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High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted *Saccharomyces cerevisiae* strain

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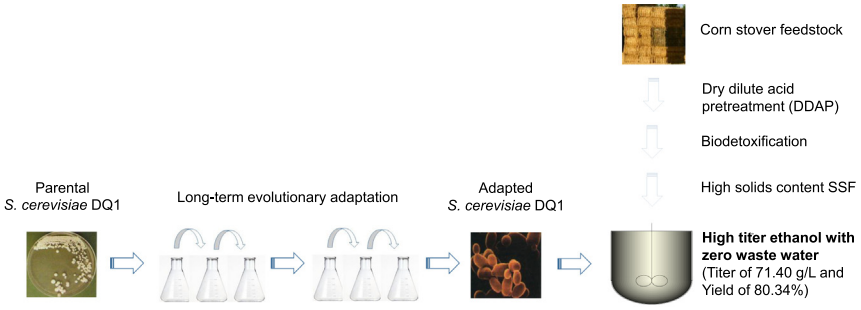
HIGHLIGHTS

- Evolutionary adaptation yields a highly robust cellulosic ethanol fermenting strain.
- High ethanol titer of 71.40 g/L and yield of 80.34% were obtained using corn stover.
- Zero wastewater generation was realized from pretreatment to fermentation.

GRAPHICAL ABSTRACT



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ABSTRACT

Ethanol fermentation was investigated at the high solids content of the dry dilute sulfuric acid pretreated corn stover feedstock using an evolutionary adapted *Saccharomyces cerevisiae* DQ1 strain. The evolutionary adaptation was conducted by successively transferring the *S. cerevisiae* DQ1 cells into the inhibitors containing corn stover hydrolysate every 12 h and finally a stable yeast strain was obtained after 65 days' continuous adaptation. The ethanol fermentation performance using the adapted strain was significantly improved with the high ethanol titer of 71.40 g/L and the high yield of 80.34% in the simultaneous saccharification and fermentation (SSF) at 30% solids content. No wastewater was generated from pretreatment to fermentation steps. The results were compared with the published cellulosic ethanol fermentation cases, and the obvious advantages of the present work were demonstrated not only at the high ethanol titer and yield, but also the significant reduction of wastewater generation and potential cost reduction.

1. Introduction

High ethanol titer in fermentation broth is crucially important for reducing the energy demand of the consequent distillation step (Galbe et al., 2007). When cellulosic ethanol fermentation is conducted using lignocellulose feedstock, the simultaneous saccharification and fermentation (SSF) technology is frequently used to lessen the strong inhibition of high sugar concentration on cellulase enzyme catalyzed cellulose hydrolysis. During SSF, high solid feedstock content is certainly required to reach the high ethanol titer. Consequently, two difficulties emerged in such a high solids content fermentation: the poor mixing of the solid feedstock majority with the liquid minority of cellulase enzyme solution and fermenting seeds broth, as well as the accumulation of inhibitory compounds with the increased pretreated solids content.

The first difficulty could be efficiently solved by proper bioreactor design, such as the horizontal rotating reactor (Jorgensen et al., 2007) or the helical ribbon stirring bioreactor (Zhang et al., 2010a). For the second difficulty, development of an inhibitor tolerant

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fermenting strain by evolutionary adaptation may provide a partial but practical solution (Heer and Sauer, 2008; Landaeta et al., 2013; Gu et al., 2014). Evolutionary adaptation allows the microorganism to grow in the inhibitors containing environment with gradually enhanced tolerance to the inhibitors, and finally the random mutations of the relevant genes at the genomic level may occur to generate a stable strain with improved ethanol fermentability. Martin et al. (2007) adapted a genetically engineered xylose-utilizing strain *Saccharomyces cerevisiae* by cultivating the cells for 15 days in the synthetic medium with increasing phenolics, furaldehydes and aliphatic acids concentrations, and the improved ethanol production was observed. Heer and Sauer (2008) carried out the adaptation of *S. cerevisiae* TMB3400 for 30 transfers in the minimal medium containing 17 mM furfural, and the lag phase of the cell growth in the hydrolysate was reduced from 90 h to 16 h. Landaeta et al. (2013) adapted a flocculent *S. cerevisiae* strain for 39 days using synthetic medium with gradual increased inhibitors concentration, and the cell growth and ethanol productivity were increased by 70% and 10%, respectively. Gu et al. (2014) developed a detailed evolutionary adaptation method for *S. cerevisiae* DQ1 in the corncob residue hydrolysate, and the ethanol titer was increased to a high level of 62.68 g/L.

Adaptation efficiency of a certain fermenting strain highly depends on the specific environment where the strain is to be used. A well-adapted strain with high fermentation performance in one specific hydrolysate may not be suitable for another hydrolysate in terms of different feedstock types, pretreatment methods, conditioning (detoxification), or hydrolysis conditions, because of the variation in the inhibitors type and concentration. In this study, a long term evolutionary adaption of a highly robust yeast strain *S. cerevisiae* DQ1 was conducted in the dry dilute sulfuric acid pretreated (DDAP) corn stover hydrolysate. After 130 transfers (taking 65 days and equivalent to 780 generations of the cell growth), a stable strain was obtained and applied to the simultaneous saccharification and fermentation at high corn stover solids content (Zhang et al., 2010a,b, 2011). Not only the high ethanol titer (71.40 g/L) and the yield (80.34%) were obtained, but also the minimum water usage and wastewater generation were reached by using the DDAP pretreatment and biodetoxification methods. The present study provides a practical approach to obtain the robust strains suitable for lignocellulose biorefinery system.

2. Methods

2.1. Raw materials

Corn stover (CS) was obtained from Dancheng County, Henan Province, China, in fall 2013. Corn stover was water-washed to remove the impurities and air-dried. The dry dilute sulfuric acid pretreated corn stover contained 39.89% of glucan and 3.04% of xylan according to the two-step H_2SO_4 hydrolysis method (Sluiter et al., 2012). The moisture content of the corn stover after drying was 7.0% (w/w). This moisture content had been taken into account for calculations of solids/liquid ratio and sulfuric acid concentration before the pretreatment operation. The corn stover was milled using a beater pulverizer to pass through the 10-mm apertures in diameter, then sealed in plastic bags and stored at room temperature until used.

2.2. Strains and enzymes

The ethanol fermenting strain *S. cerevisiae* DQ1 (CGMCC 2528) was used as the parental strain at the starting point of evolutionary adaption (Chu et al., 2012). *Amorphotheca resinae* ZN1 (CGMCC 7452) was used as the biodetoxification strain for degrading inhibitors exists in the dry dilute sulfuric acid pretreated corn stover via solid state fermentation (Zhang et al., 2010b).

The cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity of Youtell #6 was 135 FPU/g (cellulase protein equivalent to 90 mg/g DM) determined using the NREL protocol LAP-006 (Adney and Baker, 1996), and the cellobiase activity was 344 CBU/g using the method described by Sharma et al. (1991).

2.3. Pretreatment and biodetoxification operations

Corn stover was pretreated using the dry dilute sulfuric acid pretreatment (DDAP) according to Zhang et al. (2011) and He et al. (2014). Briefly, dry corn stover and dilute sulfuric acid solution at 5.0% (w/w) were co-currently fed into the reactor at a solid/liquid ratio of 2:1 (w/w) with helically stirring mixing of 50 rpm, then pretreated at 175 °C for 5 min. The solids content of the pretreated slurry was around 50% (w/w) and no wastewater was generated. The pretreated corn stover solid contained 39.89% of glucan and 3.04% of xylan based on the dry matter weight. The glucose and xylose concentrations in the liquid portion were 3.05 g/L and 12.56 g/L, respectively.

The pretreated corn stover slurry was detoxified using *A. resinae* ZN1 according to Zhang et al. (2010b). Briefly, the pretreated corn stover was neutralized with 20% (w/w) $Ca(OH)_2$ to pH value of 5–6, and then inoculated with *A. resinae* ZN1 at 10% (w/w) ratio as the seeds and incubated at 28 °C for several days to degrade the inhibitory compounds. No additional fresh water was used during biodetoxification, and the solids content of the biodetoxified corn stover material was about 50% (w/w).

2.4. Evolutionary adaptation of S. cerevisiae DQ1

The hydrolysate used for evolutionary adaptation was prepared by enzymatic hydrolysis of DDAP pretreated corn stover (without biodetoxification) at 15% (w/w) solids content, 15 FPU/g DM (dry matter) cellulase (equivalent to 10 mg protein per gram of dry matter), 50 °C, pH 4.8 and 150 rpm for 48 h in the helically stirring bioreactor (Zhang et al., 2010a). The hydrolysate was centrifuged at 16,125*g* for 10 min to remove the solid residues and the supernatant was used as adaption medium. The hydrolysate contained 60.99 g/L of glucose, 23.23 g/L of xylose, 2.28 g/L of acetic acid, 0.36 g/L of furfural and 0.21 g/L of 5-hydroxymethylfurfural (HMF).

The evolutionary adaptation of *S. cerevisiae* DQ1 was conducted by continuously transferring the cultured yeast cells from previous medium into the fresh corn stover hydrolysate. 100 mL conical flasks were used for the continuous cell transfer. In details, 10% (v/v) of the culture solution from last culture was transferred every 12 h into the fresh hydrolysate and incubated at 37 °C in the shaking incubator. At the end of each transfer, the sample was collected and used for glucose and ethanol analysis. This successive transfer process was repeated for 65 days until the fermentation performance maintained stable.

2.5. Simultaneous saccharification and ethanol fermentation (SSF)

SSF was performed in the 5-L helical ribbon stirring bioreactor using the DDAP pretreated and biodetoxified corn stover feedstock. The SSF started with 6 h prehydrolysis at 50 °C, then followed by 66 h SSF at 37 °C. Unless otherwise stated, the cellulase dosage was 15 FPU/g DM and the pH was maintained at 4.8 by automatic regulation with 5 M NaOH. The parental *S. cerevisiae* DQ1 strain was used as the control after a simple three-step treatment (Qureshi et al., 2015). The samples were withdrawn at regular intervals, centrifuged at 11,167*g* for 5 min and the supernatant was analyzed. The yeast cell viability during SSF was determined

by counting the colony forming units (CFU) on the petri dish of the diluted fermenting broth (Qureshi et al., 2015).

2.6. Analysis

Glucose, ethanol, acetic acid, lactic acid, furfural and HMF contained in the taken samples were analyzed on HPLC (LC-20AD pump, RI detector RID-10A, Shimadzu, Kyoto, Japan) with Bio-Rad Aminex HPX-87H column operated at 65 °C and 0.6 mL/min of 5 mM H_2SO_4 as the mobile phase.

Ethanol yield was calculated using the method deduced by Zhang and Bao (2012) specifically for the high solids and high ethanol titer SSF process:

$$\text{Ethanol yield} = \frac{[C_1] \times W}{976.9 - 0.804 \times [C_1]} \cdot \frac{1}{0.511 \times f \times [\text{Biomass}] \times m \times 1.111} \times 100\%$$

where $[C_1]$ was the ethanol concentration in the culture broth (g/L), W was the total water input of SSF (g), f was the cellulose fraction of corn stover feedstock, [Biomass] was the dry corn stover concentration at the beginning of SSF (g/g), m was the total weight of SSF (g), 0.511 was the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, and 1.111 is the conversion factor for cellulose equivalent to glucose.

3. Results and discussion

3.1. Evolutionary adaptation of S. cerevisiae to enhance its fermentability

The thermo- and inhibitor tolerant strain *S. cerevisiae* DQ1 was selected as the starting strain for the long-term evolutionary adaptation using the freshly prepared corn stover hydrolysate as the culture medium. The yeast cells were transferred into the fresh corn stover hydrolysate every 12 h successively until the glucose utilization rate and ethanol production rate were stable as shown in Fig. 1. The adaptation of *S. cerevisiae* DQ1 was conducted for 1560 h with 130 cell transfers (equivalent to 780 generations). The glucose consumption and ethanol production fluctuated in the first 50 transfers (approximately 300 generations) and then maintained stable with only random shifts. The ethanol yield from the glucose in the corn stover hydrolysate in Fig. 1 was relatively low (~80%), while the average ethanol yield is above 90%. The reasons for the low yield include: (1) short fermentation time in the adaptation cell transfer (every 12 h), and (2) the existence of inhibitors in the corn stover hydrolysate used evolutionary adaptation (2.28 g/L of acetic acid, 0.36 g/L of furfural, and 0.21 g/L of HMF).

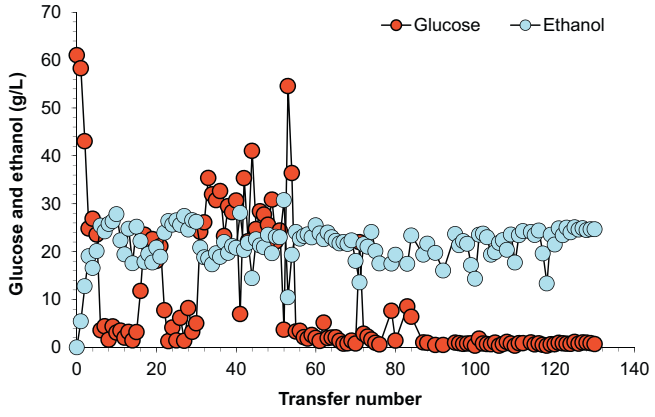

Fig. 1. Time course profile of the evolutionary adaptation of *S. cerevisiae* DQ1.

Ethanol fermentation performance of the adapted *S. cerevisiae* DQ1 was compared with the parental strain in the pure glucose solution (synthetic medium), the liquid corn stover hydrolysate, and the SSF, respectively. Fig. 2(a) shows that the ethanol titer of the adapted strain was almost same to that of the parental strain in the synthetic medium, but increased to 23.25 g/L from 12.2 g/L of the parental strain in the corn stover hydrolysate. The ethanol titer of the adapted strain was also slightly increased to 64.68 g/L from 60.33 g/L of the parental strain in the SSF, approximately 7.2% increase comparing to the parental strain. The reason for the limited improvement between the adapted and parental strains is that the inhibitors in the pretreated corn stover were almost completely removed by biodetoxification treatment. The adapted strain could play more significant roles in the case of inhibitor containing hydrolysate systems, which commonly exist in various lignocellulose biorefinery processes. The cell viability of the adapted in the corn stover hydrolysate and in SSF system was elevated by almost 50% comparing to the parental strain as shown in Fig. 2(b). This performance could also enhance the tolerance of fermenting strains in the inhibitors containing lignocellulose systems for improved ethanol fermentability.

3.2. Optimal SSF performance of the evolutionary adapted S. cerevisiae DQ1

Ethanol fermentation using the adapted *S. cerevisiae* DQ1 strain at the high solids content SSF was optimized with changing

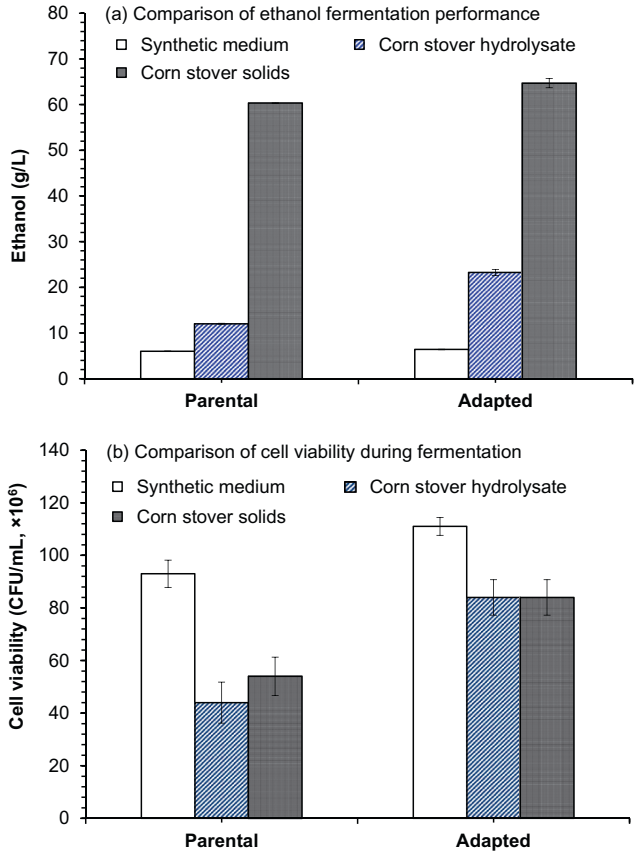

Fig. 2. Fermentation performance evaluation of the adapted *S. cerevisiae* DQ1. (a) Comparison of ethanol fermentation performance; (b) comparison of cell viability during fermentation.

Table 1
Optimization of the SSF of the pretreated corn stover using the adapted *S. cerevisiae* DQ1.

Parameters	Values	Ethanol titer (g/L)	Ethanol yield (%)	Ethanol productivity (g/L/h)
Biodetoxification time (days)	0	2.54	3.75	0.03
	3	45.36	66.44	0.63
	5	50.86	71.65	0.70
	7	55.68	79.43	0.77
	14	58.84	82.39	0.82
Solids content (%, w/w)	20	46.33	88.60	0.65
	25	55.68	79.46	0.77
	30	64.68	72.35	0.90
	35	68.21	60.36	0.95
Cellulase dosage (FPU/g DM)	7	54.71	60.90	0.76
	15	64.68	72.35	0.90
	30	71.52	79.42	0.99
Nutrients addition	0	56.05	61.79	0.78
	1 g/L YE	64.68	72.35	0.90
	1 g/L DDGS	70.52	79.25	0.98
	5 g/L DDGS	71.40	80.34	0.99

Conditions for "biodetoxification time": prehydrolysis at 25% solids content, 15 FPU/g DM cellulase dosage equivalent to 10 mg protein/g DM, 50 °C, pH 4.8 for 6 h; then SSF with 10% (v/v) yeast inoculation at 37 °C for 66 h.
Conditions for "solids content": biodetoxified for 7 days; prehydrolysis at 20%, 25%, 30%, and 35% solids content, respectively, 15 FPU/g DM cellulase, 50 °C, pH 4.8 for 6 h, 6 h, 7 h, and 12 h, respectively; then SSF with 10% (v/v) yeast inoculation at 37 °C for 66 h, 66 h, 65 h and 60 h, respectively.
Conditions for "cellulase dosage": biodetoxified for 7 days; prehydrolysis at 30% solids content, 7 FPU/g DM, 15 FPU/g DM, and 30 FPU/g DM cellulase, 50 °C, pH 4.8 for 12 h, 7 h, and 6 h, respectively; then SSF with 10% (v/v) yeast inoculation at 37 °C for 60 h, 65 h and 66 h, respectively.
Conditions for "nutrients": biodetoxified for 7 days; prehydrolysis at 30% solids content, 15 FPU/g DM cellulase, 50 °C, pH 4.8 for 7 h; then SSF with 10% (v/v) yeast inoculation at 37 °C for 65 h. The "0" indicates no additional nutrients added into the SSF; "1 g/L YE" indicates 1.0 g/L of yeast extract, 2.0 g/L of KH_2PO_4, 1.0 g/L of $(NH_4)_2SO_4$ and 1.0 g/L of $MgSO_4{\cdot}7H_2O$. Other SSF was the same to "1 g/L YE" unless stated elsewhere. The nutrients in the "1 g/L DDGS" indicate 1.0 g/L of DDGS, 2.0 g/L of KH_2PO_4, 1.0 g/L of $(NH_4)_2SO_4$ and 1.0 g/L of $MgSO_4{\cdot}7H_2O$; "5 g/L DDGS" indicate 5.0 g/L of DDGS, 2.0 g/L of KH_2PO_4, 1.0 g/L of $(NH_4)_2SO_4$ and 1.0 g/L of $MgSO_4{\cdot}7H_2O$.

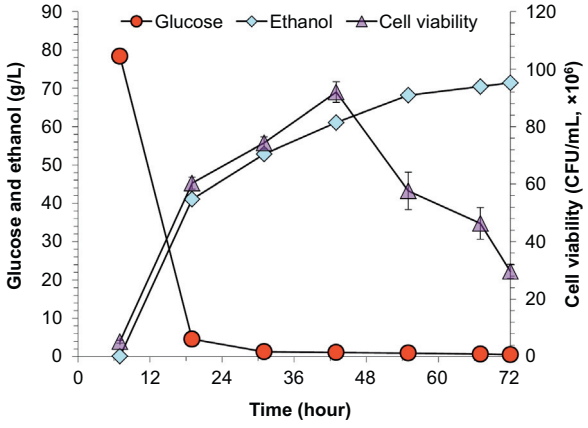

Fig. 3. Optimum SSF time course using the adapted yeast *S. cerevisiae* DQ1.

process parameters, including the biodetoxification time, solids content, cellulase dosage, and nutrient addition as shown in Table 1.

Table 1 indicates that the inhibitor concentration in the pretreated feedstock including furfural, HMF and acetic acid decreased with the prolonged biodetoxification time, leading to the increased ethanol titer, yield, and productivity. Higher solids content enhanced the ethanol titer and productivity but the ethanol yield was decreased. Cellulase dosage enhanced the ethanol titer, yield and productivity at the price of increased enzyme cost.

An important cost reduction measure was tested by replacing yeast extract (YE) with a cheap nutrient alternative usually used for animal feed, distillers' dried grains with solubles (DDGS). A better performance of DDGS usage than YE was observed, although its price was only 0.5% of the yeast extract additive (Bi et al., 2011). The time course of the SSF operation with 5 g/L DDGS addition was illustrated in Fig. 3 under the optimal biodetoxification time, solids content, and cellulase enzyme dosage. The glucose was consumed out within 12 h after yeast inoculation and ethanol steadily increased to 71.40 g/L within 72 h. The viable cells increased in the first 36 h, and then gradually decreased, probably because of the osmotic pressure of the high ethanol formation.

3.3. Evaluation of cellulosic ethanol potentials

The optimal ethanol fermentation by the adapted *S. cerevisiae* DQ1 strain using the dry dilute sulfuric acid pretreated (DDAP) corn stover as the feedstock was 71.40 g/L in the ethanol titer, 80.34% in the yield, and 0.99 g/L/h in the productivity (within the overall 72 h). More importantly, no any wastewater was generated starting from the pretreatment step to the fermentation step. This result was compared to the current high titer ethanol fermentation cases published in the academic journals as summarized in Table 2.

These cases were divided into three categories: (1) all the solids and pretreatment liquid were utilized for ethanol fermentation (the whole pretreated slurry), thus no wastewater was generated from pretreatment to fermentation; (2) the solids and the partial pretreatment liquid were utilized for fermentation, and the majority of the pretreatment liquid was released as waste water; and (3) only the solids was utilized for fermentation, and a massive amount of wastewater was generated. It should be noted that the fermentation cases were not operated at exactly same conditions such as the nutrient addition, therefore the comparison results could be considered as the reference only.

In the first category with the whole pretreated slurry used for SSF, Hoyer et al. (2013) using the steam explosion pretreated spruce as feedstock, and obtained 47.8 g/L in ethanol titer and 72% in ethanol yield at 25% solids content (13.7% water insoluble solids content). This is obviously low comparing to the present result of 71.40 g/L in ethanol titer and 80.34% in ethanol yield. Furthermore, the fresh water usage in the pretreatment step (3.1 kg/kg dry matter) was more than 3-folds greater than that of the present case (1 kg/kg dry matter).

In the second category with the utilization of all the solids and the partial pretreatment liquid, Varga et al. (2004) pretreated the corn stover with alkaline wet-oxidation pretreatment, and the cellulose rich solids were obtained after solid/liquid separation and thoroughly washed with fresh water. The washed solids fraction was used to SSF and partial pretreatment liquid was re-used to adjust the solids content. 52.3 g/L of the ethanol titer with the yield of 78.0% were obtained with 16.7 kg fresh water usage and nearly 24.7 kg wastewater generation for 1 kg of corn stover used. Lan et al. (2013) pretreated the lodgepole wood chips with SPORL at 25% solids content, and 2.3-folds of fresh water to the original dry material (in weight) was used in the disk-milling process, then the solids and partial liquid were used for SSF, leading to 47.4 g/L of ethanol and nearly 5.3 kg wastewater generation per kg of dry feedstock. Obviously, the ethanol fermentation performance in the two cases of the second category was far below that of the present case (71.4 g/L of ethanol and zero waste water generation).

For the third category with the utilization of the solids portion only, the majority of the published high ethanol fermentation studies fall into this category, not only for the solid feedstock contains high percentage of cellulose, but also the inhibitors are already

Table 2
Comparison of high ethanol titer production under high solids content.

Feedstock	Pretreatment methods	Solids content (%, w/w)	Ethanol titer (g/L)	Ethanol yield (%)	Ethanol productivity (g/L/h)	Wastewater generation[a] (kg/kg)	Sources
Whole package of the pretreated materials (solids and liquid) were used in the ethanol fermentation[b]							
Corn stover	Dilute acid with biodetoxification	30	71.4	80.3	0.99	1.0 + 0	This study
Spruce	Steam explosion	25	47.8	72.0	0.34	3.1 + 0	Hoyer et al. (2013)
Solids and partial pretreatment liquid were used in the ethanol fermentation[c]							
Corn stover	Wet oxidation with washing	17	52.3	78.0	0.43	16.7 + 8.0[c]	Varga et al. (2004)
Lodgepole wood	SPORL with wet-disk milling	20	47.4	77.9	0.33	3.0 + 2.3	Lan et al. (2013)
Solids only (after solids/liquid separation) were used in the ethanol fermentation							
Sugarcane bagasse	Formiline with washing	20	80.0	82.7	0.55	28.0[d] + 8.0[e]	Zhao et al. (2013)
Corn cob residue	Dilute acid-alkaline pretreatment with washing	20	75.0	89.4	1.04	20.0 + 8.0[e]	Lei et al. (2014)
Reed	Phosphoric acid–acetone with washing	36	69.3	74.7	0.57	104.0 + 72.0	Li et al. (2009)
Rice straw	Dilute acid and dilute alkaline with washing	16	58.7	73.4	0.49	25.0 + 8.0[e]	Sun and Tao (2013)
Palm empty fruit bunches	Alkali with washing	30	62.5	70.6	0.66	4.0 + 8.0[e]	Park et al. (2013)
Aspen wood logs	SPORL with wet disk milling	18	60.0	77.0	0.50	3.0 + 40.0	Zhu et al. (2011)
Corn stover	Steam explosion with washing	20	59.8	77.2	0.31	2.3 + 15.0	Liu et al. (2014)
Wheat straw	Steam explosion with washing	25	58.6	56.9	0.73	3.0 + 8.0[e]	Alvira et al. (2013)
Wheat straw	Dilute acid with washing	20	57.0	70.0	0.40	9.0 + 8.0[e]	Mohagheghi et al. (1992)
Eastern redcedar	Acid bilsulfite with washing	20	52.0	75.6	0.27	5.0 + 5.0	Ramachandriya et al. (2013)
Forage sorghum	Steam explosion with washing	16	50.0	85.0	0.52	1.0[f]+8.0[e]	Manzanares et al. (2012)
Corn stover	Steam explosion with washing	30	49.5	68.2	0.34	1.0 + 8.0	Lu et al. (2010)
Sweet sorghum bagasse	Hydrothermal	18	47.9	70.4	0.37	10.0 + 0	Matsakas and Christakopoulos (2013)
Spruce	SO_2 with steam explosion[g]	20	40.0	53.0	0.42	4.0 + 0	Koppram and Olsson (2014)
Rapeseed straw	Dilute acid with washing	20	39.9	57.9	0.42	16.7 + 8.0[e]	Lopez-linares et al. (2014)

[a] Waste water generation is the sum of two portions: (1) the acid or alkali or solvent solutions generated from the liquid addition or steam condensation during the pretreatment, and (2) the waste water solution generated from the washing of the pretreated lignocellulose biomass.
[b] Although the whole pretreated materials (solids and liquid) were used in the ethanol fermentation, fresh water was added into the fermentation system to adjust the solids content.
[c] Here the partial pretreatment liquid refers to the liquid generated during pretreatment, not including the wastewater generated from water-washing step.
[d] The liquid volume of the $Ca(OH)_2$ used for deformylation was unknown, so the total liquid used during pretreatment might be more than 28 kg/kg dry solid material.
[e] Because the water used for washing the solid fraction was not given. The authors just mentioned "washed to neutral pH" or "thoroughly washed", according to Lu et al. (2010), the water used for washing was at least 8.0 kg/kg dry solid material.
[f] The detailed information on steam explosion pretreatment was not mentioned in the study, so the liquid or water used was assumed to be 1 kg/kg dry solid material according to Lu et al. (2010).
[g] The pretreated slurry was solid/liquid separation, and the pretreatment liquid was used for yeast cultivation and the solids fraction was used to ethanol fermentation. Additional fresh water was added to adjust the solids content during hydrolysate preparation.

removed by thoroughly washing. The routine procedure includes the following steps: different pretreatment methods with a high liquid/solid ratio and followed by a solid/liquid separation to obtain the solid fraction; then the solid fraction was thoroughly water-washed to remove the inhibitory compounds; again the solids fraction was obtained after a solid/liquid separation step and only the solid part was used for ethanol fermentation. In this procedure, the feedstock sometimes was almost "pure cellulose" while the hemicellulose and lignin fractions were completely removed. Several studies demonstrated high ethanol fermentation performance: 80.0, 75.0, 69.3, and 58.7 g/L of ethanol with the yield of 82.7%, 89.4%, 74.7%, and 73.4% were obtained, but the high wastewater of 36, 28, 176, and 33 kg were also generated per kg dry feedstock solid utilized (Zhao et al., 2013; Lei et al., 2014; Li et al., 2009; Sun and Tao, 2013).

Other studies in the third category did not completely remove hemicellulose and lignin from the feedstock, but still only the water washed solid fraction was used for ethanol fermentation, left the waste water behind. Park et al. (2013) reached the ethanol titer and yield of 62.5 g/L and 70.6%, respectively, with 12 kg/kg wastewater generation; Zhu et al. (2011) produced 60 g/L ethanol with 43 kg wastewater per kg of feedstock; Liu et al. (2014) consumed 17.3 kg/kg water to produce 59.8 g/L ethanol; Manzanares et al. (2012) produced 50.0 g/L ethanol while 9 kg/kg wastewater was generated; Lu et al. (2010) obtained 49.5 g/L ethanol with 9 kg/kg wastewater generation; Matsakas and Christakopoulos (2013) obtained 47.9 g/L ethanol with 10 kg/kg wastewater generation; Ramachandriya et al. (2013) produced 52.0 g/L ethanol at 20% solids content, and 5 kg/kg liquid at both pretreatment and washing steps was used; Alvira et al. (2010) produced 58.6 g/L ethanol with 11 kg/kg water; Koppram and Olsson (2014) produced 40 g/L ethanol at 53% ethanol yield at the cost of 4 kg/kg wastewater generation; Lopez-linares et al. (2014) obtained the ethanol titer of 39.9 g/L and the yield of 57.9% with 16.7 kg/kg wastewater generation from pretreatment and 8 kg/kg from washing.

It is crucially important to reduce the wastewater generation in the large scale plants of cellulosic ethanol processes, because the wastewater treatment system in the farmland is not always available especially in the developing countries. In this study, the high ethanol fermentation performance by the adapted *S. cerevisiae* DQ1 strain was demonstrated. Meanwhile, the significantly less fresh water usage and wastewater generation were achieved due to the applications of new technologies such as the dry dilute sulfuric acid pretreatment, biodetoxification, and high solids content SSF in helical ribbon stirring reactors. The present study provides a practical approach for improving ethanol fermentability at high solids content and we suggest that all the microorganisms used in lignocellulose biorefinery should be experienced an evolutionary adaptation process in order to enhance their fermentation performance.

4. Conclusion

A long term evolutionary adapted yeast strain was obtained after 65 days' adaptation (equivalent to 780 generations) by successively transferring in the corn stover hydrolysate containing high inhibitor substances. The adapted yeast strain demonstrated significantly improved ethanol fermentation performance during SSF at high solids content. The ethanol titer and yield of 71.40 g/L and 80.34% were obtained, respectively, at an optimum SSF condition without any wastewater generation from pretreatment to fermentation. The present study provides a practical approach for improving fermentability of microorganisms used in lignocellulose biorefinery.

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