

In Situ Corn Fiber Conversion for Ethanol Improvement by The Addition of Novel Lignocellulolytic Enzyme Cocktail

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Research

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Abstract

Background: The technology of converting corn mashes to ethanol has been mature, but corn mashes has high-viscosity and high-sugar characteristics which hindered cellulose utilization and yeast-fermentation efficiency. The excessive viscosity of corn mash is caused by the presence of non-starch polysaccharides, such as cellulose in cereal grains. Corn kernel fiber (mostly cellulose) is typically unconverted in the process.

Results: A novel lignocellulolytic enzymes cocktail with strong substrate specificity was prepared for high-viscosity, high-sugar corn mash. The in situ conversion of corn mashes with novel lignocellulolytic enzymes at the optimum cellulase dosage of 50 FPU/L resulted in 12.4%, 12.0%, 11.8%, and 12.9% increased ethanol concentration compared with the reference mash at 0.3, 1, 5, and 70 L batch-fermentation scales, respectively. The highest yield of ethanol from corn mash digested with the prepared novel lignocellulolytic enzyme reached 117.0 ± 0.1 g/L at the 70 L batch fermentation, which was a 12.9% increase in ethanol yield. Adding the lignocellulolytic enzymes caused the greatest decrease in viscosity of corn mash by 40.9% compared with the reference mash (33.5 ± 1.5 Pa·s), whereas the residual sugars decreased by 56.3%. Simultaneously, the application of novel lignocellulolytic enzymes increased the value of dried distiller's grain with solubles by increasing the protein content and decreasing the residual cellulose and starch content.

Conclusion: The application of novel lignocellulolytic enzymes significantly improved the alcohol concentration, productivity, and yield. With the same amount of material, the application of the novel enzymes cocktail can enhance the ethanol yield by more than 10%. The in situ conversion of cellulose promoted the release of contents, including starch and protein, which can decrease the fermentation broth viscosity and improve the rheological property, thereby improving the ethanol yield. Thus, this technology can increase the net revenue of fuel-ethanol industrialization and promote the technological progress of renewable energy.

Background

Ethanol production through biotechnology is an attractive option for sustainable fuel production. Starch is the second most important and abundant source of carbon and energy in plants. Starch is an important feedstock in the fermentation industry and widely fermented to produce ethanol, which can be used as a basis for beverages or as an alternative biofuel [1, 2]. In China, the major feedstock for ethanol fuel production is corn, and new production plants tend to apply the dry milling technology more than wet milling because of lower capital costs [3]. In the dry milling process, whole corn kernels are milled and mixed with water, yielding a viscous slurry, which is liquefied by heat treatment and α -amylase. The economics of this process greatly depend on the revenue obtained by selling the main co-product known as dried distiller's grain with solubles (DDGS), which is rich in protein, fiber, and vitamins, as an animal feed [4]. Attempts have been made to develop additional co-products, such as corn oil.

Industrial corn mash has high viscosity and contains coarse particles. A problem during the processing of corn starch is the excessive viscosity of mash, which can increase the complexity of the process of starch hydrolysis and mash fermentation [5]. The excessive viscosity of mash is caused by the presence of non-starch polysaccharides, such as cellulose and hemicellulose in cereal grains [5]. The corn kernel fiber (mostly cellulose) is unutilized in the current process. Corn cellulose conversion is of great interest in the field [6]. However, most studies focused on the conversion of isolated corn fiber [7, 8]. The in situ conversion of cellulose during the dry-milling process have not been studied comprehensively. Corn consists of an outer seed cover or pericarp, which encloses the embryo and the starch-rich endosperm [9]. This structure renders them non-hydrolyzable by amylolytic enzymes to fermentable sugars. The lignocellulolytic material can serve as feedstock for additional ethanol production. The recalcitrance of corn starch requires lignocellulolytic enzymes preparation, resulting in an unacceptable and costly process.

The reported work showed lignocellulolytic enzymes could improve ethanol yield from starch due to excessive enzyme dosage. The costly process brings out inappropriate ethanol yield increase, which is unacceptable and hinder cellulase application in the field of bioethanol.

In this work, we develop a novel lignocellulolytic enzymes preparation that has a strong substrate specificity for high-sugar and -viscosity corn mash. The main objectives of this work are to convert cellulose in situ during simultaneous saccharification and fermentation (SSF) and investigate the effect of the novel lignocellulolytic enzymes addition on SSF performance, including

ethanol yield, corn mash viscosity, and residual sugars. The application of the novel enzymes cocktail can enhance ethanol yield by more than 10%, which is beneficial to bioethanol.

Results

Characteristics of lignocellulolytic enzymes

The composition of lignocellulolytic enzyme cocktail for high-viscosity corn mash would be screened in the lignocellulolytic enzyme library. The lignocellulolytic enzyme library contained the main enzyme system from *T.reesei* and the coenzyme system from *A.niger*. Although the commercial cellulase was produced from *T.reesei*, the enzyme system from *T.reesei* has some drawbacks [10]. The main enzyme system from *T.reesei* was incompleting and unbalanced, such as no genes encoding cellobiose dehydrogenases and feruloyl esterase, few monooxygenases and β -glucosidase expressed in the *T.reesei* extracellular protein [11, 12]. These key coenzymes may need to be introduced from other species to improve the enzymatic efficiency [10]. For high-viscosity, high-sugar corn mash, the coenzymes such as FAE, GH61 and β -glucosidase were overexpressed up to 15% of total extracellular enzymes for optimization of enzyme system composition. It is reported that this FAE, GH61 and β -glucosidase from *Penicillium piceum* had unique enzymology characteristics in our previous paper [13, 14]. The FAE and GH61 reduced biomass recalcitrance by increasing xylose/arabinose ratio and decreasing HBI of crude biomass, which could reduce lignocellulose degradation recalcitrance, providing favorable conditions for enzymatic hydrolysis [13,14]. The novel lignocellulolytic cocktail was balanced enzymatic ratio with filter paper activity of 40.8 FPU/mL, xylanase of 1320.6 IU/mL, and feruloyl esterase of 22.5 IU/mL and additional side activities, including amylase of 4.5 IU/mL.

Effect of lignocellulolytic enzyme loadings on corn mash fermentation

Considering the cost of using enzymes, lignocellulolytic enzyme was selected for further experiments to test at a lower enzyme dosage. Corn mash fermentation was performed at different enzyme loadings (2–200FPU/L) to facilitate the screening of optimum enzyme dosage. The ethanol concentration, sugar reduction, and viscosity of corn mash corresponding to different enzyme dosages are presented in Table 1. The ethanol yield increased with increased cellulase dosage until 50FPU/L. Further increased enzyme dosage (100–200FPU/L) did not increase the ethanol yield. The enzyme dosage ranges of 20–40FPU/L did not result in maximal promotion in alcohol concentration and yield. Therefore, 50FPU/L was selected as the optimal cellulase dosage in the SSF. At the optimum enzyme dosage of 50FPU/L mash, the ethanol yield increased by 13.3% compared with the reference mash.

The application of lignocellulolytic enzymes preparation decreased the mash viscosity and increased the yeast-fermentation efficiency. This result was consistent with a previously published report. The results of Czarnecki and Nowak indicate the beneficial effects of lignocellulolytic enzymes (e.g., xylanase, cellulase, and gluconase) on rye mashes, such as a decreased viscosity, enhanced starch saccharification, and increased productivity of ethanol [15, 16].

Effect of different fermentation tanks size on ethanol production

The novel lignocellulolytic enzymes were applied at different fermentation scales from 0.3–70 L. At the 0.3, 1, 5, and 70 L batch-fermentation scales, the reductions in corn mash viscosity were 46.3%, 31.6%, 35.5%, and 40.9% compared with the reference mash (33.5 ± 1.5 Pa·s), respectively. The treatment of corn mashes with the novel lignocellulolytic enzymes resulted in increasing concentrations of ethanol by 12.4%, 12.0%, 11.8%, and 12.9% compared with the reference mash. The highest yield of ethanol in the corn mash digested with the novel lignocellulolytic enzyme reached 117.0 ± 0.1 g/L at the 70 L batch fermentation, whereas that in the control reached only 103.6 ± 1.0 g/L. After the lignocellulolytic enzyme addition, the residual starch content decreased to $5.34 \pm 0.26\%$, whereas that without lignocellulolytic enzyme was $6.58 \pm 0.86\%$ (Table 2). The residual cellulose content of corn mash decreased to $7.48 \pm 0.10\%$, whereas that without lignocellulolytic enzyme was $12.64 \pm 0.52\%$. Approximately 1.24% of starch and 5.16% of cellulose in the corn mash were further hydrolyzed. This result indicates that a part of the residual cellulose in corn mash was mostly degradable by the lignocellulolytic enzyme. The starch conversion with the cellulase cocktail improved because cellulase disrupted the cell wall structure of the grain and promoted starch release. Furthermore, the cellulase cocktail possibly contained amylases.

Further degradation of residual SSF broth after ethanol evaporated by lignocellulolytic enzymes cocktail

To elucidate the promotion effect of lignocellulolytic enzymes on alcoholic fermentation clearly, the lignocellulolytic enzymes were added into the residual broth in SSF. The 36 h SSF broth (containing unconverted solids) with the ethanol evaporated was used for another round of separate hydrolysis and fermentation (SHF), which reduced the ethanol inhibition on the cellulase activity. As shown in Fig. 1, approximately 7.0 g/L of glucose was released from the saccharification of the residual broth with the lignocellulolytic enzymes, whereas no glucose was released from that without cellulase addition. Then, the activated yeast was added into the saccharification broth for another 36 h ethanol fermentation. Further ethanol production can yield 6.44 g/L after lignocellulolytic enzymes addition. The residual starch contents with and without lignocellulolytic enzymes addition were $5.06 \pm 0.34\%$ and $6.02 \pm 0.86\%$, respectively (Table 3). The residual cellulose contents of corn mash with and without lignocellulolytic enzymes addition were $10.07 \pm 0.10\%$ and $13.20 \pm 0.52\%$, respectively. Approximately 0.96% of starch and 3.13% of cellulose in the corn mash were further hydrolyzed. Adding lignocellulolytic enzyme resulted in 13.58 g of sugar production, including the release of 1.43 g of soluble residual sugar and 12.15 g of glucose from starch and cellulose. These results indicate that the lignocellulolytic enzymes did promote the release of more glucose from residual starch and cellulose for ethanol increase but not yeast-fermentation efficiency. Certainly, the hydrolysis efficiency of starch and cellulose in SHF was apparently lower than that in SSF.

Comparison of DDGS composition

The main co-product of corn mash fermentation was DDGS, which can increase the economics of this process. A summary of DDGS composition is provided in Table 4. The residual cellulose content of DDGS decreased from 33.12% to 21.20%, while the starch content of DDGS decreased from 14.07% to 11.26% (Table 4). These results showed that the lignocellulolytic enzymes addition did promote the further hydrolysis of residual starch and cellulose in corn mash. The crude protein in DDGS was determined to be 29.63% with the addition of the novel lignocellulolytic enzymes, and 24.12% without the novel lignocellulolytic enzymes. No difference in DDGS color was observed (Supplemental Fig.1). The value of DDGS, which is sold as animal feed, can be increased by increasing the protein content.

Techno-economic analysis of the strategy

The addition of novel lignocellulolytic enzymes for ethanol yield improvement does not require any additional equipment or control system. The only additional cost would be that of the lignocellulolytic enzymes. Ten tons of corn mash can produce one ton of ethanol. The ten tons of corn mash need add lignocellulolytic enzyme input of RMB 75 yuan (FPAase of lignocellulolytic enzyme calculated as 40 FPU/mL; RMB 6000 yuan/ton lignocellulolytic enzymes). The estimated ethanol increase can improve by 10% (conservative computation) in alcoholic industrialization, which is equivalent to increasing the income by RMB 600 yuan (6000 yuan/ton ethanol) from producing 1 ton of ethanol. The net revenue can increase to RMB 525 Yuan (600-75) from producing 1 ton of ethanol. The fuel ethanol production in Jilin Province is 0.6 million tons per year, while the fuel ethanol production in China will be approximately 10.0 million tons per year in near future. This technology can increase the net revenue of fuel ethanol in Jilin Province by RMB 3.2 billion yuan, while the net revenue of fuel ethanol in China by RMB 52.5 billion yuan per year. Moreover, the co-product will add a certain revenue due to the protein content increase of DDGS.

Discussion

Cellulolytic enzymes are believed to have common practical applications with the development of fuel ethanol production from lignocellulolytic biomass [17]. The application of cellulases is uncommon in the alcohol distilling industry, so a large amount of cellulose is unutilized. Few works have been conducted on the in situ conversion of cellulose in alcoholic fermentation.

In this study, the lignocellulolytic enzymes successfully promoted the ethanol yield from corn mash by more than 10% on the premise of the same material amount. Table 5 presents a comparison of the ethanol fermentation from starch under conditions similar to those in our studies. The previous patent showed the addition of the cellulolytic enzyme can increase ethanol yield by as much as 10% or more at the enzyme dosage in the range of 0.01–0.1% wt/wt (enzyme protein / mash solid) [18]. In our manuscript, the novel lignocellulolytic enzymes cocktail could improve ethanol yield by more than 11% at the enzyme dosage of 0.0037% wt/wt (enzyme protein / mash solid), which is lower cost and more economic.

This patent by Abbas et al. showed that the ethanol yield from corn after adding cellulolytic enzymes from *T.reesei* was 95.7 g/L at the solid concentration of 28% (w/w), while Klosowski et al. reported that when corn mash is digested with a multi-enzyme complex from *Asperigillus sp.* the ethanol concentration is 85.7 g/L [19]. Spa et al. used Multi-enzyme complex *CeluStar XL* for corn starch and obtained a high fermentation yield of 104.9 g/L [20]. Wang et al. used glucoamylase from *Rhizopus sp.* for the SSF of raw corn flour. The conversion efficiency of raw corn flour to ethanol was 94.5% of the theoretical ethanol yield [20]. Li et al. used Genecor cellulase for the SSF and obtained the ethanol efficiency of 111.3 g/L [6]. Therefore, a longer fermentation time (48–72 h) than that in our study was needed for corn starch fermentation to ethanol [6, 18, 19, 20, 21]. The work in this manuscript achieved the maximum ethanol yield of 117.0 g/L in the 36 h fermentation period at the solid concentration of 27% (w/w). Overall, the ethanol yield from corn starch reported in this study was higher than that achieved by previous studies. The lignocellulolytic enzyme cocktail application bring about lower enzyme dosage, shorter fermentation time and more significant ethanol improvement, which is more economic than the previous report.

The composition analysis of residual corn mash showed that the addition of lignocellulolytic enzyme resulted in residual starch and cellulose for further hydrolysis. A quarter of the ethanol yield increase was due to the further hydrolysis of starch, while three quarters were to the hydrolysis of cellulose.

This technology does not require any additional equipment or control system and does not modify the alcoholic fermentation technology. The lignocellulolytic enzymes with excellent properties in alcohol industrialization need minimal input. This technology will bring many benefits to the renewable energy of China.

Conclusion

A novel lignocellulolytic enzymes cocktail was prepared with a strong substrate specificity for high-viscosity and -sugars corn mash. On the premise of the same material amount, the application of the novel lignocellulolytic enzymes cocktail can enhance the ethanol yield by more than 10%. The lignocellulolytic enzymes addition for the in situ conversion of cellulose can decrease the viscosity of fermentation broth and improve the rheological property. The results provide new ideas for promoting fuel ethanol industrialization and the technological progress of renewable energy in China.

Materials And Methods

Mashpreparation

Corn mash preparation was provided by Jilin Fuel alcohol company limited. Equal amounts of tap water and back-set stillage were mixed with ground corn to a final dry matter content of 27% (w/v).

Culture condition

The method of lignocellulolytic enzyme fermentation was referring to the reported [22,23].

Enzymaticpreparations

The novel lignocellulolytic enzymatic preparation was a cocktail with the main enzyme system from *T.reesei* and a coenzyme system from *A.niger* at the ratio of 8.5:1 (v/v). The over-expression of FAE and GH61 has been performed in the coenzyme system from *A.niger*.

Yeastpreparation

Approximately 1.0% of alcohol instant active dry yeast (*S. cerevisiae*; Angel, China) was suspended in distilled water at 32 °C for 1 h. Then, 1.0 mL of yeast was added into 100 mL of corn mash with a certain quantity of lignocellulolytic enzymes.

Simultaneoussaccharificationandfermentation(SSF)ofcommashes

The SSF of corn mashes was conducted for 36 h at 32 °C. The fermentation tank was sparged with oxygen at 0.6 L/min. The samples of corn mashes were collected to detect the fermentation parameters, such as ethanol content, reducing sugars, and

viscosity.

Degradability of residual starch and residual cellulose

After SSF for 36 h, the broth without any treatments was used as the control group to study the degradability of residual starch and cellulose. To remove the ethanol effect on the cellulase activity, ethanol was evaporated from the SSF broth with a rotary evaporator at 65 °C, for 15 min, and at 0.07 MPa. The residual starch and cellulose was degraded in a 100 mL Erlenmeyer flask with the same substrate concentration of 27%. The lignocellulolytic enzymes were then added to hydrolyze at 50 °C for 40 h. Then, the yeast was added to the broth for ethanol fermentation. The residual starch and cellulose contents were then analyzed again.

Analysis of fermentation samples

The corn mash samples were analyzed. The reducing sugars and total sugar concentrations after acid hydrolysis were determined (both expressed in grams glucose per 100 mL mash) according to the Schoorl and Regenbogen method [5]. The dextrans were calculated as the difference between the total and reducing sugars considering the conversion coefficient into dextrans (0.9) and expressed in 100 mL mash.

The starch contents of the maize mash were determined using the modified Megazyme assay [24]. The method was based on the hydrolysis of starch with α -amylases and glucoamylase to produce glucose, which was subsequently calculated to the starch content of sample.

The carbohydrate in biomass was quantitatively analyzed according to the NREL Laboratory Analytical Procedures (NREL, 2006) for biomass using a two-step acid method [25]. Approximately 1 g (dry basis) of the samples was dispensed into 200 mL Erlenmeyer flasks. The samples were treated with 5 mL of 72% (w/w) H_2SO_4 at 30 °C for 2.5 h and then stirred every 15 min with a glass stirring rod. The solutions were diluted with 181.7 mL of water and then autoclaved at 121 °C for 1 h.

Residual glucose, ethanol, and other fermentation minor products were determined using high-performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) with a refractive index detector (Shimadzu) on an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The flow rate was 0.6 mL/min at 55 °C, and the mobile phase was 5 mM H_2SO_4 . Each sample was injected three times, and the average results are reported [26].

The viscosity was determined using a Hoppler viscometer type Visco Ball of Fungilab and expressed in mPa s at 20 °C. The determination principle for the viscosity of liquids using the Hoppler viscosimeter involves the measurement of the ball descent time through a constant distance in the studied liquid contained in a glass tube.

The total nitrogen was determined by the Kjeldahl method, which was calculated as crude protein content ($N \times 6.25$) [5]. The reducing sugars were determined by the DNS method according to the procedures of Song et al. [27].

Abbreviations

DDGS: Dried distiller's grain with solubles; HPLC: High performance liquid chromatography; SSF: Simultaneous saccharification and fermentation; SHF: Separate hydrolysis and fermentation; FAE : Feruloyl esterase.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its additional file).

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Authors' contributions:

LG planned and carried out the experimental work on lignocellulolytic enzyme effect on the alcoholic fermentation of corn mash, collected the data, and drafted the manuscript. SLC participated in the design of the study and helped revise the manuscript. DYZ provided the original idea, performed the statistical analysis, and helped revise the manuscript. All authors read and approved the final manuscript submitted to *Biotechnol Biofuels*, and affirmed that it is the original work of the authors.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consent to the publication of the manuscript in *Biotechnology for Biofuels*.

Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

All the supporting data are available.

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Tables

Table 1
Comparison of ethanol, reducing sugars, and viscosity at different enzyme loadings.

	20 FPU/L	30 FPU/L	40 FPU/L	50 FPU/L	100 FPU/L	150 FPU/L	200 FPU/L	CK
Ethanol (g/L)	105.1 ± 0.5	107.5 ± 0.7	112.7 ± 0.6	114.2 ± 0.5	114.6 ± 0.7	113.8 ± 0.8	112.9 ± 0.7	100.7 ± 0.8
Residual sugar (g/L)	26.0 ± 1.4	21.1 ± 1.2	19.4 ± 1.0	13.0 ± 0.7	13.2 ± 0.5	10.9 ± 0.4	11.1 ± 0.5	38.8 ± 1.7
Viscosity (Pa.s)	23.5	22.4	21.7	18.0	19.4	19.1	19.2	34.8

Table 2
Comparison of ethanol, reducing sugars, and viscosity at different fermentation tanks.

Tank size	0.3 L	1 L	5 L	70 L	Tank CK
Ethanol (g/L)	114.2 ± 0.3	113.8 ± 0.8	115.8 ± 0.5	117.0 ± 0.1	103.6 ± 1.0
Residual sugar (g/L)	16.0 ± 0.7	16.2 ± 0.5	16.4 ± 0.4	16.1 ± 0.1	36.8 ± 0.9
Viscosity (Pa.s)	18.0 ± 1.0	22.9 ± 1.2	21.6 ± 1.4	19.8 ± 1.1	33.5 ± 1.5

Table 3
The composition analysis of unutilized corn mash after SSF and SHF with/without lignocellulolytic enzymes addition.

	SSF		SHF	
	With lignocellulolytic enzymes	Without lignocellulolytic enzymes	With lignocellulolytic enzymes	Without lignocellulolytic enzymes
Residual Cellulose of corn mash (%)	7.48 ± 0.10	12.64 ± 0.52	10.07 ± 0.10	13.2 ± 0.52
Residual hemicellulose of corn mash (%)	5.07 ± 0.10	5.35 ± 0.10	5.74 ± 0.13	5.35 ± 0.10
Residual starch of corn mash (%)	5.34 ± 0.26	6.58 ± 0.86	5.06 ± 0.34	6.02 ± 0.86
Residual soluble sugar (g/L)	3.68 ± 0.86	6.42 ± 0.34	4.68 ± 0.56	6.11 ± 0.14

Table 4
Composition of DDGS with/without lignocellulolytic enzymes addition

Compositional analysis	With lignocellulolytic enzymes	Without lignocellulolytic enzymes
Crude protein	29.63	24.12
Cellulose	21.20	33.12
Starch	11.26	14.07
Ash	5.01	5.48
Xylan	5.25	8.21
Arabinan	1.89	3.19
Water extractives	14.26	6.01
Ether extractives	11.50	5.80

Table 5
Comparison alcoholic yield under different fermentation conditions

Enzymes/microorganisms	Raw sources	Solid concentration(%)	Fermentation conditions	Ethanol (g/L)	Reference
Lignocellulolytic enzymes from <i>T. reesei</i> and <i>A. niger</i> / <i>S. cerevisiae</i>	Corn	27.0	32°C, SSF, 36 h	117.0	This study
Celulolytic enzyme from <i>T. reesei</i>	Corn	28.0	30 °C, SSF, 45 h	95.7	[18]
Multi-enzyme complex from <i>Aspergillus</i> sp enzymes/ <i>S. cerevisiae</i>	Corn	28.0	32°C, SSF, 72 h	85.7	[19]
Multi-enzyme complex CeluStar XL/ <i>S. cerevisiae</i>	Corn	28.5	30 °C, SSF, 72 h	104.9	[20]
Genencor cellulase/ <i>S. cerevisiae</i>	Corn	27.0	30 °C, SSF, 48 h	111.3	[6]

Figures

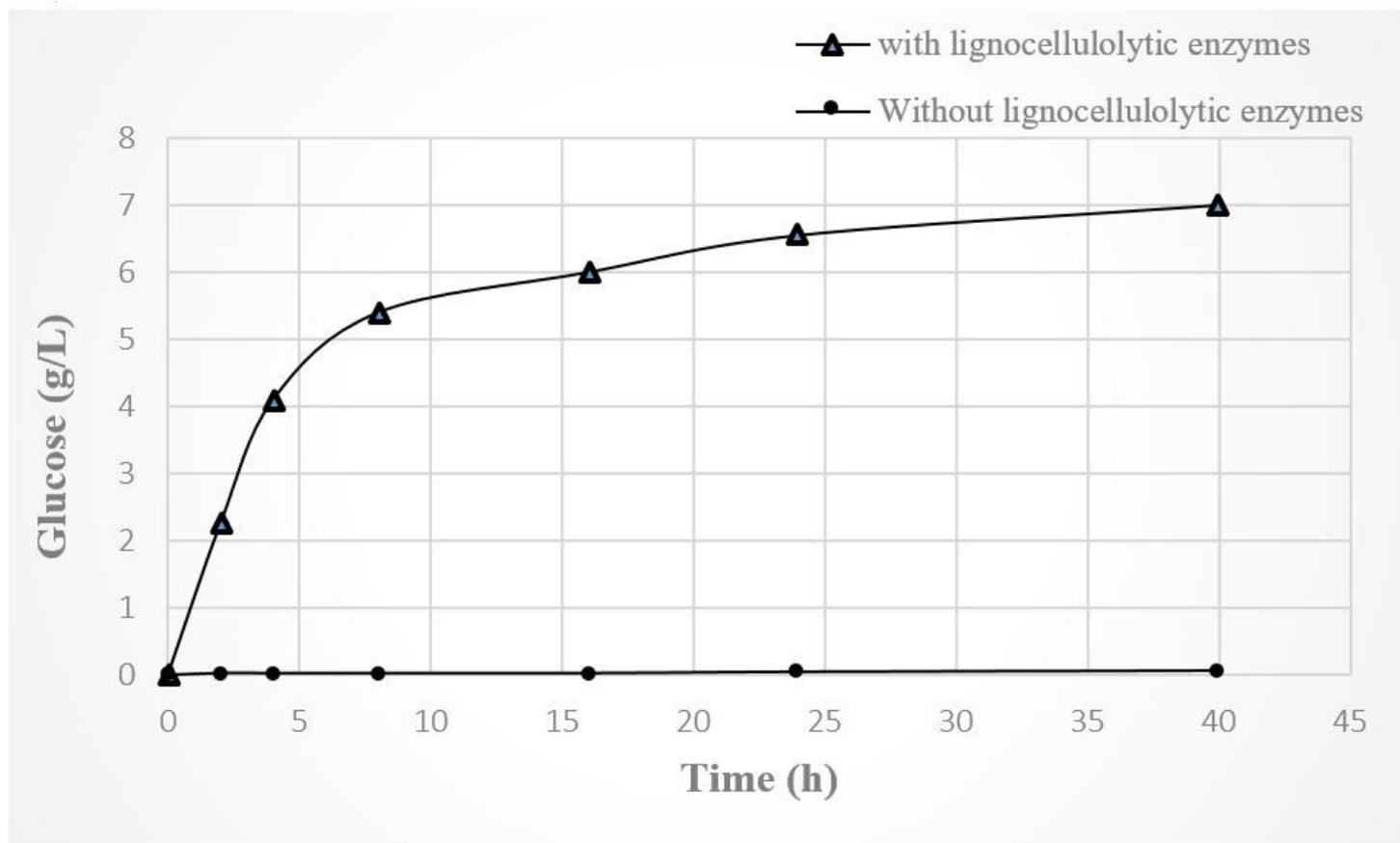


Figure 1

Further degradation of residual SSF broth after ethanol evaporated by lignocellulolytic enzymes cocktail.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalFig.1.docx](#)