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A preliminary study on L-lysine fermentation from lignocellulose feedstock and techno-economic evaluation

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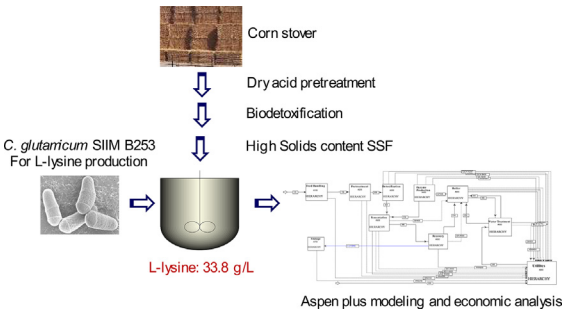
GRAPHICAL ABSTRACT



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ABSTRACT

L-Lysine is a commodity amino acid produced from starch feedstock. Various alternative feedstocks had been used for L-lysine production, but the yield was very low. This study took the first preliminary investigation on L-lysine production from lignocellulose for the replacement of food-crop starch. Corn stover was dry acid pretreated and biodetoxified, then used for enzymatic hydrolysis and L-lysine fermentation by an industrial *Corynebacterium glutamicum* strain. Various fermentation parameters, nutrient additions, and operation variables were applied and finally 33.8 g/L of L-lysine was obtained. This L-lysine titer is still below that of starch based fermentation, but already 3–5 folds greater than that of other alternative feedstocks based fermentation. A techno-economic analysis was conducted and the minimum selling price of L-lysine (hydrochloride form) was calculated to be $2.445 per kg. The cost reduction by the future improvement could fill the technical and economic gap between the cellulosic and starch based L-lysine production.

1. Introduction

L-lysine is a commodity amino acid used as the essential feed additive with the demand of 2.4 million metric tons in 2015 (Lee and Wendisch, 2017). L-lysine is produced commercially by microbial fermentation of *Corynebacterium glutamicum* from corn feedstock (Blombach and Seibold, 2010; Eggeling and Bott, 2015). Cheap substrates such as crude glycerol and silage juice were tested for L-lysine production, but very low L-lysine (less than 10 g/L) was produced (Meiswinkel et al., 2013; Neuner et al., 2013). As the most abundant and available carbohydrate resources, lignocellulose have been used for production of ethanol, lipid, and various organic acids, but few studies were concerned for L-lysine production (Liu et al., 2018; Wang et al., 2016; Zhou et al., 2017). Gopinath et al. fermented the sugars derived

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from the dilute sulfuric acid hydrolyzed rice straw and wheat bran to 6.1 g/L L-lysine by a pentose-utilizing *Corynebacterium glutamicum* (Gopinath et al., 2011). Christopher et al. produced 4.4 g/L L-lysine using the acid hydrolysate of sugarcane trash (Christopher et al., 2016).

There are two major challenges in L-lysine production from lignocellulose: one is the high toxicity of inhibitory compounds from pretreatment on fermenting microorganisms (Palmqvist and Hahn-Hagerdal, 2000), and the other is the relatively low sugar concentrations in the hydrolysate and then leads to the low product concentration with less economic significance (Zhang et al., 2010a). To give the effective solutions to these two technical barriers, this study applied a new biorefinery technology, dry acid pretreatment and biodetoxification (He et al., 2014; Zhang et al., 2010b, 2011), to obtain the intensively pretreated and inhibitor-free lignocellulose feedstock. A bioreactor fitting for handling the high lignocellulose solids was used to obtain high sugar containing hydrolysates and the reasonable L-lysine production (Zhang et al., 2010a).

The operation parameters and additives were tested for cellulosic L-lysine production in the low sugar hydrolysate. Two critical amino acids (L-threonine and L-methionine) were optimized for high titer L-lysine production in the high sugar hydrolysate. Both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) modes were designed to obtain the high L-lysine titer at high solids loading of corn stover. The Aspen Plus flowsheet model was developed and the preliminary techno-economic analysis was conducted for evaluation the potential of cellulosic L-lysine production.

2. Methods and materials

2.1. Raw materials

Corn stover was obtained in fall 2016 from Tongliao, Inner Mongolia, China. The raw corn stover contained 33.0% cellulose, 26.9% hemicellulose, 20.8% lignin, and 5.3% ash (dry weight basis) according to the two-step acid hydrolysis method in the protocols of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2012). The feedstock was coarsely milled to pass through a 10 mm diameter screens before use.

2.2. Enzymes and strains

Cellulase enzyme Cellic CTec2 was purchased from Novozymes (China), Beijing, China. The filter paper activity was 203.2 FPU/mL according to the NREL protocol LAP-006 (Adney and Baker, 2008), and the cellobiase activity was 4900 CBU/mL according to Ghose (1987). The protein content was 87.3 mg/mL using Bradford (1976).

L-lysine fermentation strain *Corynebacterium glutamicum* SIIM B253, a traditional homoserine auxotrophic mutant, was obtained from the collection center of the Shanghai Industrial Microbiology Institute (SIIM), Shanghai, China. The biodetoxification fungus *Amorphotheca resinae* ZN1 was isolated by our group in the previous study (Zhang et al., 2010b) and stored at Chinese General Microorganisms Collection Center (CGMCC), Beijing, China, with the registration number of 7452.

2.3. Dry acid pretreatment and biodetoxification processing

Corn stover was dry acid pretreated according to our previous studies (He et al., 2014; Zhang et al., 2011). Briefly, the corn stover and the dilute sulfuric acid solution were concurrently fed into the pretreatment reactor at a solid/liquid ratio of 2:1 (w/w) at the sulfuric acid usage of 3.8 mg/g corn stover. The pretreatment was operated at 175 °C for 5 min under the mild helical agitation. The solids content of the pretreated corn stover was approximately 50% (w/w) and no liquid stream was generated. The pretreated corn stover was biodetoxified by *A. resinae* ZN1 (Zhang et al., 2010b). Briefly, the pretreated corn stover was neutralized with 20% (w/w) $Ca(OH)_2$ to a pH value of 5.5, and disk milled, then inoculated with *A. resinae* ZN1 at 28 °C for 8 days to degrade the inhibitors completely.

The pretreated and biodetoxified corn stover were enzymatically hydrolyzed at the cellulase dosage of 4 mg protein/g DM (dry matter), 50 °C, pH 4.8 in a 5-L helical stirring bioreactor. The slurry was centrifuged at 10,000 rpm for 10 min to obtain the clear corn stover hydrolysate. The low sugar hydrolysate was prepared by hydrolyzing 15% (w/w) of the feedstock solids for 48 h and the composition was 68.7g/L glucose, 9.3g/L xylose, and 1.5g/L acetic acid. The high sugar hydrolysate was prepared by hydrolyzing 30% (w/w) of the feedstock solids for 72 h and the composition was 124.2g/L glucose, 16.9g/L xylose, and 2.1g/L acetic acid. The inhibitors including furfural, 5-hydroxymethylfurfural (HMF), vanillin, and syringaldehyde were undetectable.

2.4. L-lysine fermentation

C. glutamicum SIIM B253 were cultured at 30 °C for 48 h on Luria-Bertani (LB) agar plates containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 17 g/L agar. A single colony was transferred to the liquid seed culture medium containing 25 g/L glucose, 1.5 g/L KH_2PO_4, 2.5 g/L urea, 0.6 g/L $MgSO_4{\cdot}7H_2O$, 3.6 mg/L $FeSO_4{\cdot}7H_2O$, 2.0 mg/L $MnSO_4$, 25 g/L corn steep liquor (CSL) and cultured at 30 °C and 200 rpm with an initial pH of 7.0 for 12 h. Then it was activated in the fresh seed culture medium at the same conditions for 8 h before inoculated to the fermentation medium.

L-Lysine fermentation in flasks were conducted at 30 °C and 200 rpm in 250 mL flasks containing 30 mL of the low sugar hydrolysate. The nutrients addition included 1 g/L KH_2PO_4, 10 g/L $(NH_4)_2SO_4$, 0.6 g/L $MgSO_4{\cdot}7H_2O$, 3.6 mg/L $FeSO_4{\cdot}7H_2O$, 2.0 mg/L $MnSO_4$, and 20 g/L corn steep liquor (CSL). The pH was adjusted manually to 7.0 with 5 M NaOH every 4 h during fermentation. L-lysine fermentation in 3-L bioreactors were carried out at 30 °C, 600 rpm and 1.5 vvm at the constant pH of 7.0 adjusted by 25% (w/w) aqueous ammonia and 2 M H_2SO_4. The inoculation ratio was 10% (v/v).

Simultaneous saccharification and fermentation (SSF) was conducted in 5-L helical stirring bioreactors. The pretreated and biodetoxified corn stover was fed into the bioreactor to a solids loading of 30% (w/w). The prehydrolysis lasted for 24 h or 72 h at the cellulase dosage of 4 mg protein/g DM, 50 °C and pH 4.8, then the slurry was transferred into the second 5-L bioreactor mounted with a Rushton impeller. The SSF was initiated by adding the nutrients and inoculating the cultured strain seeds (10%, v/v) at the same parameters to the 3-L fermentation unless otherwise stated.

2.5. Analysis of sugars, L-lysine and inhibitors

Glucose and L-lysine were analyzed on SBA-40D biosensor analyzer (Shandong Provincial Academy of Science, Jinan, Shandong, China). Xylose, acetic acid, furfural and HMF were analyzed on HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) fitted with a Bio-Rad Aminex HPX-87H column at 65 °C and 5 mM H_2SO_4 as mobile phase at 0.6 mL/min.

2.6. L-Lysine yield calculation

L-Lysine yield from glucose (or xylose) was defined as the ratio of produced L-lysine to the total glucose (or xylose) at the beginning of the fermentation:

$$\text{L - lysine yield from glucose} = \frac{[Lys] \times V - [Lys]_0 \times V_0}{[Glu]_0 \times V_0}$$

$$\text{L - lysine yield from xylose} = \frac{[Lys] \times V - [Lys]_0 \times V_0}{[Xyl]_0 \times V_0}$$

where $[Lys]_0$ and $[Lys]$ were L-lysine concentrations at the beginning and the end of

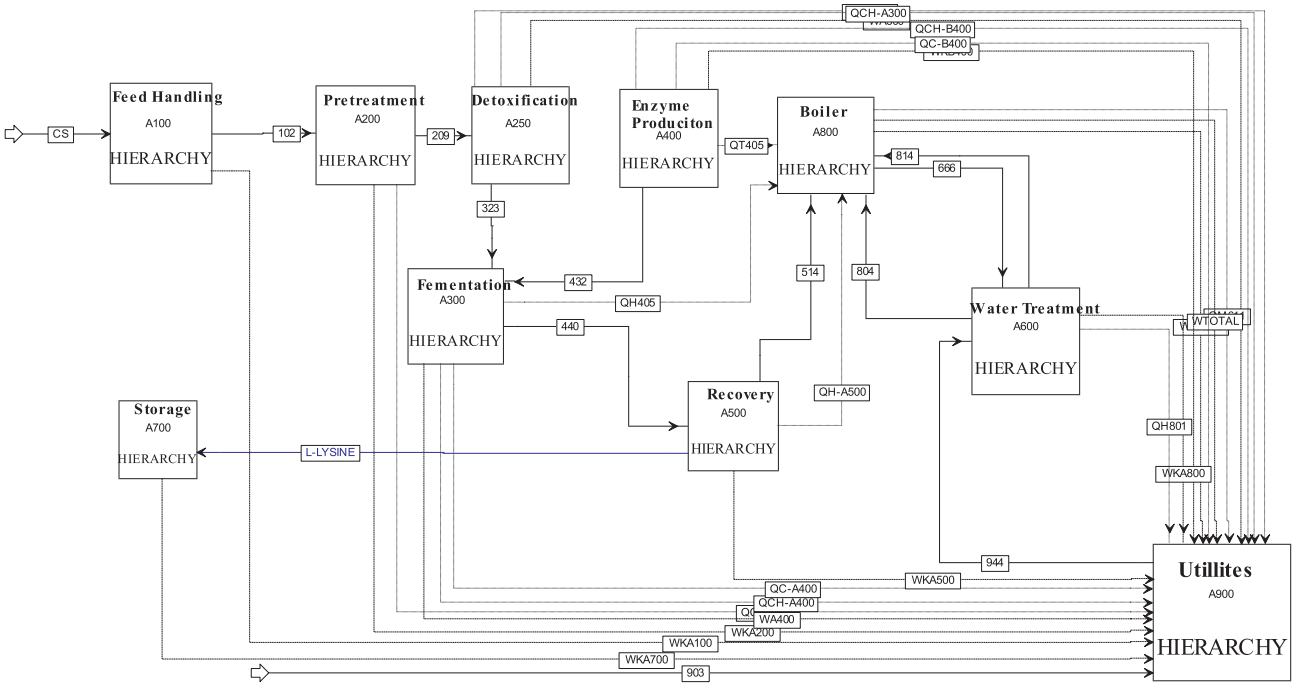

Fig. 1. Flowchart of cellulosic L-lysine production from corn stover feedstock on Aspen Plus platform.

fermentation (g/L), respectively; V_0 and V were the volume at the beginning and the end of fermentation (L) respectively; $[Glu]_0$ and $[Xyl]_0$ were the glucose and xylose concentration at the beginning of fermentation.

2.7. Process model description

Process model was built up based on the NREL design for ethanol production from corn stover using Aspen plus software (AspenTech, Cambridge, MA) (Humbird et al., 2011). The plant size was 900 tons of corn stover feedstock each day (300,000 tons annually) with an annual operation time of 8000 h. The overall flowsheet of the present model was shown in Fig. 1, including 10 operation areas of feedstock preprocessing (A100), pretreatment (A200), biodetoxification (A250), hydrolysis and fermentation (A300), cellulase fermentation (A400), product recovery (A500), wastewater treatment (A600), storage (A700), boiler (A800), and utilities (A900). Two major changes compared with the cellulosic ethanol model included:

(1) In the hydrolysis and fermentation area (A300), ammonium hydroxide was used for pH adjustment, instead of sodium hydroxide used in ethanol production. Ammonium hydroxide was also used as the nitrogen source for L-lysine fermentation.
(2) In the product recovery area (A500), the solid lignin residue and cell mass in the fermentation broth was filtrated to obtain the liquid stream and then the liquid was decolorized with activated carbon, then purified by ion exchange resins, and finally concentrated to 400 g/L by multi-effect evaporators and crystalized to obtain the L-lysine hydrochloride (98.5%, w/w) (Nagai and Carta, 2004).

2.8. Techno-economic analysis

Techno-economic analysis of cellulosic L-lysine production was performed under the assumption of "*n*th-plant" economics. The mass and energy balance data used for calculation of equipment design and chemical usage were from the Aspen Plus modeling. All costs were indexed to 2013 with the exchange rate from dollar to Chinese Yuan (CNY) of 1:6.2. The prices of pretreatment reactors, fermentation tanks and helical agitators used were quoted from manufactures in China in 2014 (Liu et al., 2015). Chemical prices were calculated according to the actual situations of China (Liu et al., 2015). The total capital investment was calculated based on the total equipment costs. The variables and fixed operating costs were determined according to the plant capacity. The discounted cash flow rate of return (DCFROR) analysis to determine the minimum L-lysine hydrochloride selling price (MLSP, $/kg) required to obtain a net present value of zero with 8% internal rate of return after taxes (Liu et al., 2015).

3. Results and discussion

3.1. L-Lysine production using corn stover hydrolysate

L-Lysine fermentation of *C. glutamicum* SIIM B253 in corn stover hydrolysate was examined as the basis of evaluation (Fig. 2). Totally 51.6 g/L glucose in the low sugar hydrolysate was converted to 7.4 g/L of L-lysine and the yield was only 0.14 g L-lysine/g glucose. The nutrient addition and fermentation parameters were adjusted to elevate the yield in flasks, and the maximum L-lysine of 14.7 g/L with the yield of 0.29 g/g was obtained. The fermenter operation with well controlled pH value did not lead to the observable improvement (13.8 g/L).

To elevate the L-lysine titer from corn stover feedstock, the high sugar hydrolysate containing 124.2 g/L of glucose was tested, but almost no L-lysine accumulation was observed (data not shown). Considering that *C. glutamicum* SIIM B253 is a homoserine auxotrophic mutant lack of the capacity of L-threonine and L-methionine synthesis (Nakayama et al., 1966; Tada et al., 2000), two amino acids on L-lysine production was examined (Fig. 3). The addition of L-threonine of 0.5 g/L showed the best cell growth and L-lysine production (Fig. 3a). The addition of L-methionine from 0.05 to 0.25 g/L was tested and 0.15 g/L addition was the optimal (Fig. 3b). Addition of L-threonine (0.5 g/L) and L-methionine (0.15 g/L) led to the accumulation of 28.4 g/L of L-lysine in high sugar hydrolysates, but the overall yield of L-lysine was similar (0.26 g/g in high sugar hydrolysates vs. 0.29 g/g in low sugar hydrolysates).

The results validated the L-lysine production using lignocellulose feedstock, but the titer and yield of L-lysine were below the starch based

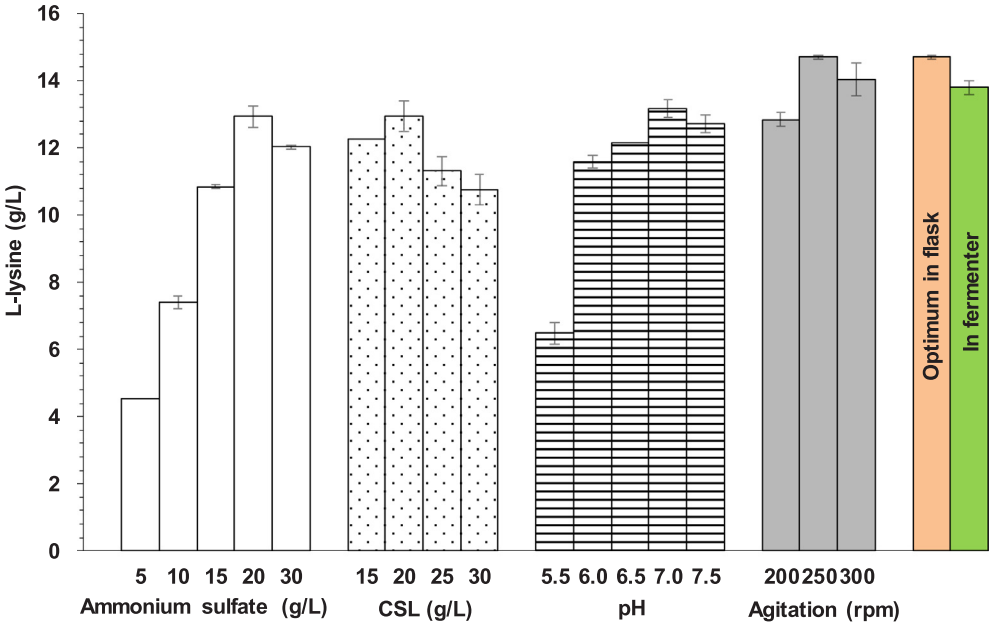

Fig. 2. Impact of nutrients and fermentation parameters on L-lysine production using low sugar hydrolysate. Conditions: 30 °C, 200 rpm and inoculum size of 10% (v/v) in 250 mL flasks for 72 h. The pH was adjusted to 7.0 every 4 h manually with 5 M NaOH unless otherwise stated. The conditions in 3-L fermenter using the low sugar hydrolysate were: 30 °C, 600 rpm, 1.5 vvm, 10% inoculation ratio with pH regulated automatically at 7.0 using 25% aqueous ammonia and 2 M H_2SO_4 for 48 h.

L-lysine production (Li et al., 2017).

3.2. Simultaneous saccharification and fermentation for L-lysine production

Simultaneous saccharification and fermentation (SSF) is an efficient way to alleviate the glucose inhibition on cellulase activity and simplify the process complexity (Sievers et al., 2014; Olofsson et al., 2008). Therefore, SSF was conducted for L-lysine production at 30% (w/w) solids loading of corn stover (Fig. 4). However, a major barrier of pH fitness was encountered between enzymatic hydrolysis (pH of 4.8–5.5) and L-lysine fermentation (pH of 7.0). To find a compromising pH value between the hydrolysis and fermentation, the pH range of 6.0–7.0 in SSF was tested and the results show that a similar L-lysine yield was obtained in SSF relative to SHF. It was worth noting that the complete glucose consumption in SSF (below 1 g/L) easily led to the microbial contamination in the neutral pH and amino acids rich environment. However, a simple extension of prehydrolysis period from 24 h to 72 h improved the L-lysine production (33.8 g/L from 28.5 g/L) and eliminated the risk of microbial contamination (Fig. 5).

L-lysine production using alternative substrates were reported by several studies. Meiswinkel et al. (2013) used crude glycerol as the sole carbon substrate and 1.6 g/L L-lysine was produced. Neuner et al. (2013) used grass or corn silage juice as feedstocks and 4.9 g/L L-lysine was obtained by *C. glutamicum*. He et al. (2015) used beet molasses and 10.7 g/L L-lysine was obtained with the engineered *E. coli* in a bath fermentation. Gopinath et al. (2011) converted acid hydrolysate of rice straw or wheat bran to 6.1 g/L L-lysine with the engineered *C. glutamicum*. Christopher et al. (2016) used the acid hydrolysate of sugarcane trash and obtained 4.4 g/L L-lysine by *C. glutamicum*. This study used corn stover feedstock and 33.8 g/L of L-lysine was obtained, 3–5 folds greater than the previous L-lysine production from alternative feedstocks. To our knowledge, this is the highest L-lysine titer using alternative carbon sources in a batch mode.

In this study, xylose was not utilized by the L-lysine producing strain. The future enabling of xylose co-fermentation to L-lysine will deliver the further improvement. Also, the future evolutionary adaptation of L-lysine producing strain at low pH values might deliver a low pH L-lysine strain to improve the SSF performance (Qureshi et al., 2015).

3.3. Techno-economic analysis of cellulosic L-lysine production

The techno-economic evaluation was carried out using the maximum L-lysine fermentation results obtained in this study (base case with the lysine yield of 0.26 g/g of glucose). Table 1 shows that the minimum L-lysine hydrochloride selling price (MLSP) was $2.445 per kg (base case), in which the cost shares of feedstock, enzyme, and non-enzyme conversion were $0.749, $0.382, and $1.314 per kg, respectively. This MLSP value was almost twofold of the market price of the feed grade L-lysine hydrochloride (~$1.4/kg, Alibaba Enterpriser, https://www.1688.com).

Since the advanced dry biorefining technology was used in this study, higher glucan and xylan conversion (~85% and ~80% correspondingly) could be reached during SSF according to our previous studies (Liu et al., 2015). The determinant factors for the high MLSP comes from the low yield of L-lysine from sugars and the improvement of L-lysine yield could lead to the reduction of MLSP. In this study, only 0.26 g of L-lysine was obtained from one gram cellulose derived glucose, and the yield was only approximately 1/3 of the starch based L-lysine fermentation. Two operation cases were assumed to show the potentials of the MLSP reduction with the improved lysine yields from sugars. Case 1 assumed that L-lysine yield from cellulose derived glucose was the same with the yield of the starch based L-lysine fermentation (0.69 g/g) (Zhai et al., 2015). Case 2 assumed that the fermenting strain utilized both glucose (at the yield of starch based glucose, 0.69 g/g) and xylose (at the L-lysine yield of 0.15 g/g xylose, the best L-lysine yield from xylose in the reported study (Henke et al., 2018). Table 1 shows that Case 1 led to almost 50% reduction of both feedstock and enzyme costs when the lysine yield from glucose was increased to 0.69 g/g from the present 0.26 g/g, and the MLSP was reduced to $1.358/kg. This selling price became comparable to the current market price of L-lysine (~$1.4/kg). Case 2 led to the further reduction of MLSP by utilizing xylose for L-lysine production at the minimum yield of 0.15 g/g from xylose. That is, if the L-lysine yield is as high as that from the starch based glucose together with the minimum xylose utilization, the MLSP could be decreased as low as $1.045/kg, a highly competing selling price to the current L-lysine product from starch based sugar.

In summary, this study showed a great potential of L-lysine production using lignocellulose feedstocks, but there is still a long way to make it cost-effective. Limit space is left to reduce the MLSP by optimizing the dry biorefining process because it has great advantages of minimal water usage, wastewater discharge and energy consumption,

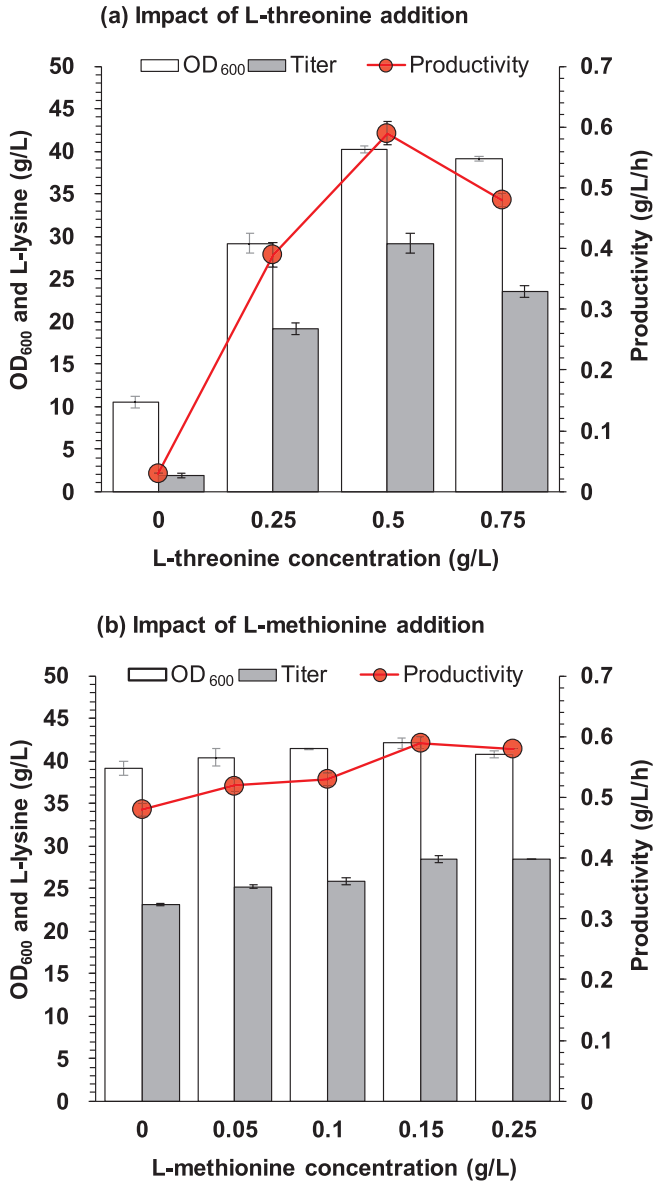

Fig. 3. Influence of L-threonine and L-methionine addition on L-lysine fermentation using high sugar hydrolysate. (a) Impact of L-threonine addition, (b) Impact of L-methionine addition. Conditions were same to those in 3-L fermenters listed under Fig. 1. When the impact of L-threonine was studied, L-methionine was fixed at 0.5 g/L. L-threonine was fixed at its optimum concentration when studied the impact of L-methionine.

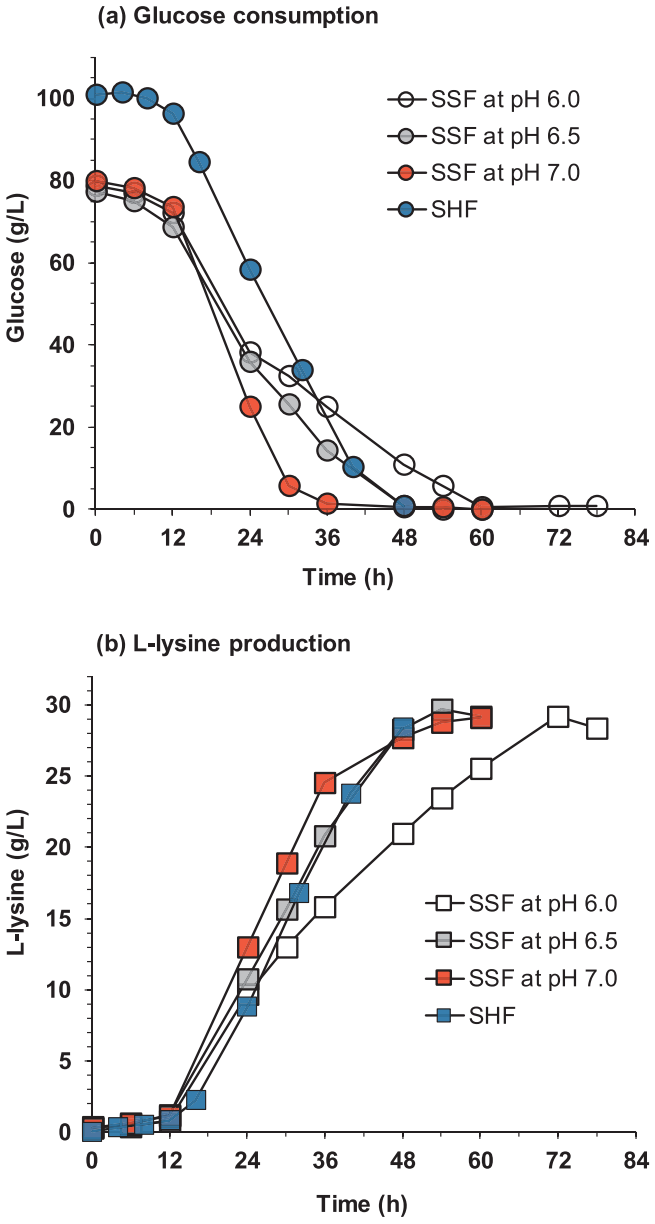

Fig. 4. Simultaneous saccharification and L-lysine fermentation (SSF) at different pH values. (a) Glucose consumption, (b) L-lysine production. Conditions: prehydrolysis at 50 °C and pH 4.8 for 24 h with 4 mg protein/g DM; SSF at 30 °C, 600 rpm, 1.5 vvm, inoculum size of 10% (v/v) with pH regulation automatically using 25% (w/w) aqueous ammonium and 2 M H_2SO_4. SHF conditions were same to the fermentation with 0.5 g/L L-threonine and 0.15 g/L L-methionine addition as shown in Fig. 3.

while keeping the high cellulose and hemicellulose conversion. Great efforts could be focused on engineering the L-lysine fermenting strains for conversion of L-lysine from glucose and xylose derived from lignocellulose efficiently, which could reduce the cellulosic L-lysine production costs significantly.

4. Conclusion

The maximum L-lysine titer of 33.8 g/L was obtained using *C. glutamicum* SIIM B253 by SSF at 30% solids loading of corn stover after dry acid pretreatment and biodetoxification. The proper concentration of L-threonine and L-methionine in the fermentation medium was crucial for L-lysine production using high sugar hydrolysate when the homoserine auxotrophic mutant strain was used. The techno-economic analysis showed that the MLSP ($2.445/kg) was relative high compared with its current market price, but a significant reduction could be reached by enhancing the yield of L-lysine from lignocellulose derived glucose and xylose.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.09.098.

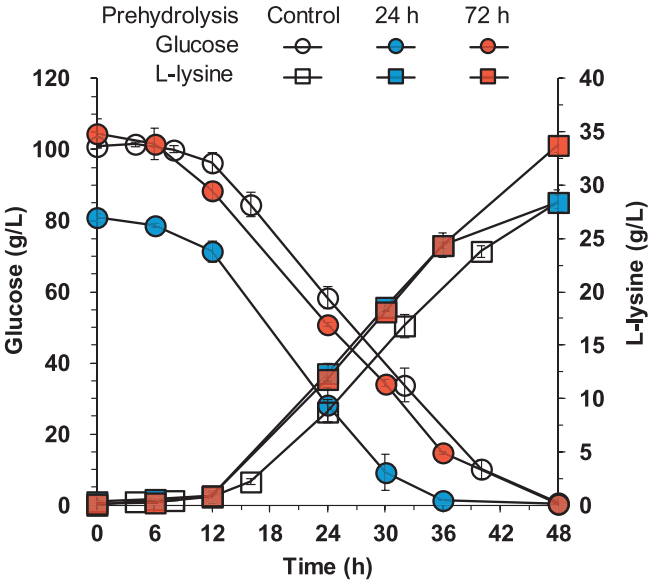

Fig. 5. Simultaneous saccharification and L-lysine fermentation (SSF) with different prehydrolysis period. Control here refers to L-lysine production by SHF. Conditions: prehydrolysis at 50 °C and pH 4.8 with 4 mg protein/g DM for 24 h and 72 h, respectively; then SSF at 30 °C, 600 rpm, 1.5 vvm, inoculum size of 10% (v/v) with pH regulation automatically at 7.0 using 25% (w/w) aqueous ammonium and 2 M H_2SO_4. SHF conditions (72 h prehydrolysis and 48 h fermentation) were same to the fermentation with 0.5 g/L L-threonine and 0.15 g/L L-methionine addition as shown in Fig. 3.

Table 1
Impact of L-lysine yield from sugars on minimum L-lysine hydrochloride selling price (MLSP) calculated based on the Aspen Plus modeling.[a]

MLSP ($/kg)	Base case[b]	Case 1[c]	Case 2[d]
L-lysine yield from glucose (g/g)	0.26	0.69	0.69
L-lysine yield from xylose (g/g)	0	0	0.15
MLSP ($/kg)	2.632	1.358	1.229
Feedstock cost ($/kg)	0.806	0.314	0.277
Enzyme cost ($/kg)	0.411	0.174	0.153
Conversion cost ($/kg)	1.415	0.870	0.799

[a] For techno-economic evaluation, glucan and xylan conversion was set at 85% and 80% during SSF at 30% solids loading according to our previous studies (Liu et al., 2015).

[b] L-lysine yield of 0.26 g/g from glucose was the results obtained in this study.

[c] L-lysine yield of 0.69 g/g from glucose was the highest yield using starch based glucose according to Zhai et al. (2015).

[d] L-lysine yield of 0.69 g/g from glucose was the highest yield using starch based glucose according to Zhai et al. (2015). In order to investigate the effect of xylose utilization on MLSP, L-lysine yield of 0.15 g/g from xylose was used in case 2 according to Henke et al. (2018).

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