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Short Communication

Cascade hydrolysis and fermentation of corn stover for production of high titer gluconic and xylonic acids

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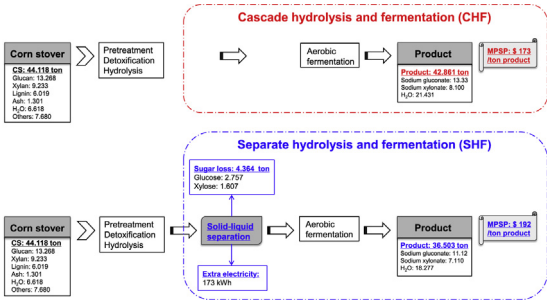
GRAPHICAL ABSTRACT



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ABSTRACT

Simultaneous saccharification and fermentation (SSF) is an efficient fermentation operation in lignocellulose biorefining. However, SSF may not be applicable when the pH values of hydrolysis and fermentation do not match, or the strong intermediate inhibitors on cellulase activity are generated. This study proposed a cascade hydrolysis and fermentation (CHF) process for cellulosic gluconic acid fermentation to overcome the inhibition of the intermediate glucono-γ-lactone on cellulase activity. The pretreated and detoxified corn stover feedstock was enzymatically hydrolyzed into hydrolysate slurry, then gluconic acid and xylonic acid fermentations were directly conducted by inoculating *Gluconobacter oxydans* strain without solid/liquid separation. The sugar loss and energy consumption were effectively avoided by moving the solid/liquid separation into the fermentation stage. The experiments and the techno-economic analysis show that the CHF is simple and cost effective fermentation operation when SSF is not applicable.

1. Introduction

Highly viscous and high solids content hydrolysis is the inevitable intermediate step for obtaining high product titer in biorefining process (Hou et al., 2017; Sievers et al., 2015). Simultaneous saccharification and fermentation (SSF) is frequently considered as an effective fermentation way to remove sugar inhibition on cellulase activity. However, SSF is not always applicable for biorefinery fermentations. When the pH value of fermentation is outside the pH scope of enzymatic hydrolysis (generally 4.8–5.5), SSF is no longer efficient in compromising the two different pH values. Another example of the failed SSF is the fermentation product or the intermediate product shows the harsh inhibition on cellulase enzyme and sometimes the inhibition is so strong that the cellulase completely loses its activity. In gluconic acid fermentation, an intermediate glucono-γ-lactone is oxidized from glucose by *Gluconobacter oxydans* strain before spontaneously hydrolyzed into

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gluconic acid, however, it strongly inhibits cellulase enzyme activity thus SSF is unable to operate for gluconic acid fermentation (Kim and Day, 2010). The pH value for gluconic acid fermentation by *G. oxydans* is 4.8, which agrees with the optimal range of cellulase enzyme. Therefore, the pH fitness is not the barrier for SSF, but the existence of intermediate inhibitor (glucono-γ-lactone) is. On the other hand, the typical separate hydrolysis and fermentation (SHF) is not a reasonable choice for the high solids content hydrolysate if SSF fails, because the solid/liquid separation of the highly viscous and high solids content hydrolysate is very energy intensive (Liu et al., 2012).

This study proposed a cascade hydrolysis and fermentation (CHF) method for the fermentations where SSF does not apply. In CHF, the enzymatic hydrolysis is conducted in the first step, then the complete hydrolysate slurry is sent for fermentation without any solid/liquid separation operation. In this way, the mismatch of pH value or the intermediate inhibition on cellulase are deleted because the hydrolysis and fermentation are conducted in two cascade way. The fermentable sugar loss and high energy input in solid/liquid separation are also avoided by sending the complete hydrolysate slurry for fermentation. The CHF operation was practiced on cellulosic gluconic and xylonic acids by *G. oxydans*, in which an intermediate glucono-γ-lactone was a strong inhibitor but it spontaneously hydrolyzed into gluconic acid in the late stage. Our previous study showed that although oxygen transfer in the highly viscous hydrolysate slurry was generally lower than that in clear hydrolysate broth, it still was sufficient to meet the need of gluconic acid fermentation using lignocellulose feedstock at moderate operation conditions (Hou et al., 2017; Hou and Bao, 2018). This study took the first investigation on cascade hydrolysis and fermentation (CHF) without solid/liquid separation and compared with conventional separate hydrolysis and fermentation (SHF) with solids/liquid separation to remove lignocellulose solids. The advantages of CHF were demonstrated by the experiments and the strict techno-economic analysis.

2. Material and methods

2.1. Raw material

Corn stover was harvested from Bayan Nur League, Inner Mongolia Autonomous Region, China in fall 2015. After collection, the materials were milled, washed then dried until constant weight. The raw corn stover contained 35.4% of cellulose and 24.6% of hemicellulose measured according to NREL protocols (Sluiter et al., 2008, 2012).

2.2. Enzymes and chemicals

Cellulase enzyme Cellic CTec 2.0 was purchased from Novozymes (China) Beijing, China. The filter paper activity was measured as 203.2 FPU/mL according to the NREL protocol LAP-006 (Adney and Baker, 1996), the cellobiase activity was 4900 CBU/mL according to Ghose (1987) the protein concentration was 87.3 mg/mL cellulase according to Bradford method using BSA as protein standard (Bradford, 1976).

2.3. Strains and media

Gluconobacter oxydans DSM 2003 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. The culture medium used for *G. oxydans* DSM 2003 included: 80.0 g/L sorbitol, 10.0 g/L yeast extract, 1.5 g/L KH_2PO_4, 1.5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$.

Biodetoxification fungus *Amorphotheca resinae* ZN1 was isolated in our previous works and stored in China General Microorganism Collection Center with the registration number 7452 (Zhang et al., 2010).

2.4. Pretreatment, biodetoxification and hydrolysis operations

Corn stover was pretreated using the dry dilute sulfuric acid pretreatment according to Zhang et al. (2011) and He et al. (2014). Briefly, dry corn stover and dilute sulfuric acid solution at 5% (w/w) were co-currently fed into reactor under the mildly helical agitation at a solid/liquid ratio of 2:1 (w/w) and pretreated at 175 °C for 5 min. The pretreated corn stover was neutralized, milled and then detoxified using *A. resinae* ZN1 for 48 h to degrade inhibitory compounds according to He et al. (2016).

The pretreated and detoxified corn stover was hydrolyzed at a 5 L bioreactor equipped with a helical ribbon impeller using solid loading of 30%, cellulase dosage of 4 mg protein/g of dry solids matter, at 50 °C and pH 4.8 for 48 h.

2.5. The co-fermentation of gluconic and xylonic acids

One vial of *G. oxydans* DSM 2003 stock was inoculated into seed medium at 30 °C, 200 rpm for 15 h. For the cascade hydrolysis and fermentation (CHF) without solid/liquid separation for production of gluconic and xylonic acids, the seed broth was directly inoculated into hydrolysate slurry without solid residue removal in a 5 L bioreactor with two Rushton impellers and fermented at 5% inoculum size, 35 °C, pH 4.8 and 1.0 vvm for 72 h. For the separation hydrolysis and fermentation (SHF) with solid/liquid separation for production of gluconic and xylonic acids, hydrolysate slurry was firstly solid/liquid separated and then inoculated by the seed broth for fermentation at the same operation conditions. The samples were withdrawn at regular intervals in both processes and centrifuged at 14,549*g* for 5 min. The supernatants were used for measuring sugars and acids.

2.6. Assay of separation efficiency

The separation operation of fermentation slurry in CHF and hydrolysate slurry in SHF both were conducted by vacuum filtration. Briefly, 40 g of the slurry was filtrated at a pressure of 0.09 MPa using a qualitative filter paper with 90 mm diameter and 20 μm maximum pore size until the pressure dropped to 0.05 MPa. After first filtration, filtration cake was resuspended using twofold water (w/w) and refiltrated. The filtration efficiency was calculated by analyzing product yield at each filtration step.

2.7. Assay of sugars and acids

Gluconic acid and xylonic acid were determined by HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Kyoto, Japan) fitted with an Aminex HPX-87H column (Bio-rad, Hercules, CA, USA) at 55 °C using a mobile phase of 5 mM H_2SO_4 at a rate of 0.4 mL/min and the detection wavelength of 210 nm. Glucose and xylose were analyzed by HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with HPX-87P column (Bio-rad, Hercules, CA, USA) at 80 °C with the sterilized deionized water as mobile phase at a flow rate of 0.6 mL/min.

2.8. Process model on Aspen plus platform and economic analysis method

The process model was developed using Aspen Plus software (AspenTech Co., Cambridge, MA, USA). The basic model was cited the NREL design report (Humbird et al., 2011), but significant changes were made in several areas, including pretreatment from dilute acid pretreatment into dry dilute acid pretreatment, detoxification from ammonia overliming into biodetoxification, saccharification and fermentation from ethanol product at 20% (w/w) solids loading into sodium gluconate and xylonate product at 30% (w/w) solids loading, product recovery from evaporation for ethanol into solid/liquid separation, decoloration and multiple evaporation steps for sodium

gluconate and xylonate. Both the solid waste (lignin residue) and the liquid waste (evaporation) were taken into the consideration of techno-economic evaluation. Gluconic acid fermentation slurry was filtrated into the liquid stream and the solid lignin residue stream. The major wastes were from the multiple evaporation steps of gluconic acid fermentation broth (liquid stream). The wastewater was sent to the wastewater treatment area and the solid lignin residue was sent to the electricity generation area (Liu and Bao, 2017). The credit and the cost of the wastes treatment were calculated based on the Aspen Plus modeling. The plant size was 300,000 metric tons processing capacity of corn stover annually. The material and energy balance data from Aspen plus modeling were used to determine equipment and chemical usage. A discounted cash flow rate of return to determine the minimum product selling price (MPSP, $/kg product) required a net present value of zero for 8% internal rate of return after taxes.

3. Results and discussion

3.1. Cascade hydrolysis and fermentation (CHF) of gluconic and xylonic acids by G. Oxydans

The cascade hydrolysis and fermentation (CHF) of the high solids content corn stover hydrolysate without solids-liquid separation for production of gluconic and xylonic acids by *G. oxydans* DSM 2003 was evaluated at the solids loading of 30% (Fig. 1). The results were compared with the separate hydrolysis and fermentation (SHF) in which the fermentation was conducted in the clear hydrolysate solution after the lignin solid residues were removed from the hydrolysate slurry. In CHF, glucose was completely converted into gluconic acid within 36 h fermentation and xylose conversion was relatively slower but completely converted after 72 h. Finally 106.2 g/L of gluconic acid and 47.9 g/L of xylonic acid were obtained. The results by CHF were very close to that of SHF only with the slight reduction on xylose conversion due to the weakened oxygen transfer from gas phase (gas) to liquid phase (hydrolysate) by the existence of solid particles. The higher solids content was tried to 35% (w/w) in CHF to yield the higher viscous hydrolysate slurry and a record high product titer was obtained at 118.9 g/L of gluconic acid and 59.3 g/L of xylonic acid after 72 h fermentation.

The CHF of gluconic and xylonic acids was further scaled up to 50 L bioreactor at the same solids loading (30%, w/w). A lag period occurred in 50 L scale fermentation perhaps due to the reduced volumetric inoculum size and mixing intension (5% in 5 L and 2% in 50 L) (Fig. 2). The final product yield at 106.0 g/L of gluconic acid and 58.5 g/L of xylonic acid reached to the similar value after the one order of magnitude scale-up.

The efficiency of solid/liquid separation for hydrolysate slurry and fermentation slurry was experimentally evaluated at the solids loading of 30% (w/w). The results show that CHF was superior to SHF in solid/liquid separation recovery yield. The solid/liquid separation of hydrolysate slurry was difficult to proceed due to the high viscosity of hydrolysate slurry and the recovery yield of glucose and xylose was 76.0 ± 2.3%. As the comparison, the recovery yield of gluconic acid and xylonic acid in CHF was 85.5 ± 3.4% because of the slurry being highly diluted and the existence of sodium gluconate and xylonate as the lubrication reagent.

3.2. Process modeling on Aspen Plus platform of the cascade hydrolysis and fermentation

The CHF of corn stover for production of gluconic and xylonic acids was detailedly compared with SHF by techno-economic analysis. In the CHF case (without the solid/liquid separation of the hydrolysate), the corn stover feedstock is hydrolyzed and then directly sent to the gluconic and xylonic acids fermentation without involving the solid/liquid separation operation. In the SHF case (with the solid/liquid separation of the hydrolysate), the corn stover feedstock is hydrolyzed and then

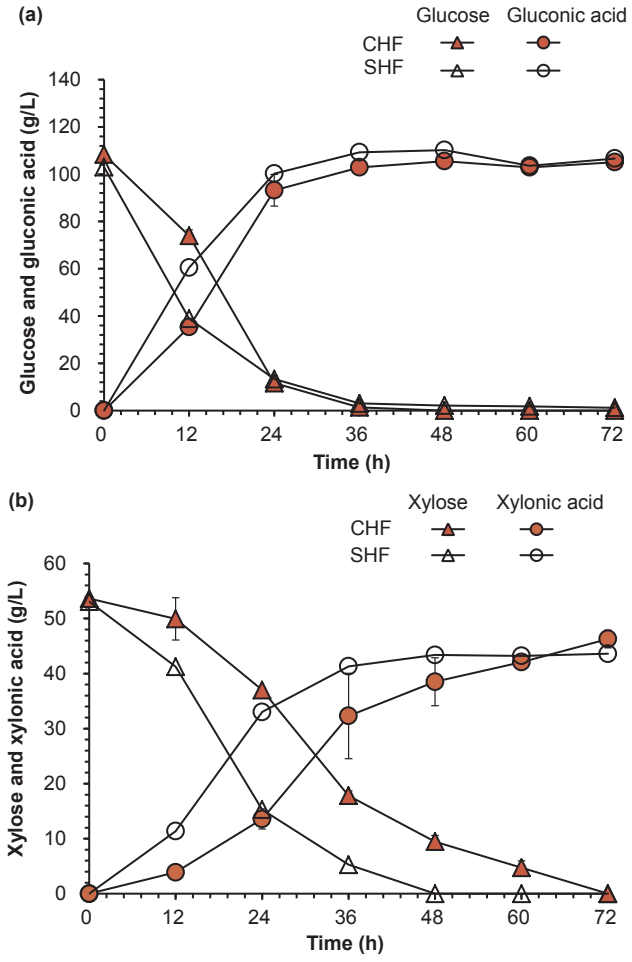

Fig. 1. Comparison of CHF and SHF for gluconic and xylonic acids fermentation. (a) Gluconic acid; (b) Xylonic acid. The hydrolysis conditions: 30% solids loading, 4 mg cellulase protein/g dry solids matter, 50 °C, and pH 4.8 for 48 h. The fermentation conditions: 5% inoculum size, 35 °C, 500 rpm, pH 4.8 and 1 vvm for 72 h.

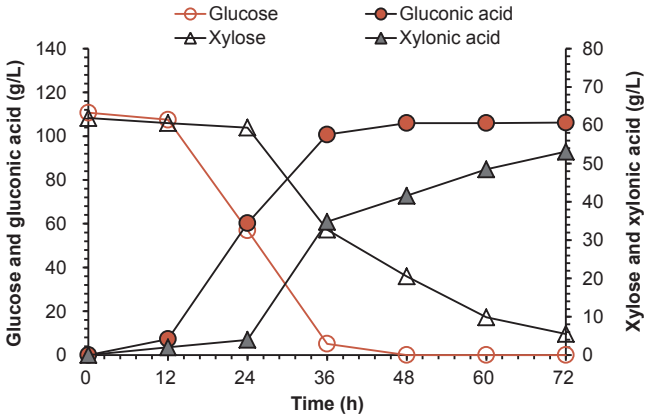

Fig. 2. Scale up of CHF for gluconic and xylonic acids fermentation to 50 L at the solids loading of 30%. The hydrolysis conditions: 4 mg cellulase protein/g dry solids matter, 50 °C, and pH 4.8 for 48 h. The fermentation conditions: 300 rpm, 1 vvm, 35 °C, inoculation ratio 2% and pH 4.8 for 72 h.

solid/liquid separated to produce the clear hydrolysate solution for gluconic and xylonic acids fermentation.

To evaluate the techno-economic performance in commercial scale, the process models of the two cases were established at the plant size of

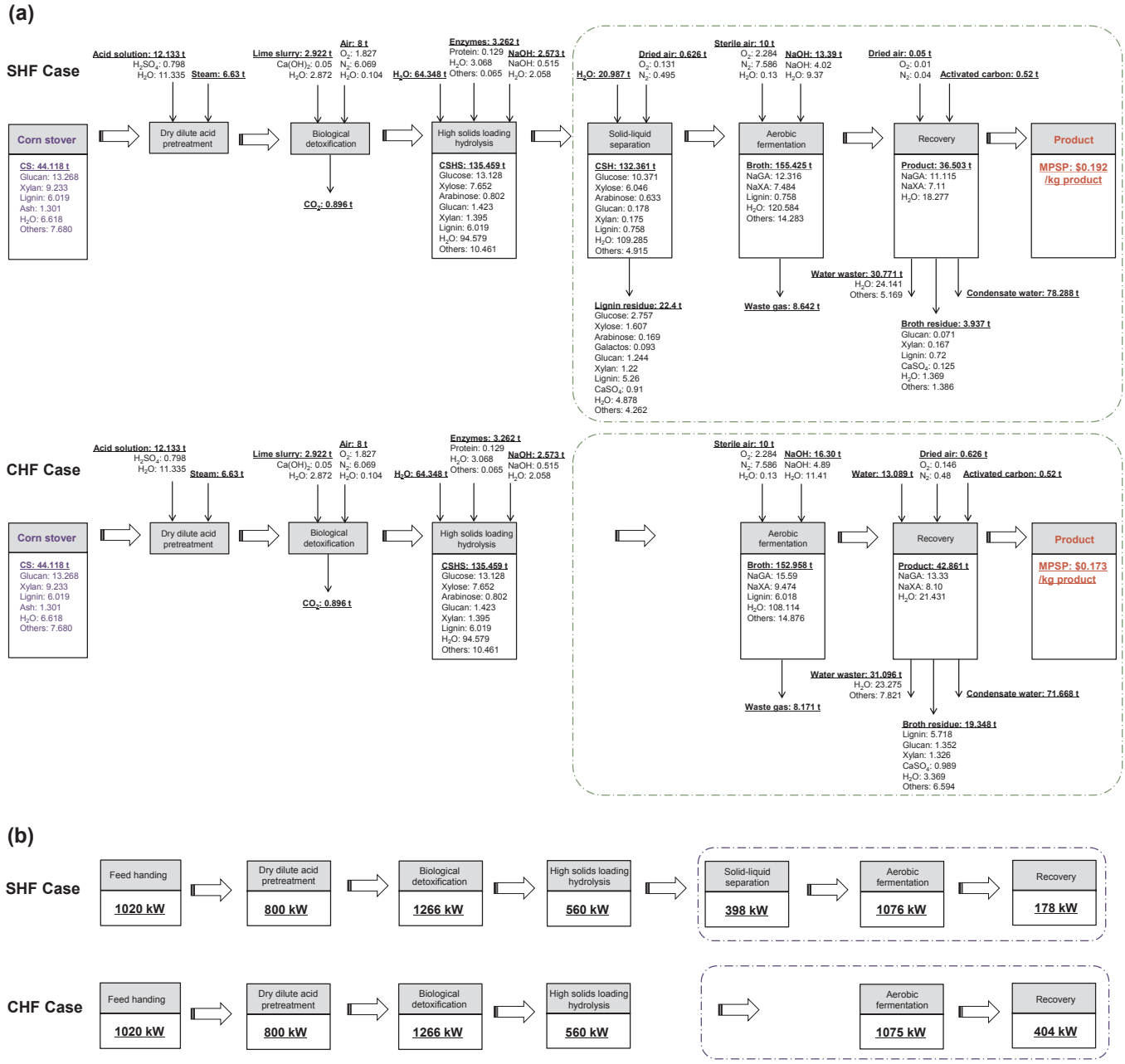

Fig. 3. The comparison of materials, energy and cost balance of cellulosic sodium gluconate and xylonate production between SHF case and CHF case. (a) Materials and cost balance; (b) Energy balance. *Abbreviations:* CS, Corn stover; CSH, Corn stover hydrolysate; CSHS, Corn stover hydrolysate slurry; NaGA, Sodium gluconate; NaXA, Sodium xylonate; MPSP, Minimum product selling price.

300,000 metric tons of dry corn stover annually (equivalently 37.5 tons dry corn stover per hour) on the Aspen Plus platform based on the following assumptions: the same feedstock is conducted by the same dry acid pretreatment and biodetoxification approach (DryPB) (Liu et al., 2018), following by the same high solids content hydrolysis (namely the same conversion yield from polysaccharides to monosaccharides) and fermentation (namely the same conversion yield from monosaccharides to products). The major difference is on the connection of hydrolysis and fermentation: CHF uses the hydrolysate directly for fermentation without the removal of solids residues by solid/liquid separation, and SHF uses the clear hydrolysate solution for fermentation after the solids residues are removed. Therefore, two solid/liquid separations are required in SHF, in which one is conducted after enzymatic hydrolysis and another is conducted after fermentation to remove the cells for gluconic acid purification while only one solid/liquid separation is required in CHF after fermentation. The yield of solid/liquid separation in both cases adopts the experimentally measured value for process simulation.

The materials balance shows that the solid/liquid separation operation in SHF led to a considerable loss at 21% of the total monosaccharides (Fig. 3a). The low product output in SHF indicated more water consumption (1.7 fold of total fresh water) and energy consumption (1.2 fold of total electricity) for producing per ton of product (Fig. 3b). Accordingly, the minimum product selling price (MPSP) of sodium gluconate and xylonate also hence increased to $0.192 per kg (SHF case) from $0.173 (CHF case), approximately 11% increase of the total cost, due to the correlative impacts caused by solid/liquid separation before fermentation. The CHF for producing cellulosic sodium gluconate and xylonate was more efficient option in industrial scale biorefining and the conclusion could be extended to other fermentation

processes in which SSF was not valid.

The major advantages of CHF were to avoid sugars loss and energy consumption in the solid/liquid separation of highly viscous hydrolysates, while its disadvantage was the reduced oxygen transfer rate. However, the reduction of oxygen transfer rate was not significant and could be compensated by the elevated agitation rate (Hou et al., 2017). The rigorous techno-economic analysis shows that CHF was a better process option for SHF in the economic aspect.

4. Conclusion

The cascade hydrolysis and fermentation (CHF) without solid/liquid separation for gluconic and xylonic acids fermentation was conducted in the highly viscous hydrolysate slurry. The very high solids loading of 35% led to a record high fermentation product based on the CHF process, including 118.9 g/L of gluconic acid and 59.3 g/L of xylonic acid. The study demonstrated the efficiency and economy of CHF by the rigorous techno-economic analysis compared with SHF. The CHF without solid/liquid separation as a practical fermentation strategy could be widely extended to other chemical productions when SSF is not valid.

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