

Original Research

Enzymatic Hydrolysis of Soybean Hull Without Pretreatment and Its Enhancement of Bioethanol Production Using Xylose-Fermenting *Escherichia coli* (FBR5)

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Abstract

Background: Lignocellulosic materials, such as soybean hulls, possess a complex and recalcitrant structure that requires efficient pre-treatment or enzymatic processing for effective conversion into valuable products. However, pretreatment processes often generate inhibitory byproducts (e.g., furfural, hydroxymethyl furfural (HMF), phenols, and lignin degradation products), which can impede enzymatic activity and increase overall production costs. This study explores soybean hulls, a byproduct of oil and meal production, as a potential high-carbohydrate biorefinery resource, assessing their chemical composition, fermentable sugar recovery, and bioethanol production potential. **Methods:** Soybean hulls (5%, w/v dry basis) were subjected to enzymatic hydrolysis at 50 °C for 72 hours, utilizing a dual impeller mixing system at 250 rpm. An enzyme load of 45 mg enzyme protein per gram of solids was applied using a combination of commercial enzyme preparations, including Cellulase Blend and Multifect Pectinase. Conversion of cellulose, xylan, and arabinan into fermentable sugars was quantified. A moderate enzyme loading of 10 mg enzyme protein/g solids was also tested for comparison. Microbial fermentation was carried out using the xylose-fermenting *Escherichia coli* FBR5 strain to produce bioethanol. **Results:** Hydrolysis of untreated soybean hulls resulted in conversion yields of 94.4% for glucan, 72.6% for xylan, and 69.3% for arabinan into glucose, xylose, and arabinose, respectively. In comparison, control experiments without cellulolytic enzymes showed significantly lower conversion yields (14.2%, 20.1%, and 15.5% for glucose, xylose, and arabinose, respectively). A moderate enzyme loading of 10 mg enzyme protein per gram of solids achieved a cellulose conversion of 90.6%, which was nearly equivalent to the conversion obtained with 45 mg enzyme protein/g solids. Microbial fermentation with *E. coli* FBR5 resulted in 94% theoretical ethanol yield, with a production rate of 0.33 g/L/h and a productivity of 0.48 g ethanol/g sugar. **Conclusions:** The study demonstrates that enzymatic hydrolysis of soybean hulls, which are rich in cellulose and hemicellulose, can be effectively conducted without the need for pretreatment. The moderate enzyme load used in this study provides a promising platform for efficient sugar release and bioethanol production, presenting a cost-effective and viable approach for utilizing soybean hulls in biorefinery applications.

Keywords: soybean hull; enzymatic hydrolysis; xylose-fermenting *Escherichia coli*; FBR5 strain; ethanol; fermentation

1. Introduction

Soybean is considered one of the most essential agricultural crops, and its production has been steadily raised to satisfy the demands for traditional food and industrial applications. Increasing attention to functional foods, natural ingredients, and dietary additives promotes worldwide soybean consumption, production, and continuous improvement in seed resources [1–3]. Soybeans are widely recognized for their high-quality protein content, which makes up about 35–40% of their dry weight. They provide a well-balanced amino acid profile, including essential amino acids like lysine and methionine. Additionally, soybean oil is primarily composed of unsaturated fatty acids, such as omega-3 and omega-6, making it an important source of essential fatty acids [1–3]. In particular, a high protein component in green soybeans (40 g per 100 g dry weight) is attractive to use in various food applications such as fat-free soybean meals, textured vegetable protein (TVP), less-

expensive source of protein for animal feedstock, and substitutions for meat and dairy foods [4,5]. According to the World Agricultural Crop Productions Report from the U.S. Department of Agriculture, annual soybean production in the U.S. is estimated to be approximately 113 million metric tons (4152 bushels) in 2023/2024, which accounted for 28.4% of the total world soybean harvest of 399 million metric tonnes and forecast to be expanded in the course of time (<https://fas.usda.gov/data/world-agricultural-production>).

Soybean hulls, the outer covering skin of soybeans, constitute around 8–10% of the soybean dry weight and are released during the soybean harvesting/residue processing steps, roughly estimating 24.8 million tons from the global soybean hull production volume [6,7]. With the high polysaccharide composition in soybean hulls, it is ordinarily ground together with outer skins (okara, soybean meal, molasses) and processed for commercial uses of supple-



mental feed and dairy cattle in pelleted and grounded forms [7–9]. Several researchers have investigated the utilization of soybean hulls as a feedstock, evaluating the effectiveness of various hydrothermal pretreatments in the presence of sulfuric acid [10] and imidazole [11], followed by enzymatic hydrolysis and bioethanol production. The addition of sulfuric acid or imidazole resulted in increased sugar concentrations, leading to bioethanol production of up to 33.9 g/L and 145 g ethanol/kg dry soybean hulls (corresponding to 0.145 g ethanol/g solids), respectively. More recent work by Vedovatto *et al.* [3] demonstrated that supercritical water hydrolysis can liberate polysaccharides into monomeric sugars, allowing for ethanol production of 3.6 g/L. Despite the considerable carbohydrate fraction remaining in soybean by-products, further utilization of soybean residues and waste materials has received limited attention due to challenges in practical techniques and economic viability at a large pilot-scale industry level. In particular, the use of chemical supplements, acids, and additional processing steps decreases overall sugar yields and increases the associated costs, which hinders the economic feasibility of these processes.

Our previous work on the low lignin lignocellulosic biomass showed that it was feasible to enzymatically hydrolyze cellulose in corn pericarp (derived from the outer skin layer of the corn kernel, <5% lignin) to glucose without pretreatment and in the presence of enzyme mixtures with an impeller mixing [12]. No pretreatment step led to relatively less release of potential inhibitors, resulting in 85% glucose conversion at 5 mg protein/g solids (22 mg/g cellulose) enzyme dosage. While several physicochemical pretreatments and attractive attempts have been studied to improve the sugar yields and further valorize the carbohydrate content in soybean hulls [2,3,5,10–12], the cellulolytic conversion of untreated soybean hulls and their utilization for producing value-added molecules remain largely unexplored. Considering the lack of this knowledge, the current work examined the chemical composition of soybean hulls, morphological/physical changes before/after hydrolysis with the impeller mixing via scanning electron microscopy (SEM), cellulose/hemicellulose conversion to fermentable sugars, and subsequent microbial fermentation of hydrolysates for ethanol production. Moreover, the xylose-fermenting *Escherichia coli* strain was applied to use both pentose and hexose sugars and increase the final ethanol yield, which was compared to those with hexose sugar fermentation.

2. Materials and Methods

2.1 Materials

The soybean hulls were donated by Peace and Plenty Farm, located in Rocky Ridge (MD, USA), after soy seed separation processing in 2019. The raw hull pieces were dried at 50 °C overnight, rolled down with a Wiley mill machine, and screened with a 30-mesh sieve (0.595 mm).

The ground samples were collected and stored in the dry oven for further use, and the moisture content of the sample was measured (<4%) using a Halogen moisture analyzer before tests (Mettler Toledo HB 43, Columbia, OH, USA). A commercial enzyme, Cellulase (Cellic CTec 2, enzyme blend SAE0020) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Multifect Pectinase was gifted from Genencor, Danisco Division (Palo Alto, CA, USA). All other chemicals and supplies used in the work were purchased from Fisher Scientific Inc. (Hampton, NH, USA) and Sigma-Aldrich (St. Louis, MO, USA).

2.2 Enzymatic Hydrolysis

The collected, processed, and screened raw soybean hulls at 5% (w/v, 5 g dry weight basis) samples were enzymatically hydrolyzed in the presence of 100 mL of 50 mM citrate buffer (pH 4.8) combined with 10 mL of enzyme preparations (1:1 ratio of Cellulase and Multifect Pectinase, corresponding from 5 or 45 mg enzyme protein/g dry basis soybean hulls). Soybean hulls were added to a 600 mL Pyrex Griffin beaker with 100 mL of enzyme solution, and enzymatic hydrolysis was conducted at 50 °C for 72 h with a dual impeller bench top mixer (IKA, Wilmington, DE, USA) at 250 rpm. The hydrolysate samples were taken and deactivated intermittently and separated into solids and liquid using a 0.45 µm nylon syringe filter (VWR catalog number: 76479-056, Radnor, PA, USA). The solid-free liquid fraction after the syringe filtration was kept at 4 °C for further sugar analysis. All hydrolysis tests were performed in triplicate. To perform ethanol fermentation, the completed hydrolysate samples were boiled for 5 min, centrifuged at 12,000 rpm for 10 min, and vacuum filtered through a Whatman microfiber filter No.1 (Millipore-Sigma catalog number: WHA1001090, St. Louis, MO, USA) to remove the released proteins (23.8 g/L) and unnecessary soluble molecules. The collected liquid samples were used for further fermentation performances.

Protein amount in enzymes was measured by a Pierce BCA protein assay kit (Thermo Fisher Scientific catalog number: A55864, IL, USA). Filter paper unit (FPU), endoglucanase, endo-xylanase, β -glucosidase, and β -xylosidase were determined using filter paper strips, CellG5, XylX6 assay kits (K-CellG5-2V, K-XylX6-2V, Megazyme, Wicklow, Ireland), para-nitrophenyl- β -D-glucopyranoside (*p*-NPG), and para-nitrophenyl- β -D-xylopyranoside (*p*-NPX) as a substrate, respectively [13–15]. Quantified enzyme activities and their generalized enzyme activities are summarized in Table 1 (Ref. [14]).

2.3 Scanning Electron Microscopy (SEM)

To evaluate the morphological/structural shifts in soybean hulls before and after hydrolysis were captured via a JCM-6000 benchtop SEM (JCM 6000-OG-2, JCM, Peabody, MA, USA). The raw soybean hulls and enzymatically hydrolyzed solids were coated with an Au/Pd (gold-

Table 1. (A) Enzyme activity profiles and (B) generalized enzyme activities at 45 mg protein/g soybean hull solids enzyme loadings.

Activity (units/mL)	Cellulase blend (Celic Ctec 2) [14]	Multipect pectinase
(A)		
protein	186.5	82
Filter paper unit (FPU)	50.4	8.8
Endo-glucanase	2179.9	577
β -glucosidase	351.6	334.5
Xylanase	288.3	974
β -xylosidase	35	76.5
(B)		
Filter paper unit (FPU)	1.5	8.4
Endo-glucanase	96.2	363.3
β -glucosidase	29.3	58.6
Xylanase	157.8	48.1
β -xylosidase	5.8	12.8

Generalized enzyme activity units (unit/g substrate solids): [enzyme activity unit/mg protein] $\times$ [enzyme dosage (mg enzyme protein)/g soybean hull solids].

palladium alloy) through a Cressington sputter coater (Ted Pella Inc., Redding, CA, USA) as described in the previous literature [5,14].

2.4 Microorganism and Inoculum Preparation

The recombinant *Escherichia coli* strain FBR5 was graciously provided by the USDA National Center for Agricultural Utilization Research (USDA-NCAUR, Peoria, IL, USA). It was pre-cultivated on a Luria Bertani (LB) agar plate at 35 °C overnight, and one of the well-isolated single colonies was inoculated to 100 mL of LB liquid medium. The LB liquid medium combined 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and additional supplements of 4 g of xylose and 20 mg of tetracycline in 1 liter of distilled water (pH 6.5). The liquid medium was cultivated in a shaking incubator at 37 °C for 24 h with agitation of 100 rpm. The supernatant of the cultured medium was removed by centrifugation (10 min, 25 °C, 12,000 rpm), and the cell pellet was washed and resuspended with distilled water to obtain the fresh cell suspension with an O.D. value of approximately 10.0 at 660 nm [16]. A 10% (v/v) inoculum of cells was added into a 250 mL Erlenmeyer flask containing 50 mL of vacuum-filtered hydrolysate liquid and tested for ethanol fermentation at 35 °C for 36 h with 100 rpm [16].

2.5 Compositional and Analytical Procedures

The chemical composition of soybean hulls was determined by the biomass compositional analysis laboratory procedures provided by the National Renewable Energy Laboratory (NREL) (Table 2A, Ref. [5,12–14,17–21]) [22]. The generations of monomeric sugars and acetic acid in the hydrolyzed samples, ethanol, and other soluble molecules from the fermentation step were analyzed by HPLC as followed by our previous works [17,23–26]. This HPLC analysis was completed using a Bio-Rad Aminex HPX-87H ion

exchange column (300 mm $\times$ 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA), Milton Roy mini pump (Milton Roy Co., Ivyland, PA, USA), Waters 717 plus autosampler, Waters 2414 refractive index detector (Waters Corp., Milford, MA, USA) connected to a Empower 2 Chromatography Data Software (Version 2.0, Waters Corp., Milford, MA, USA). The column temperature was maintained at 60 °C, and the mobile phase was at 0.6 mL/min flow rate of 5 mM sulfuric acid.

3. Results and Discussion

3.1 Chemical Composition in Soybean Hulls

Primarily given low lignin (7.0%), high polysaccharide fraction (48.2% glucan, 29.4% hemicellulose in extractives free), and other minor components (2.6% acetate and 0.2% ashes) in soybean hulls were determined by the NREL analytical methods, which is a higher composition in cellulose and similar in hemicellulose component compared to the earlier literature [27–30]. The typical range of chemical composition of soybean hulls contains 29–51% cellulose and 10–25% hemicellulose; however, it varies with seeds, geological regions, culture conditions, and different dehulling/biorefinery processes [31]. The composition of soybean hulls in the current study is commensurate with other non-edible agricultural wastes and woody plants such as corn stover, sugarcane bagasse, hemp wastes, corn pericarp, and hardwood. Previous chemical composition reports for common lignocellulosic biomass and their comparisons with soybean hulls are summarized in Table 2A.

Pretreatment plays a key role in removing hemicellulose and lignin that contributes to the breakdown of the rigid and recalcitrant structure of lignocellulose [32,33]. To improve the final sugar yield and the economic feasibility of biorefineries, various pretreatment and hydrolysis attempts have been applied to soybean hulls, which are summarized

Table 2. (A) Chemical composition of soybean hulls and their comparisons with other lignocellulosic feedstock, and (B) different pretreatment and enzymatic hydrolysis practiced in soybean hulls.

(A)				
Composition (%)				
Biomass	Cellulose	Hemicellulose	Lignin	Ref.
Soybean hull (As received)	34.2	16.5 (12.3% xylan + 4.3% arabinan)	6.7	Current work
Soybean hull (Extractives free)	48.2	29.4 (20.8% xylan + 8.6% arabinan)	7.0	Current work
Soybean straw	27.2	13.0	26.9	[5]
Hemp stem	39.6	22.3	26.0	[14]
Hemp Branch	26.0	12.0	30.6	[14]
Sugarcane bagasse	36.4–45.9	17.9–20.2	26.1–26.9	[18,19]
Corn pericarps	22.5	23.7	4.7	[12]
Corn stover	37.0	22.7–29.0	18.0–18.6	[13,17]
Hardwoods	39.8	16.6	33.1	[20]
Switchgrass	39.4	17.3	21.2	[21]
(B)				
Pretreatment conditions		Hydrolysis conditions	Glucose yield (%)	Ref.
Acid	2.7% (v/v) H ₂ SO ₄ , 118 °C, 40 min	-	59	[34]
Acid	1% (v/v) H ₂ SO ₄ , 121 °C, 40 min (<1 mm particle size)	15 FPU/g, 50 °C, 96 h	72	[35]
Acid	1% (v/v) H ₂ SO ₄ , 121 °C, 40 min (soybean hull + oat hull 1:1)	15 FPU/g, 50 °C, 96 h	81	[36,37]
Acid	0.04 M HCl, 121 °C, 30 min	1.8% (v/v) Cellic Ctec 2, 50 °C, 72 h	50	[36,37]
NaOH	1% (v/v) NaOH, 121 °C, 60 min	25 mg protein/g glucan, 50 °C, 72 h	57	[5]
H ₂ O ₂	1% (v/v) H ₂ O ₂ , 121 °C, 60 min	25 mg protein/g glucan, 50 °C, 72 h	39	[5]
Steam explosion	2% (v/v) H ₂ SO ₄ , 100 psi, 140 °C, 30 min	15 FPU/g, 50 °C, 48 h	75	[38]
Extrusion	Screw extraction at 80 °C for 30 min with 350 rpm (40% moisture content)	7 FPU/g, 50 °C, 72 h	94.8	[1]

Table 3. Comparison of ethanol fermentation from different substrates, including soybean hulls, pure xylose, wheat straw hydrolysate, corn fiber hydrolysate, and corn stover hydrolysate, using the recombinant *E. coli* FBR5 strain.

Substrate	Time (h)	Ethanol (g/L)	Productivity (g/L/h)	Yield (g/g sugars)	Yield (g/g solids)	Note	Ref.
Soybean hull hydrolysate	36	11.7	0.33	0.48	0.23	No pretreatment and supplements	Current work
Xylose	48	40.3	0.84	0.44	-	100 g xylose, 5 g/L tryptone, and 5 g/L yeast extract	[52]
Wheat straw hydrolysate	96	9.7	0.39	0.45	-	-	[54]
Corn fiber hydrolysate	31	20.1	0.65	0.42	-	10 g/L tryptone, and 5 g/L yeast extract	[55]
Corn stover hydrolysate	40	28.9	0.72	0.48	0.29	-	[53]

in Table 2B (Ref. [1,5,34–38]). Traditional acid pretreatment has been widely used with several advantages of being easy to apply, cost-effective, and expecting a high conversion yield; however, it was liable to release more potential inhibitors than other pretreatment methods with lower heat, pressure, and diluted acid. Similarly, other pretreatment approaches (sodium hydroxide, hydrogen peroxide, steam explosion, and extrusion) also had advantages/disadvantages, and their cellulose conversion yields were varied based on pretreatment and experimental conditions. Well-known inhibitors include furan aldehydes, carboxylic acids, phenolic acids, lignin-derivatives, and degraded-intermediate molecules that can cause higher enzyme loading and/or additional conditioning processes to alleviate the negative effects of undesirable compounds before/after enzyme reaction and fermentation steps [33,39,40]. In the current work, raw soybean hulls are directly used for cellulose/hemicellulose conversion into reducing sugars with dual impeller mixing; the following results are described in the next section.

3.2 Sugar Recovery From Soybean Hulls Hydrolysis

In order to enhance the cellulolytic/hemicellulolytic efficiency for C5 and C6 sugars (mainly xylose and glucose), two different types of enzymes (Cellulase blend and Multifect pectinase) were combined and used for the soybean hulls hydrolysis. This approach ensures more complete saccharification of lignocellulosic biomass, aiming for high sugar recovery with minimal chemical and enzymatic inputs. The cellulase enzyme blend contains endoglucanases, exoglucanases, and β -glucosidases, which work synergistically to depolymerize cellulose into glucose (Table 1). Meanwhile, Multifect pectinase enhances enzyme accessibility to cellulose and hemicellulose by disrupting the gel-like pectic matrix, which otherwise limits enzyme diffusion. The enzymatic degradation of pectin also reduces biomass viscosity, thereby improving substrate homogenization and facilitating more efficient mass transfer during enzymatic hydrolysis. These combined effects enhance saccharification efficiency, enable lower enzyme dosages, and ultimately make the process more cost-effective for large-scale biofuel and biochemical production [5,14,33].

The Wiley milled and screened soybean hulls at 5% (dry weight/volume, 5 g/100 mL enzyme solution) were deliberated in the presence of enzyme preparations (0–45 mg total enzyme protein/g solids) at 50 °C for 72 h with a dual head mixer impeller of 250 rpm. Dual-impeller mixing is a promising approach for enhancing the enzymatic hydrolysis of low-lignin agricultural residues, such as corn pericarp and soybean hull, particularly in the absence of conventional hydrothermal pretreatment methods. This study builds upon and modifies our previous work on corn pericarp, using two elephant ear impeller blades (2-inch, 5.08 cm) set at a 45° angle, with a 1/4-inch shaft bushing, to im-

prove mixing efficiency and substrate suspension [12]. This configuration effectively reduces biomass aggregation, ensures uniform enzyme distribution, and enhances substrate-enzyme interaction [18,39,40]. The improved mixing conditions disrupt the microstructural barriers of the biomass, allowing enzymes to penetrate and act on cellulose and hemicellulose more effectively, even in the absence of thermal or chemical pretreatment.

Control runs were performed in 50 mM citrate buffer (without enzyme) under the same experimental conditions. As expected in the control tests, low sugar yields were observed for glucose (14.2%, 2.67 g/L), xylose (20.1%, 1.37 g/L), and arabinose (15.5%, 0.36 g/L) conversion, respectively (Fig. 1). Compared to the control, initial tests with a high enzyme loading of 45 mg protein/g solids achieved 94.4% cellulose-to-glucose conversion (17.76 g/L), 72.6% xylan-to-xylose conversion (4.92 g/L), and 69.3% arabinan-to-arabinose conversion (1.63 g/L). Notably, even when the total enzyme dosage was reduced from 45 to 10 mg protein/g solids, a similar glucose recovery (90.6%, 17.1 g/L) was achieved. However, xylose (62.0%, 4.2 g/L) and arabinose (60.6%, 1.42 g/L) yields were approximately 10% and 9% lower, respectively, compared to those obtained with 45 mg protein/g solids enzyme loading. These comparable results indicated that high endo-glucanase (2179.9 units/mL) and β -glucosidase (351.6 units/mL) in Cellic Ctec2 (Cellulase blend), xylanase (947 units/mL) and β -xylanase (76.5 units/mL) activities in Multifect pectinase preserved the elevated hydrolysis efficiency. It is legitimate to mention that Cellic Ctec2 (in particular, β -glucosidase) originating from the *Aspergillus niger* strain is more resistant to potential soluble inhibitors and stable at 50 °C by maintaining enzyme activity and thermal denaturation stability during the hydrolysis [5,12,14,41]. This result is similar to the previous study of corn pericarps with low lignin (<5% lignin), presenting a 98% cellulose conversion yield without pretreatment [12].

It is noteworthy that the formation of potential inhibitors and enzyme deactivators during soybean hull hydrolysis without pretreatment was generally low or negligible. No detectable levels of hydroxymethylfurfural (HMF) or furfural were observed in the soybean hull hydrolysate, as these compounds are predominantly generated through thermal or acid-catalyzed degradation of hexoses and pentoses [27]. However, a measurable concentration of acetic acid (2.47 g/L) was identified, resulting from the deacetylation of hemicellulose. Additionally, the total phenolic acid content was relatively low (0.22 mg/L), likely due to the intact lignin-carbohydrate complex, which restricts phenolic acid release. Nonetheless, the action of cellulolytic, hemicellulolytic, and pectinolytic enzymes can partially disrupt the plant cell wall matrix, facilitating the liberation of bound phenolic acids, which may exert a mild inhibitory effect on enzymatic activity and subsequent fermentation process [17–19,27].

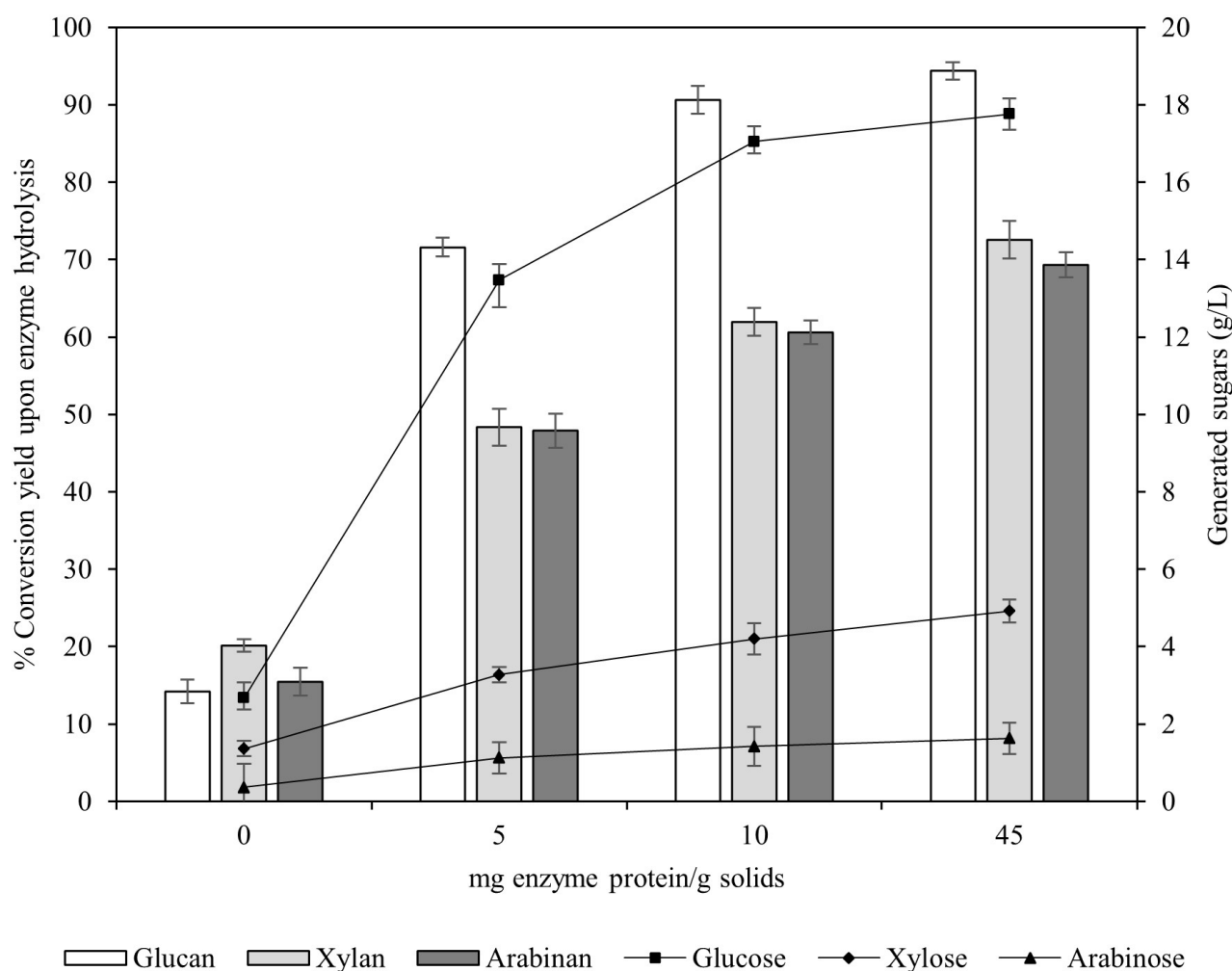


Fig. 1. Enzymatic hydrolysis of soybean hulls was conducted using different enzyme loadings ranging from 0 to 45 mg enzyme protein/g solids. The hydrolysis of 5% (w/v, dry weight basis) soybean hulls was performed in the presence of an enzyme preparation at 50 °C for 72 h, using a dual-head mixer impeller at 250 rpm. All tests were conducted in duplicate, and the average results were presented with a 95% confidence interval.

3.3 Structural/Morphological Analysis of Soybean Hulls

To examine potential morphological/structural destructions and changes in the soybean hulls, each sample, both before and after enzymatic hydrolysis, was captured and determined using scanning electron microscopy (SEM). The raw sample clearly exhibited an outstretched and flawless structure (Fig. 2A–C), whereas the hydrolyzed samples showed degraded and ruptured cellular formation (Fig. 2D–F). In general, the external lignin content and rigid crystalline structure of agricultural residues are melted and/or disrupted under harsh conditions involving heat, pressure, chemicals, pH adjustments, or other physicochemical treatments. These treatments and structural modifications are closely associated with enhancing enzyme accessibility to the exposed internal components of cellulose and hemicellulose [18,19,33,40]. Although no physicochemical treatments were applied in this study,

soybean hulls were fractured during dual impeller mixing with enzymes, resulting in disordered arrangements with enzyme-accessible holes and vacuolar spaces in the cell walls (Fig. 2F). The increased surface area and porosity in the internal structure of cellulose and hemicellulose enhanced their susceptibility to interaction with cellulolytic enzymes, thereby facilitating the degradation of exposed cellulose and hemicellulose into monomeric sugars. This observation aligns with our previous work on sugarcane bagasse pretreated with liquid hot water, ozone, or combined sequences, which demonstrated the formation of opened cells with globular shapes on the substrate's surface [18,19]. More recent works with hemp waste and soybean straw samples also showed that pretreatment with either 1% (v/v) sodium hydroxide (NaOH) or hydrogen peroxide (H₂O₂) reduced particle size and cellulose crystallinity while increasing the surface area accessible to enzyme proteins [5,18]. These changes resulted in a higher cellulose

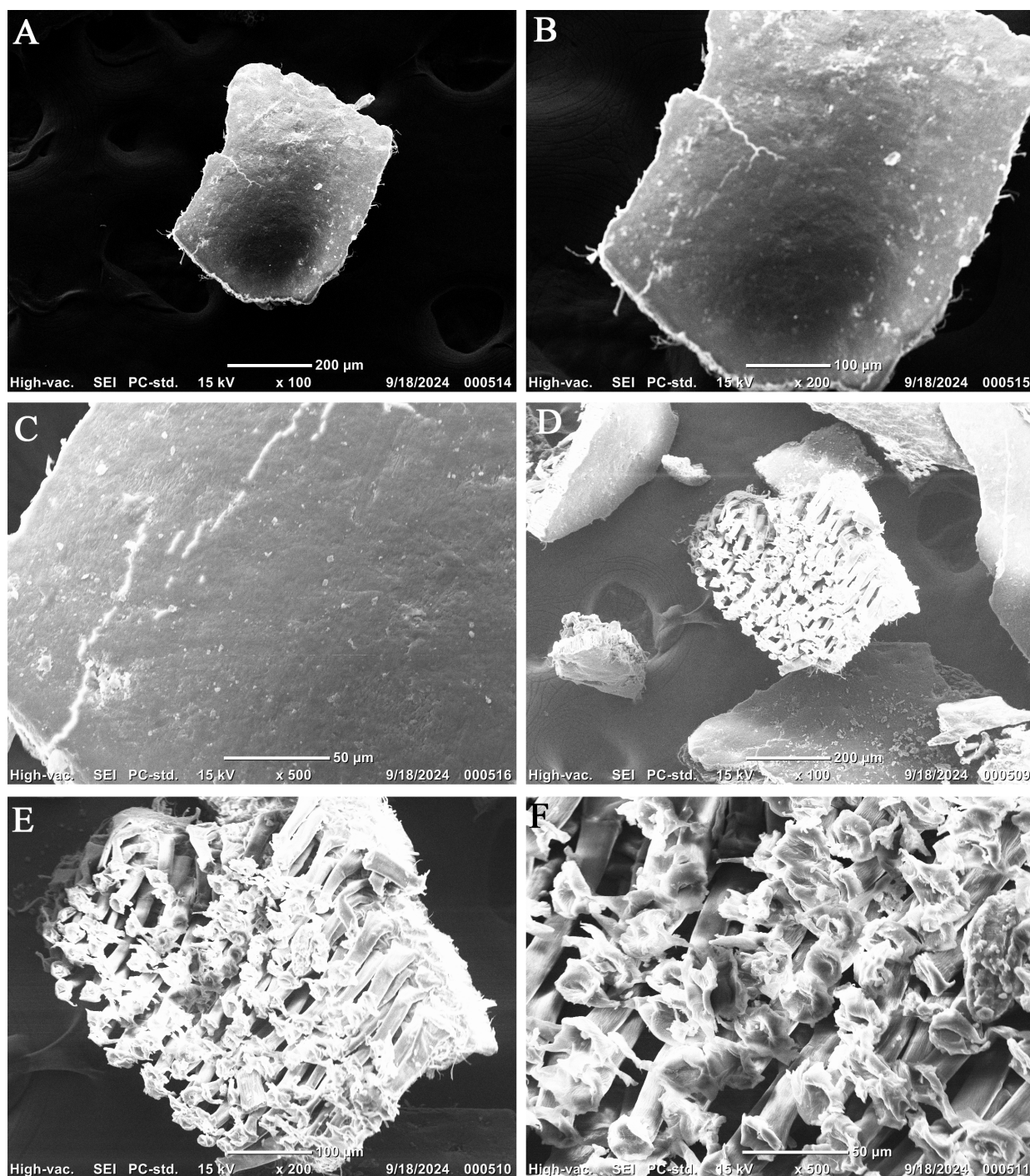


Fig. 2. Scanning electron microscopy (SEM) images of soybean hulls were captured before hydrolysis (A–C) and after hydrolysis at 50 °C for 72 h using a dual-impeller benchtop mixer at 250 rpm (D–F). Each SEM image was taken at either 100×, 200×, or 500× magnification, with scale bars and magnification levels indicated at the bottom of each image. The scale bars in Fig. 2A,D are 200 μm; the scale bars in Fig. 2B,E are 100 μm; the scale bars in Fig. 2C,F are 50 μm.

conversion yield compared to the intact surface of raw, unpretreated samples.

3.4 Fermentation of Soybean Hull Hydrolysates

One of the primary challenges in microbial fermentation processes is the limited ability to utilize C5 sug-

ars, particularly xylose. Traditional ethanol-fermenting microorganisms, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, cannot metabolize pentose sugars to produce ethanol. To address this technical limitation and enable the simultaneous utilization of both pentose and hexose sugars, several recombinant microorganisms—such as *S. cerevisiae*, *Z. mobilis*, *Escherichia coli*, and *Klebsiella*

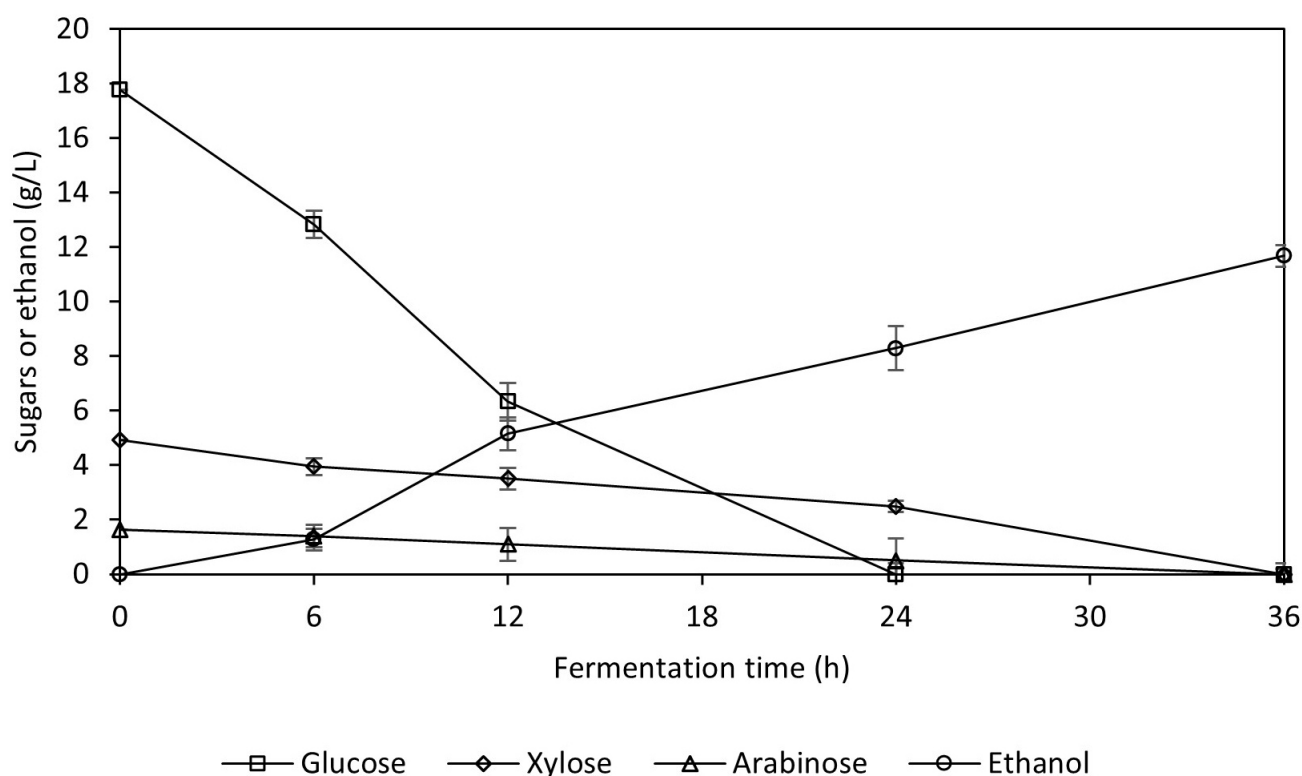


Fig. 3. Elapsed time course of ethanol fermentation via genetically modified recombinant *E. coli* FBR5 from soybean hull hydrolysates. The microbial fermentation was duplicated at 35 °C for 36 h with 100 rpm, and the averages of individual tests were presented.

oxytoca—have been developed over the past four decades [16,17,33]. To enable the utilization of both pentose and hexose sugars in soybean hydrolysates, this study employed the recombinant *E. coli* FBR5 strain, generously provided by the USDA National Center for Agricultural Utilization Research (USDA-NCAUR, Peoria, IL, USA). Briefly, the FBR5, ethanologenic bacteria, strain contains the pet operon plasmid pLOI297, which encodes the genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*), facilitating efficient ethanol production from pyruvate [42]. The presence of antibiotic resistance genes in the plasmid rendered the strain resistant to chloramphenicol, kanamycin, ampicillin, and tetracycline, allowing it to stably ferment both pentose and hexose sugars into ethanol [16].

Fermentation of soybean hull hydrolysates confirmed that the FBR5 strain can simultaneously metabolize both hexose and pentose, with a pattern of faster glucose consumption and slower arabinose and xylose metabolism. The total ethanol production reached 11.7 g/L, equivalent to 94.2% of the maximum theoretical ethanol yield. While all glucose was depleted within 24 h, approximately 30% and 51% of arabinose and xylose were consumed respectively during the same period, requiring an additional 12 hours to complete the fermentation (Fig. 3). Our results are in accordance with previous FBR5 strain fermentation

literature on mixed sugars, as well as pretreated and enzymatically hydrolyzed wheat straw, wood extract and rice hull hydrolysates, which demonstrated that the FBR5 strain preferentially utilizes glucose first, followed by arabinose, and finally xylose [43–45].

Catabolite repression is a well-known phenomenon in which glucose serves as the preferred carbon source not only in wild-type microorganisms but also in engineered recombinant strains. The presence of glucose suppresses the expression of enzymes involved in the metabolism of alternative sugars, through a mechanism governed by the cyclic AMP (cAMP) catabolite activator protein (CAP) system [46,47]. In glucose-rich conditions, intracellular cAMP levels remain low, preventing the activation of the *xylAB* and *araBAD* operons, thereby delaying the metabolism of xylose and arabinose until glucose is depleted. Once glucose is exhausted, the metabolic hierarchy shifts towards arabinose utilization, as it is typically the next most readily metabolized sugar. Arabinose is converted to xylulose-5-phosphate (X5P) via isomerization and subsequently enters the pentose phosphate pathway (PPP) [48, 49]. In contrast, xylose metabolism is more intricate, often necessitating additional enzymatic conversions through xylulose-5-phosphate or xylitol intermediates, which can introduce metabolic bottlenecks. Furthermore, glucose metabolism directly fuels adenosine triphosphate (ATP) and

nicotinamide adenine dinucleotide (NADH) regeneration, whereas xylose and arabinose metabolism channels flux through the PPP, requiring additional enzymatic processing. This complexity often results in a reduced metabolic flux, leading to a slower utilization rate and lower fermentation efficiency compared to glucose and arabinose [50,51].

4. Conclusions

Our current research and outcomes align with previous studies on *E. coli* FBR5 ethanol fermentation, which utilized various carbon substrates, including xylose and hydrolysates that were pretreated, enzymatically hydrolyzed, and subsequently fermented. A concise summary of the experimental conditions and corresponding results are summarized in Table 3 (Ref. [52–55]). Qureshi *et al.* [52] reported that a concentrated pure xylose solution (92.1 g/L) was effectively fermented into ethanol, achieving a final ethanol concentration of 40.3 g/L, an ethanol productivity of 0.84 g/L/h, and a yield of 0.44 g/g sugars. These values were notably higher than those obtained from other hydrolysates. This higher efficiency could be attributed to minimal inhibition from potential inhibitors in the medium, as well as the benefits of a concentrated initial substrate and the strain's tolerance to the accumulated ethanol concentration. However, further studies on fermentation inhibition revealed that when the xylose concentration exceeded 100 g/L, osmotic stress and ethanol toxicity combined to negatively affect cell growth and metabolism, ultimately lowering ethanol production to 36 g/L from an initial concentration of 220 g/L of xylose. As a result, ethanol productivity dropped to 0.31 g/L/h, and the yield decreased to 0.38 g/g sugars [52].

Although total ethanol production from soybean hydrolysate (11.7 g/L) was lower than that of other hydrolysate samples due to a lower initial sugar concentration, its ethanol yield was 0.48 g/g sugars (94% of the maximum theoretical yield), which was comparable to corn stover hydrolysate and slightly higher than the yields from xylose (1.09 times), wheat straw (1.07 times), corn fiber (1.14 times). This difference can be explained with the generation of potential inhibitors (e.g., lignin-derived molecules, phenolic compounds, and acids) during the pretreatment of biomass process, which hindered the enzyme saccharification as well as fermentation performance. It is valuable to acknowledge that less lignin content is favorable for suitable biorefinery/renewable energy sources because the lower level of lignin is more susceptible to being disrupted by pretreatment at relatively mild conditions and increases the fermentable sugar recovery by providing more enzyme accessibility to the exposed surface area in the cellulose [18,33]. Ladeira Ázar *et al.* [19] found that a small variation of lignin content in sugarcane bagasse samples (22.3% vs. 20.6%) could influence a significant difference in cellulose conversion yield. Enzymatic hydrolysis test with lower lignin sugarcane bagasse gave 89.5% while 68.3%

yield was obtained from the higher lignin sample. Therefore, the delignification or reduction of lignin content using effective pretreatment methods and/or approaches such as genetic/system engineering, and feedstock selection before the use of lignocellulosic biomass is important to enhance the cellulose conversion. Closely associated lignin and acetyl compounds create amorphous polyphenol polymers that provide an intrinsic resistant structure in the plant and protect the embedded cellulose-hemicellulose polysaccharide chains from most forms of microbial invasions [33,55].

By integrating our current findings and previous research, it becomes evident that the ethanol yields from FBR5 fermentation can vary significantly depending on the hydrolysates derived from different pretreatment conditions of agricultural residues. This variation is largely due to alterations in the strain's metabolic pathways and/or the impact these changes have on cell growth and overall metabolic performance. Our study primarily focused on optimizing the use of soybean hulls as a source for the conversion of cellulose and hemicellulose into monomeric sugars, followed by their efficient bioconversion into ethanol. Notably, a key outcome in this work is the demonstration that carbohydrates from low-lignin lignocellulosic biomass (e.g., soybean hulls) can be efficiently utilized under mild conditions, without the need for extreme physical or chemical pretreatment. Furthermore, the simultaneous fermentation of both C5 and C6 sugars in the hydrolysates was successfully achieved, highlighting the feasibility of this approach for bioethanol production. While the *E. coli* FBR5 strain presents advantages for sugar metabolism, its scale-up process and industrial application requires careful optimization to overcome challenges related to catabolite repression, bioreactor conditions, feedstock variability, and economic feasibility. Advanced metabolic engineering, process control strategies, and innovative fermentation technologies will be key to achieving a scalable and economically viable production process.

A wide range of feedstocks, pretreatment strategies, and microbial metabolic pathways have been investigated in previous studies (Tables 2 and 3) to enhance bioethanol production. However, further research is required to explore the potential of low-lignin residues without pretreatment, optimizing process conditions to improve yield and efficiency. Future efforts should focus on refining fermentation strategies through approaches such as optimizing inoculum medium supplementation [54], improving culture operation methods, enhancing mixing conditions, selecting suitable bioreactor configurations, utilizing engineered recombinant strains, and investigating additional factors that may enhance process scalability and economic feasibility.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

DK, investigation experimental, data curation, formal analysis, writing original draft, conceptualization, funding acquisition, project administration, supervision, and validation; EC, investigation experimental, data curation, formal analysis; EK: investigation experimental, data curation, formal analysis; SJ: investigation experimental, data curation; CGY: formal analysis, writing, methodology, supervision, and validation, formal analysis. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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