soluble solids. The measurement was conducted in the fermented liquid phase of each assay, using a portable refractometer: BRIXCO ATC 0-50%.

Acidity. A 1 mL sample was titrated with 0.1 N NaOH, utilizing phenolphthalein as an indicator (NTC 4978 of 2001). Subsequently, the percentage of acid was calculated using Eq. (1), where N_{NaOH} represents the normality of the NaOH solution used in the titration process, V_{NaOH} is the volume in liters of NaOH consumed in the titration, and PM_{eq} is the equivalent weight of NaOH.

$$\% \text{ Lactic acid} = \frac{(N_{NaOH})(V_{NaOH})\ (PM_{eq})(100)}{\text{mL of the sample}} \quad (1)$$

Dextran purification

The tibicos were washed with distilled water and ethanol and subsequently subjected to drying at 40 °C for 48 h. They were ground to achieve a particle size of 300 µm, using a high-speed multifunction grinder: HC-150 [15].

Subsequently, 100 g of clean tibicos were treated with 200 mL of a 0.1 N H_2SO_4 solution, subjected to continuous agitation and heating (100 °C) until complete dissolution was reached. After cooling to room temperature, the solution was centrifuged at 7000 rpm for 15 min at 24 °C, and its pH was adjusted to 7 using a NaOH solution. Three volumes of cold ethanol were added, and the mixture was left to rest overnight at -20°C. The following day, the supernatant was separated by decantation from the solid, moist mass, which was dried in an oven at 40° C for 72 h. Finally, it was ground until a uniform powder with a particle size of 300 µm was obtained [16].

Physicochemical characterization of dextran from water kefir tibicos

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°.

DSC and TGA thermograms of the dextran were obtained using a differential scanning calorimeter and a Setaram 1600 thermogravimetric analyzer. The temperature conditions ranged from 25 to 1000 °C at a rate of 5 °C per minute, with a nitrogen flow of 50 mL per minute.

ATR-FTIR spectra of the dextran from the water kefir tibicos were obtained using a Nicolet IS50 analytical FTIR spectrometer. 64 scans were taken at a resolution of 4 cm^{-1}, at a speed of 0.3165 stand. s^{-1}, using an aperture of 140 and covering a range from 500 to 4000 cm^{-1}.

Results and discussion

Alkaline-enzymatic pretreatment

Fig. 1 depicts the cellulose hydrolysis following the NaOH treatments. Generally, all three experiments exhibited the highest hydrolysis rate on the first day, with the highest observed in the control (18%). Subsequent days showed a slight downward trend in the values, except for the 1% NaOH-treated experiment, which experienced an increase from day 2 to day 4, although it did not reach the maximum value obtained by the control.

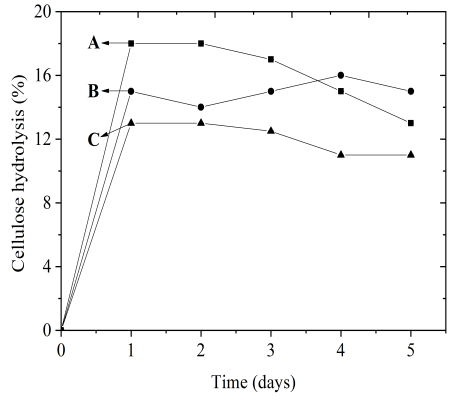


Figure 1. Cellulose hydrolysis percentage of the biomass after pretreatments with NaOH at 100 °C. a) Control, b) 1% NaOH, c) 2% NaOH.
Source: Authors

Based on these findings, it can be stated that water hyacinth does not necessitate an alkaline pretreatment with NaOH to release cellulose, and that the maximum hydrolysis reaction could be reached in 24 h.

The low values obtained in the cellulose hydrolysis percentage can be due to the structure of the cellulose substrate, which is closely related to the type of crystalline conformation of cellulose [17], [18], [19]. Specifically for water hyacinth, this might be attributed to the quantity of substrate used, to the enzyme concentration, or to the degree of cellulose crystallinity in the biomass. In some cases, it is necessary to add complementary enzymes to increase the fermentable sugar yields, as the enzymatic activities detected in most commercial cellulase preparations are either too low or not sufficiently active to achieve a representative conversion of the available cellulose [20]–[24]. Therefore, this research considered modifying the substrate quantity in an attempt to avoid inhibition.

Hydrolysis of cellulose at different substrate proportions

The reducing sugars data (Fig. 2) displayed a significant difference in the hydrolysis percentages for each substrate quantity.

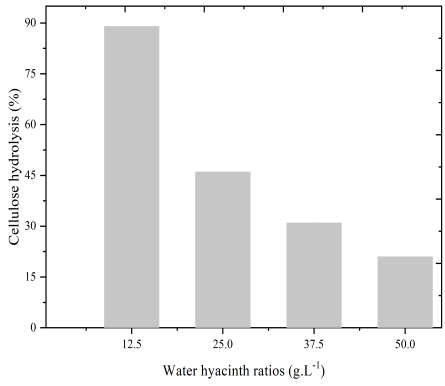


Figure 2. Cellulose hydrolysis at various ratios of water hyacinth pre-treated with distilled water at 100 °C
Source: Authors

When using a water hyacinth pre-treated solution ratio of 12.5 $g.L^{-1}$, the enzyme achieved up to 89% cellulose hydrolysis, whereas, at a ratio of 25.0 $g.L^{-1}$, the percentage significantly decreased to 46%.

This indicates that, during enzymatic catalysis, the enzyme and the substrate form a complex. Although a higher enzyme concentration increases the availability of molecules to bind to the substrate and catalyze the reaction, there might be instances where the substrate concentration is so high that it saturates all enzyme molecules, resulting in fewer available active sites. This enzyme depletion reduces the glucose release, which can be avoided by using a system based on enzyme load that relies on the available surface area instead of the cellulose mass. This approach would maintain a high enzyme concentration in the solution, preventing enzymatic depletion throughout the reaction and compensating for limited enzyme access to substrates beyond the complete coverage of adsorption sites [25], [26].

Acclimatization of the water kefir biomass

Fig. 3 depicts the biomass growth rate in g during the strain's adaptation to the culture medium. From the fourth to the 15th reseeding, the biomass stabilizes its growth at 23 g. Similarly, the ΔDO values stabilize at 0.14, the Δ°Brix at -2.03, and the ΔpH at -4.0. This demonstrates that the microorganisms adapt to the medium components after eight reseedings.

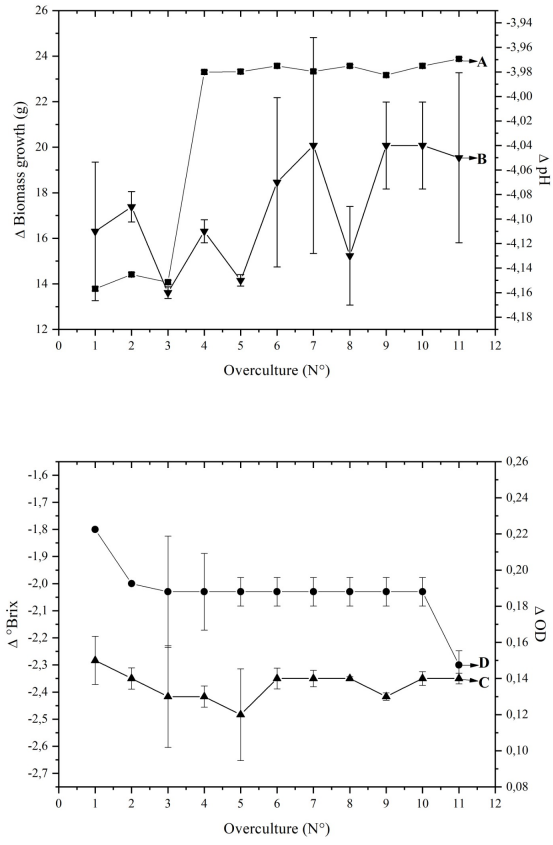


Figure 3. Biomass growth under the optimal conditions identified in phases 2 and 3. a) Δgrowth, b) ΔpH, c) ΔDO, d) Δ°Brix.
Source: Authors

Conducting the aforementioned process is crucial since it establishes the probable yield of water kefir grain production in their various stages of recultivation. This facilitates the utilization of substrates not preferred by the bacteria and aids in identifying the actual tolerance towards the adverse effects generated by fermentation by-products such as ethanol and acetic acid [27].

Evaluating the biomass growth of water kefir with water hyacinth hydrolysates

Fig. 4 illustrates the biomass growth of the water kefir tibicos. After 60 h of fermentation, among the assays without activated charcoal, there were no significant differences between conducting hydrolysis before or simultaneously with kefir fermentation, with a 300% increase concerning the initial biomass (~60 $mg.mL^{-1}$). However, notable changes were observed with the addition of activated charcoal, with a higher increase in the assay with preliminary hydrolysis, showing a 350% increase (70 $mg.mL^{-1}$).

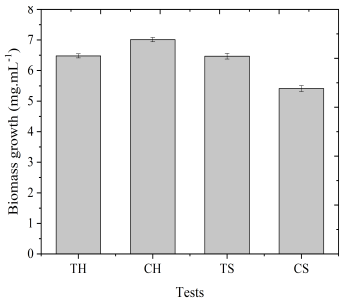


Figure 4. Growth of water kefir tibicos in different assays. TH: fermentation following hydrolysis. CH: fermentation following hydrolysis and the addition of activated charcoal. TS: simultaneous fermentation with hydrolysis. CS: simultaneous fermentation with hydrolysis and the addition of activated charcoal.
Source: Authors

Fig. 5 displays the behavior of reducing sugars over time. Overall, similar trends were observed, with an increase in values during the initial 12 h of the process, followed by a steady decrease until the 48 h mark was reached.

The assays with subsequent fermentation to hydrolysis exhibited an initial reducing sugars concentration of around 5 mg.mL^{-1}. The observed increase corresponds to the breakdown of the added sucrose, indicating that, within 12 h, there was a preliminary consumption of the available sugar, as the maximum initial total sugar (20 mg.mL^{-1}) was not achieved [13], [28], [29]. At the end of the process (60 h), the assays without and with the addition of activated charcoal showed a reduction of 61.82 and 66.93% in total sugars.

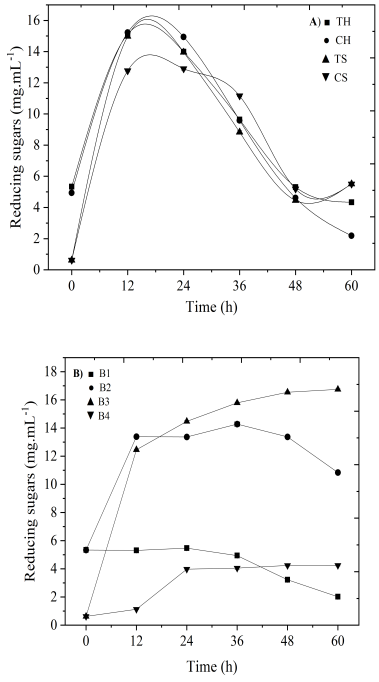


Figure 5. Behavior of reducing sugars vs. time in the assays (a) and the controls (b). TH: fermentation following hydrolysis. CH: fermentation following hydrolysis and the addition of activated charcoal. TS: simultaneous fermentation with hydrolysis. CS: Simultaneous fermentation with hydrolysis and the addition of activated charcoal.
Source: Authors

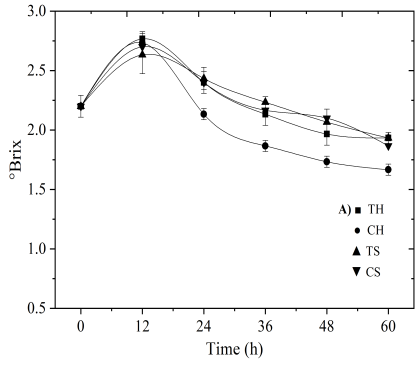
The assays with simultaneous fermentation and hydrolysis showed an increase in reducing sugars concentration at 12 h, but, by the end of the fermentation time, the levels decreased to 5.52 and 5.51 mg.mL^{-1}.

The °Brix values support the depletion of sugar. The microbial consortium metabolized the available reducing sugars to a lesser extent during the 36-60 h period.

On the other hand, control 1 (subsequent fermentation without sucrose) indicated a consumption of the hydrolysates produced before fermentation, as they decreased over time to about 60% due to the presence of water kefir tibicos. In addition, the slight increase in reducing sugars for control 3 (simultaneous fermentation without sucrose and water kefir tibicos) reflects the typical behavior of cellulase hydrolysis (Figs. 1 and 2), reaching a peak at 24 h.

Controls 2 (subsequent fermentation with sucrose and without water kefir tibicos) and 4 (simultaneous fermentation with sucrose, without water kefir tibicos or enzyme) showed an increase in reducing sugars, similar to that observed in the assays with water kefir tibicos. This increase could be due to the effects of the sterilization process (121 °C) on water hyacinth, which might have generated sufficient organic acids to react with the available sucrose, breaking its bonds into fructose and glucose.

Fig. 6 illustrates the behavior of dissolved solids (°Brix) over time. Overall, all four assays exhibited similar patterns, starting around 2.2 and finishing below 2. The initial increase in the first 12 h may be related to the non-sugar soluble compounds remaining in the water hyacinth, which leached out during this period. This can be corroborated by the values observed in the controls during the same period (Fig. 6). Nevertheless, the subsequent decrease indicates that the microbial consortium consumes sucrose, which metabolized glucose and fructose over a specific period from 24 to 48 h, as suggested by the data in the graph [29].



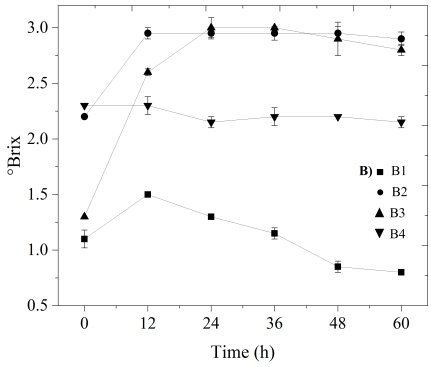


Figure 6. Suspended solids behavior over time in the assays (a) and the controls (b). TH: fermentation subsequent to hydrolysis. CH: fermentation subsequent to hydrolysis with the addition of activated charcoal. TS: simultaneous fermentation during hydrolysis. CS: simultaneous fermentation during hydrolysis with the addition of activated charcoal.
Source: Authors

Fig. 7 depicts the production of lactic acid, showing a continuous increasing trend throughout the process. By correlating this with the observations in Fig. 4, it could be stated that this parameter impacts the growth of the water kefir tibicos.

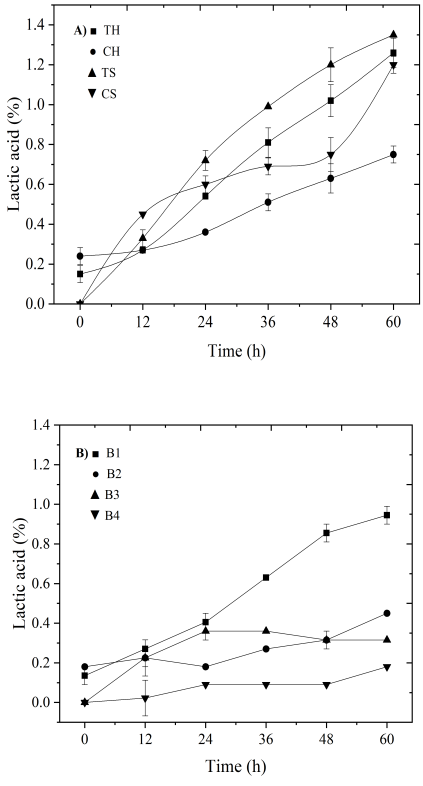


Figure 7. Behavior of produced lactic acid vs. time in the assays (a) and the controls (b). TH: Fermentation following hydrolysis. CH: fermentation following hydrolysis and the addition of activated charcoal. TS: simultaneous fermentation with hydrolysis. CS: simultaneous fermentation with hydrolysis and the addition of activated charcoal.
Source: Authors

This EPS is synthesized through the use of glucose by extracellular enzymes, *i.e.*, glucansucrases, whose optimal activity occurs at a pH range of 4.3 to 4.6. Their activity drastically diminishes when the pH decreases to 3.6-3.2, hindering the production of glucansucrases more than the enzymatic activity itself. When the growth of the biofilm declines, less glucose gets integrated into the matrix, increasing the availability of sugar for the production of acids by acetic acid bacteria and yeasts, thereby elevating the acid stress within the medium. Additionally, the low pH values may be associated with the limited growth of the biofilms [12], [30]–[33].

However, the slight increments in acidity in the controls imply a correlation with that shown in Fig. 3 regarding the hydrolysis of sucrose in the absence of biofilms. This can be correlated with processes observed in pyrolysis or hydrothermal treatments for this type of biomass, aiming to obtain chemical platforms. Although the operating conditions for the pre-cultivation sterilization of biofilms are lower in temperature (121 °C) when compared to those used in such treatments (200-700 °C), it is possible that 121 °C was sufficient to generate tiny amounts of organic acids (*e.g.*, lactic acid). These acids could have reacted with the available sucrose, cleaving its bonds into fructose and glucose [34], [35].

Fig. 8 illustrates the pH behavior over time. Overall, all assays had a decreasing trend, starting from a neutral pH and culminating in an acidic pH, confirming the acidification of the medium (Fig. 7). This correlates with the growth of microorganisms, as the controls in the absence of biofilms (B2, B3, and B4) maintained their values above 7.

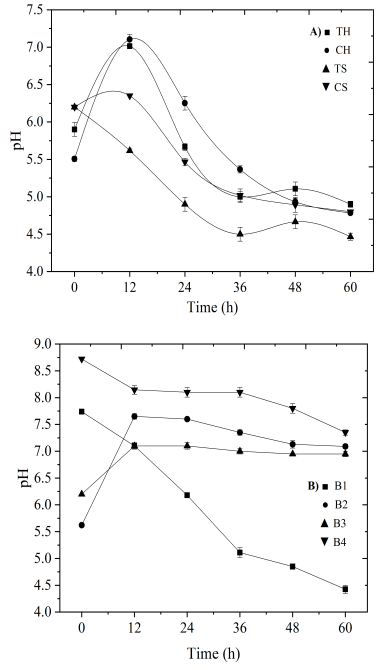


Figure 8. pH behavior over time in the assays (a) and the controls (B). TH: fermentation subsequent to hydrolysis. CH: fermentation subsequent to hydrolysis the addition of activated charcoal. TS: simultaneous fermentation during hydrolysis. CS: simultaneous fermentation during hydrolysis with the addition of activated charcoal.
Source: Authors

According to the aforementioned observations, the most significant biofilm growth occurred when utilizing the culture medium with activated charcoal-treated water hyacinth hydrolysate. Studies have shown that the addition of this compound promotes the growth of plants and microorganism populations due to its ability to adsorb inhibitory substances, growth regulators, or organic components [36]–[42]. Moreover, there is a possibility that activated charcoal may gradually release some of the adsorbed reagents. Thus, the organism would have a long-term source of the reagent, enhancing its response in the culture [36]–[42].

Physicochemical characterization of dextran from water kefir tibicos

Fig. 9 shows the FTIR spectra of the tibicos from the four tests with water hyacinth.

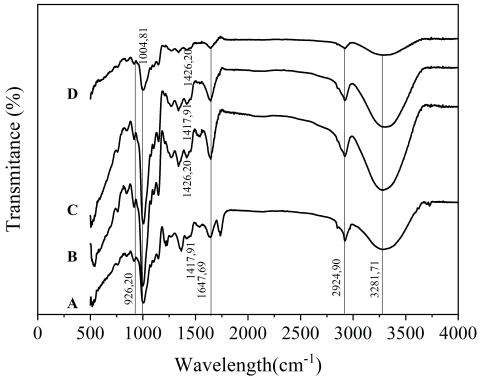


Figure 9. FTIR spectrum of dextran from water kefir tibicos: a) fermentation subsequent to hydrolysis, b) fermentation subsequent to hydrolysis with the addition of activated charcoal, c) simultaneous fermentation during hydrolysis, d) simultaneous fermentation during hydrolysis with the addition of activated charcoal.
Source: Authors

The analysis of the data revealed that the obtained polysaccharide corresponds to dextran, as the values assigned to each functional group are characteristic of it, coinciding with those reported in the literature. For example, peaks at 1640 and 1648 cm^{-1} indicate the stretching vibration in the C=O bond [43]. Polysaccharides contain a considerable number of hydroxyl groups (O-H), and the stretching vibrations of O-H usually appear as a broad and intense absorption peak between 3200 and 3600 cm^{-1} [44]. Therefore, the broad and intense band at 3274 cm^{-1} can be attributed to the stretching vibration of the O-H bond, characteristic of polysaccharides. The strong peaks at 2930.06 cm^{-1} are attributed to C-H stretching vibrations [45].

Hence, the bands at 2919 and 2904 cm^{-1} are associated with the stretching vibration of the C-H bond present in the isolated organic compound. Meanwhile, the bands between 1000 and 1416 cm^{-1} represent the symmetric stretching vibration band of the free -COO- bond [46].

The sharp peaks at 1108.48 cm^{-1} and 1147.75 cm^{-1} indicate a highly flexible chain around α-(1→6) linkages [47]. The absorption peaks at 908.67 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} is indicative of an α-glycosidic configuration [48]. The additional peaks at 754.02 and 746.49 cm^{-1} are due to the stretching of the C-C bond in the molecule [49]. The profile of the functional groups indicates that the spectra correspond to dextran [50].

Although the dextran isolated from the biofilms obtained in four different assays did not exhibit significant variations in its base structure, it is important to highlight that, visually, the addition of activated charcoal demonstrates a uniform incorporation of this material within the polymers, resulting in a black coloration, which remained consistent during the biofilm grinding and analysis. This could be relevant for the potential use of this material as an encapsulant or particle immobilizer.

The subsequent characterization's results relate to the water kefir biofilms produced after fermentation and hydrolysis. Fig. 10 presents a comparison between purified and unpurified water kefir biofilms. The biopolymer was not affected by the techniques used for isolation, as no significant differences were observed between the two spectra.

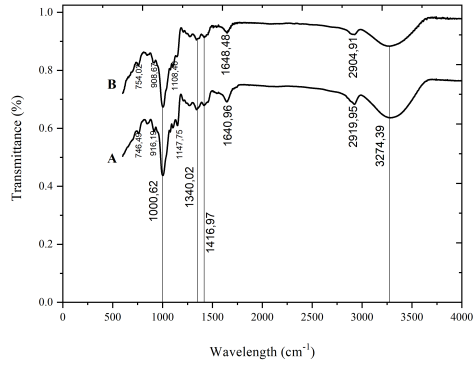


Figure 10. FTIR spectrum of dextran from water kefir tibicos: a) purified, b) unpurified
Source: Authors

According to the XRD profile observed in Fig. 11 for unpurified and purified polymers, the dextran extracted from water kefir biofilms exhibits an amorphous structure, as it lacks well-defined crystallinity peaks. This is characteristic of the polymeric nature of the exopolysaccharide, as observed in previous studies [51].

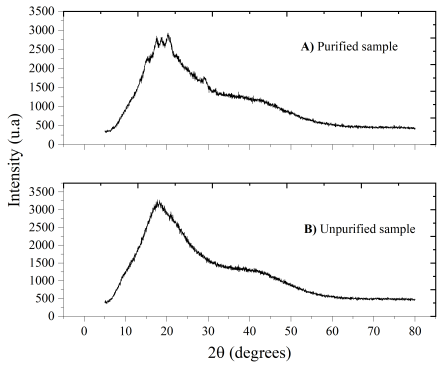


Figure 11. X-ray diffractogram of dextran samples from purified (a) and unpurified (b) water kefir
Source: Authors

However, the purification of the isolated dextran reveals the formation of more defined crystallinity peaks alongside the broad peak at 2θ°, in the range of 15-21°, which is consistent with that observed in the crystallinity profiles of commercial dextran samples and those from other sources, such as the strains of *Lactobacillus mali* CUPV271 *and* *Leuconostoc carnosum* CUPV411 [52], [53].

The DSC results (Fig. 12) show endothermic peaks at 295 and 288°C in the purified and unpurified samples. The temperature at which this glass transition occurs (Tg) is relatively high compared to other biopolymers, likely due to the presence of strong hydrogen bonds between the dextran macromolecules of water kefir. Previous studies have shown high Tg values for dextran produced by lactic acid bacteria, including *Leuconostoc pseudomesenteroides* (278.4 °C), which is part of the microbial consortium of interest [54]–[55].

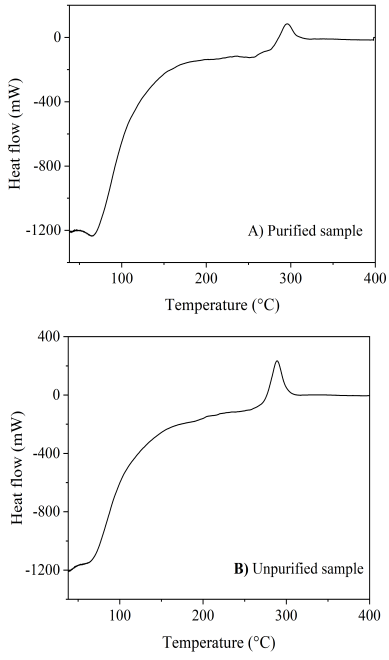


Figure 12. DSC of purified (a) and unpurified (b) water kefir dextran samples
Source: Authors

[55] displays the curves of TGA and their respective derivative. Both figures exhibit the same thermal behavior, showing two mass losses: one between 40 and 130 °C and a second one in the range of 250 to 350°C, with a peak at 295 °C for the purified biopolymer and at 288 °C for its unpurified counterpart. The initial mass loss is related to the decrease in free water, associated with the material's structures and the condensation of hydroxyl groups on the surface. The second mass loss has to do with the degradation of the polysaccharide structure, due to the depolymerization resulting from the breakdown of the C—C and C—O bonds present in the sugar and to the release of small molecules during this process [56], [57].

The observed variations between both samples relate to the presence of other components in the unpurified polymer. Regardless of this, the TGA results suggest that the biopolymer's structure is dextran [58].

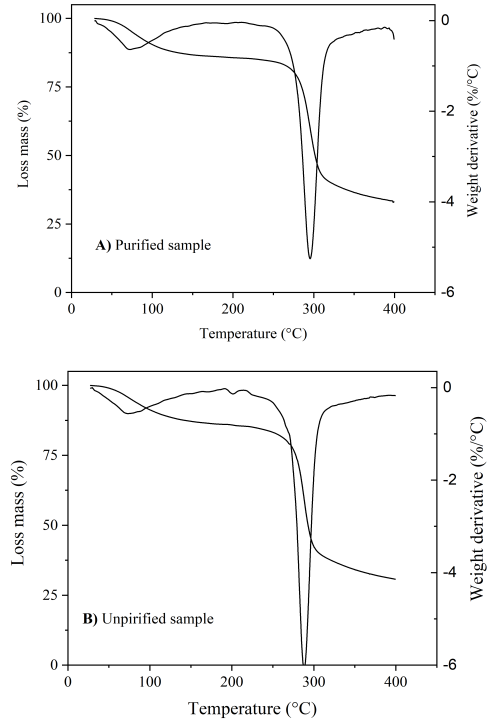


Figure 13. TGA and DSC of dextran samples from purified (a) and unpurified (b) water kefir
Source: Authors

The thermal stability observed in both the DSC and TGA supports the potential application of this dextran as a candidate for thermally processed formulations [53]–[55]. The high Tg, associated with strong hydrogen bonding between α-(1→6)-linked glucose units, indicates a compact and stable polysaccharide structure. Furthermore, the incorporation of activated charcoal suggests physical or chemical interactions with the polymer matrix. These interactions may contribute to enhanced homogeneity and thermal behavior, making the composite more suitable for encapsulation systems, scaffolds, or other biomedical applications requiring stability at moderate to high processing temperatures [58].

It is important to highlight the interesting thermal characteristics of the biopolymers obtained through both processes, given their potential in industrial applications, where processing temperatures reach approximately 150 °C. For example, in the pharmaceutical industry this includes autoclave sterilization (15 min at 121 °C), spray drying (<200 °C), granulation, and fluid bed drying (<200°C), among others [59].

Conclusions

The results obtained and discussed allow answering the research question: the conditions required for the water kefir microbial consortium to produce dextran through fermentation processes using substrates derived from the aquatic plant *E. crassipes* must consider that the alkaline pretreatment applied to water hyacinth does not enhance cellulase hydrolysis, indicating the avoidance of compounds like NaOH. Nevertheless, the amount of substrate used is indeed relevant, exhibiting a proportional relationship between substrate quantity and the percentage of cellulose hydrolysis achieved. The findings suggest that 1.25 g of water hyacinth in 100 mL of solution yield effective hydrolysis results, preventing high substrate concentrations from saturating enzyme molecules, which tends to reduce the glucose release. In summary, these findings have crucial implications for the development of more efficient and sustainable methods for enzymatic cellulose hydrolysis.

Additionally, while the production of EPS by the water kefir microbial consortium shows no significant changes regardless of whether it is performed before or during hydrolysis, the presence of other compounds during biofilm formation, such as activated charcoal, does suggest the that each process should be conducted independently. This helps to reduce the potential interference of byproducts from one process with the development of the other.

The effect of activated charcoal on biomass growth should also be highlighted. While its extrapolated ability to adsorb inhibitory substances, growth regulators, or organic components could promote microorganism growth, its inclusion in such cultures generates composite materials that capitalize on the properties of both components, resulting in a stable, water-insoluble material.

The dextran purification processes did not exhibit significant physical or chemical differences in the samples. Physicochemical characterization techniques such as FTIR, XRD, DSC, and TGA confirmed their chemical identity. This indicates robustness and stability in the polysaccharide molecule, even after processing, which is crucial in the fields of chemistry and biotechnology. Moreover, other components from the microorganisms involved in the formation of the biofilms were found in negligible quantities, implying potential applications without purification in some processes.

The foregoing validates the alternative hypothesis: the fermentation of a culture medium with *E. crassipes* hydrolysates obtained from sequential and simultaneous pretreatments with cellulase and the inclusion of activated charcoal suspensions (using sucrose as a cosubstrate) has an effect on the formation of kefir tibicos.

The results obtained in this research allow for some recommendations concerning future work and/or perspectives when it comes to obtaining biopolymers via the microbial fermentation of plant biomass. The raw material composition (*i.e.*, the percentage of cellulose, hemicellulose, and lignin) could be analyzed in different sources before general sampling, in order to select the biomass with the highest quantity of the desired component—in this case, cellulose—and identify enzyme activity while considering a broader range of variables such as pH, substrate quantity, and enzyme quantity.

As a future perspective, the utilization of plant biomass for the production of biopolymers with promising industrial applications is intriguing, as it would allow reducing of the environmental impacts of the invasive growth of some plant species.

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CRediT author statement

Author 1 conceived the idea and was entrusted with background research, data curation, and formal analysis. *Authors 2* and *3* were in charge of funding acquisition, resources, supervision, and validation. *Author 2* was in charge of project administration. All authors contributed to the investigation process, the methodology, and the original draft.

Access to research data

The datasets generated and/or analyzed during this study are available from the authors upon reasonable request.

Conflicts of interest

The authors declare that they have no conflict of interest.

Statement on artificial intelligence

The authors did not use IAG. The authors take full responsibility for the contents of this publication.

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