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Study on the Sugar-Producing Effect of High-Temperature Anaerobic Straw Biosaccharification Strain

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Abstract: The utilization of straw waste cellulose will be beneficial by economic, social, and environmental means. The present study sought to screen the high-efficiency cellulose sugar-producing strain from corn straw. The 16S high-throughput sequencing method and the combination of morphological, physiological, and biochemical characteristics of the strain confirmed the strain to be *Clostridium thermocellum*, which was named *Clostridium thermocellum* FC811. Moreover, the single factor experiment was conducted to investigate the effect of environmental factors on saccharification efficiency. The optimal saccharification conditions of cellulose saccharification of FC811 strain selected through response surface analysis were as follows: temperature of 58.9 °C, pH of 7.21, culture time of 6.60 d, substrate concentration of 5.01 g/L, and yeast powder concentration of 2.15 g/L. The soluble sugar yield was 3.11 g/L, and the conversion rate of reducing sugar was 62.2%. This study will provide a reference for resource and energy utilization of straw materials, simultaneous fermentation of sugar and hydrogen production, and their large-scale production and application.

Keywords: bio-saccharification; reducing sugar accumulation; biological pretreatment of straw; high temperature anaerobic bacteria; response surface

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1. Introduction

In recent years, traditional fossil energy has been facing serious social problems [1]. Moreover, the global water resources are also polluted by the digestion of traditional fossil energy [2,3]. The lignocellulosic raw material is the most abundant and cheapest renewable resource on earth, of which 89% are unutilized by humans. According to reports, the global annual cumulative production reaches 200 billion tons [4–6]. Some studies find that mealworms are capable of digesting lignin, hemicellulose, and cellulose in rice straw, corn straw, wheat straw, and bran [7,8]. Therefore, the conversion of lignocellulose into clean energy substances, such as hydrogen, ethanol, and butanol, through biotechnology could effectively reduce CO_2 emission and synergistically solve environmental and energy problems by ecological means [9–12].

Lignocellulosic saccharification has always been a bottleneck in the efficient utilization of fibrous raw materials, such as straw [13,14]. The keys to converting lignocellulose into energy substances include the destruction of lignin, the release of cellulose and hemicellulose, and the hydrolysis of these substances into small monosaccharides [15,16]. At present, the lignocellulosic saccharification treatment can be divided into three ways: physical, chemical, and biological [17,18]. Although the physical and chemical methods can effectively saccharify lignocellulose, the production of monosaccharides through the hydrolysis process is often accompanied by the generation of by-products, contributing to secondary

environmental pollution [19,20]. However, the biological saccharification method characterized by mild conditions, low energy consumption, and green environmental protection has attracted the attention of researchers worldwide [21–23]. It is an important pathway to promote the lignocellulosic biomass resource utilization by converting cellulose, hemicellulose, and other macromolecule carbohydrates into fermentable sugars using saccharifying strains, or enzymes.

Cellulase is widely distributed in nature and can be obtained from many microorganisms, plants, and insects [24–26]. It has the advantage of decomposing cellulosic raw materials. However, at present, the cost of cellulase acquisition is high, and the enzymatic hydrolysis efficiency is generally low, far less than amylase and protease, which greatly affects the mass production and wide application of cellulase [27,28]. Therefore, screening the saccharifying strains that can degrade lignocellulose efficiently in the environment for biosaccharification would be a feasible research direction.

However, the glucose effect is the primary bottleneck in the biosaccharification process using strains. Most of the cellulose degrading bacteria can metabolize the reducing sugar obtained through the biosaccharification process but cannot accumulate the reducing sugars. Therefore, exploring and domesticating cellulose biosaccharifying strains with high-efficiency, reducing sugar tolerance, continuously improving and optimizing their culture conditions, and further fermenting to produce hydrogen, ethanol, butanol, and other energy substances are the keys to the efficient utilization of cellulose resources. The study will provide a theoretical basis for the application of biological refining pathway for energy production from organic wastes.

2. Materials and Methods

2.1. Strain Source

In this study, rotten bark (Yichun, Heilongjiang, 128.92 E, 47.73 N, 290 m, pH 7.4) was used as the source of strain. The compost samples were put into anaerobic sterile water with several glass beads and vibrated in an oscillator for 1 h under the protection of nitrogen. The shear force of the glass beads was used for crushing the compost sample to fully disperse the microorganisms in the system. The mixed system was then inoculated in corn straw culture medium with 10% inoculation amount, enriched, and in static at 35 °C for 10 d. The inoculation was transferred twice to accumulate the cellulose degrading strains greatly.

The original bacterial solution was transferred to a 100 mL anaerobic flask containing the modified ATCC 1191 (MA) medium for culture [29]. The working volume was 50 mL, the inoculation amount was 10%, and the culture time was 5 d. After inoculation in the continuous flow domestication reactor, the hydraulic retention time was 7 d. Then, the fresh corn straw culture solution was added every 24 h and continued to use the corn straw as the sole carbon source to culture in a 35 °C incubator. Later, the samples were taken every 24 h, and the reducing sugar concentration, pH, and other indicators were determined using DNS colorimetry under the conditions of anaerobic culture until the compound bacteria tend to be stable. When the reducing sugar yield increased, the culture system with the dilution degree was diluted to 10^{-3}–10^{-9} and then inoculated to the fresh straw culture medium for liquid culture. In this way, continuous dilution was used to obtain a high sugar yield compound bacteria. In order to verify the cellulose degradation ability of the compound bacteria, they were added into the fermentation medium with straw and filter paper as the only carbon source, respectively, to observe the degradation effect.

2.1.1. Preparation of Culture Medium

Modified MA medium: NaCl 1.0 g/L, NH_4Cl 1.0 g/L, K_2HPO_4 1.5 g/L, KH_2PO_4 3.5 g/L, $MgCl_2$ 0.5 g/L, KCl 0.2 g/L, yeast extract 2 g/L, peptone 2 g/L cysteine 0.6 g/L, trace element 5 mL/L, vitamin 0.5 mL/L, 0.1% (*w*/*v*) resazurin 0.2 g/L, microcrystalline cellulose (PH101) 5 g/L, distilled water 1000 mL, and pH was adjusted to 7.

Straw medium: carbon source was corn straw; other components were the same as in the MA medium.

Microcrystalline cellulose rolling tube medium: NaCl 1.0 g/L, NH_4Cl 1.0 g/L, K_2HPO_4 1.5 g/L, KH_2PO_4 3.5 g/L, $MgCl_2$ 0.5 g/L, KCl 0.2 g/L, yeast extract 2 g/L, peptone 2 g/L cysteine 0.6 g/L, trace element 5 mL/L, vitamin 0.5 mL/L, 0.1% (*w*/*v*) resazurin 0.2 g/L, microcrystalline cellulose (PH101) 5 g/L, agar 20 g, distilled water 1000 mL, and pH was adjusted to 7.

2.1.2. Preparation of Reagent and Buffer Solution

Trace element: $FeCl_2$ 1.5 g/L, $ZnCl_2$ 70.0 mg/L, H_3BO_3 6.0 mg/L, $MnCl_2$ $4H_2O$ 0.1 g/L, $CuCl_2$ $2H_2O$ 2.0 mg/L, $CoCl_2$ $6H_2O$ 0.19 g/L, $NiCl_2$ $6H_2O$ 24.0 mg/L, Na_2MoO_4 H_2O 36.0 mg/L, Na_2WO_4 15.0 mg/L, Na_2O_4Se $5H_2O$ 15.0 mg/L, and distilled water 1000 mL.

Vitamins: Lipoic acid 50.0 mg/L, biotin 20.0 mg/L, niacin 0.35 g/L, thiamine hydrochloride 5.0 mg/L, p-aminobenzoic acid 50.0 mg/L, folic acid 20.0 mg/L, calcium pantothenate 50.0 mg/L, Vitamin B12 1.0 mg/L, pyridoxine hydrochloride 100.0 mg/L, distilled water 1000 mg/L, and pH was adjusted to 6.8–7.0.

DNS solution: 6.3 g of 3,5-nitrosalicylic acid was dissolved in 262 mL of 2 mol/L NaOH solution, and then the mixed solution was slowly added to the pre-prepared hot water solution containing 185 g of sodium potassium tartrate, 5 g of phenol, and 5 g sodium sulfite. Later, the solution was thoroughly stirred and dissolved. After the solution was cooled, distilled water was added to a constant volume of 1000 mL, transferred to a brown flask, and refrigerated at 4 °C for 7 days before use.

1 mg/mL glucose standard solution: accurately 100 mg of pure analytical glucose was weighed, dried in an oven at 80 °C to a constant weight, placed in a small beaker, added a small amount of ultra-pure water to dissolve, then transferred to a volumetric flask of 100 mL. Then, ultra-pure water was added to a constant volume of 100 mL, shaken up, and stored in a refrigerator at 4 °C for later use.

2.2. Isolation, Purification, and Identification of High-Efficiency Cellulose Degrading Sugar-Producing Strain

The compound strain was domesticated by the continuous transfer method and inoculation using different carbon sources. The single strain colony was isolated and obtained by the multiple gradient dilution method. Then, the single bacteria with crystalline cellulose as the substrate was used for fermentation. The cellulose degradation and reducing sugar yield rates were recorded to screen high-efficiency degrading bacteria. The bacterial colonies cultured for 5 d were selected, stained by the Gram staining method, and bacteria color was observed under an oil microscope (CX23LEDRFS1C 100X/1.25). The morphological characteristics were identified by scanning electron microscope. Then, the physiological and biochemical reaction characteristics of glucose, microcrystalline cellulose, cellulose-disaccharide, mannose, mannitol, fructose, filter paper, xylose, pentose, and starch were identified according to the previously reported methods. Finally, molecular biological identification was carried out. The genome of the target strain was extracted using the SK8255 Ezup column bacterial genome DNA extraction kit. 27F-AGTTTGATCMTGGCTCAg and 1492R-GGTTACCTTGTTACGACTT were used as the primers for V3-V4 fragment amplification of the 16SrDNA gene. Taq Plus DNA Polymerase was used for PCR amplification. The PCR reaction conditions were set as follows: DNA Template 1 μL, 10× Buffer (with Mg^{2+}) 11.3 μL, dNTP 1 μL, enzyme 0.2 μL, primer F (10 μM) 1 μL, primer R (10 μM) 1 μL, added double-distilled H_2O to 25 mL, and stored at 4 °C. The products obtained from this process were purified and detected by 1% agarose gel electrophoresis [30]. The amplification products were sent to Sangon Biotech Co. Ltd. (Shanghai, China). The data were processed using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 November 2020) [31]. The obtained sequences were uploaded to Gen Bank (www.ncbi.nlm.nih.gov, accessed on 1 November 2020) [32] for similarity retrieval, and the strain with the most similar strain sequences was obtained. Finally, MEGA7.0 software (Version 7.0.14, Sudhir

Kumar, Koichiro Tamura, and Masatoshi Nei, USA)was used for phylogenetic tree analysis [33]. The taxonomic status of the strain was determined according to its genetic position, morphological, and physiological characteristics in the phylogenetic tree [34].

2.3. Effect of Environmental Factors on Sugar Production Efficiency of High-Efficiency Cellulose Degrading Strains

2.3.1. Temperature

Approximately 45 mL of the fermentation medium containing 5 g/L microcrystalline cellulose was added into a 100 mL anaerobic flask. After sterilization, the cellulosic saccharifying bacterium FC811 was injected into the anaerobic flask with 10% inoculation amount (v/v) and cultured in a water bath constant temperature oscillator at pH 7.0 and 130 rpm under the conditions of 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, and 75 °C. Three parallels were set for each temperature. Later, the samples were taken after 6 d culture, and the biomass of FC811 strain and soluble sugar accumulation in the solution was determined.

2.3.2. Initial pH

Approximately 45 mL of the fermentation medium was added into a 100 mL anaerobic flask. The concentration of microcrystalline cellulose was 5 g/L, and the pH was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 with 1 mol/L HCl and NaOH. The cellulose saccharifying bacterium FC811 was injected into the fermentation medium with 10% inoculation amount (v/v) and cultured in a water bath constant temperature oscillator at 60 °C with 130 rpm. Three parallels were set for each pH. Later, the samples were taken after 6 d culture, and the biomass of FC811 strain and soluble sugar accumulation in the solution was determined.

2.3.3. Culture Time

Approximately 45 mL of the fermentation medium containing 5 g/L microcrystalline cellulose was added into a 100 mL anaerobic flask. The cellulosic saccharifying bacterium FC811 was injected into an anaerobic flask with 10% inoculation amount (v/v) and cultured in a water bath constant temperature oscillator at 60 °C with pH 7.0 and 130 rpm. The culture time was 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, and 8 d, and 3 parallels were set for each culture time. Later, the samples were taken after 6 d culture, and the biomass of FC811 strain and soluble sugar accumulation in the solution was determined.

2.3.4. Substrate Concentration

Approximately 45 mL of the fermentation medium was added into a 100 mL anaerobic flask, and then the concentration of the microcrystalline cellulose in the fermentation medium was adjusted to 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 g/L. The cellulosic saccharifying bacterium FC811 was injected into the anaerobic flask with 10% inoculation amount (v/v) and cultured in a water bath constant temperature oscillator at 60 °C with pH 7.0 and 130 rpm. Three parallels were set for each concentration. Later, the samples were taken after 6 d culture, and the biomass of FC811 strain and soluble sugar accumulation in the solution were determined.

2.3.5. Yeast Powder Content

Approximately 45 mL of the fermentation medium containing 5 g/L microcrystalline cellulose was added into a100 mL anaerobic flask, and then the concentration of yeast power in the fermentation medium was adjusted to 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 g/L. The pH was 7, the temperature was 60 °C, and the inoculation amount of the FC811 strain was 10%. The strain was cultured in a water bath constant temperature oscillator 130 rpm for 6 d. Three parallels were set for each concertation. Later, the samples were taken after 6 d culture, and the biomass of FC811 strain and soluble sugar accumulation in the solution was determined.

2.4. Optimization of Experimental Conditions for Sugar Production Efficiency of High-Efficiency Cellulose Degrading Strain

2.4.1. Response Surface Optimization Design for Saccharification Conditions of Microcrystalline Cellulose

Based on the single factor experiment results, the Central Composite Design (CCD) was used to optimize the conditions. Temperature, pH, substrate concentration, culture time, and yeast powder concentration were selected as the independent variables, and the yield of soluble sugar was taken as the only dependent variable. Three parallels were set for each group of experiment, and the factors and levels are listed in Table 1. The polynomial regression method was adopted, and Minitab software (Version17.1.0, Minitab Inc., Philadelphia, PE, USA) was used for quadratic polynomial fitting of experimental data of CCD design. The equation was as follows [35]:

$$Y = A + \sum_{i=1}^{k} A_i X_i + \sum_{j=1}^{k} A_{jj} X_j^2 + \sum_{i<j}^{k} A_{ij} X_i X_j$$

where:

Y: response model A: model constant

A_i: Linear coefficient of the model A_{jj}: Square coefficient of the model

A_{ij}: Interactive coefficient of two factors of the model

Table 1. Five factors and level design.

Factor	Name	Unit	Low Level (−1)	Medium Level (0)	High Level (1)
A	Temperature	°C	55	60	65
B	pH		6.5	7	7.5
C	Culture time	d	5	6	7
D	Substrate concentration	g/L	4	5	6
E	Yeast powder concentration	g/L	1.5	2	1.5

2.4.2. Verification Experiment of Saccharification Ability of High-Efficiency Cellulose Degrading Strain to Microcrystalline Cellulose

Approximately 45 mL of the fermentation medium was added into a 100 mL anaerobic flask, and the bacterial solution of *Clostridium thermocellum* FC811 at 10% inoculation was injected into the fermentation medium. The optimal saccharification conditions optimized by CCD were used for the culture. Three parallels were set, and the samples were taken after culture to determine soluble sugar accumulation in the solution.

2.5. Determination of Reducing Sugar Content

Take 1.00 g of fermented residue into a 50 mL centrifuge tube, add 9.00 mL of sterile water to the centrifuge tube, vortex shake for 0.50 h, stand still, suck the suspension at 3000 g, centrifuge at 4 °C for 5 min, take 1.00 mL of supernatant and add it to a 25 mL graduated tube, at the same time, suck 1.00 mL of supernatant without fermented straw after the same treatment into a 25 mL graduated tube as a blank control, add 1.00 mL of citric acid buffer and 1.50 mL of DNS solution. After mixing, heat it in boiling water for 5 min, cool it, and then fix the volume to the scale line. After fully shaking, measure the absorbance at the wavelength of 540 nm, and measure the reducing sugar concentration through the glucose standard curve.

3. Results and Discussion

3.1. Screening and Morphological Identification of Strains

Figure 1A depicts the changes in the fermentation medium before and after the degradation of unpretreated straw by the compound bacteria, i.e., the straw was significantly degraded by the compound bacteria. It was found that many yellow affinity substances (YAS) were produced in the serum flask when the strain used microcrystalline cellulose as the substrate. These substances are considered to be carotenoids, which can promote the degradation of microcrystalline cellulose [36,37]. In this study, two strains were preliminarily named FC721 and FC811 after the purification of the compound bacteria. YAS was used to characterize the degradation of crystalline fiber, and the more the plus sign, the more obvious the degradation. The results showed that the FC811 had a good effect on cellulose crystal fiber degradation (Table 2). Transparent hydrolysis circles were formed on the microcrystalline cellulose rolling tube medium. After a period of time of culture in a constant temperature water bath shaker at 60 °C, the hydrolysis circles became significantly larger. Meanwhile, the yellow colonies were observed in the center of the hydrolysis circle, which was non-transparent with a smooth surface and a diameter of about 0.5–2 mm (Figure 1B). Therefore, this bacterium was selected for subsequent research. FC811 strain showed purple color after gram staining, confirming it to be a gram-positive bacterium (Figure 1C). The scanning electron microscopy results showed that the bacterium was rod-shaped, sporous, and flagellated, capable of movement and single growth (Figure 1D).

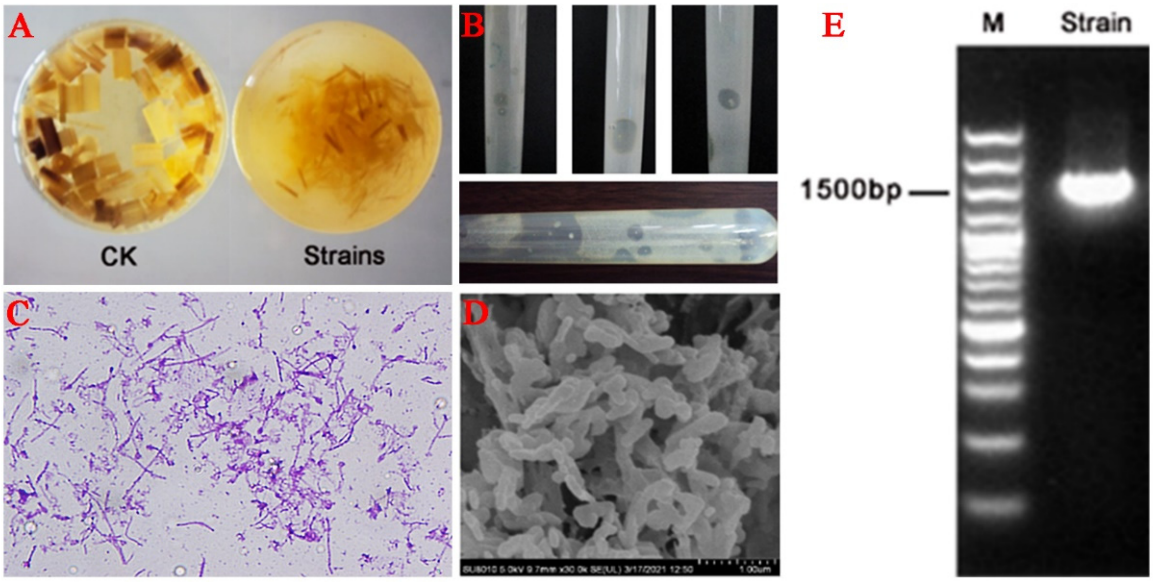

Figure 1. High-efficiency cellulose degrading sugar-producing strain: (**A**). Changes in straw fermentation medium before and after degradation (CK: straw fermentation medium without compound bacteria, Strains: straw fermentation medium with compound bacteria); (**B**). Hydrolysis circles formed in microcrystalline cellulose rolling tube of FC811; (**C**). Morphology of gram-stained cells of XF811; (**D**). SEM image of FC811; (**E**). Electrophoresis of PCR products of FC811 strain.

Table 2. Degradation of crystalline cellulose and yield of reducing sugar.

Strain	YAS	Yield of Reducing Sugar (mg/L)
FC721	+	230.6
FC811	+++	2764

Note: "+" indicates the degradation of crystalline fiber, and the more the plus sign, the more obvious the degradation.

3.2. Physiological and Biochemical Characteristics and Molecular Biological Identification of FC811

The decomposition ability of the nutrient matrix is different due to the different enzyme systems of various bacteria. The metabolic effect of bacteria, metabolites, and metabolic types on various substrates was detected by the biochemical experiment method. The physiological and biochemical characteristics of the FC811 strain are summarized in Table 3. The results indicate that the FC811 strain could use glucose, microcrystalline cellulose, cellulose-disaccharide, mannose, mannitol, fructose, and filter paper but could not ferment xylose, pentose, or starch. PCR amplification products of the anaerobic FC811 strain are depicted in Figure 1E. The sequencing verification results showed that the PCR amplification band size of the FC811 strain was about 1.5 kb, and the length of the 16S rDNA sequence of the FC811 strain was 1464 bp. The gene was sequenced by the similarity retrieval analysis using the GenBank database. The sequence with higher homology of the FC811 strain 16S rDNA was obtained, as shown in Figure 2. The phylogeny of the FC811 strain and the obtained highly homologous sequence were constructed using MEGA7 software (Version 7.0.14, Sudhir Kumar, Koichiro Tamura, and Masatoshi Nei, Philadelphia, PE, USA) (Figure 3). The similarity between FC811 strain and *Clostridium thermocellum* strain JN4 was 100%. The morphological observation, physiological, and biochemical analysis results confirmed FC811 to be *Clostridium thermocellum*, which was named *Clostridium thermocellum* FC811.

Table 3. Physiological and biochemical characteristics of FC811 strain.

Characteristics	Results	Characteristics	Results
Glucose	+	Mannose	+
Microcrystalline cellulose	+	Mannitol	+
Cellobiose	+	Filter paper	+
Fructose	+	Sucrose	+
Lactose	−	Maltose	+
Xylose	−	Starch	−
Xylan	+	Anaerobic growth	+
Growth at 60 °C	+	Gelatin experiment	+
Fluorescent pigment	−	CMC-Na	+
Nitrate reduction experiment	−	Citrate	−

Note: "+" indicates that it can be used; "−" indicates that it cannot be used.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Hungateiclostridium thermocellum strain JCM 9322 16S ribosomal RNA, partial sequence	Acetivibrio thermocellus	2704	2704	100%	0.0	100.00%	1489	NR_113157.1
Clostridium thermocellum strain JN4 16S ribosomal RNA gene, partial sequence	Acetivibrio thermocellus	2704	2704	100%	0.0	100.00%	1516	EF680276.1
Acetivibrio thermocellus ATCC 27405 chromosome, complete genome	Acetivibrio thermocellus AT…	2704	10790	100%	0.0	100.00%	3843301	CP000568.1
Ruminiclostridium thermocellum DSM 2360, complete genome	Acetivibrio thermocellus DS…	2699	10779	100%	0.0	99.93%	3577579	CP016502.1
Ruminiclostridium thermocellum AD2, complete genome	Acetivibrio thermocellus AD2	2699	10779	100%	0.0	99.93%	3554854	CP013828.1
Acetivibrio thermocellus DSM 1313 chromosome, complete genome	Acetivibrio thermocellus DS…	2699	10774	100%	0.0	99.93%	3561619	CP002416.1
Uncultured Firmicutes bacterium gene for 16S ribosomal RNA, partial sequence, clone: B09	uncultured Firmicutes bacte…	2695	2695	99%	0.0	99.93%	1463	AB874488.1
Hungateiclostridium thermocellum strain ATCC 27405 16S ribosomal RNA, partial sequence	Acetivibrio thermocellus	2693	2693	100%	0.0	99.86%	1521	NR_074629.1
Clostridium thermocellum gene for 16S ribosomal RNA, partial sequence, strain: JCM 9323	Acetivibrio thermocellus	2693	2693	100%	0.0	99.86%	1489	AB588017.1
Clostridium thermocellum partial 16S rRNA gene, strain GP1	Acetivibrio thermocellus	2691	2691	99%	0.0	99.93%	1489	FN555230.1
Uncultured bacterium partial 16S rRNA gene, isolate G40_3a	uncultured bacterium	2687	2687	100%	0.0	99.80%	1522	FN868409.1
Uncultured bacterium clone EUB_37 16S ribosomal RNA gene, partial sequence	uncultured bacterium	2687	2687	100%	0.0	99.80%	1488	FJ189558.1
Uncultured bacterium partial 16S rRNA gene, isolate G40_19	uncultured bacterium	2686	2686	100%	0.0	99.73%	1524	FN868428.1

Figure 2. 16SrDNA sequence source and database registration number.

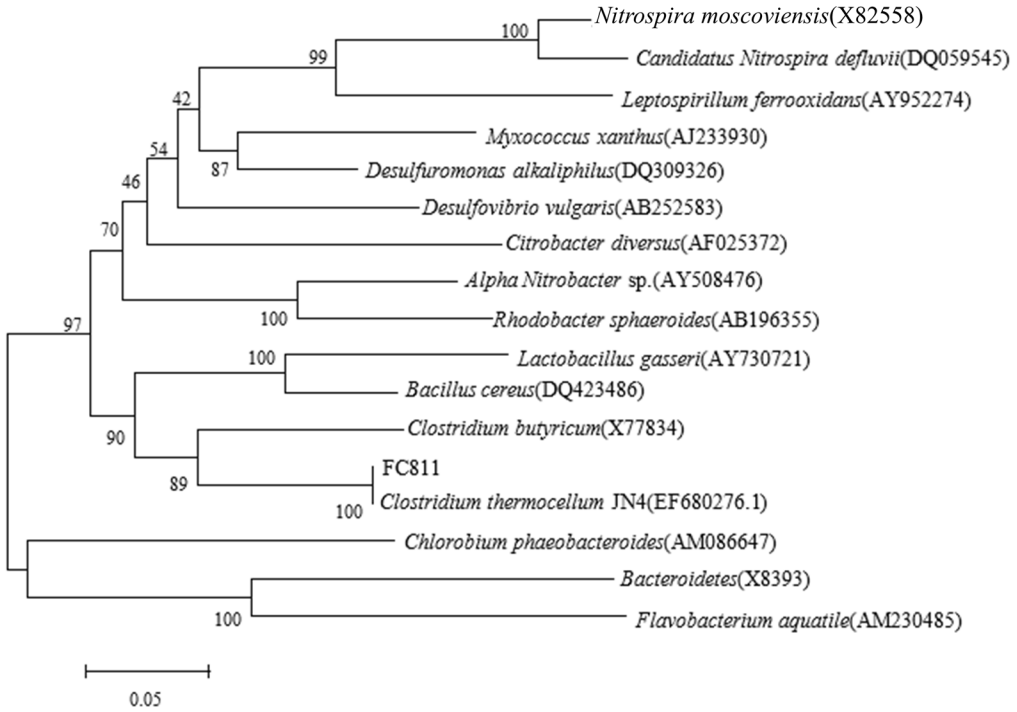

Figure 3. Phylogenetic tree of cellulose saccharifying bacterium FC811.

3.3. Factors Influencing Cellulose Saccharification Effect of FC811 Strain

Microorganisms are tiny single-cell organisms that are in direct contact with the external environment; thus, the physiological activities of microbial populations are closely related to the changes in the environmental factors, such as temperature, pH, and nutrients. The data of soluble sugar accumulation are obtained through a wet test.

3.3.1. Effect of Temperature on the Growth of FC811 Strain and Saccharification Effect of Microcrystalline Cellulose

Temperature is one of the essential factors affecting the growth and reproduction of microorganisms. In a certain range, the physiological and biochemical reactions of the strain increase with the increase of temperature, but if the temperature continuously increases and reaches a certain extent, the metabolic capacity of the strain decreases sharply, leading to cell death. As depicted in Figure 4A, the biomass of the FC811 strain, the soluble sugar accumulation, and the saccharification rate increased with the increase of temperature from 45 to 60 °C. When the temperature was 60 °C, the soluble sugar accumulation of the FC811 strain reached the maximum, which was 577.20 mg/g microcrystalline cellulose and the saccharification rate was 57.72%. However, the soluble sugar accumulation began to decrease with the increase in temperature. When the temperature was increased to 70 °C, the soluble sugar yield was 81.87 mg/g of microcrystalline cellulose, and the saccharification rate was less than 10%. Compared with 60 °C, the biomass of the strain was also significantly reduced, indicating that the FC811 strain grew slowly or stopped growing when the temperature was 70 °C. The results suggested that the FC811 strain had the highest saccharification efficiency on microcrystalline cellulose under the survival environment of 60 °C. Therefore, 60 °C was the optimum temperature for the growth and saccharification of the FC811 strain.

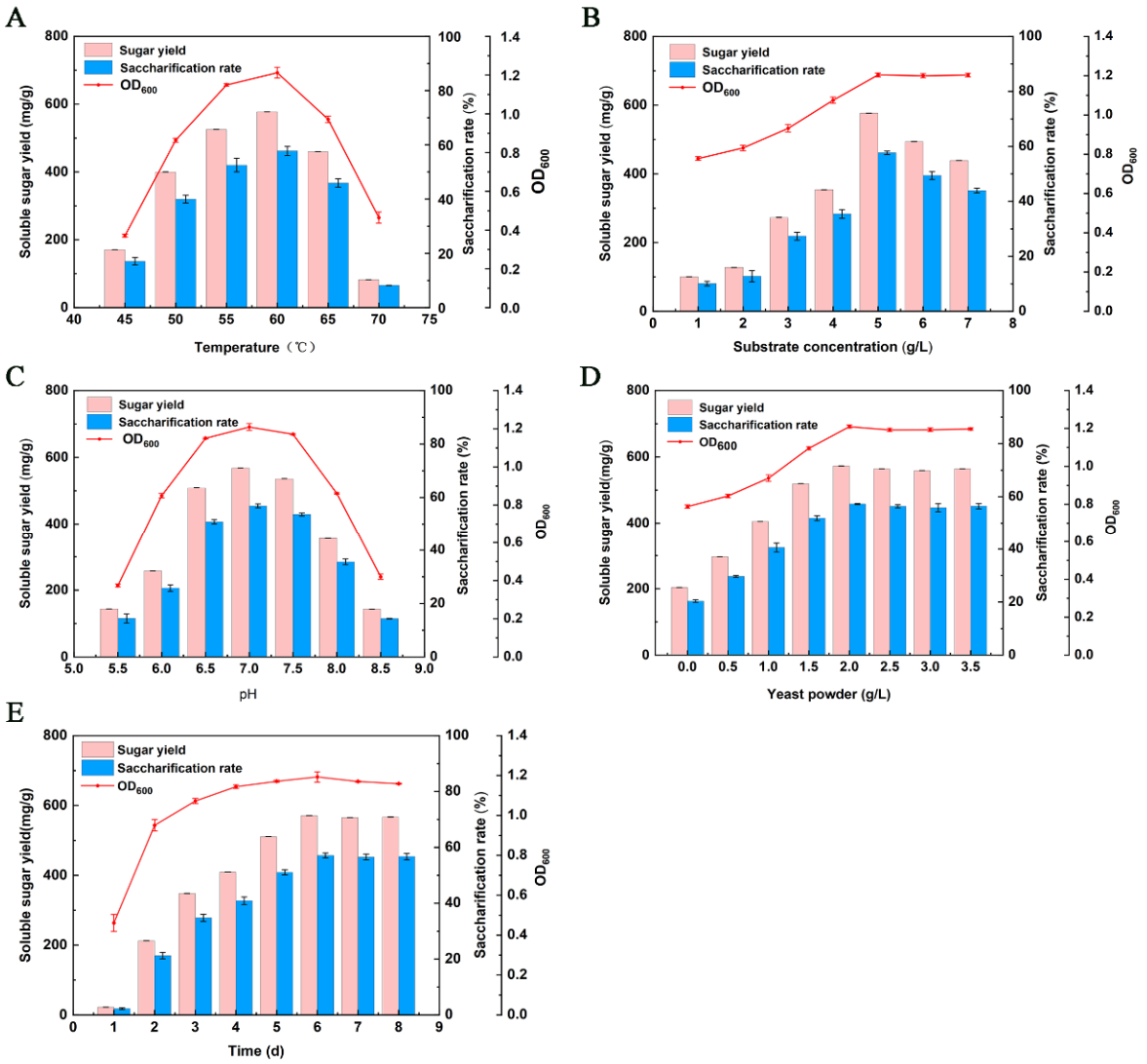

Figure 4. Sugar production effect of saccharifying strains under different culture conditions: (**A**). Effect of tmperature; (**B**). Effect of substrate concentration; (**C**). Effect of pH; (**D**). Effect of yeast powder; (**E**). Effect of time.

3.3.2. Effect of Initial pH on the Growth of FC811 Strain and Saccharification Effect of Microcrystalline Cellulose

Too high or too low pH will affect the metabolic ability of microorganisms, leading to survival difficulty. Different microorganisms have different requirements for pH [38]. As depicted in Figure 4B, the FC811 strain was in the initial pH range of 5.5–7.0. With the increase in pH, the saccharification effect of FC811 strain on microcrystalline cellulose and its biomass increased. When the pH was 7.0, the soluble sugar accumulation of the FC811 strain reached the maximum, which was 568.13 mg/g of microcrystalline cellulose, and the saccharification rate was 56.81%. When pH exceeded 8.0, the biomass of the FC811 strain, the soluble sugar accumulation, and saccharification rate decreased significantly (Figure 4). When the pH increased to 9.0, the FC811 strain could not grow, and almost no soluble sugar could be detected in the anaerobic flask. It might be that too high pH inhibited the activity of the strain and prevented the strain from metabolizing, making the strain unable to grow or saccharify microcrystalline cellulose. The results showed that the biomass of the FC811 strain, the soluble sugar accumulation, and the saccharification rate reached a peak when pH was 7.0. Therefore, the initial pH of 7.0 was the optimal initial pH for the growth of the FC811 strain and saccharification of microcrystalline cellulose.

3.3.3. Effect of Substrate Concentration on the Growth of FC811 Strain and Saccharification Effect of Microcrystalline Cellulose

According to the Michaelis–Menten equation, the substrate concentration has no effect on enzyme activities of metabolic capacity (FC811 strain in this study); rather, it only affects the enzymatic reaction rate [39]. With the increase of substrate concentration, the enzymatic reaction rate gradually increases. After reaching a certain value, it does not increase with increasing concentration. As depicted in Figure 4C, when the initial concentration of microcrystalline cellulose was 5 g/L, the biomass of the FC811 strain was 1.204 (OD_{600nm}), and the accumulation of soluble sugar reached the maximum, which was 576.67 mg/g microcrystalline cellulose, and the saccharification rate was 57.67%. When the initial concentration of microcrystalline cellulose was in the range of 1–5 g/L, the saccharification rate and the biomass of the FC811 strain increased with the increase of microcrystalline cellulose concentration. When the concentration of microcrystalline cellulose was continuously increased, it was found that the accumulation of soluble sugar increased a little, and the saccharification rate gradually decreased. This might be because cellulase could not fully interact with the substrate, affecting the saccharification effect. Therefore, 5 g/L microcrystalline cellulose concentration was selected as the optimal concentration.

3.3.4. Effect of Yeast Powder Content on the Growth of FC811 Strain and Saccharification Effect of Microcrystalline Cellulose

The concentration of yeast powder, one of the nutrients of strains, will directly nourish the biomass accumulation and reproduction capacity of strains [40]. As depicted in Figure 4D, when the yeast powder concentration was in the range of 0–2 g/L, the biomass of the FC811 strain, the soluble sugar accumulation, and saccharification rate increased with the increase of yeast powder concentration. When the yeast powder concentration was 2.0 g/L, the biomass of the strain was 1.209, the soluble sugar accumulation was 572.6 mg/g, and the saccharification rate was 57.26%. When the yeast powder concentration was more than 2.0 g/L, the biomass of the FC811 strain and soluble sugar accumulation changed a little. Therefore, considering the economic benefits, the yeast powder concentration of 2.0 g/L was selected as the optimal concentration for the experiment.

3.3.5. Effect of Culture Time on the Growth of FC811 Strain and Saccharification Effect of Microcrystalline Cellulose

The growth rate of different strains is different, and the biomass in the reactor is not enough if the time is too short. If the time is too long, the strains will age and even show apoptosis. As depicted in Figure 4E, when the culture time was 1 d, the biomass of FC811 strain, soluble sugar accumulation, and saccharification rates were all low. When the culture time was 2 d, the FC811 strain grew rapidly, its biomass was 0.951, and the soluble sugar accumulation was 212.6 mg/g. After 3–8 d of culture, the FC811 strain grew slowly, but the soluble sugar accumulation and saccharification rate reached the maximum value on 6 d, which were 570.93 mg/g and 57.09%, respectively. Therefore, 6 d was the optimal culture time for the growth and saccharification of the FC811 strain.

3.3.6. Optimization of the Growth of FC811 Strain and Saccharification Effect of Microcrystalline Cellulose

In this study, temperature (A), pH (B), culture time (C), substrate concentration (D), and yeast powder concentration (E) were used as independent variables, and soluble sugar yield was used as the response value. In terms of single factor experiment results, it was determined that $55 < A < 65$, $6.5 < B < 7.5$, $5 < C < 7$, $4 < D < 6$, and $1.5 < E < 2.5$. The optimal saccharification condition of the strain and the effect of two factors' interaction on the soluble sugar yield were determined using the Minitab software (Version17.1.0, Minitab Inc., Philadelphia, PE, USA). Tables 4 and 5 summarize the design and response surface CCD experimental results for microcrystal cellulose saccharifying strain *Clostridium thermocellum* FC811.

Table 4. Response surface CCD experiment design of *Clostridium thermocellum* FC811 strain.

Number	Temperature (°C)	pH	Culture Time (d)	Substrate Concentration (g/L)	Yeast Powder Concentration (g/L)	Soluble Sugar Yield (g/L)	
						Actual Value	Predicted Value
1	60	7	6	5	2	2.87	2.87
2	55	7.5	5	4	1.5	1.25	1.23
3	55	6.5	5	4	2.5	1.23	1.21
4	65	6.5	7	4	2.5	1.10	1.12
5	60	7	6	5	2	2.83	2.87
6	55	6.5	5	6	1.5	2.18	2.18
7	65	7.5	5	6	1.5	1.97	1.95
8	65	7.5	5	4	2.5	1.20	1.23
9	65	6.5	5	6	2.5	2.14	2.17
10	60	7	6	5	2	2.86	2.87
11	65	7.5	7	6	2.5	2.47	2.46
12	65	7.5	7	4	1.5	1.29	1.29
13	55	6.5	7	6	2.5	2.53	2.57
14	65	6.5	7	6	1.5	2.01	2.03
15	55	6.5	7	4	1.5	1.13	1.12
16	55	7.5	5	6	2.5	2.50	2.46
17	55	7.5	7	6	1.5	2.38	2.36
18	60	7	6	5	2	2.85	2.87
19	65	6.5	5	4	1.5	1.24	1.22
20	55	7.5	7	4	2.5	1.26	1.23
21	60	7	6	5	2	2.82	2.84
22	60	7.5	6	5	2	2.78	2.82
23	65	7	6	5	2	2.60	2.60
24	60	7	6	5	2	2.89	2.84
25	60	7	5	5	2	2.72	2.75
26	60	7	6	4	2	1.63	1.66
27	60	7	6	5	2	2.89	2.84
28	60	7	6	5	2	2.82	2.84
29	60	7	6	5	1.5	2.56	2.59
30	60	7	7	5	2	2.83	2.82
31	60	6	6	5	2	2.54	2.54
32	60	7	6	6	2	2.73	2.72
33	55	7	6	5	2	2.69	2.71
34	60	7	6	5	2	2.91	2.84
35	55	7.5	7	4	1.5	1.24	1.26
36	65	6.5	5	6	1.5	1.94	1.95
37	65	7.5	5	4	1.5	1.32	1.30
38	60	7	6	5	2	2.87	2.91
39	55	6.5	5	6	2.5	2.51	2.50
40	60	7	6	5	2	2.93	2.91
41	65	7.5	7	6	1.5	2.18	2.20
42	55	6.5	7	4	2.5	1.15	1.16
43	65	7.5	7	4	2.5	1.32	1.31
44	55	7.5	5	6	1.5	2.18	2.21
45	55	6.5	5	4	1.5	1.25	1.27
46	65	6.5	7	4	1.5	1.18	1.18
47	60	7	6	5	2	2.95	2.91
48	65	6.5	7	6	2.5	2.38	2.35
49	60	7	6	5	2	2.92	2.91
50	55	7.5	7	6	2.5	2.67	2.71
51	65	6.5	5	4	2.5	1.23	1.21
52	65	7.5	5	6	2.5	2.25	2.25
53	55	6.5	7	6	1.5	2.34	2.30
54	55	7.5	5	4	2.5	1.24	1.25

After the strain *Clostridium thermocellum* FC811 was inoculated in the anaerobic fermentation medium, the soluble sugar accumulation in the solution ranged from 1.101–2.950 g/L, indicating that the five factors had a significant impact on soluble sugar yield. The soluble sugar yield measured in the experiment was consistent with the predicted result of the model, implying that the regression equation had high accuracy.

Minitab17.0 software was used for statistical analysis of the experimental data, and the results are summarized in Table 5. The coefficient of determination (R^2) and adjusted coefficient (Adj. R^2) of the experimental model were 99.85% and 99.75%, respectively. The *p* value of the response surface regression model was less than 0.0001, indicating that the regression model had a high fitting degree and could be used to predict the response value.

Table 5. Response surface CCD statistics and analysis results.

Source	Coefficient	Degree of Freedom	Sum of Square	Mean Sum of Square	F Value	*p* Value
Model		22	24.1737	1.09880	946.52	<0.0001
Linear		5	9.9579	1.99158	1715.56	<0.0001
A	−0.05576	1	0.1057	0.10573	91.08	<0.0001
B	0.03615	1	0.0446	0.04464	38.46	<0.0001
C	0.03329	1	0.0377	0.03769	32.47	<0.0001
D	0.53215	1	9.6281	9.62814	8293.74	<0.0001
E	0.06614	1	0.1407	0.14071	121.21	<0.0001
Square		5	8.8270	1.76540	1520.72	<0.0001
A^2	−0.1835	1	0.0815	0.08152	70.22	<0.0001
B^2	−0.05790	1	0.0509	0.05093	43.87	<0.0001
C^2	−0.0555	1	0.0075	0.00745	6.42	0.017
D^2	−0.6480	1	1.0167	1.01668	875.78	<0.0001
E^2	−0.1861	1	0.0512	0.05124	44.14	<0.0001
Interaction of two factors		10	0.4281	0.04281	36.88	<0.0001
AB	0.01147	1	0.0042	0.00421	3.63	0.066
AC	0.00828	1	0.0022	0.00219	1.89	0.179
AD	−0.06497	1	0.1351	0.13507	116.35	<0.0001
AE	−0.00559	1	0.0010	0.00100	0.86	0.360
BC	0.02484	1	0.0198	0.01975	17.01	<0.0001
BD	−0.00128	1	0.0001	0.00005	0.05	0.833
BE	0.00259	1	0.0002	0.00022	0.19	0.670
CD	0.04916	1	0.0773	0.07732	66.61	<0.0001
CE	0.00528	1	0.0009	0.00089	0.77	0.387
DE	0.07653	1	0.1874	0.18743	161.45	<0.0001
Residual		31	0.0360	0.00116		
Lack of fit		21	0.0242	0.00115	0.98	0.539
Pure error		10	0.0118	0.00118		
Total		53	24.2097			

Note: R^2 = 0.9985; Adj. R^2 = 0.9975; A, B, C, D, and E denote temperature, pH, culture time, substrate concentration, and yeast powder concentration, respectively. $p < 0.05$ indicates significant, $p < 0.0001$ indicates extremely significant, and $p > 0.05$ indicates not significant.

The fitting regression optimization equation of *Clostridium thermocellum* FC811 strain with soluble sugar yield as the response value was obtained as follows:

$$Y = -55.40 + 0.897A + 2.733B - 0.015C + 7.208D + 2.342E - 0.007339A^2 - 0.2316B^2 - 0.0555C^2 - 0.6480D^2 - 0.744E^2 + 0.00459AB + 0.00166AC - 0.01299AD - 0.00224AE + 0.0497BC - 0.0026BD + 0.0104BE + 0.04916CD + 0.0106CE + 0.1531DE$$

where Y denotes the accumulation of soluble sugar; A, B, C, D, and E denote temperature, pH, culture time, substrate concentration, and yeast powder concentration, respectively.

As summarized in Table 5, the interactions between temperature and substrate concentration, pH and culture time, culture time and substrate concentration, and substrate concentration and yeast powder concentration were extremely significant ($p < 0.0001$).

Combined with the above results, the response curve of the two factors were extremely, significantly affecting the soluble sugar yield of microcrystalline cellulose saccharified by *Clostridium thermocellum* FC811 strain that was plotted (Figure 5). Based on the regression model equation analysis, the optimal saccharification conditions for *Clostridium thermocellum* FC811 were set as follows: temperature of 58.94 °C, pH of 7.21, culture time of 6.60 d, substrate concentration of 5.01 g/L, yeast powder concentration of 2.15 g/L, and soluble sugar yield of 3.03 g/L. Further experiments were carried out to verify the accuracy of the model prediction. The anaerobic culture results showed that the soluble sugar yield was 3.11 g/L under the optimal saccharification conditions, which was consistent with the predicted value of the model.

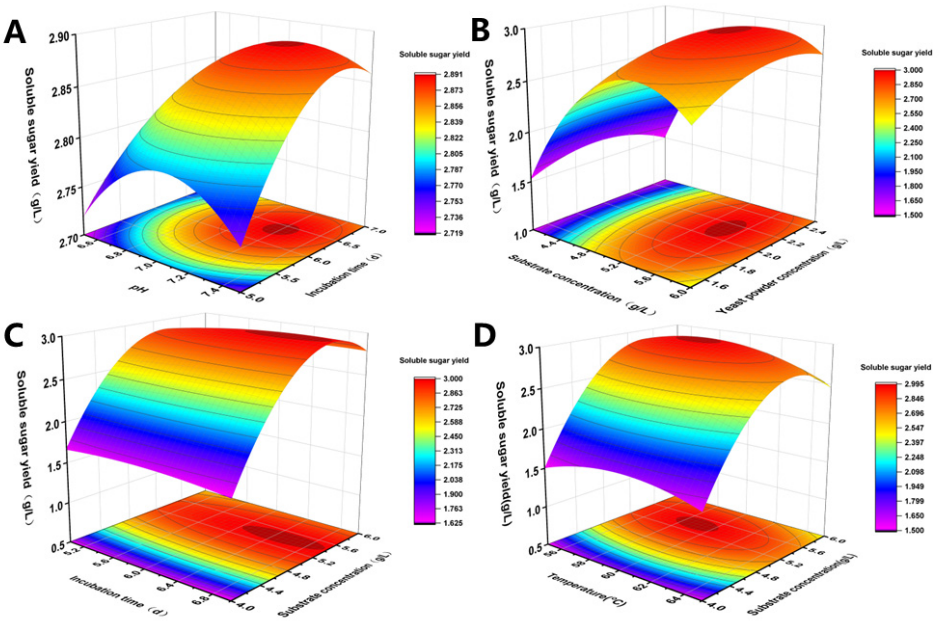

Figure 5. Surface results of effects on sugar production yield under different culture conditions: (**A**). Effect of pH and incubation time; (**B**). Effect of substrate concentration and yeast powder concentration; (**C**). Effect of incubation time and substrate concentration; (**D**). Effect of temperature and substrate concentration.

In this study, two cellulose saccharification strains were isolated from rotten bark. The FC811 strain was identified as *Clostridium thermocellum*, a Gram-positive strain named *Clostridium thermocellum* FC811 with the strongest cellulose saccharification effect. It also showed a strong utilization capacity of glucose, microcrystalline cellulose, cellobiose, mannose, mannitol, and filter paper. The key factors affecting FC811 for fiber saccharification, such as fructose as temperature, initial pH, substrate concentration, yeast powder content, and culture time, were verified through the single factor experiment. The optimization results were obtained through response surface analysis: temperature of 58.94 °C, pH of 7.21, culture time of 6.60 d, substrate concentration of 5.01 g/L, and yeast powder concentration of 2.15 g/L. The soluble sugar accumulation and saccharification rates were 622 mg/g and 62.2%, respectively.

4. Conclusions

Due to the depletion of global energy reserves and continuous environmental pollution by straws, hydrogen production by anaerobic fermentation of corn straw has significant development potential. In this study, the compound bacteria screened from rotten wood were inoculated in the straw medium. The sugar production characteristics were determined from two perspectives of nutrient factors and ecological factors. It was proved that the optimal conditions for sugar production of FC811 strain were a temperature of 58.9 °C, pH of 7.21, culture time of 6.60 d, substrate concentration of 5.01 g/L, and yeast powder concentration of 2.15 g/L. The yield of soluble sugar was 3.11 g/L, and the conversion rate of reducing sugar was 62.2%, which is much higher than the previous [41]. The present study results will provide a theoretical basis for subsequent synchronous fermentation of sugar and hydrogen production.

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