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Artículo de investigación

Metabolic dynamics of bacterial strains in *eichhornia crassipes* hydrolysate: a comparative study of growth and lactic acid production

Dinámica metabólica de cepas bacterianas en el hidrolizado de *eichhornia crassipes*: estudio comparativo del crecimiento y la producción de ácido lácticoO

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Resumen

En la presente investigación, se comparan las condiciones de crecimiento en un proceso de fermentación a 35 °C, 180 rpm durante 60 h, de cuatro cepas de bacterias comerciales DRI SET 432, DRI FAS 992, *Bacillus subtilis*, y Kéfir de agua en dos medios de crecimiento (M1 medio de crecimiento base y M2 medio suplementado con hidrolizado de *E. crassipes*). La respuesta metabólica de las cepas en los medios se monitoreó y evaluó mediante la producción de biomasa por el método turbidimétrico de la escala de Mc Farland (células.mL^{-1}), consumo de azúcares (g.L^{-1}) y producción de ácido láctico (%), cada 12 h. Las cepas evaluadas presentaron su fase exponencial a las 12 h, en M1 y M2, encontrando una disminución en la producción de biomasa y ácido láctico, en los procesos de fermentación con M2 para DRI SET 432, DRI FAS 992 y kéfir de agua, y un mayor crecimiento con *Bacillus subtilis* (32 x 10^8 células/mL) a las 60 h. Por otra parte, el mayor rendimiento de ácido láctico se presentó con la cepa FAS 992 (*estreptococos salivarius sub. thermophilus*) con 1.390 g de ácido láctico/ g de sustrato consumido en M1 y en el Kéfir de agua con 0.753 g de ácido láctico/ g de sustrato consumido para M2.

Palabras Clave: bacterias ácido lácticas, *Bacillus sp.*, Eichhornia crassipes, hidrólisis ácida, pretratamiento alcalino.

Abstract

In the present investigation, the growth conditions in a fermentation process at 35 °C, 180 rpm for 60 h, of four strains of commercial bacteria DRI SET 432, DRI FAS 992, *Bacillus subtilis*, and water kefir were compared in two growth media (M1 base growth medium and M2 medium supplemented with *E. crassipes* hydrolysate). The metabolic response of the strains in the media was monitored and evaluated by means of biomass production by the Mc Farland turbidimetric method (cells.mL^{-1}), sugar consumption (g.L^{-1}) DNS method and lactic acid production (%) NTC 4978, these controls were performed every 12 h. The strains evaluated presented their growth phase in the growth media. The strains evaluated presented their exponential phase at 12 h in M1 and M2. We found a decrease in biomass and lactic acid production in the fermentation processes with M2 for DRI SET 432, DRI FAS 992 and water kefir, and a greater growth with *Bacillus subtilis* (32 x 10^8 cells/mL) at 60 h. On the other hand, the highest lactic acid yield was presented with strain FAS 992 (*streptococcus salivarius sub. thermophilus*) with 1.390 g lactic acid/ g substrate consumed in M1 and in Water kefir with 0.753 g lactic acid/ g substrate consumed for M2.

Keywords: acid hydrolysis, alkaline pretreatment, *Bacillus sp.*, *Eichhornia crassipes*, lactic acid bacteria.

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1 Introduction

Among the alternatives for harnessing energy resources from aquatic plant biomass, such as water hyacinth (*Eichhornia crassipes*), are acid hydrolysis processes, which yield high-value products like sugars and organic acids. This process occurs when H_2SO_4 breaks the polysaccharide bonds (cellulose and hemicellulose), releasing fermentable sugars and other compounds resulting from polysaccharide degradation. These hydrolysates are of significant importance in the production of biofuels, chemicals, and biodegradable materials, as they provide raw materials with various industrial applications [1, 2].

However, prior to this process, it is necessary to perform delignification or removal of undesirable compounds through alkaline methods, which promote the separation of cellulose from the rest of the material, primarily lignin. This enhances the yields of products such as cellulose and hemicellulose [3].

The biomass availability of the species *Eichhornia crassipes* in the department of Boyacá is widely distributed in locations such as the Playa reservoir in the municipality of Tuta, the north and south cooling pools of the 'Termopaipa' thermoelectric plant in Paipa, and the Palagua wetland in Puerto Boyacá [4]. This invasive species can disrupt the biological processes of water sources, leading to eutrophication and water contamination. Additionally, studies have reported the use of other aquatic plants in lactic acid production, such as sugars derived from the microalga *Nannochloropsis salina*, mediated by *Lactobacillus pentosus* (ATCC-8041), achieving a yield of 0.45 $g{\cdot}L^{-1}$/h for the microalgal extract [5, 6].

Due to the limited information on acid hydrolysis processes and the production of valuable products from aquatic plant hydrolysates, specifically from water hyacinth (*E. crassipes)*, this research focuses on evaluating the growth of consortia of commercially available microbial strains, such as *Bacillus subtilis* (an aerobic bacterium that does not belong to the lactic acid bacteria (LAB) group but has industrial applications), lactic acid bacteria (LAB) (a diverse group of facultative anaerobes primarily used in food fermentation), and water kefir (a fermented beverage resulting from the interaction of yeasts and LAB, promoted as a probiotic food). The evaluation is carried out through a fermentation process using basic and supplemented culture media with acid hydrolysates from *E. crassipes* biomass, pretreated with NaOH, aiming to obtain key metabolites for industry, such as lactic acid, among others [5, 6].

The objective is to exploit the lignocellulosic resource represented by E. crassipes biomass and thus provide a solution to the in-situ degradation of this aquatic plant in water sources, which negatively impacts ecosystems.

2 Materials y methods

2.1 Sampling and Preparation of Water Hyacinth (*Eichhornia crassipes*)

The biomass collection of water hyacinth (E. crassipes) was conducted at the northern cooling ponds of the "Termopaipa" thermoelectric plant, located in the municipality of Paipa, Boyacá (N 5°46'8.3" W 73°08'40.3", 42 km from Tunja, the capital of the department). The drying process of the samples was performed at room temperature. After this stage, the particle size of the material was reduced using a High-speed Multifunction Grinder HC-150, followed by sieving with a Tyler #30 sieve, obtaining a final particle size of 0.5 mm (500 μm). To ensure the sample was representative of the batch, successive quartering was carried out to minimize any bias in the selection of the final sample. The percentage composition of E. crassipes was determined by applying the following analytical methods, with each analysis performed in triplicate. Extractives were determined using the TAPPI T 204 os-46 method [7]; while α-cellulose, holocellulose, hemicellulose, and lignin were assessed following the TAPPI T202 standard [8]; Moisture and ash content were measured according to AOAC methods [9].

The samples selected for acid hydrolysis underwent an alkaline pretreatment using a 2 % w/v NaOH solution at a temperature of 80 °C. This pretreatment was carried out in a batch reactor with agitation, with a liquid capacity of 3 L. A ratio of 1:20 (*Eichhornia crassipes*: NaOH solution) was employed, and the process lasted for 1 hour. This procedure was repeated until a total of 500 g of biomass was processed.

Once the alkaline pretreatment process was completed, the samples were filtered, and successive washes with water were performed on the wet material. This was done to remove any excess sodium hydroxide present in the samples [7]. Subsequently, the cellulose purity percentage was calculated using Eq. (1), taking into account the initial weight, final weight, and the initial percentage of cellulose in the sample.

$$\%purity = \frac{final\ weight * \%cellulose\ in\ the\ sample}{inicial\ weight} * 100 \quad (1)$$

2.2 Acid Hydrolysis of Water Hyacinth (*Eichhornia crassipes*) Cellulose

Once the alkaline pretreatment stage was completed, acid hydrolysis of the cellulose was carried out to obtain fermentable sugars. A 250 mL Erlenmeyer flask with an effective volume of 100 mL was used in an autoclave, employing a 1:15 ratio of E. crassipes to 2% w/v H_2SO_4. The hydrolysis was performed at 121°C for 30 minutes. The liquid fraction (containing the reducing sugars) was recovered using a vacuum pump, washing the wet solid fraction to remove excess trapped sugars. The wet biomass was dried at 105°C. The sugar concentration was determined using the DNS method for reducing sugars [10]. The efficiency of the acid hydrolysis process for converting cellulose into fermentable sugars was calculated using Eq. (2), considering the concentration of fermentable sugars (AF), the volume used in the hydrolysis process (VSLH), and the percentage of cellulose purity.

$$EH\% = \frac{AF * VSLH}{\%purity} * 100 \quad (2)$$

2.3 Purification of Cellulose Hydrolysates from Water Hyacinth (*Eichhornia crassipes*)

To carry out the purification or clarification process of the cellulose hydrolysates, activated carbon was used at a concen-

tration of 2% (w/v). The mixture of hydrolysate and activated carbon was kept in contact for 30 minutes, allowing sufficient time for the activated carbon to adsorb and purify the microbial growth inhibitors present in the solution. The liquid fraction was recovered through vacuum filtration. [11, 12].

2.4 Microorganisms preparation

The following commercial strains were used: lactic acid bacteria DRI SET 432 (*Streptococcus salivarius subsp. thermophilus, Lactobacillus delbrueckii subsp. bulgaricus*), DRI FAS 992 (*Streptococcus salivarius subsp. thermophilus*) from the VIVOLAC brand, *Bacillus subtilis*, and water kefir. The culture media for the strains were prepared according to the nutritional requirements of each microorganism. The culture medium containing all the necessary nutrients for each microorganism will be referred to as M1, while the culture medium supplemented with water hyacinth hydrolysate (*E. crassipes*) will be designated as M2.

For the lactic acid bacteria, a culture medium with the characteristics of MRS broth (De Man Rogosa Sharpe Medium) was used, with the following composition: dextrose (20 $g.L^{-1}$), tryptone (5 $g.L^{-1}$), sodium acetate (5 $g.L^{-1}$ $C_2H_3NaO_2$), dipotassium phosphate (2 $g.L^{-1}$ K_2HPO_4), magnesium sulfate (0.05 $g.L^{-1}$ $MgSO_4$), manganese sulfate (0.2 $g.^{-1}$ $MnSO_4$), B-complex vitamins (28.9 $mg.L^{-1}$), and yeast extract (5.0 $g.L^{-1}$). Since water kefir is a community of bacteria and yeasts, including those from the lactic acid bacteria group, the same culture medium was used, except for sodium acetate. In the MRS medium, sodium acetate regulates pH and consequently inhibits the growth of other microorganisms, such as yeasts. For this reason, it was excluded from the water kefir culture [13, 14].

For the growth of the *Bacillus subtilis* strain, a medium was used that ensured a source of carbon and nitrogen, in addition to salts that supported the strain's metabolic functions [2, 3] as well as microelements and salts present in the lactic acid bacteria medium supplemented with (1.2 $g.L^{-1}$ $(NH_4)_2SO_4$) [15]. Each culture medium underwent sterilization in an autoclave at 121°C for 15 minutes. Additionally, a pre-adaptation process was carried out for each microorganism in both culture media (M1) and (M2). The adaptation phase was considered complete when no variations in substrate consumption were observed, as measured by the DNS method for reducing sugars, across replicates for both M1 and M2 media.

2.4.1 Fermentation process in culture media (M1)

After the adaptation process, each strain was inoculated in sterile conditions into the M1 culture media at a concentration of 10% v/v. The fermentation process was carried out in an orbital shaker at 35°C and 180 rpm for 60 hours. Parameters such as substrate consumption, using the DNS method for reducing sugars[10], biomass growth through turbidimetric analysis [16-18] and lactic acid quantification (NTC 4978) [19] were measured.

2.4.2 Turbidity analysis

The spore concentration was determined using the Mc Farland turbidimetric method [18] which measures the growth of a microorganism suspension through a standard, considering that absorbance is directly proportional to the spores/mL present. A photometer calibration curve was used for each assay, employing theoretical cell concentrations ranging from 1.5 to 30 x 10^8 $spores.mL^{-1}$; (spores/mL = (18.138 × absorbance +1.2212) x dilution factor x 10^8; R^2 = 0.9957). For this analysis, distilled water was initially established as the standard. During the inoculation stage, the samples were collected in triplicate by taking 0.5 g of koji corn, which were dissolved in 5 mL of distilled water. Subsequently, the sample was vortexed for 1 minute and then it was left to decant for 15 minutes. The suspensions obtained were analyzed using a Hanna Instruments HI 801 iris photometer based on the absorbance at the selected optical density. Eq. (3) was used to determine spores in the solid phase growth (X, $spores.g^{-1}$), where S are the spores/mL in suspension, VD is the volume of distilled water used (mL), MD are the grams of sample used in the dilution (g), and H is the humidity of microbial strains.

$$X = \frac{S * V_D}{M_D} \tag{3}$$

2.5 Fermentation trials with hydrolyzed Water Hyacinth (Eichhornia crassipes) medium (M2)

The composition of the MRS culture medium described in Section 2.4 (M1 medium) was followed for each of the previously mentioned strains, replacing the carbon source with the hydrolyzed E. crassipes. The carbon source was adjusted to 20 $g.L^{-1}$ with dextrose, and the procedure outlined in Sections 2.4.1 and 2.4.2 was followed accordingly.

2.6 HPLC analysis

The organic acids extracted from the fermentation process of each microbial strain were separated using a reverse-phase column. For the analysis, 20 µL of the prepared sample was injected into a Kanauer Azura liquid chromatograph equipped with a UV-visible detector with a xenon lamp and refractive index detection. The samples were analyzed using a Nova-Pak Waters C-18 column, operated at 30°C. The mobile phase (flow rate 0.5 $mL.min^{-1}$) consisted of distilled water acidified to pH 2.24 with 0.5% w/v $(NH_4)_2HPO_4$ and phosphoric acid. The mobile phase was filtered, ultrasonicated, and degassed before use. The eluted compounds were detected at 214 nm. The optimal pH obtained was 2.24 at 30°C.

3 Results and Discussion

3.1 Characterization of Water Hyacinth (*Eichhornia crassipes*

Table 1 presents the percentage composition of hemicellulose, lignin, moisture, ash, and extractable compounds (surface tannins, resins, and other impurities) in the leaves and stems of water hyacinth (*E. crassipes*) relative to the percentage of cellulose.

Table 1. Physicochemical composition of Water Hyacinth (*Eichhornia crassipes*)

Parameter	Percent (%)
Moisture	7.79 ± 0.120
Ashes	7.87 ± 0.002
Cellulose	23.73 ± 0.120
Hemicellulose	44.46 ± 0.750
Lignin	14.35 ± 0.870
Extractables	2.39 ± 0.148

Following alkaline pretreatment in the reactor, the cellulose content increased to 62.15 %, indicating partial removal of lignin. This removal reduces the protective barrier around polysaccharides, including cellulose and hemicellulose, present in water hyacinth (*E. crassipes*) biomass. This resulted in the purification of these components and facilitated depolymerization, leading to the formation of reducing sugars through acid hydrolysis [20].

The cellulose content approaches the values reported by authors such as Ospino et al., [22] who recorded a value of 19.61%; and Tejada Tovar et al, [21] who reported values ranging from 24.71 ± 0.72% to 26.94 ± 0.62% in leaves and stems, respectively. Additionally, they found a hemicellulose content of 20.42 ± 0.87% and a lignin content of 12.91 ± 0.49 % w/v. Reales et al. reported lignin contents of 28.8% [11].

The results confirmed the lignocellulosic material content comparable to that reported in other studies [23]. For instance, corn waste was found to contain 29.1% cellulose, 39.7% hemicellulose, and 11.4% lignin. In the specific case of water hyacinth (*E. crassipes*), cellulose, hemicellulose, and lignin contents were reported as 34.6%, 29.3%, and 21.4%, respectively Gao et al. [24], Brundu et al. [25], e Istirokhatun et al. [26].

It was found that this water hyacinth (*E. crassipes*) has a high content of cellulose and hemicellulose, with low lignin content; these results can be compared to those reported by Sornvoraweat et al., [27] and Ma et al., [28] in 2010, where the cellulose, hemicellulose, and lignin values were lower. However, more recent results, such as those reported by Teixeira, et al., [29] indicate elevated levels of cellulose and lignin alongside low hemicellulose content. This variation may be attributed to factors such as the pretreatment applied to the water hyacinth biomass (*E. crassipes*) when determining its composition. Nonetheless, the high levels of cellulose and hemicellulose facilitate the extraction of greater concentrations of fermentable sugars, such as glucose or xylose, which can be converted into lactic acid or other organic acids through microbial action [29].

3.2 Acid Hydrolysis of Water Hyacinth (*(Eichhornia crassipes)*

A concentration of fermentable sugars of 30.940 ± 0.447 $g{\cdot}L^{-1}$ was achieved, representing a hydrolysis efficiency of 74.69 % in converting cellulose to fermentable sugars. Efficient fermentable sugar production from cellulose requires careful control of variables including temperature, reaction time, pretreatment type, and lignocellulosic biomass particle size. In this study, these factors were crucial for the acid hydrolysis of water hyacinth (*E. crassipes*), favoring the production of reducing sugars like glucose. An alkaline pretreatment was used, as several studies have employed alkali solutions such as sodium hydroxide or ammonia at different concentrations (5 % or 10 % w/w), with reaction times ranging from 30 to 60 minutes. This process ensures the safety of the biomass, which can later be neutralized with water. However, despite not generating toxic by-products, its efficiency, compared to other pretreatment methods, is lower, yielding between 50 and 70 % w/w in the case of *E. crassipes* [3].

In this study, the cellulose content increased to 62.15 %. The low productivity may stem from the alkaline substance causing biomass swelling, which does not necessarily promote hydrolysis or significantly alter the chemical composition of *E. crassipes* biomass. This effect primarily impacts lignin, with minimal influence on hemicellulose or cellulose. Specifically, treatment with 2% NaOH causes swelling that increases the internal surface area of the biomass. As a result, the degree of polymerization and crystallinity rise, as well as the separation of structural bonds between lignin and carbohydrates, and the breakdown of the lignin structure [33, 34]. This explains why working with low NaOH concentrations (2 % w/v) in this study achieved such delignification yields or cellulose recovery after the alkaline process.

However, research indicates that increasing the temperature and reaction time, or using a strong acid, can lead to the formation of degradation products from glucose, which ultimately suppresses the concentration of reducing sugars [20, 35].

Through acid hydrolysis, the total available fermentable sugar can reach 70% to 90% or higher of the theoretical value. It is reported that sugar recovery is the main advantage of this process, for both xylose and glucose [36]. Diluted acid hydrolysis requires temperatures between 120°C and 200°C and pressures ranging from 1.0 atm to 5.0 atm, with reaction times between 30 minutes to 2 hours in continuous processes [37]. Concentrated acid processes can successfully yield such sugar levels when using 60% sulfuric acid under mild temperatures and moderate pressures [36]. However, preliminary research on cellulose hydrolysis into fermentable sugars has reported conversion rates of up to 24 and 29.09 % at 120 °C with a reaction time of 30 minutes and 1 % v/v of diluted acid, or 140°C with a reaction time of 3 minutes, respectively [38]. Jongmeesuk et al., [39] proposed the following three operating conditions: 1). 10 g of *E. crassipes* sample treated with 2 % H_2SO_4, autoclaved at 121°C for 15 minutes, followed by filtration and drying at 60°C for 48 hours, yielding 15.6 $g{\cdot}L^{-1}$ of reducing sugars. 2). 10 g of *E. crassipes* sample treated with 2% NaOH, autoclaved at 121°C for 15 minutes, followed by filtration and drying at 60°C for 48 hours, yielding 2.4 $g{\cdot}LL^{-1}$ of reducing sugars. 3). 2.0 g of pretreated *E. crassipes* sample with 2 % H_2SO_4, subjected to 0.3 mL of cellulase per gram of sample, incubated in a water bath at 50°C for 48 hours, and centrifuged at 1800 rpm for 15 minutes, yielding 11.9 $g{\cdot}L^{-1}$ of reducing sugars.

In this study, a similar procedure was carried out using a 1:15 ratio of *E. crassipes* and 2% w/v H_2SO_4 in a 100 mL solution. Under these conditions, a significant conversion of cellulose

into reducing sugars was achieved, reaching up to 74.69 %, while operating at 120°C for 30 minutes. This improvement in conversion is likely attributed to the implementation of physicochemical pretreatment on the *E. crassipes* sample. It has been previously observed that reducing the particle size of this biomass through mechanical grinding can alter cellulose crystallinity, reduce its degree of polymerization, and increase its specific surface area. This process enhances access to the chemical bonds within cellulose, promoting more effective acid hydrolysis and leading to a more efficient release of reducing sugars. However, it is important to note that this approach involves high energy consumption and, in many cases, faces limitations in the efficient removal of lignin. In fact, due to its limited capacity to eliminate lignin, the applicability of this method is restricted in certain cases [40, 41].

On the other hand, the use of sodium hydroxide also deserves attention, as its application has been extensively researched over time. It has been demonstrated that this compound can alter the lignin structure in lignocellulosic biomass, which subsequently increases the accessibility of homogeneous solvents such as H_2SO_4 or enzymes to the cellulose and hemicellulose present in the sample [42]. However, the achievement obtained in this study regarding the recovery of reducing sugars from water hyacinth (Eichhornia crassipes) is noteworthy, especially when compared to yields reported in predominant chemical processes as well as in biological approaches involving fungi, bacteria, or enzymes acting on the cellulose from the same biomass. These alternative methods, which have been widely explored, have consistently shown lower results, with values around 32 % in terms of reducing sugar recovery [43].

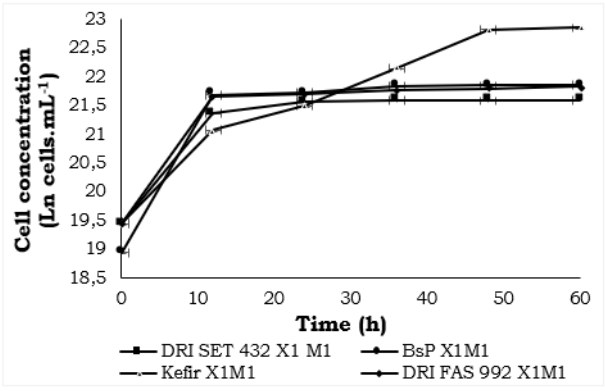
3.3 Fermentation Process and Microbial Biomass Growth

Figure 1 illustrates the microbial biomass growth of the evaluated strains in growth media M1 and M2 over time.

Figure 1a illustrates the biomass growth in M1 for the strains DRI SET 432, DRI FAS 992, water kefir, and Bacillus subtilis. It shows the absence of a lag phase, followed by the exponential phase at 12 hours. The Bacillus subtilis strain reached a cell concentration of 25×10^8 cells.mL^{-1}.; the DRI SET 432 and DRI FAS 992 strains exhibited cell concentrations of 19×10^8 cells.mL^{-1} each, while the water kefir strain recorded the lowest growth, with a cell concentration of 14×10^8 cells.mL^{-1}.

Conversely, the DRI FAS 992 and Bacillus subtilis strains continue in the growth phase, significantly decreasing their cell generation rate, reaching cell concentrations of up to 30×10^8 cells.mL^{-1} and 31×10^8 cells.mL^{-1} for Bacillus subtilis and DRI FAS 992, respectively. The DRI SET 432 strain seemingly entered the stationary phase at 48 hours into the process; however, it exhibited an increase in biomass, achieving a final concentration of 24×108 cells.mL^{-1}. In contrast, the water kefir behavior shows a continuous increase over time without evidence of a stationary phase, ultimately achieving the highest biomass concentration among the evaluated strains, reaching up to 84×108 cells.mL^{-1}.

Similarly, Figure 1b presents the cellular development response of the strains in the medium supplemented with the hydrolysate of water hyacinth (*E. crassipes*) (M2). A behavior similar to that observed with M1 is noted for the DRI SET 432, DRI FAS 992, water kefir, and Bacillus subtilis strains, with a biomass decrease observed at 60 hours of the process compared to the trials conducted in M1. The most significant difference in cell concentration occurred with the water kefir strain, which reached 28×10^8 cells.mL^{-1} by the end of the process. In contrast to previous cases, the Bacillus subtilis strain demonstrated a greater affinity for M2, achieving a concentration of 32×10^8 cells.mL^{-1} at 60 hours.



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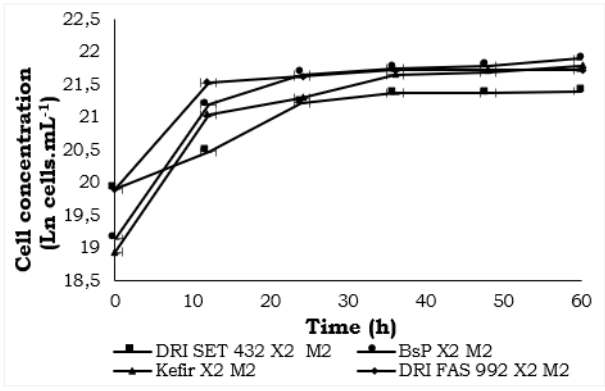


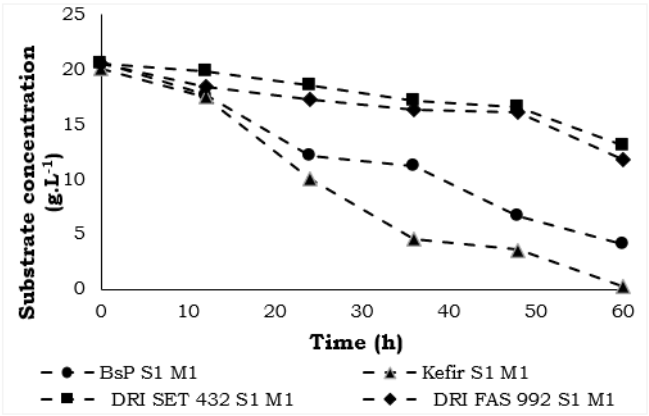
b.

Figure 1: (a) Cell concentration vs. Time under the evaluated growth media M1 and M2. (b) Cell concentration vs. Time under the evaluated growth media M1 and M2.

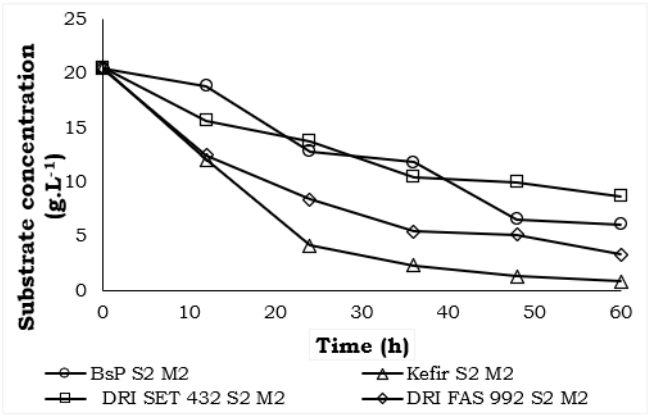
On the other hand, Figure 2 illustrates substrate consumption in the growth media M1 and M2 over time.

In Figure 2a, the BAL strains in M1 exhibited slow substrate consumption until 48 hours, followed by an increase in the consumption rate from 48 to 60 hours, possibly associated with the onset of a new exponential growth phase of the bacteria. However, these strains utilized less than 50 % of the carbon source throughout the process, a behavior linked to the low biomass productivity. In the case of *Bacillus subtilis*, more than 80 % of the initial substrate was consumed.

In Figure 2b, the assays conducted with M2 show that the DRI SET 432 and DRI FAS 992 strains exhibited greater sub-



a.



b.

Figure 2: (Substrate concentration (g.L^{-1}) vs. Time (h) during the fermentation process in M1. (b) Substrate concentration (g.L^{-1}) vs. Time (h) during the fermentation process in M2.

strate affinity compared to M1; however, this consumption does not directly correlate with biomass productivity. The consumption trends for the *Bacillus subtilis* strains and the water kefir were similar to those observed in M1.

Additionally, Table 2 presents the biomass yields and lactic acid production in the fermentation media M1 and M2. Overall, microorganisms cultivated in the experiments conducted with M1 demonstrated higher yields of biomass and lactic acid, except for the *Bacillus subtilis* strain, which exhibited greater yield for both the acid product and biomass in the assays with M2.

Table 2. Biomass and lactic acid yields for each evaluated strain in M1 and M2.

Strain	$Y_{(X/S)}$ $1x10^8$ cell.mL^{-1}/g.L^{-1} M1	$Y_{(P/S)}$ M1	Lactic acid g.L^{-1} M1	$Y_{(X/S)}$ $1x10^8$ cell.mL^{-1}/g.L^{-1} M2	$Y_{(P/S)}$ M2	Lactic acid g.L^{-1} M2
Bacillus subtilis	1.80	0.12	2.07	2.11	0.14	2.08
Water Kefir	4.12	0.75	16.57	1.37	0.75	15.27
DRI SET 432	2.86	1.02	7.61	1.29	0.65	7.81
DRI FAS 992	2.93	1.39	12.94	1.33	0.73	12.62

Analyzing the growth evolution and substrate consumption of different microbial strains in the culture media M1 and M2 over 60 hours reveals that, after 12 hours, discernible differences in growth patterns (Figure 1) and substrate consumption (Figure 2) between both media are absent. For all strains, substrate consumption begins to decline after 12 hours, leading to an increase in cellular concentration. This similarity in growth and substrate consumption can be attributed to the well-known influence of available nutrients in the culture medium, particularly carbon and nitrogen, as well as favorable environmental conditions that stimulate or inhibit the metabolism and development of microorganisms. However, it is common for the concentration of the carbon source to act as a limiting factor in these processes.

During the growth of the strains in M1 and M2, it is generally observed that the strains share similarities in the stages of cellular development, at least up to 24 hours, but differ in the final biomass concentration up to 60 hours. By this time, Water Kefir, for example, tends to grow more (84×10^8 cell.mL^{-1} in M1 and 30×10^8 cell.mL^{-1} in M2) while consuming more reducing sugars (0.24 g.L^{-1} in M1 and 0.81 g.L^{-1} in M2) in each culture medium [44].

The exponential phase in growth and substrate consumption for the Water Kefir strain in M1 can be explained by findings from authors such as López et al. [14] in 2017, who reported similar yields, achieving a biomass increase of up to 74.6% relative to the initial value. This behavior attributes to the fact that during fermentation, the Water Kefir tibicos form dextran (a polysaccharide composed of D-glucose units) with the sugars present in the medium, which explains the growth and consumption of Water Kefir due to the availability of glucose in M1, facilitating biomass production up to 60 hours [14]. Furthermore, this strain's ability, along with the yeasts in the culture medium, to ferment various sugary liquids allows it to utilize sugars for the production of lactic acid, acetic acid, ethanol, carbon dioxide, or other secondary products [45].

The biomass growth of the DRI SET 432 and DRI FAS 992 strains in M1 and M2 reaches the exponential phase at 12 hours into the process, consistent with the findings reported by Gámez et al., [39]. Similarly, Montes et at., [46] indicated that in a fermentation lasting 12 hours using a medium based on molasses and powdered milk with *Lactobacillus casei subsp. rhamnosus* culture, the maximum growth peak of the bacteria occurred at 11 hours, establishing the harvesting time with a biomass of 9.3×10^{11} CFU.mL^{-1}. Finally, it is essential to consider that the fermentation curve depends on factors such as the type of strain, timing and evaluation periods, medium, and cultivation conditions. In addition to the nutritional requirements of lactic acid bacteria (LAB), temperature serves as a limiting factor in their metabolism. This is supported by Serna et al., [47] who reported optimal growth for *Streptococcus salivarius* at 10 hours at 32 °C, a temperature very close to that established in the present study. Although this temperature is 2 °C higher, the strain still achieves its exponential phase at 12 hours.

The biomass growth in M1 and M2 for the DRI SET 432 and DRI FAS 992 strains is comparable to product yields of up to

0.83 g/g reported for *Streptococcus salivarius* [47]. Additionally, according to Jurado-Gámez et al., [48] and Ramirez et al., [49] lactic acid bacteria (LAB) require sugars such as lactose and glucose, along with amino acids, vitamins, and other growth factors for their multiplication. However, milk serves as a special and satisfactory medium for the proliferation of this type of bacteria, while most species also require several amino acids and B vitamins (riboflavin, thiamine, biotin, nicotinic acid, pantothenic acid, folic acid) [50].

The substrate consumption by the DRI SET 432 and DRI FAS 992 strains in M1 and M2 begins after 10 hours of the process. At 60 hours in M1, DRI FAS 992 shows the lowest consumption among all strains (11.73 $g.L^{-1}$), followed by DRI SET 432 (13.06 $g.L^{-1}$). Notably, in the LAB strains (DRI SET 432 and DRI FAS 992) and water kefir, during the fermentation process with M2, substrate consumption exceeds 80 %, yet results in low microbial biomass productivity compared to M1. This behavior may be attributed to the presence of cell growth inhibitors generated during the acid hydrolysis of lignocellulosic material, such as phenolic compounds, acetic acid, hydroxymethylfurfural, and furfural. Chandler et al., [35] indicate the production of these species during acid hydrolysis treatments of cellulosic materials, where good yields of lactic acid were obtained with LAB strains; Laopaiboon et al., [51] reported 10.85 $g.L^{-1}$ of lactic acid.

This may also be attributed to the fact that when the microorganisms in water kefir consume glucose and decompose it through fermentation processes, this consumption of sugars, which provides the necessary energy for their growth and reproduction, becomes exhausted. Consequently, the microbial strain depletes all available reducing sugars in the medium and cannot grow further. Furthermore, as the microorganisms metabolize glucose, the fermentation of glucose produces lactic acid, which can lower the pH of the medium. This, along with the production of metabolites such as ethanol, may affect the sugar concentration and, consequently, the growth of the strain [52]. The highest productivity of lactic acid in the evaluated media occurred in water kefir, with lactic acid production directly related to biomass concentration, as reported by López et al., [14] who documented concentrations of 0.477 g/100 mL of lactic acid for water kefir.

In the Bacillus subtilis strain grown in M2, the increase in biomass correlates with acid production, indicating a favorable yield. Authors such as Castells et al., [55] report a conversion of 0.065 g of lactic acid per g of sugar, while Román et al., [53] document L-lactate production with conversion yields exceeding 80 %, using glucose as a carbon source. The exponential growth phase for the Bacillus subtilis strain in M1 and M2 aligns with the behavior reported by Pulido et al., [54] where no lag phase occurs, and the exponential growth phase begins at hour 0, reaching its peak at 10 hours. It is noteworthy that this strain exhibited superior performance in the M2 growth medium compared to M1, particularly at 60 hours. This improvement may be attributed to a more favorable carbon(C:N) ratio in the nutrient concentrations present in the *E. crassipes* hydrolysate of M2. The C:N ratio plays a crucial role in substrate utilization rate, considered optimal when approaching a value of 20:1. Although not evaluated in this experiment, analyzing the C:N ratio in future assays would be a valuable suggestion for subsequent research [55, 56].

Additionally, another reason why *Bacillus subtilis* may have halted its growth in both M1 and M2 is the inhibition caused by the accumulation of acidic products resulting from metabolism, which alters the pH of the growth environment. It has been demonstrated that H^+ ions can act as non-competitive inhibitors in various enzymatic reactions. However, in the context of this study, the influence of pH was not considered. Therefore, it is particularly recommended to monitor the pH value during the growth process of this bacterium for a comprehensive and accurate analysis [55].

During the conducted assays, the consumption of reducing sugars throughout the fermentation process was also evaluated. At 33 °C, an average yield of 83 % was achieved. This may be due to Bacillus subtilis consuming a greater amount of sugar to reach the stationary phase and subsequently converting the excess reducing sugars into polymers. Given its slower metabolism, it is possible that the inhibition by certain compounds in the hydrolysate does not significantly affect its growth. The substrate consumption of Bacillus subtilis in M2, compared to the other strains cultivated in both M1 and M2, may be attributed to the inherent adaptive characteristics of this microorganism in fermentation processes.

Bacilli are commonly associated with a variety of fermented products, characterized by high growth rates that enable short fermentation cycles and a significant capacity to secrete enzymes into the extracellular medium. Most Bacillus species can produce extracellular amylases, such as α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), and glucoamylases (EC 3.2.1.3). Bacillus subtilis can produce amylases, particularly α-amylase. Lignocellulosic biomasses, such as barley and peas, are the primary cereals used in fermentation processes due to their potential as good carbon sources for growth and their high content of hydrolyzable starch and cellulose. Starch and cellulose can be degraded to cellobiose and maltose, and subsequently to glucose by Bacillus using its extracellular enzymes [57].

3.4 HPLC analysis

Table 3 presents the various acids separated from the fermentation process using M1 and M2 through HPLC. These acids were identified by comparing their retention times. The butyric, propionic, succinic, acetic, lactic, and oxalic acids were determined simultaneously, with retention times of 6.90, 2.00, 1.60, 1.30, 1.20, and 0.95 minutes, respectively. They exhibited their peaks at 214 nm.

The production of organic acids during the fermentation process with various microbial strains using dextrose (M1) and water hyacinth hydrolysates (*E. crassipes*) (M2), evaluated via HPLC, varied within a range of 0.18 to 41.01 % (w/v) (see Table 3). This variation reflects the synthesis of organic acids other than lactic acid during fermentation, attributed to the diversity of microbial strains employed.

Organic acids arise from bacterial development and the transformation of high molecular weight compounds into

Table 3. Recovery of organic acids from the fermentation of Eichhornia crassipes hydrolysates using different microorganisms in M1 and M2 via HPLC.

Strain and Culture Medium	Recovery of Organic Acids (% w/v)					
	Butiric	Propionic	Succinic	Acetic	Lactic	Oxalic
***Bacillus subtilis* M2**	-	-	-	23.69	-	-
DRI FAS 992 M2	-	0.18	-	-	-	0.70
Water Kefir M2	-	0.37	-	-	11.24	0.08
DRI SET 432 M2	18.07	41.01	-	-	-	-
***Bacillus subtilis* M1**	-	-	2.48	-	9.83	3.19
DRI FAS 992 M1	1.70	-	8.38	-	13.12	10.52
Water Kefir M1	-	-	-	-	3.68	1.34
DRI SET 432 M1	-	-	2.96	-	5.45	5.02

fermented products. Additionally, they are generated during fermentation due to hydrolysis, biochemical metabolism, and microbial activity [58]. It has been documented that during the incubation of *Bacillus sp.*, this bacterium utilizes glucose as a substrate to generate various organic acids, including malic, lactic, acetic, citric, succinic, propionic, and butyric acids. These findings are supported by research employing advanced high-resolution liquid chromatography techniques to analyze the microbiota composition of Daqu, a traditional Chinese beverage. In this context, Bacillus was identified as the predominant microorganism in this beverage. Understanding the production of organic acids by *Bacillus sp.* is highly relevant in the context of incubating various fermentation processes, as these changes in microbiota directly impact those processes [59].

In the present study, the *Bacillus subtilis* strain in M2 exhibits a high presence of acetic acid (23.69 % p/v), which can be associated with the intrinsic growth of its biomass due to the strain's ability to produce acids as part of its metabolism. The release of these acidic metabolites during growth and the production of enzymes occur as byproducts of the degradation of sugars from the E. crassipes hydrolysates. Conversely, the *Bacillus subtilis* strain cultivated in M1 shows a high content of lactic acid, along with lower concentrations of succinic and oxalic acids (2.48 % and 3.19 % p/v, respectively). The elevated concentration of lactic acid (9.83 % p/v) results from the fact that B. subtilis does not produce endotoxins, making it an attractive cell factory for producing various organic acids, sugars, and proteins. The biological production of lactic acid has garnered significant interest, as it is possible to produce this acid with high optical purity through fermentation with concentrations of 100 $g.L^{-1}$ or higher. Additionally, lactic acid can be applied to the synthesis of biopolymers, such as polylactic acid (PLA), food ingredients, and pharmaceutical and medical precursors [61].

On the other hand, the production of lactic acid in lactic acid bacteria (LAB) strains (DRI SET 432, DRI FAS 992, and water kefir) in M1 and M2 reflects the fermentation of sugars by LAB. Lactic acid is considered a metabolite generated by certain microorganisms and is classified as GRAS (Generally Recognized As Safe) by the FDA (Food and Drug Administration) for use as a food additive [50]. The production of lactic acid occurs through the fermentation of hexoses to lactic acid via the Embden-Meyerhof pathway. Furthermore, the potential for lactic acid production, as well as hydrogen peroxide and bacteriocins primarily from probiotic strains, is demonstrated by their bactericidal role, which acts by targeting the metabolism of pathogenic bacteria or their structures, such as the cytoplasmic membrane [62]. In a study, the production of organic acids by a strain of Lactobacillus casei in an MRS culture medium at 24 hours and 37 °C was investigated. HPLC analysis indicated that the L. casei strain is homofermentative, exhibiting a lactic acid production exceeding 80%, which could contribute to lowering the pH of the medium and inhibiting pathogenic microorganisms. The remaining 20 % corresponded to citric acid (11.92 %), succinic acid (1.97 %), acetic acid (3.76 %), and ethanol (2.34 %) [39].

For the DRI FAS 992 strain in M2, a recovery of 0.18% w/v of propionic acid and 0.70% w/v of oxalic acid was observed. This is attributed to its ability to oxidize the sugars from *E. crassipes* hydrolysates through specific metabolic pathways. For instance, propionic acid can be formed through mixed glucose fermentation or ethanol carboxylation, while oxalic acid can be produced by the oxidation of ethylene glycol or the dehydrogenation of glycolate [64].

In the DRI SET 432 strain cultivated in M2, a high production of propionic acid (41% w/v) and a lower production of lactic acid (7.82 $g.L^{-1}$) were observed. This behavior could be related to the acidification that occurs during the fermentation of dairy products, which depends on a limited number of lactic acid bacteria (LAB) species that ferment lactose into lactic acid, often added to milk as starter cultures. Strains of *Streptococcus salivarius subsp. thermophilus* are primarily used in thermophilic starter cultures for the production of hard cheeses, but also in other dairy products [65-67]. They tend to be metabolically active at the beginning of cheese-making processes, rapidly fermenting lactose and acidifying their environment [65]. This characteristic, along with the ability to ferment galactose, makes *S. salivarius subsp. thermophilus* valuable to the cheese industry by reducing production time and limiting the growth of undesirable pathogenic and spoilage bacteria [68–70]. Additionally, some strains contribute to aroma and flavor development during the maturation of products like cheese due to their proteolytic activity, influencing the texture of fermented products through the production of exopolysaccharides [70] They also produce health-beneficial compounds such as B vitamins

[71], and even exhibit probiotic attributes [72].

4 Conclusions

The production of biomass and organic acids through the utilization of aquatic plants is feasible, despite the fact that trials with M2 showed low yields, offering a viable alternative for utilizing hydrolysates from the aquatic plant E. crassipes.

Regarding biomass growth, it can be concluded that, in general, there is an inhibition of cell development in the M2 strains. This behavior is largely attributed to the lack of an adaptation process to the new growth medium. Nevertheless, some strains demonstrated good product yields. It is important to highlight that the increase in these metabolites depends directly on the nature of the strain being evaluated. For example, higher yields were achieved for the LAB strains and the water kefir strain.

The recovery of organic acids resulting from the fermentation of *E. crassipes* hydrolysates using different microbial strains (DRI SET 432, DRI FAS 992, *Bacillus subtilis*, and water kefir) was successfully separated and analyzed by HPLC with satisfactory repeatability. Butyric, propionic, succinic, acetic, lactic, and oxalic acids were simultaneously determined and eluted within 60 minutes. Considering the sample preparation and the proposed procedure, this method can be regarded as precise and efficient for determining these organic compounds.

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.

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Conflict of Interest Statement

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

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