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Biohydrogen Production and Quantitative Determination of Monosaccharide Production Using Hyperthermophilic Anaerobic Fermentation of Corn Stover

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Abstract: Second-generation biofuels from lignocellulosic biomass remain critical and require several challenges due to lignin compounds' inefficient degradation and recalcitrate characteristics. In this regard, this study focuses on enzymatic technology as a promising treatment that is beneficial in breaking down the biomass's hemicellulose and cellulosic parts. Thermostable bacterial species owe thermostable enzymes that are able to degrade complex carbohydrate compounds and produce efficient hydrogen production. The present study investigates the direct utilization of ligninolytic enzymes such as cellulase and xylanase derived from the hyperthermophilic bacteria *Thermotoga maritima* (ATCC 43589 strain). The results show that xylanase and cellulase enzymes extracted from *Thermotoga maritima* could depolymerize the lignin bonds of corn stover substrate and release monomers such as Galactose in the media. In conclusion, this study can open a new advanced research window on directly applying a hyperthermophilic consortium of enzymes capable of hydrolyzing lignocellulose material toward hydrogen production.

Keywords: biohydrogen; hyperthermophile; Thermotoga maritima; biofuels

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1. Introduction

The industrial revolution has increased greenhouse gas (GHG) emissions by more than 40% [1]. The continuous increase of GHG emissions due to the increased demand for fossil fuel consumption requires an urgent need for a green replacement solution. In this regard, biofuel generation from different waste resources is among the strategies that benefit from being a renewable source and providing clean energy (with no harmful emissions). Reusing agricultural and forestry residues to produce biofuels is an approach towards the second generation of biofuels [2]. In recent decades, due to its abundant generation, there has been a growing initiative to produce biohydrogen from woody biomass with a lignocellulose foundation. The global annual production of such waste is estimated to increase to more than 220 billion tonnes, equal to 60 billion tons of crude oil [3].

Moreover, yearly demands for biohydrogen as a clean energy source are increasing by 10% compared to the last decade, and it is estimated that biohydrogen will represent 80% of total energy demand by 2025 [4]. However, the degradation of complex lignocellulose structures from different plants has remained challenging. One of the major components of lignocellulose biomass is hemicellulose, made of xylan compounds with a backbone of β-1,4- linked with xylose sugar (Figure 1), which makes it difficult to break down in nature.

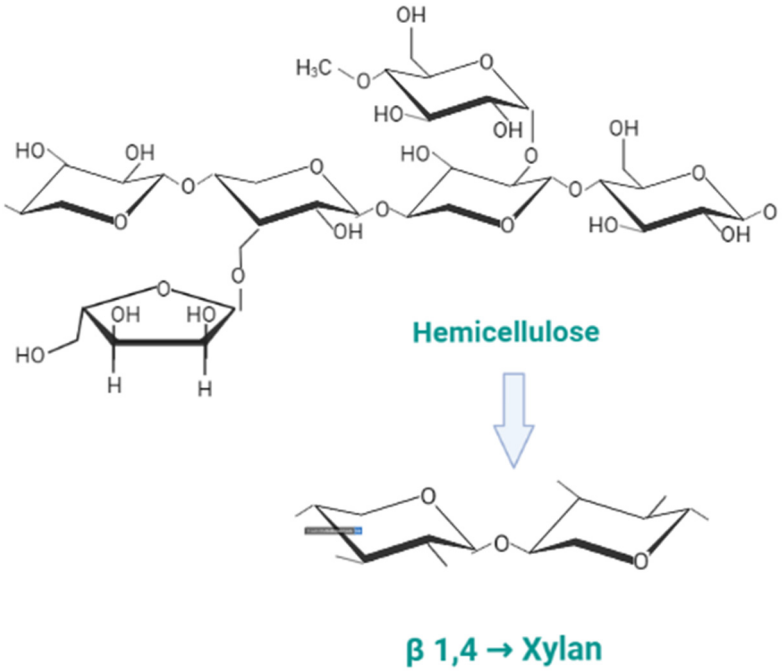


Figure 1. Hemicellulose structure with xylan bonds.

Biohydrogen (H_2) production from lignocellulosic compounds through biological processes has garnered significant scholarly interest. Notably, there is a growing emphasis on advancing fermentation techniques, with in-depth investigations into feedstock utilization, reactor design, and optimizing H_2 production within this system. From a technological point of view, a study on the fermentative hydrogen-producing bacteria could help implement large-scale reactors that benefit the country's economy. Hence, bioconversion of lignocellulose material into H_2, using bacterial and enzymatic degradation, is the key focus. However, there are ongoing concerns regarding the production of lignocellulose degrading enzymes due to its economical viability and practical application [5].

Among various hydrogen-producing bacteria, hyperthermophiles have become a central focus for numerous industries because these microorganisms can continue growing under elevated temperatures ≥80 °C. Several hyperthermophiles have been isolated from various sources, such as thermal natural habitats or hot industrial environments. Most isolated hyperthermophiles belong to archaea, while the Thermotogales family is the only hyperthermophilic bacterial order so far discovered [6]. The interesting fact is that Thermotogales roots in extreme thermophiles, which can grow up to 95 °C. According to the phylogenetic tree of Thermotogales, *Thermotoga maritima*, and *T. naepolitina*, with 96% strain sequence similarity, have optimum growth at 80 °C. Hence, they can serve as the best option for the efficient production of H_2. Shao et al. [7] reported that *T. maritima* (*T.ma*) can efficiently produce hydrogen from simple and complex carbohydrates. The authors studied the optimal *T.ma* growing culture that results in higher growth of the bacteria under 80 °C temperature. Until now, there is limited information on using *T.ma* bacteria to successfully degrade lignocellulosic compounds. To the best of our knowledge, a few studies [8–10] are available using the pure substrate to analyze biohydrogen production using *T.ma*.

Saidi et al. [11] examined cellulosic material, such as kitchen and garden waste, in which 285 mmol/L hydrogen yield was achieved. The effective breakdown of lignocellulosic biomass has emerged as a significant bottleneck in industrial processes. However, no study has reported the application of such complex materials with successful hydrogen production without prior substrate pretreatment. Biochemical conversion of biomass is considered a leading technology to overcome this issue by facilitating the breakdown of complex carbohydrate compounds into monomeric sugar compounds. Enzymatic technology has received enormous attention due to eliminating the acidic/alkaline chemicals that

could harm the environment. Previous studies reported hydrogen production is higher in Thermophilic bacteria than in mesophiles [12,13]. Their operation at high temperatures minimizes contamination risks and enhances pathogenic destruction while also improving reaction rates, lowering viscosity, and facilitating better media mixing [12,14]. Therefore, in-depth research is vital to explore the efficient use of such hyperthermophiles and take advantage of their enzymes to degrade complex lignocellulose structures. Therefore, in-depth research is vital to explore the efficient use of such hyperthermophiles and take advantage of their enzymes to degrade complex lignocellulose structures.

Energy crops such as maize (*Zea mays* L.), wheat (*Triticum*), Oat (*Avena sativa*), or other woody plants like oak (*Quercus*) need special delignification pretreatment before the fermentation process. Maize is the dominant substrate among energy crops; however, it can only produce 205–450 methane yield m^3 per tonne of volatile solids [15]. Thus, this study will fill in the gap in knowledge of efficient H_2 production from corn stover (cobs of maize) using thermostable enzymes derived from a thermophilic bacterium. For optimal utilization of source material, comprehending the pattern of the lignocellulosic wall and identifying target bonds are crucial. This knowledge enables maximum penetration into the cell wall, leading to accelerated degradation. In this regard, the identification of sugar in lignocellulose is of great interest in exploring which enzymes can degrade the cell wall of the biomass. Both hemicellulases and cellulases are important enzymes in biorefinery processes. Wu et al. [16] showed that xylanase from *T.ma* depicted the highest activity at the extreme temperature >95 °C. The importance of xylanase over other enzymes is its ability to break down the xylan into smaller monomers. Therefore, the current research will investigate the application of thermostable enzymes such as xylanase and cellulase from *T.ma* to examine the feasibility of biodegradation of corn stover to produce biohydrogen under dark fermentation without any prior pretreatment process.

2. Materials and Methods

2.1. Chemicals and Reagents

Carboxymethyl cellulose (CMC) (CAS No: 9004-32-4), xylan from beechwood (CAS No: 9014-63-3), 3,5-dinitrosalicylic acid (CAS No: 609-99-4), and 2,20-Azino-bis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (CAS No: 30931-67-6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade. Corn stover was purchased from a local farm in Zhenjiang, Jiangsu province, P.R. China. The samples were shredded, sieved (<2 mm), and stored at room temperature. Further chemicals and reagents used in this experiment are as follows: D (+)- Glucose (CAS No: 50-99-7), D (+)- Xylose (CAS No:58-86-6), D (+)- Galactose (CAS No: 59-23-4), and Galacturonic Acids (CAS No: 91510-62-2), Sodium cyanoborohydride ($NaBH_3CN$) (CAS No: 25895-60-7), dimethyl sulfoxide (DMSO) (CAS No: 67-68-5), 4-Aminobenzoic acid (*p*-ABA) (CAS No: 150-13-0), ammonium formate (CAS No: 540-69-2), calcium chloride dihydrate (CAS No: 10035-04-8), and tris(hydroxymethyl) aminomethane (CAS No: 77-86-1), Formic acid (HCOOH), sodium hydroxide and hydrochloric acid, were purchased from Sigma-Aldrich. Acetic acid Glacial (CAS No: 64-19-7) and methanol (CAS No: 67-56-1) (HPLC grade, >99% purity) were purchased from Merck (Darmstadt, Germany).

2.2. Strain, Media, and Growth Conditions

The microorganism used for H_2 production in this approach was *T. maritima* strain (ATCC 43589). The strain was preserved in 15% glycerol at −80 °C, according to Leonard et al. [17]. *Thermotoga maritima* basal (TMB) medium with the following composition per liter: glucose, 1.5 g KH_2PO_4; 4.2 g $Na_2HPO_4.12H_2O$ (22 mM PO_4); 0.5 g NH_4CL; 0.2 g $MgCL_2$. 6 H_2O (1 mM); 27 g NaCl; 4 g yeast extract [18] was used for the optimum growth of *T. maritima*. The media was prepared in anaerobic conditions by flushing with sterile nitrogen gas and Na_2S (0.4 g L^{-1}) while the pH was adjusted at 7.0 with 1 M NaOH. After inoculation with *T. maritima* (10% *v*/*v*), the serum bottles were incubated at 80 °C in a static water bath. These precultures were used to inoculate the bioreactor [18].

2.3. Ezymes Extraction from T. maritima Bacterium

A novel plasmid pHsh was used to construct the expression vector pHsh-*TmaxynlnB* [19]. The recombinant plasmid was transformed into *E. coli* JM109 using the heat shock method [20]. The transformed cells were then grown on Luria broth (L.B.) agar plate and incubated at 30 °C for 24 h. One colony was transferred to a fresh 200 mL of L.B. media supplemented with 100 µg/mL of antibiotic ampicillin and incubated at 30 °C with shaking at 200 rpm to an OD_{600} of 0.8 before heat shock and incubated further at 42 °C, 200 rpm for 8 h for protein expression. Successively, the cells were harvested by centrifugation at 7000× *g* for 20 min at 4 °C, the supernatant was discarded, and the obtained cell pellets were re-suspended in binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris) pH 8.0. The cells were disrupted by sonication for half an hour. This cytoplasmic extract was heat treated for 15 min at 75 °C, centrifuged at 10,000× *g* for 30 min at 4 °C and supernatant was loaded on Nickle affinity column, and eluted using elution buffer (500 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl). The same method was used to express and purify cellulase, xylanase, and pectinase from pHsh-*T.ma.* The purified enzymes were conserved in the stock solution (25 mM pH 6.8 PI buffer, 20% glycerol, 1 mM EDTA, and 0.02% sodium azide) for future use.

2.4. Experimental Setup-Batch Fermentation Process

The batch fermentation experiment of *T. maritima* for H_2 production was conducted under anaerobic conditions. The reaction mixture consisted of 1 g of sieved substrate in 100 mL flat-bottomed flasks tightly covered with rubber stoppers. The headspace air and trace amounts of oxygen in the fermentation medium were displaced by nitrogen gas to ensure anaerobic conditions. The bioreactors were designed as triplicates and incubated in two batches: (1) 12 h incubation and (2) 24 h. in a water bath with an exact degree of 80 °C. After the pre-incubation period (12 or 24 h), 5 mL of fresh *T. maritima* culture (OD:0.5) and 500 µL of extracted enzymes (cellulase and xylanase) were added to start the fermentation process. Samples at different time intervals (2, 4, 6, 8, 10, and 12 h) were collected to measure the enzymatic activity and hydrogen volume production.

2.5. Enzyme Activity Essay

The activity of the purified enzyme was determined by calculating the amount of reducing sugars released during substrate hydrolysis using the 4-hydroxybenzoic acid hydrazide method [21]. The reaction mixture, containing 100 µL of 0.5% (*w*/*v*) substrate in water, 95 µL of phthalate-imidazole (P.I.) buffer (25 mM, pH 6.0), and 5 µL diluted enzyme, was incubated at a desired temperature for 5 min. The reaction was stopped by adding 600 µL of 4-hydroxybenzoic acid hydrazide. This solution was boiled for 10 min, cooled, and the absorbance was measured at a wavelength of 410 nm. All the enzymatic activity assays were performed in triplicates, and data were reported as mean ± standard deviation. One unit (U) of enzyme activity is defined as the amount of enzyme needed to release 1 µmol of reducing sugars per minute [22]. The total protein of the enzymes was assessed using the Bradford method, and the absorbance was recorded at 510 nm wavelengths.

2.6. HPLC Analysis of Inhibitory Products

The HPLC system (SHIMADZU, made in Kyoto, Japan) consisted of a low-pressure quaternary gradient unit (model LG-1580-04) with an in-line degasser (model DG-1580-54) and an auto-sampler (model AS-950). The system had a photodiode array detector (model SPD-20A UV/vis multiwavelength detector). The mobile phase consisted of (A) water and (B) methanol, both containing 0.1% of formic acid and ammonium formate (20 mmol L^{-1}) in gradient mode (100% to 65% of A in 40 min followed by an equilibration step to 100% of A in 20 min). The flow rate was 0.3 mL min^{-1}, and the detection wavelength of the D-galacturonic acid (G.A.) derivative was 304 nm. A total of 20 µL of syringe-filtered sample was injected into the column that was kept at room temperature. An 8-points calibration curve was used to measure the calibration curve of each sugar in HPLC. The analyte in each sample was identified by comparing its retention time (Rt ≈ 40 min) and

U.V.–vis spectra with those of standard solutions. Peak purity was checked to exclude any contribution from interfering peaks.

Derivatization Process

The procedure employed for derivating the sugars with *p*-ABA was carried out following the method described by Moreira et al. [23]. Reductive amination of the analyte with *p*-ABA (0.35 M) was performed in a DMSO/acetic acid solution (70:30 *v*/*v*). Every time, the reagent solution was prepared fresh. An aliquot (200 µL) of the standard solution or sample, the *p*-ABA solution (500 µL), and $NaBH_3CN$ (10 mg) were mixed in 10 mL polyethylene vials. The vials were tightly capped and heated for 15 min at 60 °C. Then, the vials were cooled to room temperature, and the reaction mixtures were dissolved in water (6.3 mL) containing 0.1% of formic acid and ammonium formate (20 mmol L^{-1}), filtered through 0.45 µm Nylon membranes analyzed by using HPLC.

3. Results and Discussion

3.1. Physical Monitoring of the Substrate during the 80-Degree Incubation

The cell wall of the cornstover consists of different microfibrils, such as cellulose, hemicellulose, lignin, and pectin (Figure 2). Hence, breaking down each bond requires selecting one or consortia of enzymes. For instance, the degradation of the complex chemical composition of xylan requires the synergistic action of several extracellular enzymes that help to increase the yield of fermentable glucose and xylose [24].

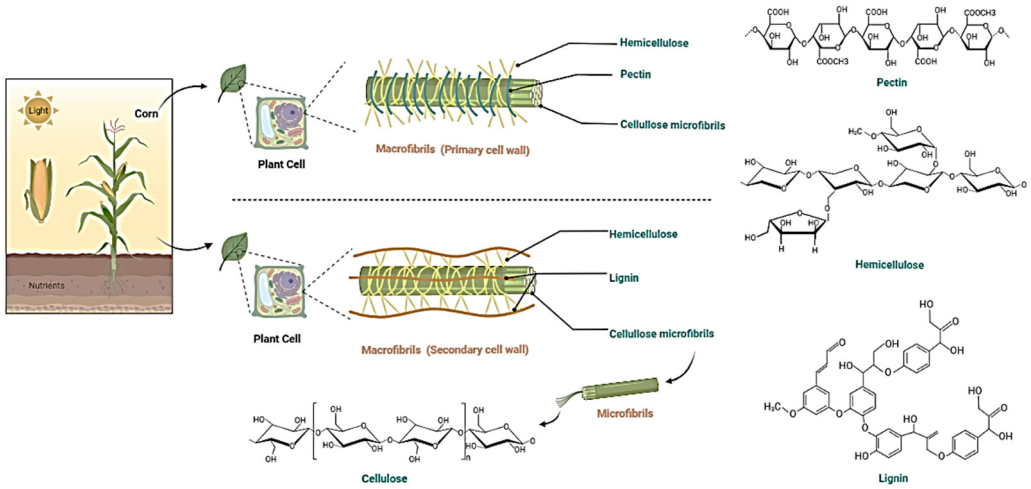


Figure 2. A schematic wall structure of corn stover cell wall and structural bonds (Figure created with BioRender.com).

With the awareness that the primary cell wall of corn stover primarily comprises hemicellulose and pectin as the main constituents, three key hyperthermophilic enzymes, specifically cellulase, xylanase, and pectinase, were isolated from *T.ma* and employed to assess their hydrolytic impact on corn stover. Xylan is a polysaccharide made up of β-1,4-xylose units or β-1,4-mannose units [25], and the synergistic action of endo and exo-acting enzymes will help in cleaving the glycosidic bonds and releasing these sugar monomers. For instance, endo-1,4-β-xylanase (simply referred to as xylanase) is an essential enzyme that acts on the xylan backbone of the hemicellulose with high specificity, negligible substrate loss, and side products [25].

The core objective of this study was to study the biological degradation of corn stover without prior pretreatment processes such as alkaline or acidic treatments. Hence, to create an environment conducive to the growth of hyperthermophilic bacteria, the bioreactors were incubated in a water bath at 80 °C for a duration of up to 24 h before introducing the enzymes. Figure 3 shows the progression of the bioreactors during the 12 and 24 h incubation time. Interestingly, the physical changes in the substrate's structure became

highly evident over the 24 h period, with a gradual buildup of dark brown substances at the bottom of the bioreactor. These observable changes can be attributed to the initial delignification of the lignocellulose substrate. Liu et al. [26] stated that, with heat preservation of corn stover for a minimum of 1 hr, the glucan conversion increases from approximately 42% to 83%. This could elucidate the emergence of dark substances in our sample bioreactors. The partial degradation of the intricate polymers might be further intensified through enzymatic hydrolysis, wherein the specialized enzymes can permeate the cell wall of the corn stover and liberate the sugar monomers. Several other studies have also demonstrated strong positive correlations between delignification and sugar released from enzymatic hydrolysis [27].

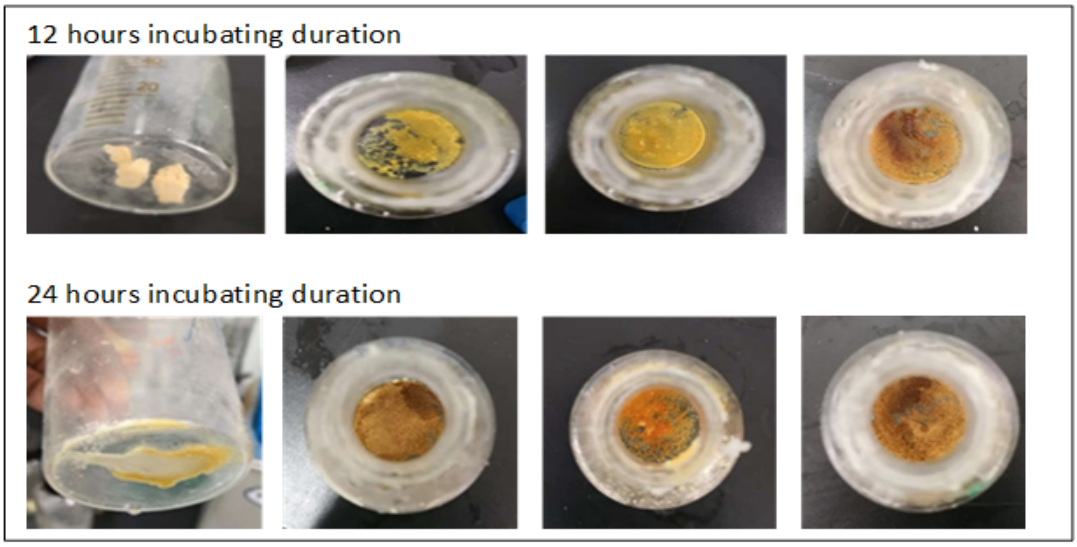


Figure 3. Corn stover batch bioreactors during the 12 and 24 h of water bath incubation at 80 °C. Photo has been taken from the top of the bioreactor for clear visibility of degradation.

3.2. Enzymatic Degradation during the Dark Fermentation of Corn Stover Using T. maritima

This experiment assessed the effects of pre-incubating lignocellulosic substances for 12 and 24 h in an 80 °C water bath before the addition of hyperthermosable enzymes. After the heat incubation (80 °C), bacterial inoculum and purified hyperthermophile *T.ma* enzymes (xylanase and cellulase) were added to the bioreactors. The hydrolysis effect and hydrogen production were analyzed for a period of 96 h (Figure 4). The findings from the 24 h incubation at 80 °C indicate a continuous increase in cellulase and xylanase enzyme production in comparison to the 12 h incubation period, as depicted in Figure 3. The results align with the observed rise in protein concentration in the samples incubated for 24 h. Thus, it can be concluded that pre-incubating lignocellulosic substances for up to 24 h before inducing the enzymes enhances the production of ligninolytic enzymes, thereby resulting in degradation.

As stated in Section 3.1, a substantial amount of brownish substances was identified at the bottom of the bioreactor bottles during fermentation, as illustrated in Figure 3. These brownish substances were developed as the experiment progressed up to 96 h. This coincided with a decreasing pattern in enzyme development and total protein concentration. Therefore, the decline in enzymatic growth throughout the 96 h process could be attributed to delignification and the production of sticky substances resulting from the breakdown of the lignocellulose compound. A similar pattern was observed previously by Agbor et al. (2011) [28], and the authors concluded that the sticky material acts as the inhibition factor, which diversely affects the enzymatic growth scale. The results of the protein concentration between the two sets of pre-incubation suggest that 24 h of incubation has possibly eased to cleave the cellulosic bonds of the substrate and a better exposure of the enzymes to the substrate and increase in releasing the sugar content. Hence, it is vital

to study which sugar monomers will be released into the matrix during heat incubation. Understanding the released sugars will help in the selection of the enzymes that can attack the particular compounds, leading to increased degradation proficiency and biohydrogen yields. Therefore, a modified HPLC method has been applied to detect the low sugar molecular weight, according to Fischer et al. [29].

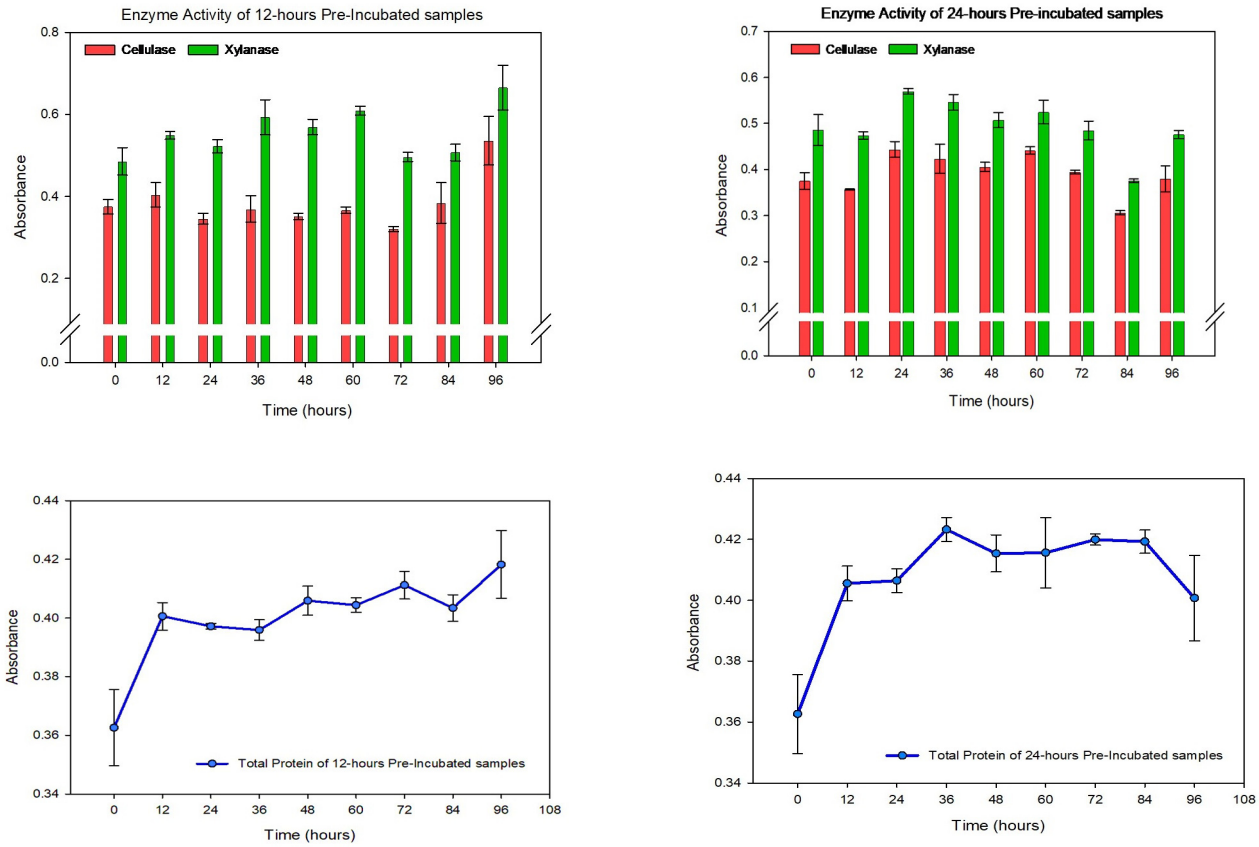


Figure 4. Protein concentration and enzyme activity of Cellulase and Xylanase during 12 and 24 h pre-incubation.

Figure 5 illustrates the volume of hydrogen production per g substrate. According to the results, the maximum hydrogen production was observed at the 12th hour, attributed to the addition of enzymes at that time. However, the trend of hydrogen production decreased over the subsequent 96 h. Similarly, when enzymes were added after 24 h of incubation (dark green), the maximum hydrogen production occurred at the 24th hour, reflecting the immediate action of enzymes. It is noteworthy that although hydrogen development decreased over time in both reactors (12 and 24 h of incubation), the hydrogen production volume in the 24 h incubation was higher than that in the 12 h incubation. Ligninolytic enzymes play a crucial role in the degradation of lignocellulosic substrates [30], and 24 h of pre-incubation has facilitated the breaking down of the lignocellulosic bonds and eased the hydrolysis process.

According to Akinbomi, J. and M.J. Taherzadeh [31], a decrease in H_2 production throughout the fermentation process could be related to the presence of alcoholic, aldehydes or lactones compounds released during the process, which inhibit the growth of many microorganisms. Since this preliminary study aims to address the knowledge gap on lignocellulose breakdown using enzyme treatment technology, the successful generation of biohydrogen demonstrates a promising application of this strategy. However, definitive conclusions cannot be drawn at this stage. Moreover, further investigation is necessary to analyze potential inhibitors of biohydrogen production, necessitating a more in-depth examination.

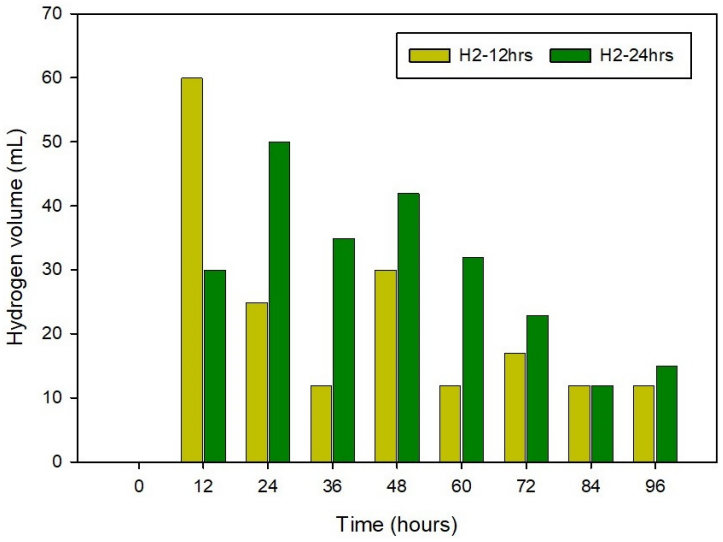


Figure 5. Volume of hydrogen production in 12 and 24 h pre-incubation.

3.3. Determination of Sugar Monomers of Delignified Corn Stover

An investigation into the separation of ABA-sugar derivatives of specific monosaccharides using a C18 column in conjunction with stationary and mobile phases, monitored via a UV system, was conducted accordingly. The monosaccharides are not able to separate on the C18 column using RID due to the fact that the chromatic peaks are too broad. Hence, this work aimed to develop a rapid, reproducible analysis method for quantifying different monosaccharides released during the fermentation process using *T.ma.* An experimental established practice named the "derivatization method" can improve the chromatographic properties of the analyte [32]. The derivation by reductive amination helps the compounds with low absorption in the U.V. region to be able to transform into compounds of high absorption. Researchers have used different reagents for chemical derivatization, such as 8-aminopyrine-1,3,6-trisulfonate (APTS) [33] and Fmoc-hydrazine [34], (*para*-aminobenzoic acid) [35,36], etc. Among them, derivatization with *p*-ABA is the most convenient as it does not require special equipment. Moreover, *p*-ABA is one of the popular labels that react with reducing carbohydrates [29].

The calibration curve of p-ABA and the standards used in this study were performed to detect the exact peak area (Figure 6). This figure provides a clear depiction of the HPLC chromatogram, indicating the occurrence of amination at 35 min, with the glucose standard detected within the 30 to 35 min timeframe. To validate our findings, the subsequent phase involved identifying the necessary standards for the quantitative analysis of our samples, including xylose, Galactose, and galacturonic acids.

The optimized HPLC procedure involved varying the hydrolysis time, specifically 15, 30, 45, and 60 min, to assess the suitability of the suggested method. Peak identification was accomplished by comparing the retention times with standard reference samples. Figures 7 and 8 display the chromatograms obtained from the corn stover sample at the initial time, before any treatment was applied, and contrast it with the sample after 24 h of incubation in an 80 °C water bath.

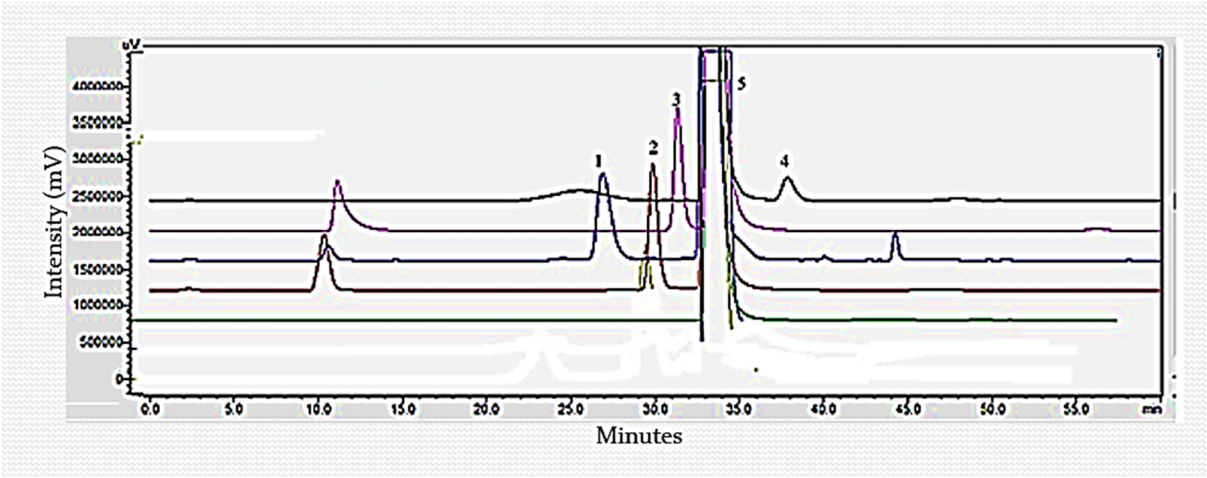


Figure 6. Chromatogram obtained in HPLC for (1) Galacturonic acid, (2) Galactose, (3) Glucose, (4) Xylose, and (5) reductive amination of p-ABA.

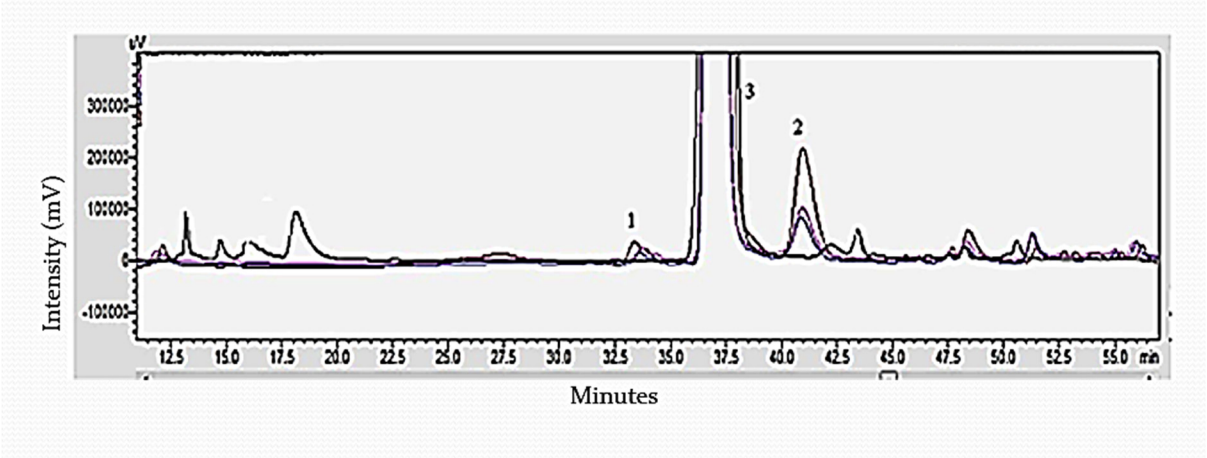


Figure 7. Chromatograms were obtained in HPLC for the control samples at different hydrolyzed times: 15, 30, 45, and 60 min. (1) Glucose, (2) Xylose, and (3) reductive amination of p-ABA.

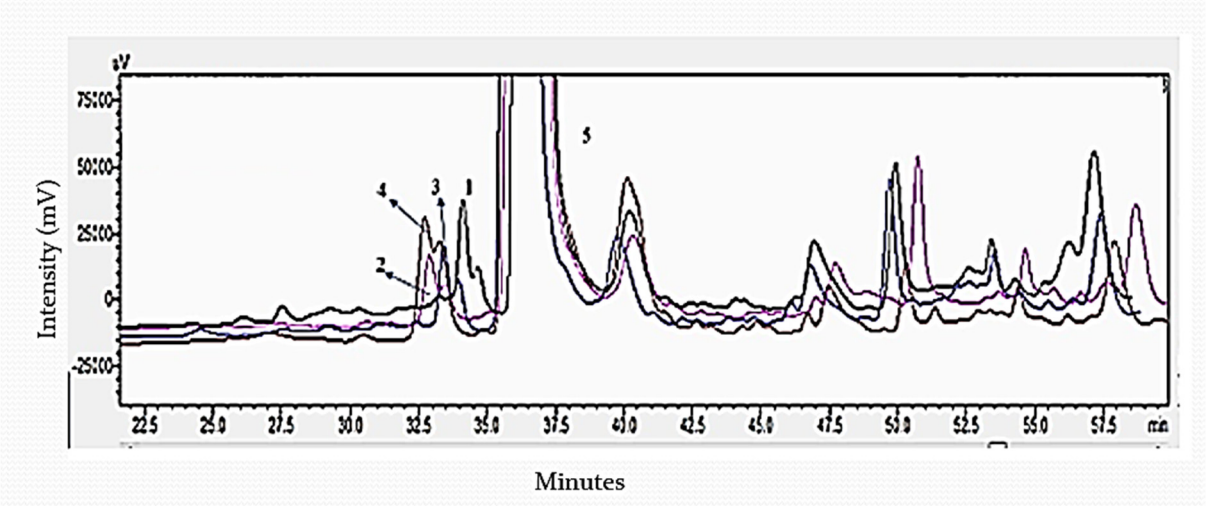


Figure 8. Chromatogram obtained in HPLC for the 24 h incubated samples at different hydrolyzed times 15, 30, 45, and 60 min. (1) Galacturonic acid, (2) Galactose, (3) Glucose, (4) Xylose, and (5) reductive amination of p-ABA.

As illustrated in Figure 7, the chromatogram obtained from the sample at time zero displayed very minimal peaks. This can be attributed to the intricate polysaccharide structure of corn stover, rendering it scarcely detectable. Notably, peak number 2 directly corresponds to the xylose standard peak, indicating the substantial presence of Xylan in the mixture. In contrast, Figure 8 reveals the samples after the incubation period, and it is evident that monosaccharides have been released, becoming detectable in the chromatogram. During the 15 min hydrolysis, only glucose and xylose were discernible in the mixture. However, by extending the hydrolysis time to 30 and 45 min, the chromatogram obtained from HPLC exhibited the presence of xylose, galacturonic acid, and glucose.

The chromatogram results obtained from the samples after 24 h of pre-incubation showed the release of Galacturonic acid and Galactose in the mixture.

This observation aligns with prior literature, which indicates that *T.ma* has the metabolic capability to process a wide range of substrates, encompassing simple sugars polysaccharides spanning from hexose and pentose monomers to starch and xylan polymers [37].

The findings of this study confirm the necessity of introducing particular enzymes capable of deconstructing the intricate lignocellulosic composition of corn stover. For instance, an enzyme responsible for breaking down the Galacturonic acid structure is essential in addition to xylanase. Fundamentally, the Galacturonic acid chain forms the homologous backbone of pectin. Consequently, forming the pectin structure necessitates using degradative enzymes capable of breaking down the pectate matrix. Therefore, it is imperative to delve into more comprehensive investigations regarding the structure and function of pectin-degrading enzymes derived from hyperthermophilic bacteria like *T.ma* to facilitate efficient lignin degradation for successful biohydrogen production.

4. Conclusions

Thermostable enzymes capable of functioning at elevated temperatures, typically 45 to 100 °C, have long been sought after in industrial production and bioprocessing research. In this study, *T.ma*, a hyperthermophilic anaerobic bacterium, was employed to target the degradation of lignocellulosic biomass, such as corn stover. The complex polysaccharide structure of corn stover necessitates the action of cell wall lytic enzymes secreted by microorganisms to break down plant cell walls into simpler sugars efficiently. Consequently, the use of an enzymatic consortium with varying substrate specificities is imperative for effective deconstruction.

Our experiment revealed that a 24 h incubation at 80 °C before introducing the bacterial inoculum provides an alternative method to facilitate the breakdown of complex hydrocarbon cell walls, enabling enzymes to access the intricate carbon chains. The HPLC results show the presence of galacturonic acid as one of the primary monomers released during the delignification process. Observing galacturonic acid in the media ensures the successful delignification process. Further study is necessary to investigate the addition of pectinase enzyme in the enzyme consortia and evaluate the hydrogen production. These results pave the way for potential industrial applications of crude enzymes derived from hyperthermophiles in the pursuit of sustainable biofuel production. In summary, enzymatic treatments play a vital role in converting lignocellulosic biomass into fermentable sugars, which are subsequently used in biofuel production.

Author Contributions: Methodology, W.S.; Validation, W.S.; Investigation, P.F.R.; Data curation, B.S.; Writing—original draft, P.F.R.; Writing—review & editing, R.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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