A remixed-fermentation technique for the simultaneous bioconversion of corncob C6 and C5 sugars to probiotic *Bacillus subtilis*

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Abstract

The probiotic strain of Bacillus subtilis presents a promising application potential for the value-added bio-utilization of lignocellulosic carbohydrates. By the combined acidolysis pretreatment and enzymatic hydrolysis, hemicellulose and cellulose constituents of corncob were efficiently converted respectively into fermentable C5 and C6 sugars, mainly including xylose and glucose. *B. subtilis* grew well in xylose solution while it was hindered completely in the acidolysis broth because of the bio-toxicity of degraded chemicals derived from corncob. A mixed-fermentation technique was therefore developed and performed to blend the acidolysis broth and enzymatic hydrolysis slurry together, by which C5 and C6 sugar molecules were successfully fermented and efficiently utilized for the growth of *B. subtilis* cells with a yield of 0.33 g cells/g sugar consumed. A net amount of 186.1 ± 0.9 g of *B. subtilis* powder was obtained from 1000 g of corncob that could improve the economic benefits of the process to around 5–7 times.

1. Introduction

Bacillus subtilis being the most popular probiotic, is an indispensably beneficial microorganism in animal husbandry. *B. subtilis* can regulate the gastrointestinal micro-ecological balance and enhance the immune performance of animals, thus playing an important role in promoting animals' health and reproduction [1]. Besides being used as a probiotic in fish breeding and poultry raising, *B. subtilis* is also utilized in water purification, green aquaculture, soil improvement, ecological remediation, *etc* [2–4]. At present, *B. subtilis* is mainly produced through the fermentation of starchy materials at an average market price of 55,000 yuan/ton. Therefore, exploring the cheap and non-food sources for the commercial production of *B. subtilis* probiotics is of greater concern.

Large amount of agro-forest residues are well-known cheap and readily available biomass resources for fermentable C6 and C5 sugars supply, which certainly interests other microbiological fermentation applications besides *B. subtilis* proliferation [5, 6]. Compared with the solo C6 sugar- glucose, present in starchy sources, mannose and galactose are also present in the fermentation broth of agro-forest lignocellulosic residues. Moreover, the C5 sugars- xylose and arabinose are usually inert and hardly fermentable [7–9]. Among different kinds of agro-forest residual biomass, corncob is a typical xylan-rich lignocellulosic waste and thus the biorefinery technology development of corncob provides a general reference for xylan fermentation [10].

B. subtilis is reported to have potential for glucose and xylose fermentation except for crude lignocellulose derivate sugar broth [11]. The bioconversion of agro-forest lignocellulosic biomass requires at least three pretreatment processing steps, enzymatic hydrolysis and microbial fermentation [12, 13]. The initial pretreatment step inevitably releases various complexly degraded chemicals during acidic, hot-water or steam explosion process, which might be lethal for microorganisms and fermentation [14, 15]. In this study, corncob was acidolyzed with dilute sulfuric acid because of its outstanding pretreatment performance and assistance to enzymatic hydrolysis [16, 17]. A mixed-fermentation technique was

therefore developed to proliferate and harvest *B. subtilis* from the hydrolyzed crude corncob broth, in which various C6 and C5 sugars were co-fermented readily and effectively to high-value added bacterial probiotics.

2. Materials And Methods

2.1 Materials and strains

Corncob was collected from the local farmer at Jiangsu province in China, and ground to 20–40 mesh powder. The composition of corncob was determined by the National Renewable Energy Laboratory protocol to be 348.6 ± 0.6% glucan, 314.2 ± 0.4% xylan and 37.0 ± 0.2% araban. Cellulase reagent (Cellic CTec2, Novozymes) and other chemicals were purchased from Sigma-Aldrich Company Co. Ltd. The strain *B. subtilis* CICC 10071 was stored on the agar plate at 4°C.

2.2 Fermentation by Bacillus subtilis

The strain employed was bacillus subtilis CICC 10071. The cells were first cultivated in solid YPD medium. One single colony transferred into the preculture medium (YPD medium) and cultured at 50°C, 150 rpm for 24 h. The seed cells were cultivated in 250 mL conical flask bottles with 50 mL liquid medium. After 24 h, cells were collected and inoculated into the fermentation media. The initial seed in fermentation media was OD600 = 0.5.

The fermentation medium contained 5 g/L yeast extract and 10g/L corn extract based on dried weight. Xylose, arabinose or glucose was added to the stimulated medium in designed titer.Bacterial fermentation experiments were performed in 250 mL shaking Erlenmeyer flaks containing 50 mL medium, and cultured at 150 r/min and 50°C. The initial bacterial inoculation was at the cell density of OD 0.5, and cell pellet was harvested by 2 g centrifugation for 5 min.

2.3 H_2SO_4 acidolysis pretreatment

1000 g corncob powder was mixed with 1% (w/w) H_2SO_4 at a solid-liquid ratio of 1:10, and acidolyzed in a 15-L stainless steel rotary boiler reactor at 150°C and 80 rpm for 30min. The acidolyzed mixture was cooled down in air and centrifuged. The solid residue was stored in a refrigerator at 4°C for later enzymatic hydrolysis.

2.4 Enzymatic hydrolysis

Solid residue weighing 250 g (ODW) was enzymatically hydrolyzed in a 10-L stainless steel and stirred tank reactor at 50°C and 300 rpm for 48 h. The solid residue, 5 % (w/v), was mixed with 20 FPIU/g cellulase reagent to produce glucan at pH 4.80. To this, 0.2% (w/v) tetracycline was added to prevent bacterial contamination [18].

2.5 Hydrolysate fermentation and enzymatic hydrolysis fermentation

Hydrolysate fermentation is the direct use of undetoxified corncob sulfuric acid hydrolysate for fermentation. Fermentation was carried out in a 250mL conical flask at 50℃ and 150rmp, and PH was

adjusted to neutral by 1mol /L NaOH. In order to compare the growth of Bacillus subtilis and the utilization rate of sugar in the hydrolysate with different concentrations of sugar, two concentration gradients were set up in this experiment: the diluted hydrolysate and the original hydrolysate. In order to monitor the change of sugar concentration and the growth of bacteria in the hydrolysate in real time, samples were taken every 3 hours and fermented continuously for 24 hours.

Enzymatic fermentation is similar to hydrolysate fermentation. Fermentation was carried out in a 250mL conical flask at 50℃ and 150rmp, and PH was adjusted to neutral by 1mol /L NaOH. In this experiment, two concentration gradients were set up: the diluted enzyme hydrolysate and the original enzyme hydrolysate. Similarly, samples were taken every three hours and fermented continuously for 24 hours.

In order to simplify the experimental steps and improve the utilization rate of sugar, we mixed the hydrolysate and enzymatic hydrolysate in the same proportion for fermentation. Because the sugar concentration of the mixed solution is high, and the mixed solution contains both pentose and hexose, we also set up the mixed solution with two concentration gradients to compare the growth of Bacillus subtilis and the utilization rate of sugar.

2.6 Analytical methods

Glucose, xylose, arabinose and inhibitors were analyzed by a high performance liquid chromatograph (HPLC) (Agilent 1260, USA) equipped with an Aminex Bio-Rad HPX-87H column (Bio-Rad Laboratories, USA). The HPLC was operated at 50°C, and 0.005mol/L sulfuric acid was used as the eluent at a flow rate of 0.6 mL/min. The samples were centrifuged, and the supernatant was analyzed by HPLC system. The data was expressed as the mean values and represented in figures. The bars in the figures indicate the ranges of the standard deviation.

The growth and proliferation of *B. subtilis* was monitored by ultraviolet spectrophotometer (Spectrumlab752s, Leng Optical Technology Co. LTD, Shanghai, China). The turbidimetric method was used as follows: 1 ml fermentation broth was centrifuged in 1.5 ml centrifuge tube at 6000 r/min for 5 min, and the supernatant was removed, washed with normal saline, and the mycelium was re-suspended. After dilution with distilled water, the absorbance was determined with a 0.5 cm optical diameter cuvette at the wavelength of 600 nm of spectrophotometer. The obtained absorbance was multiplied by the dilution ratio to obtain the optical density (OD) bacteria. The cell density was converted into cell dry weight by the relationship curve between bacterial dry weight (y) and OD600nm (y = 1.0000x-0.2006, R2 = 0.999).

3. Results And Discussion

3.1 Comparison of various monosaccharide fermentability by *Bacillus subtilis*

The *B. subtilis* fermentation of solo C5 sugars (xylose and arabinose) and C6 sugars (glucose) was compared. The experimental results showed that *B. subtilis* could uptake efficiently the three individual monosaccharides and use them for cells proliferation (Fig. 1). Among the three sugars, the utilization rate

of glucose was higher and reached 3.34 g/L/h, while that of two pentose sugars was observed to be relatively slower at 0.82 g/L/h of xylose and 1.22g/L/h of arabinose.

By comparing individual sugar utilization kinetics, we also found for the first time a very interesting phenomenon that the arabinose utilization rate was somewhat higher than that of xylose. This finding may provide a novel solution for the efficient fermentation and bioconversion of inert arabinose present in other carbohydrate rich biomasses. Glucose, xylose and arabinose reached the same maximum cell titer value of around 4.5 g/L and 0.33g cell/ g consumed sugar, despites the varied kinetics of sugar consumption. When the cell titers increased to 4.5 g/L, sugar consumption was still maintained while no cell proliferation occurred or cell titer remained stable. Moreover, we could hardly detect any fermentation products such as organic acid, acidic acid or proteins. We speculate the threshold effect for cell titer during fermentation of above-mentioned sugars by *B. subtilis* in shaking flask, and it remains to be further studied.

3.2 Direct fermentation of crude xylose-broth from corncob acidolysis step

Corncob was pretreated with hot sulfuric acid to produce crude xylose broth, which contained mainly 30.68 g/L xylose, the rest 3.01 g/L glucose and 3.68 g/L arabinose. However, no fermentation was detected after 12 hours in the crude xylose broth since all the bacterial cells died. Therefore, we stimulated the fermentation by mixing the three sugars together making it similar to the crude xylose broth. As shown in Fig. 2a, B. *subtilis* consumed almost simultaneously C5 and C6 sugars of xylose for the proliferation of cells. Here, the dominant xylose component presented similar fermentation kinetics as that of glucose, while arabinose showed a weak tendency that was different from individual sugar fermentation and was ascribed logically to the metabolic competition among sugar carbon sources. Compared with the individual sugar fermentation, mixed sugars decreased cell yield to 20.1% because of the mentioned speculated threshold effect of *B. subtilis*.

For identification of the bio-inhibition factors in the crude xylose broth, we determined and presented the rest of the components other than sugars in Table 1, such as furfural (furfural, 5-hydroxymethyl furfural (HMF)), phenols of vanillin, weak acids of acetic acid and formic acid [19]. These degraded chemicals usually cause intracellular acidification, energy loss and active oxygen accumulation, and inhibit or kill microorganisms [20]. In order to avoid these negative and inhibitory effects on bacterial fermentation, the detoxification step must be integrated into the biomass-biorefinery process. The common detoxification methods include physical (evaporation, membrane filtration), chemical (resin exchange, alkali detoxification, activated carbon adsorption) and biological (microbial and enzyme) methods [21, 22]. Anyway, these added-detoxification operations improves processing-units, cost and environmental problems of industrial production [23]. For overcoming the fermentation inhibition due to bio-toxicity, we tested the basic ‘detoxification method’ by water dilution [24]. *B. subtilis* yield reached 36.6% by the in-situ and half dilution of crude xylose broth with water (Fig. 2b). Compared with other detoxification methods[25], water-dilution always presents overwhelming advantages of being a simple process, being operation ready and low cost for commercial production.

Table 1
Chemical components of hydrolysate and enzymatic hydrolysate

Sample	Chemical components (g/L)						
	Glucose	Xylose	Arabinose	Acetic acid	Fomic acid	HMF	Furfural
Hydrolysate	2.35	20.48	3.05	3.42	—	0.12	1.31
Enzymatic hydrolysate	21.92	10.64	—	—	3.11	—	—
Mixed hydrolysate	11.42	15.53	1.76	1.88	1.52	0.06	0.72

3.3 Mixed-fermentation of corncob C5 and C6 sugars to *B. subtilis*

Solid-liquid separation followed by acidolysis pretreatment is a necessary and effective step before carrying the enzymatic hydrolysis with respect to the inhibitory effects of various degraded chemicals, such as lignin fractions and mono-/oligo-saccharides. The lignin fractions could adsorb and deactivate enzyme-protein components, and mono-/oligo-saccharides works as end-product inhibitors of enzymatic hydrolysis reaction. The separated solids from the pretreatment step were then hydrolyzed effectively to fermentable glucose with 20 FPIU/g of cellulase loading. We obtained 318.4 ± 0.3 g/L glucose slurry at 88.9 ± 0.2% of enzymatic hydrolysis yield based on the residual cellulose content. This was increased by 27% when compared with the yield of 70% obtained with pretreated materials with no solid-liquid separation step.

Based on the dilution fermentation result, we designed a mixed-fermentation technique for the proliferation of *B. subtilis* from corncob, by mixing the crude xylose broth with the enzymatic hydrolysis slurry containing 336.6 g/L C6 sugar and 221.7 g/L C5 sugars (xylose + arabinose) and was named as mixed-C5/C6 broth. The mixed-C5/C6 broth bioconversion performed almost similar to the enzymatic hydrolysate during shaking flask fermentation (Fig. 3). *B. subtilis* effectively utilized xylose just like glucose, but failed to utilize arabinose. For just one day fermentation, 558.3 g/L of C5 and C6 sugars were converted to 186.1 g/L bacterial cells with a yield of 33.3%. Mixed-fermentation technique thus works effectively for the bio-conversion of corncob C5 and C6 sugars to probiotic *B. subtilis*.

Based on a full-processing test of 1000 g corncob, we proposed the integration technique and the mass-balance result in Fig. 4 as follows: First, corncob powder was acidolyzed with 1.0% H_2SO_4 with the broth comprising of 18.2 ± 0.2 g glucose, 196.4 ± 0.5 g xylose and 20.2 ± 0.2 g arabinose. Second, the rest solid portion was hydrolyzed to 318.0 ± 3.0 g glucose during enzymatic hydrolysis. Finally, 185.0 ± 9.0 g dried cell powder of *B. subtilis* probiotic was harvested. By a brief techno-economic analysis (TEA), we could improve around 5–7 times the economic income through the bioconversion of corncob to *B. subtilis*. Here, TEA was based on the average market price of 5000 dollars/t of bacterial probiotics and 100 dollars/t of corncob, as well as the processing cost of around 30% of product's cost. It is noted that the

mixed-fermentation technique dominated the integration process because of the critical bio-toxicity and inhibition on the C5/C6 sugars fermentation by *B. subtilis*.

4. Conclusion

B. subtilis probiotic cells were proliferated and harvested from corncob by the combined technology of acidolysis pretreatment and enzymatic hydrolysis. To develop a simple and efficient process, C5 and C6 sugars were remixed by blending the acidolysis broth and enzymatic hydrolysis slurry together that caused an effective and in-situ dilution of bio-toxic inhibitors during *B. subtilis* fermentation. We harvested 186.1 ± 0.9 g of *B. subtilis* powder from 1000 g of corncob, and improved the economic benefits around 5–7 times by the integrated bioprocess. This study could provide technical developments for the bioconversion of agro-forest lignocellulosic biomass, especially to high-value added bacterial probiotics.

Declarations

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Conflicts of interest/Competing interests The authors declare that they have no conflict of interest.

Availability of data and material Not applicable.

Code availability Not applicable.

Authors' contributions XuTong Ma and Yong Xu conceived and designed the study.

Ethics approval Not applicable.

Consent to participate The authors declare that they consent to participate.

Consent for publication The authors declare that they consent for publication.

References

[1] Suva, M.; Sureja, V.; & Kheni, D. (2016). Novel insight on probiotic Bacillus subtilis: Mechanism of action and clinical applications. *Journal of Current Research in Scientific Medicine, 2(2),* 65-72.

[2] Elshaghabee, F. M. F.; Rokana, N.; Gulhane, R. D.; Sharma, C.; & Panwar, H. (2017). Bacillus As Potential Probiotics: Status, Concerns, and Future Perspectives. *Frontiers in Microbiology, 8,* 01490.

[3] Olmos, J. (2014). Bacillus subtilis A Potential Probiotic Bacterium to Formulate Functional Feeds for Aquaculture. *Journal of Microbial & Biochemical Technology, 06(07),* 361-365.

[4] Anwar, Z.; Gulfraz, M.; & Irshad, M. (2019). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. *Journal of Radiation Research and Applied Sciences, 7(2),* 163-173.

[5] Chang, Y.; Chang, K.; Lee, C.; Hsu, Y.; Huang, C.; & Jang, H. (2015). Microbial lipid production by oleaginous yeast Cryptococcus sp. in the batch cultures using corncob hydrolysate as carbon source. *Biomass and Bioenergy, 72,* 95-103.

[6] Liu, Q.; Zhang, T.; Liao, Y.; Tan, J.; Wang, T.; Qiu, S.; He, M.; & Ma, L. (2017). Production of C5/C6 Sugar Alcohols by Hydrolytic Hydrogenation of Raw Lignocellulosic Biomass over Zr Based Solid Acids Combined with Ru/C. *ACS sustainable chemistry & engineering, 5(7),* 5940-5950.

[7] Martinez, R.; Flores, A. D.; Dufault, M. E.; & Wang, X. (2019). The XylR variant (R121C and P363S) releases arabinose-induced catabolite repression on xylose fermentation and enhances coutilization of lignocellulosic sugar mixtures. *Biotechnology and Bioengineering, 116(12),* 3476-3481.

[8] Phaiboonsilpa, N.; Chysirichote, T.; Champreda, V.; & Laosiripojana, N. (2020). Fermentation of xylose, arabinose, glucose, their mixtures and sugarcane bagasse hydrolyzate by yeast Pichia stipitis for ethanol production. *Energy Reports, 6,* 710-713.

[9] Granados-Arvizu, J. A.; Madrigal-Perez, L. A.; Canizal-García, M.; González-Hernández, C.; García-Almendárez, B. E.; & Regalado-González, C. (2018). Effect of cytochrome bc1 complex inhibition during fermentation and growth of Scheffersomyces stipitis using glucose, xylose or arabinose as carbon sources. *FEMS yeast research, 19(2),* 1-8.

[10] Gullón, P.; Eibes, G.; Lorenzo, J. M.; Pérez -Rodríguez, N.; Lú - Chau, T. A.; & Gullón, B. (2020). Green sustainable process to revalorize purple corn cobs within a biorefinery frame: Co-production of bioactive extracts. *Science of The Total Environment, 709,* 136236.

[11] Zhang, B.; Li, X.; Fu, J.; Li, N.; Wang, Z.; Tang, Y.; & Chen, T. (2016). Production of Acetoin through Simultaneous Utilization of Glucose, Xylose, and Arabinose by Engineered Bacillus subtilis. *Plos One, 11(7),* e159298.

[12] Nguyen, T. Y.; Cai, C. M.; Kumar, R.; & Wyman, C. E. (2017). Overcoming factors limiting high-solids fermentation of lignocellulosic biomass to ethanol. *Proceedings of the National Academy of Sciences, 114(44),* 11673-11678.

[13] Gandla, M.; Martín, C.; & Jönsson, L. (2018). Analytical Enzymatic Saccharification of Lignocellulosic Biomass for Conversion to Biofuels and Bio-Based Chemicals. *Energies, 11(11),* 2936-2936.

[14] Bhatia, S. K.; Kim, S.; Yoon, J.; & Yong, Y. (2017). Current status and strategies for second generation biofuel production using microbial systems. *Energy Conversion and Management, 148,* 1142-1156.

[15] Wang, G.; Tan, L.; Sun, Z.; Gou, Z.; Tang, Y.; & Kida, K. (2014). Production of bioethanol from rice straw by simultaneous saccharification and fermentation of whole pretreated slurry using Saccharomyces cerevisiaeKF-7. *Environmental Progress & Sustainable Energy, 34(2),* 582-588.

[16] Lee, I.; & Yu, J. (2020). The production of fermentable sugar and bioethanol from acacia wood by optimizing dilute sulfuric acid pretreatment and post treatment. *Fuel, 275,* 117943.

[17] Ouyang, S.; Qiao, H.; Xu, Q.; Zheng, Z.; & Ouyang, J. (2019). Development of two-step pretreatment of Chinese fir sawdust using dilute sulfuric acid followed by sodium chlorite for bioethanol production. *Cellulose, 26(15),* 8513-8524.

[18] Guo, J.; Cao, R.; Huang, K.; Zhou, X.; & Xu, Y. (2020). Comparison of selective acidolysis of xylan and enzymatic hydrolysability of cellulose in various lignocellulosic materials by a novel xylonic acid catalysis method. *Bioresource Technology, 304,* 122943.

[19] Jönsson, L. J.; & Martín, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology, 199,* 103-112.

[20] Moreno, A. D.; Carbone, A.; Pavone, R.; Olsson, L.; & Geijer, C. (2019). Evolutionary engineered Candida intermedia exhibits improved xylose utilization and robustness to lignocellulose-derived inhibitors and ethanol. *Applied Microbiology and Biotechnology, 103(3),* 1405-1416.

[21] Fernanda, R.; Anil, K.; Deb, P.; Sushil, A.; Hui, L.; & Samir, K. (2017). Environmental application of biochar: Current status and perspectives. *Bioresource Technology, 246,* 110-122.

[22] Farmanbordar, S.; Amiri, H.; & Karimi, K. (2018). Simultaneous organosolv pretreatment and detoxification of municipal solid waste for efficient biobutanol production. *Bioresource Technology, 270,* 236-244.

[23] Romaní, A.; Ruiz, H. A.; Pereira, F. B.; Teixeira, J.; & Domingues, L. (2014). Integrated approach for effective bioethanol production using whole slurry from autohydrolyzed Eucalyptus globulus wood at high-solid loadings. *Fuel, 135,* 482-491.

[24] Anwar, Z.; Gulfraz, M.; & Irshad, M. (2019). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. *Journal of Radiation Research and Applied Sciences, 7(2),* 163-173.

[25] Kumar, V.; Sandhu, P. P.; Ahluwalia, V.; Mishra, B. B.; & Yadav, S. K. (2019). Improved upstream processing for detoxification and recovery of xylitol produced from corncob. *Bioresource Technology, 291,* 121931.

Figures

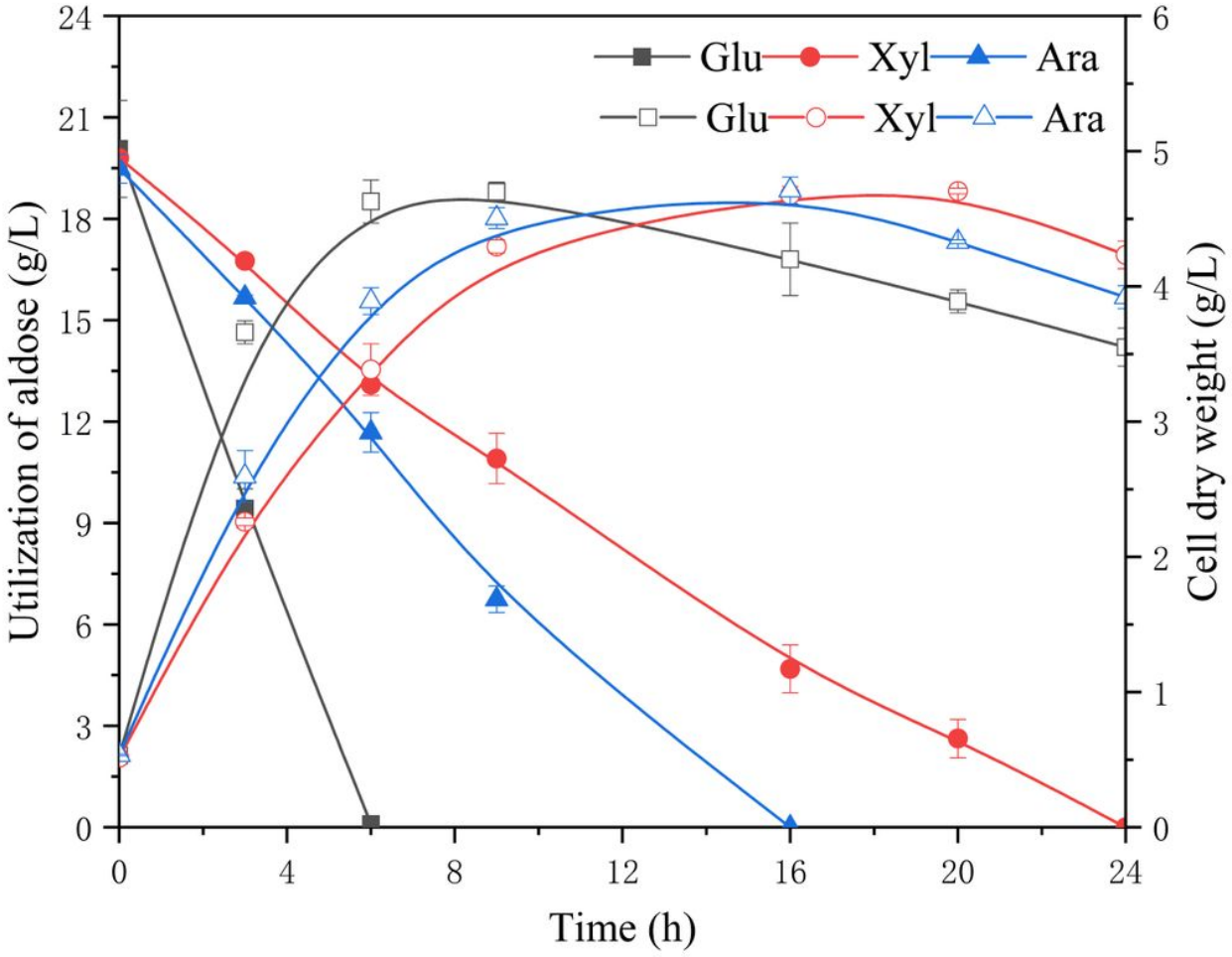

Figure 1

Comparison of cell growth and sugar utilization during individual fermentation of B. subtilis

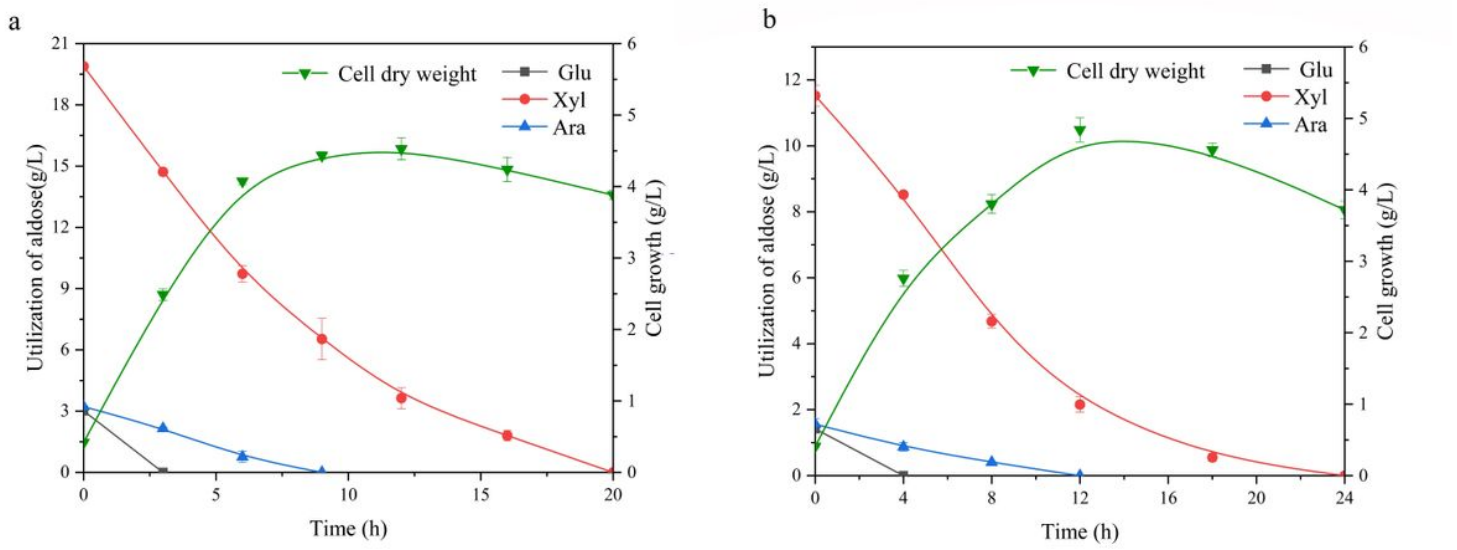

Figure 2

B. subtilis fermentation of the mixed-sugars in the stimulated broth (a) vs the dilute crude xylose-broth of corcob (b)

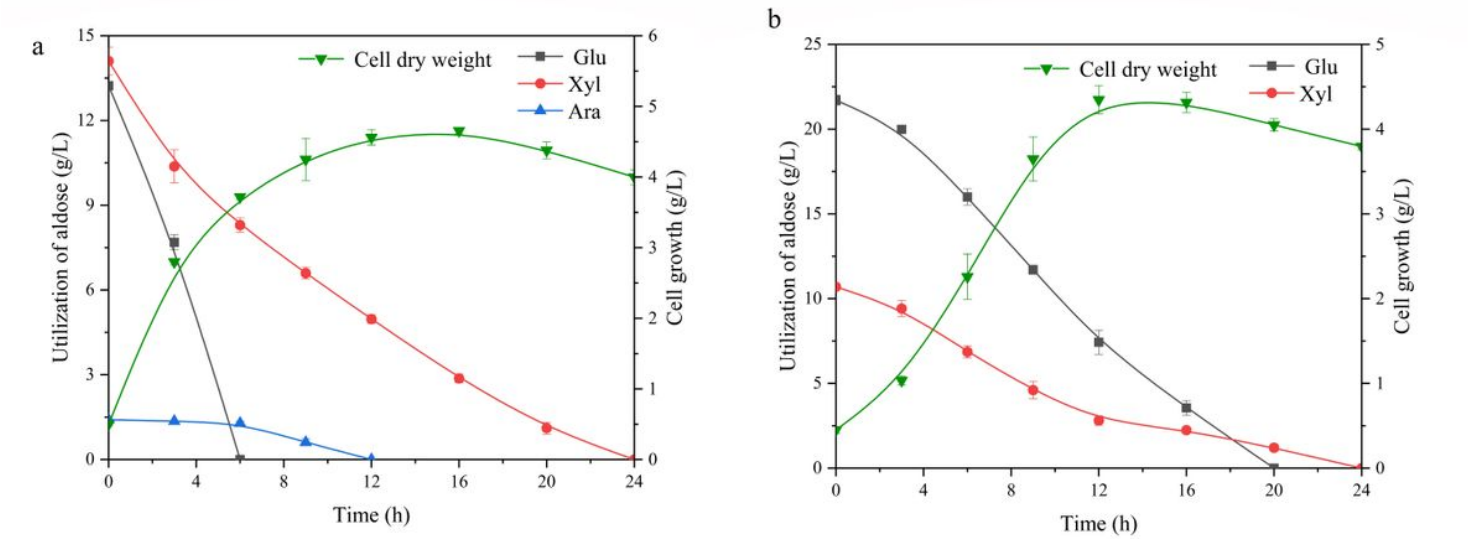

Figure 3

B. subtilis proliferations in the mixed-C5/C6 broth (a) and the enzymatic hydrolysate (b)

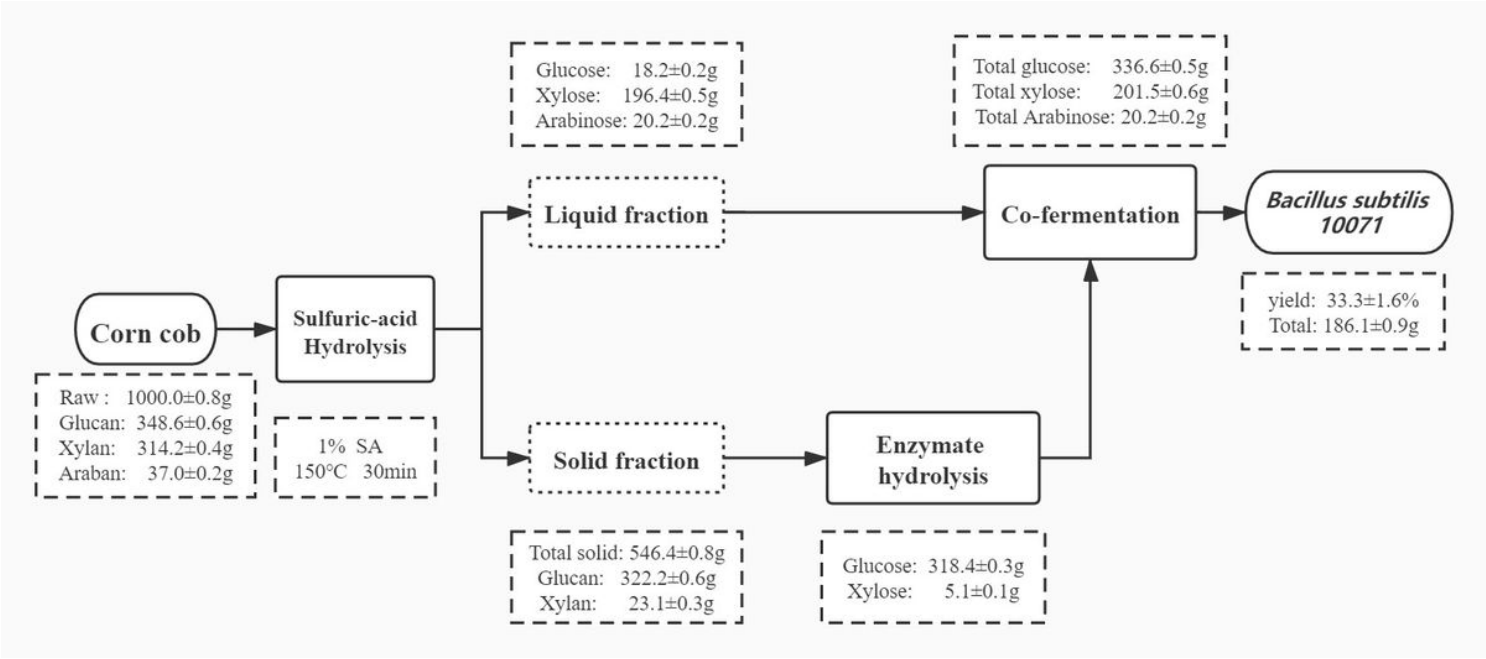

Figure 4

Integration process and mass balance of B. subtilis bioproduction from corncob