Research Article

Microwave Synthesis of Butyl Levulinate Using a Keggin Heteropolyacid from *Eichhornia crassipes* Cellulose Obtained by Pretreatment with *Bacillus sp.*

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Received: 19 September 2024; Revised: 5 November 2024; Accepted: 20 December 2024; Published online: 14 January 2025

Abstract
Underutilized lignocellulosic biomass, such as aquatic plants, faces sustainability challenges due to energy-intensive treatments. This study investigates the use of *Bacillus* sp. for the biological pretreatment of water hyacinth (*Eichhornia crassipes*) aiming to improve the efficiency of alkyl levulinate (AL) production, which are valuable compounds used as fuel additives, solvents, and plasticizers. The pretreatment with *Bacillus* sp. resulted in stable bacterial growth and a significant increase in cellulose content (up to 27%) compared to untreated samples. X-ray diffraction revealed a higher crystallinity index (63%) in the treated cellulose, while morphological analysis confirmed microbial degradation. Thermogravimetric analysis showed improved thermal stability, consistent with the higher crystallinity, and FTIR spectra indicated successful delignification. The microwave-assisted synthesis of butyl levulinate yielded 30% from *Bacillus* sp.-treated cellulose, compared to 11% from untreated cellulose, highlighting the potential for biomass valorization through biological pretreatment.

Keywords: Alkyl-levulinate, *Bacillus* sp. pretreatment, Cellulose crystallinity, Heteropolyacid catalysts, Water hyacinth

1 Introduction

Underexplored sources of lignocellulosic biomass include aquatic plants, primarily because their traditional utilization has involved treatments that are not sustainable from an energy and environmental standpoint, thereby hindering their commercial scaling. Biomass-derived from water hyacinth (*Eichhornia crassipes*) could be a valuable precursor for obtaining high-interest industrial products,

contributing to the conservation of aquatic ecosystems affected by its uncontrolled growth [1].

Aquatic plants like *Eichhornia crassipes* (water hyacinth), *Salvinia minima*, *Pistia stratiotes*, *Lemna spp.*, and others thrive in diverse water environments, from brackish to polluted waters, due to their adaptability and low maintenance needs. These species rapidly grow under high nutrient levels, generating biomass rich in carbohydrates, lipids, and proteins. This biomass can be fermented into biogas, biohydrogen, and liquid biofuels (e.g., bioethanol, biomethanol, and biobutanol) and converted into high-value chemicals and materials [2].

Among these, *E. crassipes* stands out for its invasive potential and extensive study as a feedstock for biofuels and other products. Its applications span from biohydrogen and bioplastics to biochar, animal feed, compost, xylitol, and even household items like baskets and hygiene products [3]. Between those compounds, the alkyl levulinates (AL) found wide applications, including fuel additives, solvents, and plasticizers. Given their high miscibility with diesel or gasoline, certain levulinate esters can be incorporated into these fuels, helping to reduce the emission of pollutants such as NO_x into the atmosphere.

Alkyl levulinates are produced from cellulose using heteropolyacids (HPAs) [4], [5]. These catalysts are particularly effective for the alcoholysis of more resistant cellulose and even raw biomass. HPAs offer a number of advantages that make them very attractive as catalysts in a variety of homogeneous or heterogeneous liquid phase reactions and have been widely used in reactions involving cellulose obtained from pretreated biomass sources. These advantages include low volatility, low corrosivity, high acidity, high activity, flexibility, and thermal stability. For example, solids such as $H_3PW_{12}O_{40}$ and $H_4SiW_{12}O_{40}$, even without any support, show remarkable activity in alcohol dehydration reactions, despite having a small surface area, about 1-5 m^2/g. This is because these reactions follow a bulk type I (pseudo-liquid) catalysis mechanism, in which all protons, both surface and internal, participate in the acid catalysis. In this context, the surface area loses relevance [6]. Zhao *et al*., [7] replaced a proton in the Keggin-type $H_3PW_{12}O_{40}$ with larger monovalent cations, such as K^+ ($KH_2PW_{12}O_{40}$) and Ag^+ ($AgH_2PW_{12}O_{40}$), reducing the initial Brønsted acidity and rendering the catalyst insoluble. $KH_2PW_{12}O_{40}$ emerged as the most effective catalyst, achieving a comparable maximum EL yield to that of $AgH_2PW_{12}O_{40}$, but with the advantage of using a more cost-efficient KCl precursor instead of $AgNO_3$. Additionally, the authors demonstrated that adding toluene significantly increased the EL yield from fructose, boosting it from 51 mol % (with pure EtOH) to 69 mol %. This improvement was attributed to the extraction of EL into the toluene phase, which prevented product degradation. High EL yields were also obtained from inulin and sucrose, although glucose and cellulose again resulted in unsatisfactory EL yields [7]. Pinheiro *et al*.,[8] prepared various tin salts of $H_4SiW_{12}O_{40}$, which exhibited minimal solubility in alcohol-based media, and evaluated their catalytic efficiency in EL synthesis using different saccharides as starting materials. Their findings indicated that $Sn_2SiW_{12}O_{40}$ was the most effective catalyst, delivering promising EL yields from fructose, sucrose, and inulin. The exchange of protons with Sn^{2+} notably improved the selectivity for EL. The particularly high yield is obtained from sucrose (78 mol %). A disaccharide composed of fructose and glucose was attributed to the glucose unit, which, after being released at the catalytic site, undergoes direct isomerization to fructose and is subsequently converted to EL [8].

The conversion of lignocellulosic and cellulose biomass into alkyl levulinates typically demands significant quantities of homogeneous acids, with the required pretreatment posing a major challenge to the valorization of these biomass types. Studies on the butanolysis of cellulose, conducted in the presence of dilute sulfuric acid (around 0.2 $mol \cdot L^{-1}$), have yielded promising results, achieving approximately 40 mol % of butyl levulinate (BL) under reaction conditions similar to those used for shorter-chain alkyl levulinates [9]. Given the characteristics of this type of lignocellulosic biomass, such as being a highly structured and highly resistant material to biological degradation, it requires a pretreatment stage before the hydrolysis stage, where fermentable sugars are obtained [5]. The primary goal of pretreatment is to alter the biomass structure and disrupt the crystalline arrangement of cellulose, thereby enhancing the substrate's accessibility for chemical or enzymatic hydrolysis [6]. Various pretreatment techniques have been explored, each with specific advantages and disadvantages [5], [6], [8], [9]. The main disadvantages found in the chemical pretreatments are the high consumption of reagents, corrosion of equipment, high energy costs, requiring high temperatures between 60 and 120 °C, low yields of

sugars, and formation of humins as undesirable products after the reaction is obtained [10].

Therefore, biological pretreatment is an ecological approach to pretreatment of lignocellulosic biomass that involves the use of microorganisms, such as fungi or bacteria [10]. These microorganisms synthesize lignin-degrading enzymes (including lignin peroxidases, polyphenol oxidases, and laccases), quinone-reducing enzymes, hemicellulases and cellulases. An attractive genus microorganism for this process is *Bacillus*, which can produce extracellular enzymes such as cellulases, amylases, and xylanases to convert carbohydrate polymers into monomers such as simple sugars or glucose [11]. Finally, the genus *Bacillus* is recognized as safe (GRAS) and has outstanding physiological characteristics and a highly adaptable metabolism, which makes it easy to grow on inexpensive substrates [11]–[13].

Biological pretreatment with fungi plays a significant role in maximizing lignin production, preventing holocellulose degradation and enhancing glucose yield during enzymatic hydrolysis is crucial. Sari *et al.*, [14] reported that lignin degradation reached 51.55 % after six weeks of incubating water hyacinth with *Phanerochaete chrysosporium* [14]. In contrast, Jongmeesuk *et al.*, [15] observed that an enzyme concentration of 0.30 $mL \cdot g^{-1}$ of water hyacinth, combined with a 48-hour incubation period, yielded the highest concentration of reducing sugars, measuring 11.95 ± 0.22 $g \cdot L^{-1}$. Nevertheless, the efficiency of cellulose hydrolysis in pretreated substrates is modulated by several factors, such as enzyme dosage, biomass concentration, incubation time, and surfactant presence [15].

Our study targets the underutilized lignocellulosic biomass from *Eichhornia crassipes* (water hyacinth) for producing high-value compounds like alkyl levulinates (ALs), which are utilized as fuel additives, solvents, and plasticizers. While conventional lignocellulosic treatments are energy-intensive and unsustainable, biological pretreatment using *Bacillus* sp. offers a more sustainable approach. This microorganism, recognized as GRAS, produces extracellular cellulases, amylases, and xylanases, enhancing cellulose conversion while growing on low-cost substrates.

Pretreating water hyacinth with *Bacillus* sp. increases cellulose content by 27% compared to untreated samples, achieving a crystallinity index of 63%, as X-ray diffraction confirms. Morphological analysis reveals microbial degradation in treated samples, while untreated samples remain structurally compact. The thermogravimetric analysis further shows improved thermal stability in treated cellulose, corresponding with enhanced crystallinity. FTIR spectra confirm successful delignification, with reduced O-H stretching and diminished aromatic peaks.

Following biological pretreatment, the cellulose catalyzed with phosphotungstic acid ($H_3PW_{12}O_{40}$) yields butyl levulinate at 30%, compared to only 11% from untreated cellulose. This highlights the efficacy of biological pretreatment in optimizing biomass conversion, reducing energy, and lowering acid consumption. We believe this approach enhances the commercial feasibility of alkyl levulinate production and similar bioproducts.

While aquatic plants have been explored for biomass applications, their traditional use involves treatments that are not sustainable, limiting their commercial scalability. This study stands out by utilizing *Bacillus* sp. as a biological pretreatment for water hyacinth (*Eichhornia crassipes*), addressing the limitations of conventional chemical pretreatments that are energy-intensive, costly, and produce undesirable by-products like humins. The novelty of our approach lies in combining this biological pretreatment with subsequent catalysis using phosphotungstic acid ($H_3PW_{12}O_{40}$) to produce butyl levulinate, an important industrial compound used as a fuel additive, solvent, and plasticizer. Biological pretreatment not only enhances cellulose accessibility but also significantly reduces reagent consumption and energy costs, offering a more sustainable and efficient method for lignocellulosic biomass valorization. By improving the yields of cellulose for conversion into high-value products, this study presents an innovative and eco-friendly approach that could be transformative in the field of biomass utilization.

The use of *Bacillus* sp. for the pretreatment of *Eichhornia crassipes* (water hyacinth) aims to enhance cellulose and hemicellulose accessibility, facilitating the production of bioproducts like alkyl levulinates. *Bacillus* species produce enzymes that efficiently degrade lignocellulosic biomass, overcoming the high cellulose and hemicellulose content bound by lignin [16]. The pretreatment reduces biomass recalcitrance and improves enzymatic hydrolysis efficiency, with studies indicating that microbial pretreatment, specifically with *Bacillus* sp., significantly enhances hydrolysis by breaking down cellulose and hemicellulose into fermentable sugars [17]. This process is crucial, as

untreated water hyacinth's dense structure impedes microbial fermentation [18]. Additionally, *Bacillus* sp. pretreatment increases fermentable sugar yields and overall bioconversion efficiency, supporting the production of polyhydroxybutyrate (PHB), bioethanol, and enhancing biogas yield in anaerobic digestion [19], [20].

2 Materials and Methods

2.1 *Materials*

Keggin heteropolyacid ($H_3PW_{12}O_{40}$) and Butanol were purchased commercially. Water hyacinth samples were collected in the north and south cooling pools of the "Termopaipa" thermoelectric plant located in the municipality of Paipa, Boyacá, Colombia (N °5 46' 8.3" W 73° 08' 40.3"). The drying process of the samples (leaves and stems of water hyacinth) was conducted at room temperature. Once this stage was completed, the particle size of the material was reduced using a high-speed multifunction grinder HC-150, followed by a sieving process with a Tyler # 30 sieve until a particle size of 500 µm was obtained.

2.2 *Water hyacinth pretreatment by Bacillus sp.*

A modified MRS liquid culture medium was prepared, composed of 20 $g.L^{-1}$ glucose as the main carbon source, 5.0 g. L^{-1} yeast extract and Tryptone as nitrogen source and protein source and as micronutrients 0.5 $g.L^{-1}$ of K_2HPO_4, 5.0 $g.L^{-1}$ of NaCl, 1.0 $g.L^{-1}$ of $MgSO_4$, 0.1 $g.L^{-1}$ of $FeSO_4$ and 0.1 $g.L^{-1}$ of $CaCl_2$. In 250 mL Erlenmeyers, 10 mL of inoculum (0.3 g of commercial *Bacillus* sp. in 100 mL of sterile distilled water) were added to 90 mL of culture medium to a total liquid volume of 100 mL. The fermentation was evaluated over 72 h at 30 °C with agitation at 200 rpm in a Centricol orbital shaker. The culture was regrowth several times until OD, pH and °Brix do not present significant variations. After completing the acclimatization phase, assays were prepared using the same culture medium and consistent time and temperature conditions, but with varying proportions of tryptone and yeast extract: 0:10, 2.5:7.5, 7.5:2.5, 5.0:5.0, and 10:0.

Finally, an assay was conducted under the best conditions identified for glucose concentration and nitrogen source, with the addition of 5 g of water hyacinth (*E. crassipes*). Each assay was prepared in triplicate and included a negative control. The fermentation for pretreatment was evaluated over 15 days at 30 °C with agitation at 200 rpm in a Centricol orbital shaker. The liquid phase was subsequently separated from the solid phase through vacuum filtration and dried at 60 °C for 24 h in a Memmert UN75 universal oven [21].

2.3 *Physicochemical analysis of the culture medium using water hyacinth (Eichhornia crassipes) pretreated with Bacillus* sp. *strain*

°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 h. The dissolved solid decrease (DS) value was calculated by the difference between initial and final values.

The pH was determined according to NTC 3651 of 1994, directly in the fermented liquid phase obtained every 72 h, with an ORION 8107UWMMD ROSS Ultra pH/ATC triode electrode, Thermo scientific. The pH decrease value (pH_v) was calculated by the difference between initial and final values.

To measure reducing sugars, 1 mL of the diluted supernatant sample (fermented culture medium) was mixed with 1 mL of DNS reagent. Subsequently, it was heated at 90 °C for 10 min in a water bath and allowed to boil for 5 min. Then, 8 mL of distilled water was added. After the time elapsed, it was transferred to an ice bath until room temperature. Finally, it was stirred and read at 540 nm in a Hanna HI 801 iris spectrophotometer, having beforehand a calibration curve prepared to find the reducing sugars ($mg.mL^{-1}$) concentration [22]. The decrease value (RS) was calculated by the difference between initial and final values.

For 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. ΔOD increase was calculated by the difference between final and initial values.

The organic acids extracted from the fermentation process using the commercial *Bacillus sp.* 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 a refractive index detector. The samples were analyzed using a Nova pack Waters C-18 column. The column was operated at a temperature

of 30 °C. The mobile phase (flow rate of 0.5 mL.min^{-1}) consisted of distilled water acidified and adjusted to a pH of 2.24 with 0.5 % w/v $(NH_4)_2HPO_4$ and phosphoric acid, filtered, ultrasonicated, and degassed prior to use. The eluted compounds were detected at 214 nm.

To obtain bleached cellulose, an additional treatment with a 1.5 % w/v glacial $NaClO_2/CH_3COOH$ solution was applied to the samples obtained with each of the pretreatments. The ratio of the treated water hyacinth biomass mass solution to the $NaClO_2/CH_3COOH$ solution was 1:20. The mixture was heated in a water bath at 75 °C for 2 h. During the 2-h period, the pH was maintained at around 4.0. The samples were thoroughly washed until a neutral pH was achieved. If the resulting sludge was not pure white, the treatment with the $NaClO_2$ and glacial CH_3COOH mixture was repeated. The final white samples were then dried in a Memmert oven at 40 °C for 48 h [23].

2.4 *Physicochemical characterization of cellulose extracted from water hyacinth (Eichhornia crassipes) with Bacillus* sp. *pretreatment and untreated sample*

The ATR infrared spectra of biomass and cellulose extracted from water hyacinth (*E. crassipes*) were obtained using a Nicolet IS50 analytical FTIR spectrometer, 64 scans were taken with a resolution of 4, at a speed of 0.3165 stand. s^{-1}, with an aperture of 180 and a range of 500-4000 cm^{-1}. The crystallinity index (%CrI) of the obtained cellulosic material was determined by Nelson and O'Connor method [24], applicable for cellulosic polymers consisting of crystallized cellulose I or II and blends of both constituents. For this purpose, the study focused on the calculation and analysis of the ratios of the band intensities defined by the 1375/2902 cm^{-1} ratio (total crystallinity index, ICT) can be used for the determination of the crystallinity of cellulosic materials, with the advantage, compared to the previous ratio, that it can be applied for both types of crystallized cellulose I and II, as well as for mixtures of both constituents [25].

XRD patterns were obtained using a Malvern Panalytical Empyrean 2012 X-ray diffractometer, equipped with a Pixel 3D detector and a Cu source (λ = 1.5453 Å) at 45 kV and 40 mA. The step size was 0.02°, with a dwell time of 50 s per step. Scanning electron microscopy (SEM) analyses were conducted using a JEOL JSM-6490LV scanning electron microscope. Images of the cellulose surfaces resulting from pretreatment with *Bacillus* sp. were taken mounted on conductive tape and coated with palladium gold, using a voltage of 20 KV [26]. DSC thermograms of cellulose from *Bacillus* sp. strain pretreatment were obtained using a differential scanning calorimeter and a Setaram 1600 thermogravimetric analyzer. The temperature conditions were from 0 to 400 °C with a ramp of 5 °C.min^{-1}, with nitrogen flow of 50 mL.min^{-1}.

2.5 *Synthesis of butyl levulinate*

Butyl levulinate synthesis was used as a test reaction. The reactions were conducted using an Anton Paar Monowave 400 microwave reactor. Briefly, 0.3 g of water hyacinth cellulose, 0.15 g of heteropolyacid, 2 mL of anhydrous butanol, 2 mL of chloroform, and 0.5 mL of octane as internal standard was added to the sealed reactor. The mixture was then heated to 170 °C with agitation at 300 rpm for up to 90 min. After the reaction, the reactor was rapidly cooled under chilly water, and the product was collected. Separation of the liquid-solid mixture was achieved using a Power Spin LX Centrifuge. Reaction monitoring was conducted via gas chromatography using a Shimadzu 2014 chromatograph with an SPB-1 column (30 m, 32 mm, 1.00 μm), equipped with an FID detector [27]. The moles (n_{AL}), grams (g_{AL}), and yield (% Y_{AL}) of alky levulinates were calculated using Equations (1)–(3):

$$n_{AL} = \frac{C_{AL} \times V_s}{1000} \quad (1)$$

$$g_{AL} = n_{AL} \times M_{AL} \quad (2)$$

$$\%\, Y_{AL} = \frac{g_{AL}}{g_{cell}} \times 100 \quad (3)$$

Where C_{AL} is the initial concentration of alky levulinate calculated with a calibration curve with an internal octane standard. V_s is the volume of reaction solution in mL. g_{cell} is the gram of cellulose, and M_{AL} is the molar mass of AL.

3 Results and Discussion

3.1 *Bacillus* sp. *pretreatment*

3.1.1 Evaluation of the growth of the Bacillus sp. *strain at different concentrations of nitrogen source.*

Figure 1 shows the variation in physicochemical parameters for the assays with different proportions of nitrogen sources (tryptone and yeast extract) in the culture medium over a 72-hour period.

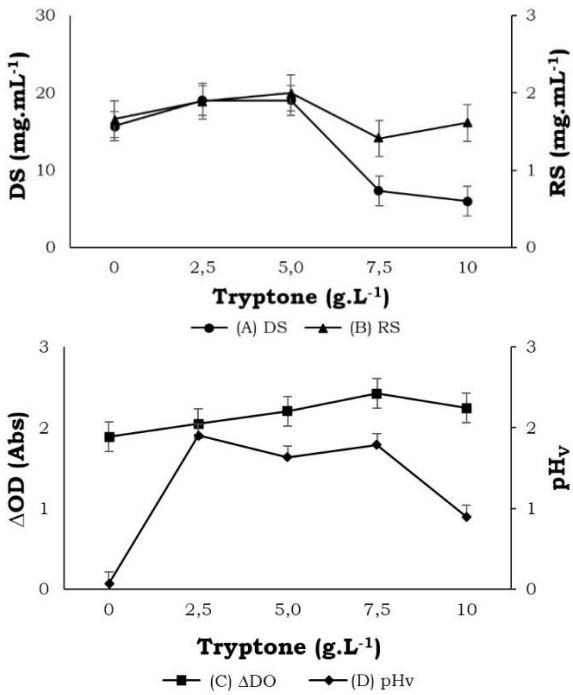

Figure 1: Evaluation of the growth of the *Bacillus* sp. strain at different nitrogen source concentrations (tryptone and yeast extract: 0:10; 2.5:7.5; 5.0:5.0; 7.5:2.5; and 10:0) with 20 $g.L^{-1}$ glucose at 72 h of fermentation. (A) Dissolved solid (DS) (Initial: 25.20 ± 5.07 $mg.mL^{-1}$). (B) Reducing sugars (RS) (Initial: 2.66 ± 0.54 $mg.mL^{-1}$). (C) Optical density (ΔOD) (Initial: 0.01 ± 0.01 Abs). (D) pH_V (Initial: 6.45 ± 0.14).

Figure 1 results demonstrate that varying tryptone and yeast extract concentrations in the culture medium significantly affect pH, substrate consumption, and bacterial growth. Higher tryptone levels (7.5 $g.L^{-1}$ and 10 $g.L^{-1}$) and lower amounts of yeast extract (2.5 $g.L^{-1}$ and 0 $g.L^{-1}$) reduce dissolved solids (DS) and reducing sugars (RS), while the opposite trend is observed with lower tryptone (0 $g.L^{-1}$ and 2.5 $g.L^{-1}$) and higher yeast extract (10 $g.L^{-1}$ and 7.5 $g.L^{-1}$). This pattern aligns with previous studies on nitrogen source impacts on bacterial metabolism.

Additionally, higher tryptone and lower yeast extract levels increase oxygen uptake, indicating enhanced bacterial growth. The pH trends show alkalinization at elevated tryptone concentrations, while tryptone-to-yeast extract ratios of 2.5:7.5 and 7.5:2.5 provide the best balance, with 2.5:7.5 achieving optimal substrate consumption and an acidic pH.

Complex nitrogen sources like tryptone and peptone are shown to boost enzyme production (e.g., cellulases, xylanases) critical for lignocellulosic biomass degradation in *Bacillus* sp. Additionally, glucose (20 $g.L^{-1}$) plays a key role in regulating pH and DS levels, promoting microbial growth and organic acid formation essential for biomass pretreatment. This optimal 2.5:7.5 ratio combined with glucose creates favorable conditions for effective water hyacinth pretreatment.

The results in Figure 1 suggest the assay with the 2.5:7.5 ratio enhances microbial activity and subsequent organic acid production, supporting water hyacinth pretreatment for alkyl levulinate production without causing alkalinization of the culture medium. Studies have reported the influence of adding tryptone and/or yeast extract to the culture medium when working with *Bacillus* sp. strains to produce specific chemical compounds. For example, it has been reported that the addition of yeast extract, beef extract, and/or amino acids enhances cell growth. Additionally, complex nitrogen sources like tryptone or peptone have been found to be efficient in the synthesis of penicillin G acylase (PAC) in *Bacillus* sp. Furthermore, tryptone and peptone not only increase PAC productivity and cell growth but also enhance specific activity [28].

Similarly, it has been demonstrated that the addition of peptone (1.0 %) and yeast extract (0.5 %) to the culture medium of *Bacillus* sp. shortens the lag phase, improves growth, and enhances α-amylase synthesis. On the other hand, it has been reported that the addition of glucose to the culture could significantly reduce α-amylase synthesis, indicating that glucose has some effect on this microorganism [29].

In all the assays, bacterial growth remained stable after 72 hours of fermentation, as indicated by the lack of an increase in the OD value. This could be explained by the bacteria's faster metabolism, which depletes essential nutrients, such as the nitrogen source, before achieving a high concentration of microorganisms. The limitation of essential nutrients, despite the availability of glucose, appears to be a key factor hindering further growth [30]. Thus, the acidity, substrate consumption, and microbial growth

provided by the 2.5:7.5 tryptone-to-yeast extract ratio may more effectively support the pretreatment of water hyacinth.

Studies have shown that pH levels and peptone concentration significantly influence bacterial cell growth dynamics, crucial for optimizing conditions for biomolecule production. Peptones, as nitrogen sources in culture media, affect growth rates and metabolic activities. For instance, Kwon *et al*., found that 50 $g.L^{-1}$ peptone maximized nattokinase activity in *Bacillus subtilis*, illustrating nitrogen's role in enhancing enzyme production [31]. Similarly, Gray *et al.*, and Kujawska *et al*., reported increased bacterial and *Schizochytrium* sp. growth with higher peptone concentrations due to better nutrient availability [32], [33].

pH also plays a critical role in microbial growth and enzyme activity. Cheng *et al*., showed that pH 5.5–6.0 optimized proteinase activity in *Lactobacillus plantarum*, while Pham highlighted that optimal pH during fermentation enhances cell density, suggesting a synergistic effect with nutrient concentration [34], [35].

Sulaiman *et al*., reveal that pH and peptone concentration interact to affect growth, with specific peptone levels supporting optimal *Bacillus velezensis* growth at certain pH ranges [36]. Additionally, the buffering capacity of peptone helps maintain stable pH, crucial for releasing proteinases associated with microbial metabolism, as observed by Turbay *et al.* [37].

3.1.2 Growth of the Bacillus sp. *with the best identified conditions of protein and carbon source in the culture medium using water hyacinth (Eichhornia crassipes).*

Figure 2 shows the increase and decrease of DS, RS, OD, and pH during the adaptation of the *Bacillus* sp. to 50 $g.L^{-1}$ of water hyacinth (*E. crassipes*). From the third to the fifth subculture, the values of dissolved solids (Figure 2(A)) and reducing sugars (Figure 2(B)) remained constant without significant variations. However, the values of optical density (OD) (Figure 2(C)) and pH (Figure 2(D)) decreased.

The decrease in microorganisms in the assays without water hyacinth, compared to those that included it, can be explained by several factors. In the media containing water hyacinth (*E. crassipes*), the optical density (OD) and pH parameters decreased, suggesting that the microorganisms adapted to the more acidic environment and possibly utilized the compounds present in the hyacinth for growth. This acidic environment, generated by fermentation byproducts such as organic acids, promotes bacterial growth and contributes to the breakdown of the aquatic plant [38]. The reduction in OD and pH indicates that the microorganisms were actively metabolizing the available nutrients, creating a favorable environment for the degradation of the hyacinth and, consequently, the process's efficiency.

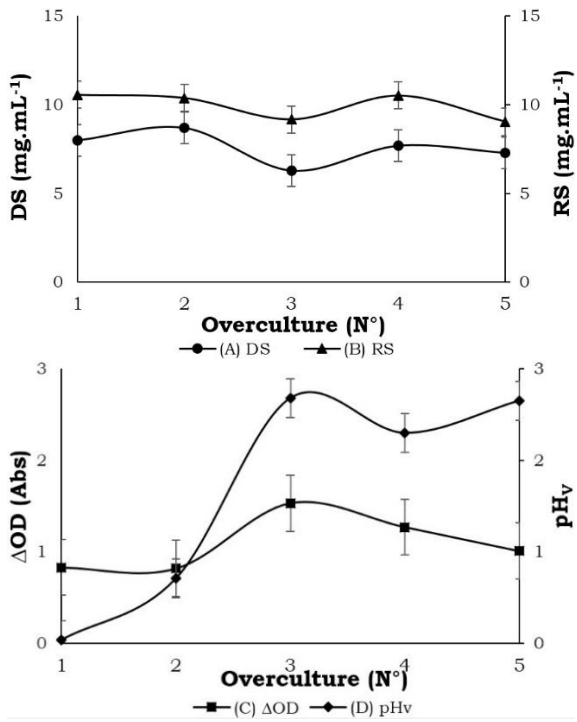

Figure 2: Evaluation of the growth of the *Bacillus* sp. strain under the optimal nitrogen source and glucose conditions in the culture medium using water hyacinth (*E. crassipes*). (A) Dissolved solids (DS) (21 ± 0.00 $mg.mL^{-1}$). (B) Reducing sugars (RS) (19.54 ± 0.00 $mg.mL^{-1}$). (C) Optical density (ΔOD) (3.10 ± 0.00 Abs). (D) pH_V (Initial: 6.19 ± 0.00)

In contrast, in the assays without hyacinth, an increase in both OD and pH were observed, indicating that microbial growth was less efficient in terms of adaptation to the medium. In this case, the absence of hyacinth may have resulted in a less acidic environment, which is less favorable for fermentation and microbial growth. Despite the increase in OD, the lack of a suitable environment for sustained growth, such as the one provided by the hyacinth compounds, may have led to less controlled and efficient growth [39].

However, the decrease in microorganisms in the assays with hyacinth was not detrimental. On the contrary, the decline in OD and pH suggests a controlled and suitable environment for bacterial growth, preventing overpopulation and rapid depletion of medium resources. Additionally, the acidity and compounds contributed by the hyacinth may have inhibited the growth of undesirable microorganisms, favoring process stability and efficiency in the breakdown of the plant material [38]. Therefore, although there was a reduction in microbial population, this was beneficial in maintaining balance and treatment efficiency.

On the other hand, glucose is a primary substrate for *Bacillus* species, essential for metabolism and byproduct production. Increased glucose concentration can enhance bacterial fermentation rates, boosting growth and density, which benefits the pretreatment of *Eichhornia crassipes* (water hyacinth) by facilitating biomass conversion. However, excessive glucose may cause osmotic stress, inhibiting microbial activity, while low glucose levels can limit fermentation rates and metabolite production.

Glucose consumption, integral to *Bacillus* growth, directly influences bacterial population dynamics. Through fermentation, glucose is metabolized to release energy that supports cellular activity, fostering bacterial growth beneficial for biomass pretreatment. Notably, glucose consumption intensifies by the third inoculation cycle, impacting the pH of the culture medium due to organic acid production. These organic acids lower pH and reduce dissolved solids (DS) and reducing sugars (RS), as observed through decreasing °Brix values. This reduction in available sugars slows microbial growth over time, with pH alterations from acid accumulation further affecting bacterial metabolism and sugar utilization efficiency.

Figure 3 shows the bacterial growth rate expressed as DS and RS (mg.mL^{-1}), DO, and pH under the optimal fermentation conditions identified during the 15-day pre-treatment and fermentation process of 50 g.L^{-1} of water hyacinth (*E. crassipes*): 20 g.L^{-1} glucose, 2.5 g.L^{-1} tryptone, and 7.5 g.L^{-1} yeast extract in the culture medium. It can be observed that starting from the third day and continuing until the fifteenth day, the biomass stabilizes its growth with DO values at 4.0, DS at 13 mg.mL^{-1}, RS at 12 mg.mL^{-1}, and pH at 4.0. This demonstrates that the microorganisms adapted to the components of the culture medium over 15 consecutive days.

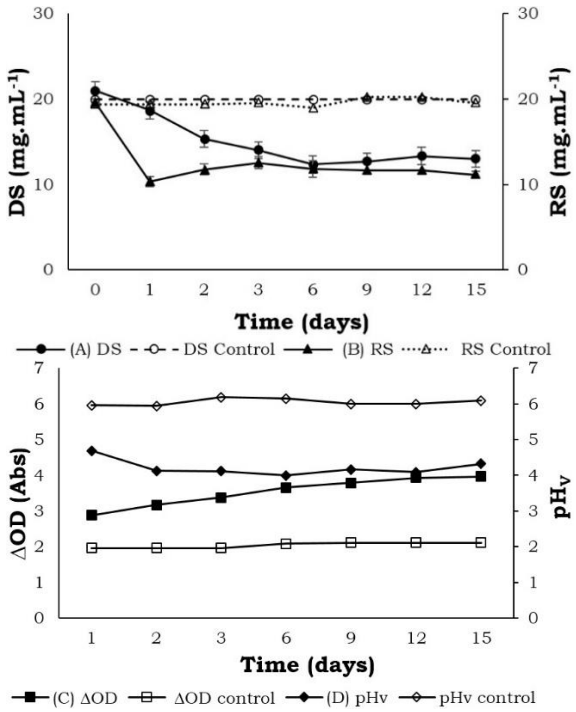

Figure 3: Evaluation of the growth of the *Bacillus sp*. strain under the optimal glucose and nitrogen source conditions during fifteen days using water hyacinth (*E. crassipes*). (A) Dissolved solids (DS) (mg.mL^{-1}). (B) Reducing sugars (RS) (mg.mL^{-1}). (C) Optical density (ΔOD) (Abs). (D) pH$_V$.

It can be inferred that the higher the number of cells present in the medium, the lower the dissolved solids and reducing sugars will be, and vice versa, leading to the acidification of the culture medium. This acidification promotes the production of acids such as lactic and formic acid by the end of the process (day 15), as shown in Table 1 [40].

Table 1: Concentration (g.L^{-1}) of various organic acids found by HPLC in the pretreatment of water hyacinth (*E. crassipes*) using the commercial strain of *Bacillus* sp.

Organic acids	Concentration (g.L^{-1})
Propionic acid	1.04
Acetic acid	0.22
Lactic acid	3.73
Succinic acid	0.41
Formic acid	1.74

Bacillus sp. is generally associated with neutral pH, but Calvo and Zúñiga *et. al.*,[41] in 2010, found that some strains exhibit better growth under acidic

conditions. The higher growth of *Bacillus* sp. observed in this experiment could be due to the addition of water hyacinth (*E. crassipes*), along with the consumption of nitrogen sources and glucose from the culture medium. Previous studies have shown that this microorganism could adapt to this type of biomass [30].

Organic acids arise from bacterial development and the transformation of high molecular weight compounds into fermented products [40]. Additionally, they are generated during the fermentation process due to hydrolysis, biochemical metabolism, and microbial activity [42]. Studies have shown that during the incubation of *Bacillus* sp., the bacterium metabolizes glucose to produce a range of organic acids, including lactic, propionic, malic, acetic, butyric, citric and succinic acids. These findings are corroborated by research utilizing advanced reverse-phase high-performance liquid chromatography to analyze the composition of Daqu, a traditional Chinese beverage, where *Bacillus* sp. was identified as the dominant microorganism.

The fermentation of water hyacinth (*Eichhornia crassipes*) creates an acidic environment that supports the growth of *Bacillus* species by increasing nutrient availability and facilitating the decomposition of complex organic materials. This process generates organic acids, primarily lactic acid, which reduce the pH of the medium. *Bacillus subtilis* and related species, adapted to acidic conditions, utilize these byproducts to proliferate, gaining a selective advantage in organic-rich environments such as fermented water hyacinth. This approach enhances the utility of water hyacinth for potential applications in aquaculture and other fields, underscoring the role of fermentation in resource optimization.

It has been reported that in *Bacillus subtilis* 168 cultures with glucose concentrations exceeding 10 $g \cdot L^{-1}$, the primary secondary metabolites identified via HPLC analysis were acetic acid, formic acid, lactic acid, and acetoin. Formic acid was detected at approximately 1.25 $g \cdot L^{-1}$, and its toxicity to cells was suggested to play a significant role in the cessation of growth observed in media with high substrate concentrations. Consequently, it can be inferred that the *Bacillus* sp. strain used in this study demonstrated tolerance to formic acid, despite its higher concentration (1.74 $g \cdot L^{-1}$) following the water hyacinth pretreatment. This tolerance is supported by stable growth, substrate consumption, and acidification of the culture medium after the third day (Figure 3) [43].

The chemical composition of water hyacinth (*E. crassipes*) was evaluated over a 15-day pretreatment period, as shown in Figure 4. Table 2 describes the dry-based chemical composition of the water hyacinth and the crystallinity index (% CrI) after 15 days of pretreatment process using the commercial strain of *Bacillus* sp., compared to the untreated sample.

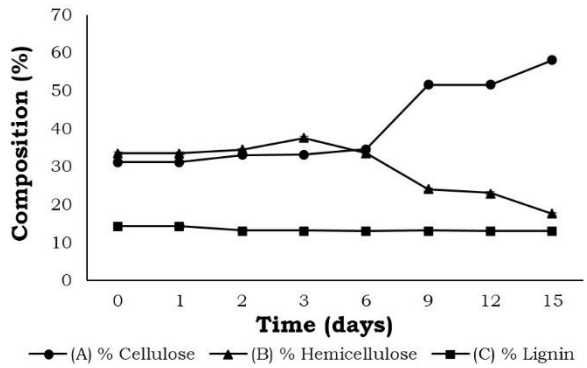

Figure 4: Evaluation of the chemical composition of water hyacinth (*E. crassipes*) during 15 days of pretreatment with *Bacillus* sp. (A) Cellulose, (B) Hemicellulose, (C) Lignin.

Table 2: Chemical composition on dry basis of water hyacinth pretreated by *Bacillus* sp. for 15 days.

Parameters	**Pretreatment**	
%	**Untreated**	***Bacillus* sp.**
Moisture	7.79 ± 0.12	5.96 ± 0.60
Ash	7.87 ± 0.002	5.07 ± 0.02
Cellulose	23.73 ± 0.12	57.98 ± 0.72
Hemicellulose	44.46 ± 0.75	17.51 ± 0.32
Lignin	14.35 ± 0.87	13.13 ± 0.05
Extractables	2.39 ± 0.15	2.37 ± 0.15
ICr	47	63

This observation could be attributed to the low temperature and the conditions of the culture medium in which the pretreatment process with *Bacillus* sp. was conducted. These conditions might promote the partial removal of lignin from the external surface of the plant while targeting other components. This process could involve the generation of necessary enzymes such as xylanases, which facilitate the partial depolymerization of hemicellulose into xylose, thereby inhibiting the dehydration of sugars into furfural. Consequently, this promotes the recovery of reducing sugars (Figure 3(B)) and, therefore, an increase in cellulose, as reported for other types of biomass using a strain of the same microorganism [38].

Conversely, comparing the cellulose content of water hyacinth obtained at the end of the pretreatment process (day 15) with values reported in the literature reveals that in some cases, the percentages are higher (35% [44] and 15%–25% [15]) while in others, they are lower (28.3%–93.3%) [45]. This suggests that the pretreatment with *Bacillus* sp. applied to water hyacinth is effective for cellulose recovery.

3.2 *Physicochemical characterization of cellulose extracted from water hyacinth (Eichhornia crassipes) with Bacillus* sp. *pretreatment and untreated sample*

The X-ray diffraction patterns obtained from *Bacillus* sp. and untreated pretreatments are shown in Figure 5. The cellulose obtained from pretreatment with *Bacillus* sp. exhibits diffraction peaks at 2θ at 21.6° corresponding to reflections of the crystallographic plane (020) characteristic of cellulose I, and the band at lower intensity at 38.5° and 32.5 2θ that could be related to the structure of cellulose Iβ [46]. Amorphous cellulose, however, contributes three distinct signals to the diffraction pattern, notably at 20.5° and 38.9° 2θ. This differs from earlier studies, which predominantly identified a single amorphous peak centered around 20° [47]. Additionally, studies on aquatic plants report multiple diffraction patterns on treated biomass; for example, pretreated duckweed shows a broad amorphous peak at 2θ = 22.1° and several peaks at 2θ = 27.0° and 29°, demonstrating changes in peak intensity [48]. For the untreated sample, the crystalline peak predominates and is well defined on a common scale. The untreated samples have a high content of crystalline cellulose. Besides, *Bacillus* sp. treated sample has a crystallinity index higher than that of the untreated sample, as can be seen in Table 2.

Previous XRD studies on aquatic plants, such as those by Bhetalu *et al*. [48], describe diffraction patterns in treated biomass, showing duckweed with a broad amorphous peak at 2θ = 22.1° and additional peaks at 2θ = 27.0° and 29°, indicating intensity changes. Rangel *et al*. [49] also detailed the thermal decomposition of *Eichhornia crassipes*, identifying four phases in its thermal degradation. Initial fiber weight loss occurs between 50 °C and 100 °C due to moisture and volatile component removal, with a small peak around 75–100 °C linked to water vaporization from cellulose, attributed to its OH group's hydrophilic nature [49]. These findings align with the present study.

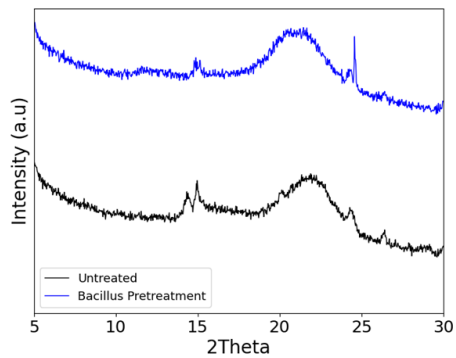

Figure 5: X-ray diffraction patterns of the cellulose from water hyacinth (*E. crassipes*) obtained from untreated sample and *Bacillus* sp. pretreatment.

The pretreatment of *Eichhornia crassipes* cellulose with *Bacillus* sp. influences cellulose crystallinity and composition due to the cellulolytic activity of *Bacillus* species, which enzymatically degrade and alter cellulose structure. Crystallinity, a crucial determinant of enzymatic saccharification efficiency, is notably affected by such treatments. Xu *et al*., reported that changes in crystallinity directly impact hydrolysis rates, as evaluated through X-ray diffraction (XRD) [50]. Similarly, studies by Akhtar *et al*., demonstrate that *Bacillus* sp. can disrupt crystalline structures in biomass, improving enzyme accessibility [51], [52]. The transformation of crystalline cellulose (cellulose I) into amorphous structures (cellulose II) during pretreatment enhances cellulose reactivity and increases its susceptibility to enzymatic hydrolysis [53].

Bacillus sp. also degrades lignin and hemicellulose, enhancing cellulose composition by improving enzyme access and purity, and optimizing it for biofuel production and other applications [54], [55]. The change in morphology of the cellulosic material obtained with the *Bacillus* sp. pretreatment and the untreated sample is reported in Figure 6.

The cellulose procured from *Bacillus* sp. pretreatment (Figure 6(A)) exhibits expansive longitudinal tracheids characterized by arrays of conspicuously flat-bordered pits and pit apertures devoid of or adorned with rudimentary vestures. These morphological features are typically observed in plant specimens renowned for their capacity to store substantial quantities of water. Although cellulose fibers are not observed, degraded parts due to

microbial action are evident. On the other hand, the image obtained from the surface of the untreated sample revealed a very compact and ordered morphological characteristic (Figure 6(B)), compared to the image obtained after pretreatment with *Bacillus* sp. [56], [57].

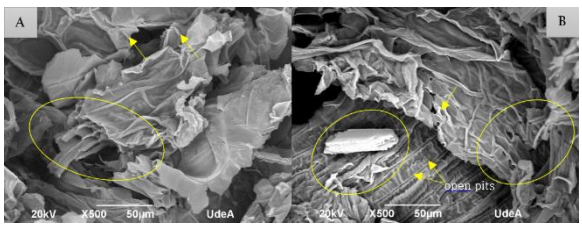

Figure 6: SEM images of cellulose at 500X derived from water hyacinth (*E. crassipes*) biomass. (A) Untreated. (B) *Bacillus* sp.

In general, the thermal degradation of water hyacinth (*E. crassipes*) cellulose, both pretreated with *Bacillus* sp. and untreated, unfolds in four sequential phases as depicted in Figure 7. The initial weight loss of the fibers within the temperature range of 50 °C to 100 °C is attributed to the expulsion of moisture and certain volatile constituents from the fibers [58]. The minor temperature peak observed around 75–100 °C is correlated with the vaporization of water within the cellulose structure, a phenomenon stemming from the hydrophilic nature of the hydroxyl (OH) functional group inherent to cellulose [49].

The weight loss resulting from water evaporation was comparable across all pretreatments, including cellulose obtained without pretreatment. This observation suggests a high moisture content within the cellulose fibers, attributable to the increased hydrophilic propensity of the crude fiber [47]. Regarding the second phase, the weight loss observed between 150 and 250 °C was associated with the degradation of amorphous hemicellulose components and cellulose glycosidic bonds. Hemicellulose exhibits the lowest thermal stability among the fiber constituents, undergoing rapid degradation within the temperature range of 200–350 °C [59]. Conversely, cellulose demonstrates greater thermal stability in comparison to hemicellulose. Lignin, characterized by its aromatic structure, exhibits the highest thermal stability among the components. Its thermal decomposition occurs within the temperature range of 160–900 °C, with the most rapid decomposition occurring between 310 and 420 °C [60].

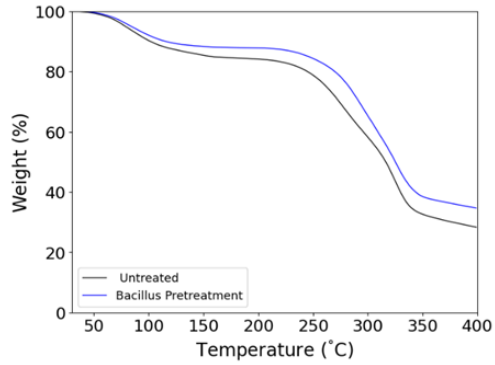

Figure 7: Thermogravimetric analysis (TGA) curves of the cellulose from water hyacinth obtained from the untreated sample and *Bacillus* sp. pretreatment.

The cellulose obtained with *Bacillus sp.* pretreatment and untreated cellulose started to decompose easily and the weight loss occurred between 100 °C and 325 °C. With the remarkable difference in cellulose obtained with *Bacillus* sp., pretreatment shows the splitting of cellulose signal between 290–325 °C, which could be due to the degradation of the amorphous components of the glycosidic bonds of both hemicellulose and cellulose [60]. This behavior may be related to the degree of crystallinity, and a higher thermal resistance of the cellulose obtained by this treatment. The values of the maximum heat flux corroborate this same behavior, with a higher heat release for the cellulose obtained, due to its less crystalline and ordered structure facilitating the thermal degradation process.

Figure 8 shows the representative DSC thermograms of the cellulose fibers obtained by pretreatment with *Bacillus* sp. and untreated at 400 °C. The results obtained indicate that the cellulose obtained with *Bacillus* sp. is the one that presents superior thermal stability, with a degradation onset temperature of 3 °C and 2 °C higher than the untreated cellulose. This behavior can be related to the degree of crystallinity since an increase or decrease in this property increases or decreases the thermal resistance of the celluloses. A higher heat release can be observed for the cellulose obtained without treatment than with the cellulose obtained with *Bacillus* sp. pretreatment, because its structure, less crystalline and ordered, facilitates the thermal degradation process [25].

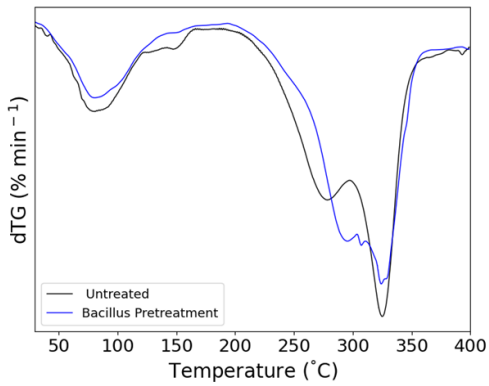

Figure 8: Differential Scanning Calorimetry (DSC) of the cellulose from water hyacinth (*E. crassipes*) obtained from untreated sample and *Bacillus* sp. pretreatment.

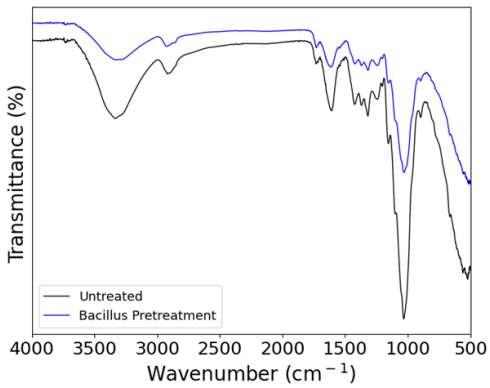

Figure 9: FTIR spectra of purified cellulose from water hyacinth obtained from untreated sample and *Bacillus sp.* pretreatment.

Figure 9 shows the FTIR spectra for the untreated cellulose and the cellulose obtained with *Bacillus* sp. pretreatment. It can be observed that there is variation between the intensity of the bands found in the range of 3453 cm^{-1} to 3200 cm^{-1}, which are indicative of O-H stretching vibrations for the cellulose of water hyacinth (*E. crassipes*) obtained untreated and by pretreatment with *Bacillus* sp. Particularly, for the cellulose obtained by the *Bacillus sp*. pretreatment, there was a decrease in the band located precisely at 3333 cm^{-1}, suggesting a low frequency of tension in the hydrogen bonds of the -OH group, due to the breaking of bonds by solvents in the cellulose, there was a decrease in the band located precisely at 3333 cm^{-1}, suggesting a low strain frequency in the hydrogen bonds of the -OH group, due to bond breaking by solvents in cellulose [61], [62].

For both celluloses, a peak around 1550 cm^{-1} to 1500 cm^{-1} corresponds to the C-H stretching of the aromatic structure of lignin [63]. However, a decrease in the peaks is observed in the samples pretreated with *Bacillus* sp. implying a delignification effect. Moreover, in cellulose obtained from *Bacillus* sp. pretreatment, this peak was eliminated, whereas in untreated cellulose, it persists slightly [64]. An increase in the intensity of the bands related to CH_2 stretching was found around 1450 cm^{-1} to 1300 cm^{-1} for untreated cellulose, which is also associated with the crystalline structure of cellulose. The bands between 1227 cm^{-1}, 1159 cm^{-1} and 1028 cm^{-1} show vibrations of the C-O-C, C-OH and C-O bonds belonging to cellulose, to the β-glucopyranose ring of cellulose characteristic of pyranose sugars. The peak in the region 1614 cm^{-1} is observed with different intensities for untreated cellulose and that obtained with *Bacillus* sp. pretreatment indicative of the O-H group of the adsorbed water. Finally, two peaks between 893 cm^{-1} and 953 cm^{-1} represent the vibration of the CH_2OH bond [65].

For both celluloses, a peak around 1550 cm^{-1} to 1500 cm^{-1} corresponds to C-H stretching of the aromatic structure of lignin. However, a decrease in the peaks is observed in the samples pretreated with *Bacillus* sp. implying a delignification effect. Moreover, in cellulose obtained from *Bacillus* sp. pretreatment, this peak decreases, whereas in untreated cellulose, it increases [65]. An increase in the intensity of the bands related to CH_2 stretching was found around 1450 cm^{-1} to 1300 cm^{-1} for untreated cellulose, which is also associated with the crystalline structure of cellulose. The untreated cellulose is less crystalline than the cellulose obtained with *Bacillus* sp. pretreatment. The bands between 1227 cm^{-1}, 1159 cm^{-1} and 1028 cm^{-1} show vibrations of the C-O-C, C-OH and C-O bonds belonging to cellulose, to the β-glucopyranose ring of cellulose characteristic of pyranose sugars. The peak in the region 1614 cm^{-1} is observed with different intensity for untreated cellulose and that obtained with *Bacillus* sp. pretreatment indicative of the O-H group of adsorbed water, is mostly observed in untreated cellulose. Finally, a peak between 893 cm^{-1} and 953 cm^{-1} represents the vibration of the CH_2OH bond, which decreases in the cellulose obtained with the *Bacillus* sp. Pretreatment [46].

3.3 *Synthesis of butyl levulinate*

Figure 10 shows the butyl levulinate yields from microwave-assisted reactions of cellulose obtained through different pretreatments for up to 90 min using a Keggin-type HPA ($H_3PW_{12}O_{40}$) as an acid catalyst. The Brönsted sites of the heteropolyacid effectively promote the conversion of cellulose to butyl levulinate [66]. The yield trend of butyl levulinate follows the order of cellulose obtained from *Bacillus* sp. pretreatment and the untreated sample.

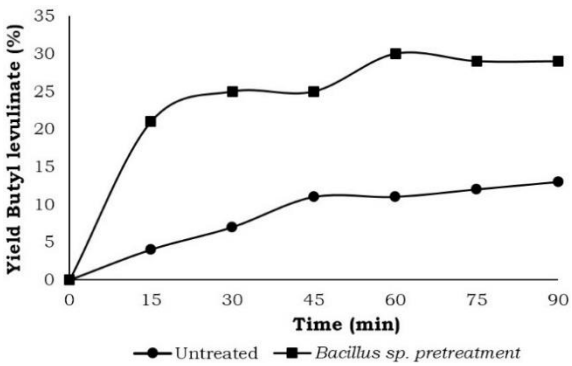

Figure 10: Yield to butyl levulinate of the cellulose from water hyacinth obtained from untreated sample and *Bacillus* sp. pretreatment.

This study demonstrates that *Bacillus* sp. pretreatment of *Eichhornia crassipes* enhances cellulose properties such as increased crystallinity, reduced water content, and lower hemicellulose and extractives which are crucial for efficient butyl levulinate (BL) production. The rise in crystallinity improves reactivity by increasing catalytic site accessibility during hydrolysis, resulting in higher BL yields. Reduced hemicellulose and water content further optimize the substrate by minimizing reactant dilution and unwanted byproducts [67].

The Keggin-type heteropolyacid (HPA) catalyst, specifically phosphotungstic acid, markedly enhances BL production due to its strong Brönsted acidity and distinctive polyoxometalate structure, which provides a high density of active sites. This unique structure, combined with the thermal stability and phase adaptability of HPAs, contributes to their exceptional catalytic efficiency. Furthermore, supporting HPAs on mesoporous materials has significantly improved their activity and selectivity in BL synthesis [67].

This order depends on the characteristics of the cellulose and suggests that cellulose with a higher crystallinity index (CrI), lower water adsorption, lower hemicellulose content, and reduced extractive proportion, influenced by *Bacillus* sp. ability to degrade various lignocellulosic components, could enhance butyl levulinate production by effectively promoting cellulose hydrolysis.

Reducing hemicellulose and extractives is critical for enhancing butyl levulinate (BL) yields from lignocellulosic biomass, as these components interfere with cellulose conversion processes. Hemicellulose hydrolyzes into sugars such as xylose, which dehydrate into furfural, a by-product that hinders BL production. This competitive reaction pathway, combined with the structural susceptibility of hemicellulose to premature degradation, generates complex mixtures that complicate product separation and lower levulinic acid and BL yields [68], [69].

Selective hemicellulose removal through treatments such as hydrothermal, biological, or acidic ionic liquids can produce a concentrated cellulose feedstock better suited for conversion. Biological pretreatment with *Bacillus* sp. enhances BL production by reducing lignin and hemicellulose content and increasing cellulose accessibility. The enzymatic activity of *Bacillus* sp., particularly xylanases, reduces cellulose crystallinity, improving hydrolysis and boosting levulinic acid and ester yields by breaking cellulose into fermentable sugars. This approach highlights the potential of biological pretreatment to optimize BL yields and advance lignocellulosic biomass valorization.

Guo *et al*., [70] investigated the laccase production and hydrolytic capabilities of diverse bacterial genera, including *Bacillus*, *Pseudomonas*, *Exiguobacterium*, and *Aeromona*s, within the context of *Miscanthus* pretreatment. *Miscanthus* was subjected to a 96 h pretreatment process by introducing the substrate into the culture medium containing the selected microorganism. Following pretreatment, the solid material was washed and subsequently subjected to enzymatic hydrolysis for a duration of 72 h, culminating in a lignin removal of approximately 30–60% [70].

The production of hydrolytic enzymes by bacterial genera, particularly the *Bacillus* family, plays a pivotal role in biotechnological processes such as waste degradation and bioremediation. Moreno *et al*., [71] characterized hydrolytic bacterial communities in heavy-metal-contaminated soils, highlighting the prevalence of Gram-positive bacteria, primarily from the *Bacillaceae* family, due to their robust enzyme production. Guo *et al*., [70] identified *Bacillus* strains

capable of secreting laccase, an enzyme essential for lignin degradation. Similarly, Zhang *et al*., [16] isolated strains such as *Pseudomonas extremorientalis* and *Bacillus tequilensis*, demonstrating that bacterial laccase production can surpass fungal production rates, offering significant advantages for bioprocessing applications.

These studies collectively emphasize the importance of bacterial hydrolytic capabilities and highlight how environmental conditions and carbon sources influence enzymatic production. While Moreno *et al*., [72] examined the ecological aspects of hydrolytic bacteria in contaminated soils, Guo *et al*., [70] and Zhang *et al*., [16] focused on practical applications in biomass degradation. Methodologically, Moreno *et al*., [72] used 16S rRNA sequencing for community characterization, whereas Guo *et al*., [70] and Zhang *et al*., [16] relied on enzyme assays, underscoring the multifaceted approach in hydrolytic enzyme research.

Together, these studies underline the biotechnological potential of hydrolytic bacterial genera, particularly *Bacillus*, in waste management and bioremediation applications.

Transforming the invasive *Eichhornia crassipes* (water hyacinth) into valuable compounds such as butyl levulinate (BL) presents significant environmental and economic advantages. As a disruptive aquatic plant, water hyacinth depletes oxygen levels and clogs waterways, causing ecological harm. Repurposing this biomass for BL production mitigates these impacts while reducing dependence on expensive removal methods. Economically, the plant's abundance and lignocellulosic composition make it an affordable and sustainable feedstock for chemical synthesis.

This study employs *Bacillus* sp. pretreatment and microwave-assisted reactions with Keggin-type heteropolyacid (HPA), showcasing a scalable, cost-efficient approach to industrial compound production with reduced energy consumption. Microwave-assisted treatment enhances energy efficiency (0.8 kWh/gEL compared to 2.94 kWh/gEL in batch reactors), shortens reaction times, and simplifies processes, thereby lowering the carbon footprint and promoting sustainable practices. Furthermore, biomass with higher crystallinity and reduced water absorption significantly improves BL yield, underscoring the importance of feedstock selection in optimizing both economic and environmental outcomes.

4 Conclusions

This study highlights the effectiveness of *Bacillus* sp. pretreatment in enhancing cellulose extraction from *Eichhornia crassipes* and its subsequent conversion into butyl levulinate (BL) through microwave-assisted acid hydrolysis using a Keggin-type heteropolyacid catalyst. Cellulose pretreated with *Bacillus* sp. achieved the highest BL yield, attributed to improved cellulose properties such as increased crystallinity and reduced impurities. Optimal growth conditions for *Bacillus* sp. were identified (glucose: 20 g/L, tryptone: 2.5 g/L, yeast extract: 7.5 g/L), with the acidic environment of water hyacinth fermentation promoting microbial adaptation and resulting in a 27% increase in cellulose content. These findings demonstrate the potential of biological pretreatment as a sustainable approach for biomass valorization, enabling greener and more efficient methods for converting water hyacinth into valuable industrial compounds. Future research should prioritize optimizing microbial pretreatment processes and scaling them for industrial applications.

Acknowledgments

A.F.M., G.A.C., J.J.M. acknowledge the financial support from Vicerrectoría de Investigaciones-Universidad Pedagógica y Tecnológica de Colombia and research group Procesos Ambientalmente Amigables (PROAM) (GRANT SGI 3651 project). GPR and AGS gratefully acknowledge the financial and scientific support of Universidad Nacional de La Plata (UNLP), Argentina, el Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-PIP 0111) y al FONDAGRO (Secretaría de Bioeconomía por el apoyo financiero). GPR y AGS are members of CONICET.

Author Contributions

G.P.R. and A.G.S.: conceptualization, reviewing and editing; G.A.C. and A.F.M.: investigation, methodology, writing an original draft; J.J.M.: data analysis. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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