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Inhibitor analysis and adaptive evolution of *Saccharomyces cerevisiae* for simultaneous saccharification and ethanol fermentation from industrial waste corncob residues

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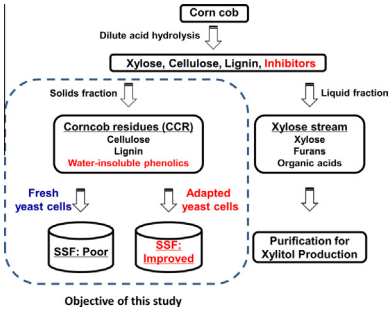
HIGHLIGHTS

- High level of water-insoluble phenolics is responsible for poor fermentation of CCR.
- Evolutionary adaptation of *Saccharomyces cerevisiae* improved fermentability of CCR.
- High ethanol titer and productivity without pretreatment and detoxification of CCR.

GRAPHICAL ABSTRACT



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ABSTRACT

Industrial waste corncob residues (CCR) are rich in cellulose and can be hydrolyzed directly without pretreatment. However, a poor fermentation performance was frequently observed in the simultaneous saccharification and ethanol fermentation (SSF) of CCR, although the furans and organic acid inhibitors were very low. In this study, the high level of water-insoluble phenolic compounds such as 2-furoic acid, ferulic acid, *p*-coumaric acid, guaiacol, and *p*-hydroxybenzoic acid were detected in CCR and inhibited the growth and metabolism of *Saccharomyces cerevisiae* DQ1. An evolutionary adaptation strategy was developed by culturing the *S. cerevisiae* DQ1 strain in a series of media with the gradual increase of CCR hydrolysate. The high ethanol concentration (62.68 g/L) and the yield (55.7%) were achieved in the SSF of CCR using the adapted *S. cerevisiae* DQ1. The results provided a practical method for improving performance of simultaneous saccharification and ethanol production from CCR.

1. Introduction

High processing cost is still the major technical barrier to cellulosic ethanol production at commercial scales (Lynd et al., 2008). One reason for the high cost is the high steam energy consumption in the distillation of fermentation broth with low ethanol titer when lignocellulose materials are used as feedstock (Galbe et al., 2007). Increasing the cellulose content in the fermentation system is a direct way for increasing ethanol titer and the utilization of the lignocellulose material with high cellulose content is a proper option.

Currently, more than 20 million tons of corncobs are produced annually in China and considerable amount of them are used as feedstock for xylitol and furfural production due to their high xylan content (Bai et al., 2008; Bu et al., 2011; Cheng et al., 2010). One

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negative outcome is that approximately half million tons of corncob residues (CCR) are generated as solid wastes annually (Tang et al., 2011). On the other hand, CCR is rich in cellulose (up to 50%), and the cost intensive pretreatment is not necessary for enzymatic hydrolysis, because the upstream dilute acid hydrolysis of corncob has deeply extracted its hemicellulose (xylan) and destroyed its lignocellulose structure (Liu et al., 2010). For these merits, CCR has been used for ethanol production in many previous studies (Cheng et al., 2011; Liu et al., 2010, 2012; Tang et al., 2011).

In previous studies, the long lag phase and poor fermentation performance of *Saccharomyces cerevisiae* had been observed when CCR was used for ethanol fermentation (Cheng et al., 2011; Tang et al., 2011). The phenomenon generally appears when inhibitory compounds such as acetic acid, formic acid, furfural, 5-hydroxymethylfurfural (HMF), and phenol derivatives exist in lignocellulose feedstock (Almeida et al., 2007). However, all the studies (including present one) showed that most of these inhibitors were water-soluble and had been sufficiently removed from CCR during the multiple washing steps of xylan extraction. On the other hand, phenolic compounds from lignin degradation usually have low solubility or even insolubility in water (Palmqvist and Hahn-Hägerdal, 2000) and could possibly precipitate and accumulate on CCR even after multiple washing steps. These slightly water-soluble compounds could gradually release from the solid phase to the liquid phase during the processes of hydrolysis and ethanol fermentation (Thomsen et al., 2009). The long lag phase and poor fermentation of CCR could be related to the existence of the water-insoluble phenolic compounds.

At present, there is no effective way to remove the water-insoluble phenolics from CCR, because common detoxification methods such as overliming, electrochemical and ion exchange treatments are only applicable for liquid (Mancilha and Karim, 2003; Zhang et al., 2012). Biodetoxification works on solids, but the growth of detoxification strain (such as Amorphotheca resinae ZN1) on CCR is poor because the soluble components (as nutrients) are removed in the washing process (Zhang et al., 2010b). In this study, an evolutionary adaptation method was investigated for improving the tolerance of the ethanol fermenting strain *S.* cerevisiae DQ1 to phenolics. Specifically, the yeast strain was cultured in a series of adaptation media prepared by CCR hydrolysate before it was finally inoculated into the SSF of CCR. After this adaptation procedure, the yeast strain presented the higher inhibitor tolerance, shorter lag phases and greater ethanol fermentation performance. Finally, the optimized process of SSF was performed in the 5 and 50 L bioreactors for ethanol production and the satisfactory results were obtained. This study provided a simple and practical procedure for high performance of ethanol fermentation using CCR as feedstock.

2. Methods

2.1. Raw materials

CCR was provided by Longlive Biotechnology Co., Yucheng, Shandong, China. The CCR contained 66.3% of water, and the dry CCR contained 56.5% of glucan and 2.6% of xylan, which was determined using the protocol of the National Renewable Energy Laboratory (Sluiter et al., 2012).

The commercial cellulase enzyme Youtell #6 was kindly provided by Hunan Youtell Biochemical Co., Yueyang, Hunan, China. The filter paper activity was 145 FPU per gram, which was analyzed using the protocol of NREL LAP-006 (Adney and Baker, 1996). The cellobiase activity was 344 IU per gram, which was analyzed according to the method in Ghose (1987).

Corn steep liquor was purchased from Xiwang Sugar Co., Zouping, Shandong, China. Yeast extract was purchased from Angel Yeast Co., Yichang, Hubei, China. 2-furoic acid, guaiacol, *p*-hydroxybenzoic acid, *p*-coumaric acid and ferulic acid were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. All other chemicals and reagents were analytical pure and obtained from Lingfeng Chemical Reagent Co., Shanghai, China, including glucose, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, NaOH, H_2SO_4 and DMSO.

2.2. Strain and culture medium

S. cerevisiae DQ1 used for ethanol fermentation was stored in Chinese General Microorganisms Collection Center, Beijing, China, with the registration number of CGMCC 2528 (Chu et al., 2012; Guo et al., 2013; Zhang et al., 2010a). The culture solution was aliquoted into 2.0 ml vials containing 30% (w/w) glycerol and stored at −80 °C freezer. The dry cell weight was calculated according to the calibration in Chu et al. (2012) and 0.5 g/L dry cell weight corresponding to the optical density at 600 nm was 1.0.

The CCR hydrolysate was prepared by enzymatic hydrolysis with 15% and 25% (w/w, dry base) of CCR loading in overall hydrolysate at 50 °C, pH 4.8 for 48 h with enzyme dosage of 15 FPU/g CCR dry matter (DM). The water insoluble solid in the CCR hydrolysate was centrifuged at 16,125*g* for 10 min, and the supernatant was collected and autoclaved at 115 °C for 20 min, then filtered through sterilized filter paper in clean bench and stored at 4 °C. Culture media used in this study included:

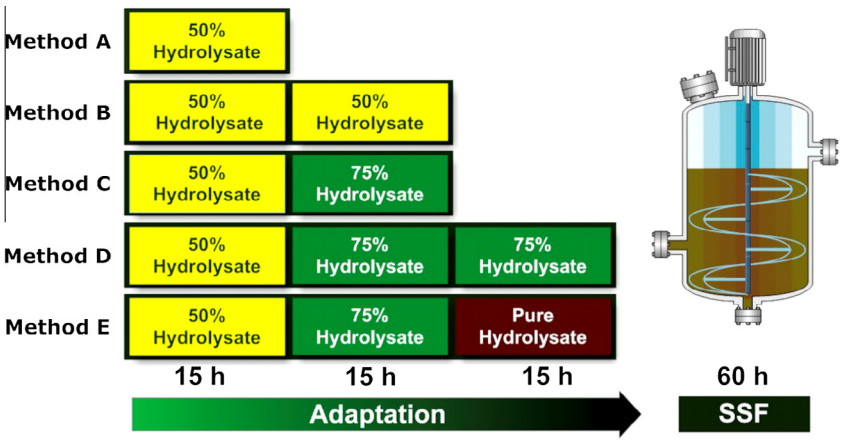


Fig. 1. Schematic illustration of the short term adaptation strategy.

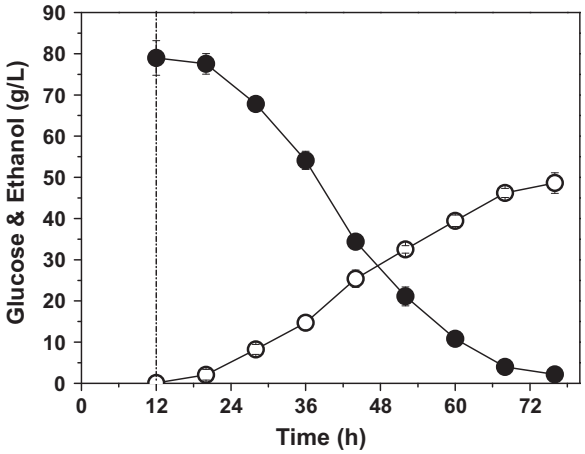


Fig. 2. Fermentation performance of *S. cerevisiae* DQ1 in SSF using CCR. Conditions: Prehydrolysis stage: solids loading of 25% (w/w), the enzyme dosage of 15 FPU/g DM, 50 °C and pH 4.8 for 12 h. SSF stage: the strain was inoculated after prehydrolysis stage at 37 °C and pH 5.5.

(1) Synthetic medium: 20.0 g/L of glucose, 2.0 g/L of KH_2PO_4, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4{\cdot}7H_2O$ and 1.0 g/L of yeast extract.
(2) Inhibitor medium: 2-furoic acid, guaiacol, *p*-hydroxybenzoic acid, *p*-coumaric acid and ferulic acid were added into synthetic medium separately with different concentrations. The stock solutions of inhibitors were prepared in aqueous solution with 30% (v/v) of DMSO and sterilized by filtering through 0.22 μm filter. The final concentration of DMSO in the inhibitor medium was below 0.7% (v/v) and no impact on the growth of *S. cerevisiae* DQ1 was observed.
(3) Adaptation medium: CCR hydrolysate prepared at the solids loading of 15% (w/w) was diluted by deionized water to 50% (hydrolysate:water = 50:50, v/v) and 75% (hydrolysate:water = 75:25, v/v), respectively. Three kinds of adaptation medium were prepared by supplementing the inorganic salts and nutrients to the diluted (50% and 75%, v/v) and pure hydrolysate at the same concentration as the synthetic medium.
(4) SSF medium: 2.0 g/L of KH_2PO_4, 1.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of $MgSO_4{\cdot}7H_2O$ and 1.0 g/L of yeast extract were added into the SSF fermentation system.

2.3. Adaptation of S. cerevisiae DQ1 to CCR hydrolysate

One vial of *S. cerevisiae* DQ1 was taken from the −80 °C freezer and inoculated into a 100 ml flask containing 20 ml of sterilized synthetic medium, cultured in a shaking incubator at 30 °C, pH 6.0, for 18 h as the seeds culture.

The adaptation procedure was carried out by sequentially transferring cultures to the adaptation medium with diluted CCR hydrolysate. Five adaption methods preformed for preparation of adapted yeast seeds were tested as shown in Fig. 1:

Method A: the yeast seeds were inoculated into a 500 ml flask containing 200 ml of adaptation medium with 50% (v/v) of hydrolysate;

Method B: the first step was same with the method A, then inoculated the adapted culture to 200 ml of adaptation medium with 50% of hydrolysate;

Method C: the first step was same with the method A, then inoculated the adapted culture to 200 ml of adaptation medium with 75% (v/v) of hydrolysate;

Method D: the first two steps were same with the method C. Then, inoculated the two-step adapted culture to 200 ml of adaptation medium with 75% of hydrolysate;

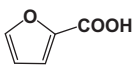
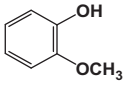
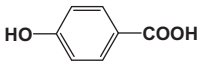
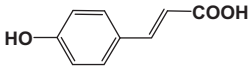
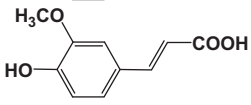
Method E: the first two steps were same with the method C. Then, inoculated the two-step adapted culture to 200 ml of adaptation medium with pure hydrolysate;

All of the adaptations were carried out at 30 °C, pH 6.0 for 15 h with a 10% (v/v) of inoculation ratio. After each adaptation methods, the adapted seeds at 10% inoculum were transferred to SSF at the solids loading of 25% (w/w) for ethanol fermentation.

2.4. Simultaneous saccharification and ethanol fermentation (SSF)

The SSF operation was performed in two bioreactors (5 and 50 L) equipped with helical ribbon impeller (Baoxing Biotechnology Equipment Co., Shanghai, China) (Zhang et al., 2010a). The SSF was divided into two stages, the prehydrolysis stage and the

Table 1
GC–MS analysis of composition and structure of phenolic monomers in hydrolysate of CCR.

Trimethylsilylatd (tms) compounds	Structure of compounds	RT (min)	MW	Quantification ions	Solubility* (g/100 ml)
tms 2-furoic acid		7.88	169	95,125,169	2.71[a]
tms guaiacol		9.78	196	166,181,196	1.70[b]
tms *p*-hydroxybenzoic acid		16.53	282	223,267,282	0.50[b]
tms *p*-coumaric acid		20.66	308	294,293,308	0.10[c]
tms ferulic acid		22.50	338	293,323,338	0.59[d]

* Solubility of the original compounds without silylation in water at 25 °C.
[a] Harrisson, R.J., Moyle, M., 1956. 2-FUROIC ACID. Org. Synth., 36, 36.
[b] Yalkowsky, S.H., He, Yan., 2003. An extensive compilation of aqueous solubility data for organic compounds extracted from the AQUASOL DATAbASE. In: Yalkowsky, S.H., He, Yan. (Eds.), Handbook of Aqueous Solubility Data. CRC Press LLC, Boca Raton, FL., pp. 377–398.
[c] Tetko, I.V., Tanchuk, V.Y., Kasheva, T.N., Villa, A.E., 2001. Estimation of aqueous solubility of chemical compounds using E-state indices. J. Chem. Inf. Comput. Sci., 41, 1488–1493.
[d] US EPA; Estimation Program Interface (EPI) Suite. Ver.3.12. Nov 30, 2004. Available from, as of Oct 27, 2008: <http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm>.

SSF stage. The prehydrolysis stage started when the CCR, nutrients, and cellulase enzyme were fed into the bioreactor and lasted for 12 h at 50 °C and pH 4.8; then the SSF stage began when the adapted *S. cerevisiae* DQ1 seeds were inoculated and lasted for 60 h at 37 °C and pH 5.5. The pH was maintained during the prehydrolysis and SSF stages by addition of 5 M NaOH solution. All experiments were performed in duplicate, except the SSF in 50 L bioreactor which was performed only once. The results shown in the figures were expressed as the mean and standard deviation of the twice independent experiments.

2.5. Analysis of total phenolic content

The total phenolic content in the whole slurries (solid and liquid) of CCR hydrolysate were measured by a modified Folin & Ciocalteu method (Ainsworth and Gillespie, 2007) with optimized concentration of the Folin & Ciocalteu reagent and Na_2CO_3. The phenolic compounds were extracted from 200 mg samples by 20 ml of 95% (v/v) methanol at ambient temperature for 48 h. Then the samples were centrifuged at 11,167*g* for 5 min to obtain the supernatant. 500 μl of the supernatant and 1 ml of 15% (v/v) Folin

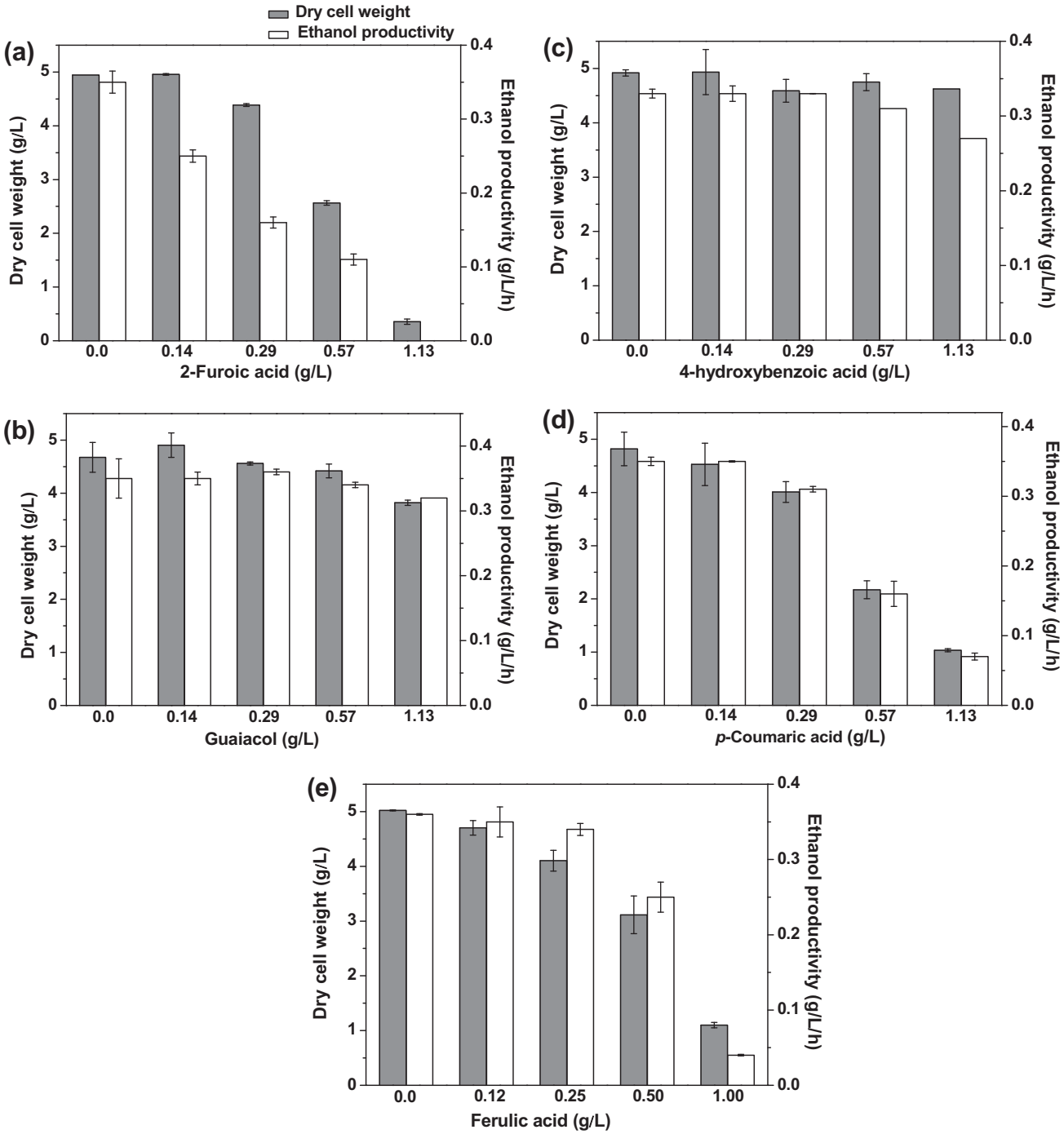


Fig. 3. Inhibition of phenolic compounds in CCR on the growth and fermentability of *S. cerevisiae* DQ1. Conditions: 250 ml flask containing 40 ml of the synthetic medium with the individual inhibitors at various concentration, 10% (v/v) inoculation ratio, at 30 °C for 24 h. All experiments were performed in duplicate. The phenolic monomers include: (a) 2-furoic acid, (b) guaiacol, (c) *p*-hydroxybenzic acid, (d) *p*-coumaric acid, (e) ferulic acid.

& Ciocalteu reagent to a 10 ml tube and vortexed thoroughly, then added 4 ml of 0.7 M Na_2CO_3. The assay tube was incubated at ambient temperature for 2 h, then the absorbance was measured at 765 nm. Each sample was detected in duplicate. The concentration of total phenolic compounds was determined from a calibration curve based on gallic acid.

2.6. Analysis of phenolic compounds in CCR using GC–MS

The phenolic compounds in the CCR hydrolysate were qualitatively analyzed using the GC–MS. First, the hydrolysate was selectively extracted using solid phase extraction on polystyrene divinylbenzene polymer columns: Poly-Sery PSD SPE Tubes 250 mg/3 ml (CNW Technologies GmbH, Germany). The extracted samples were silylated with *N,O*-bis (trimethylsilyl) trifluoroacetamide, then analyzed by GC–MS according to the method described in the previous researches (Klinke et al., 2002; Varga et al., 2004). GC–MS analysis was performed on an Agilent 6890 GC–MS fitted with a HP-5 MS column (30 m × 0.25 mm × 0.25 μm) under the following temperature program: from 80 °C (held for 4 min) to 280 °C at 8 °C/min. 1 μl sample was injected and detected under splitless condition.

2.7. HPLC analysis

Glucose, ethanol, furfural, HMF, acetic acid, and levulinic acid, were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) fitted with a Bio-Rad Aminex HPX-87H column at 65 °C. The mobile phase was 5 mM H_2SO_4 at 0.6 ml/min (Zhang et al., 2010a). 4-hydroxybenzaldehyde, vanillin and syringaldehyde were analyzed using HPLC fitted with UV/Vis detector SPD-20A (Shimadzu, Kyoto, Japan) set at 270 nm and YMC-Pack ODS-A column (YMC Co., Japan) at 35 °C. The mobile phase was water: acetonitrile (70:30, v/v) at 1.0 ml/min (Khoddami et al., 2013). All samples were centrifuged at 11,167*g* for 5 min to remove the water insoluble substances and the supernatant filtered through a 0.22 mm filter before analysis.

2.8. Calculations

The ethanol yield was calculated according to the modified method taking into account the volume change by ethanol formation and the mass balance due to water loss in the SSF system (Zhang and Bao, 2012).

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

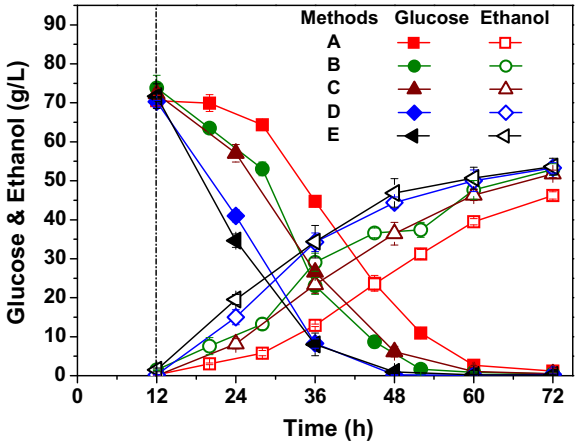
where [*C*] is the concentration of ethanol in the fermentation broth at the end of the SSF (g/L), *W* is total water input into the SSF system (g), *f* is the cellulose content in the dry CCR (g/g), [CCR] is the dry CCR loading (g/g), *m* is total weight of the SSF system at the beginning of the operation, 976.9 is the correction factor, 1.111 is the conversion factor for cellulose to equivalent glucose, 0.511 is the conversion factor for glucose to ethanol based on the stoichiometric biochemistry of yeast.

3. Results and discussion

3.1. Fermentation performance of S. cerevisiae DQ1 using CCR as feedstock

The SSF of CCR by *S. cerevisiae* DQ1 was performed at the solids loading of 25% (w/w) for evaluation of its ethanol fermentation performance. The results in Fig. 2 showed that the strain *S. cerevisiae* DQ1 had the long lag phase and poor fermentability during the SSF using CCR as the feedstock. The glucose generation included the initial glucose of 78.98 g/L in the 12 h prehydrolysis and the glucose released in the 64 h SSF. The time for the complete consumption of the glucose took 76 h. This fermentation time (76 h) was extraordinarily long, and almost 50 h than the usual fermentation time using corn stover (18 h), spruce (24 h) or corncob (18 h) by *S. cerevisiae* species with the similar initial glucose. Meanwhile, the ethanol productivity was only 0.61 g/L/h in the first 24 h of fermentation, which was 3–5 folds lower than those in the previous reports (Hoyer et al., 2013; Jin et al., 2013; Zhang et al., 2010c).

To investigate the reasons of the long lag phase and poor fermentation performance, the typical inhibitors in the CCR hydrolysate at solids loading of 25% (w/w) was measured. However, the concentrations of furfural, HMF, formic acid, vanillin, syringaldehyde, and 4-hydroxybenzaldehyde were found below 0.005 g/L; the concentrations of acetic acid and levulinic acid were at around 0.20 g/L. These inhibitor levels were not high enough to give obvious inhibition on ethanol fermenting strains (Klinke et al., 2004; Palmqvist et al., 1999). The further searching of inhibitors at low concentrations in species in the CCR hydrolysate was carried out by the enrichment method as described in the Section 2.6, followed by the then silanization and qualitative analysis by GC–MS. Five phenolic compounds were found in the CCR hydrolysate, including 2-furoic acid, guaiacol, *p*-hydroxybenzoic acid, *p*-coumaric acid,



Adaptation process	Productivity (g/L/h)	* Ethanol titer (g/L)	Yield (%)
Method A	0.35	48.60	63.1
Method B	0.75	52.90	68.9
Method C	0.68	51.76	67.4
Method D	1.23	53.32	69.5
Method E	1.50	53.62	69.9

* Productivity was calculated for the initial 12 h after inoculation.

Fig. 4. Adaptation on fermentability of *S. cerevisiae* DQ1 during SSF. Method A: the strain was adapted in the adaptation medium with 50% (v/v) of the CCR hydrolysate; Method B: the strain was successively cultured in the adaptation medium with 50% of hydrolysate for two-step adaptation; Method C: the strain was successively cultured in the adaptation mediums with 50% and 75% (v/v) of hydrolysate for two-step adaptation; Method D: the strain was successively adapted in adaptation mediums with 50%, 75% and 75% of hydrolysate for three-step adaptation; Method E: the strain was successively adapted in adaptation mediums with 50%, 75% and pure hydrolysate for three-step adaptation. Conditions: SSF at solids loading of 25% (w/w). Prehydrolysis stage: the enzyme dosage of 15 FPU/g DM, 50 °C and pH 4.8 for 12 h. SSF stage: each of the adapted yeast seeds underwent different adaptation methods were inoculated after prehydrolysis stage and cultured at 37 °C and pH 5.5.

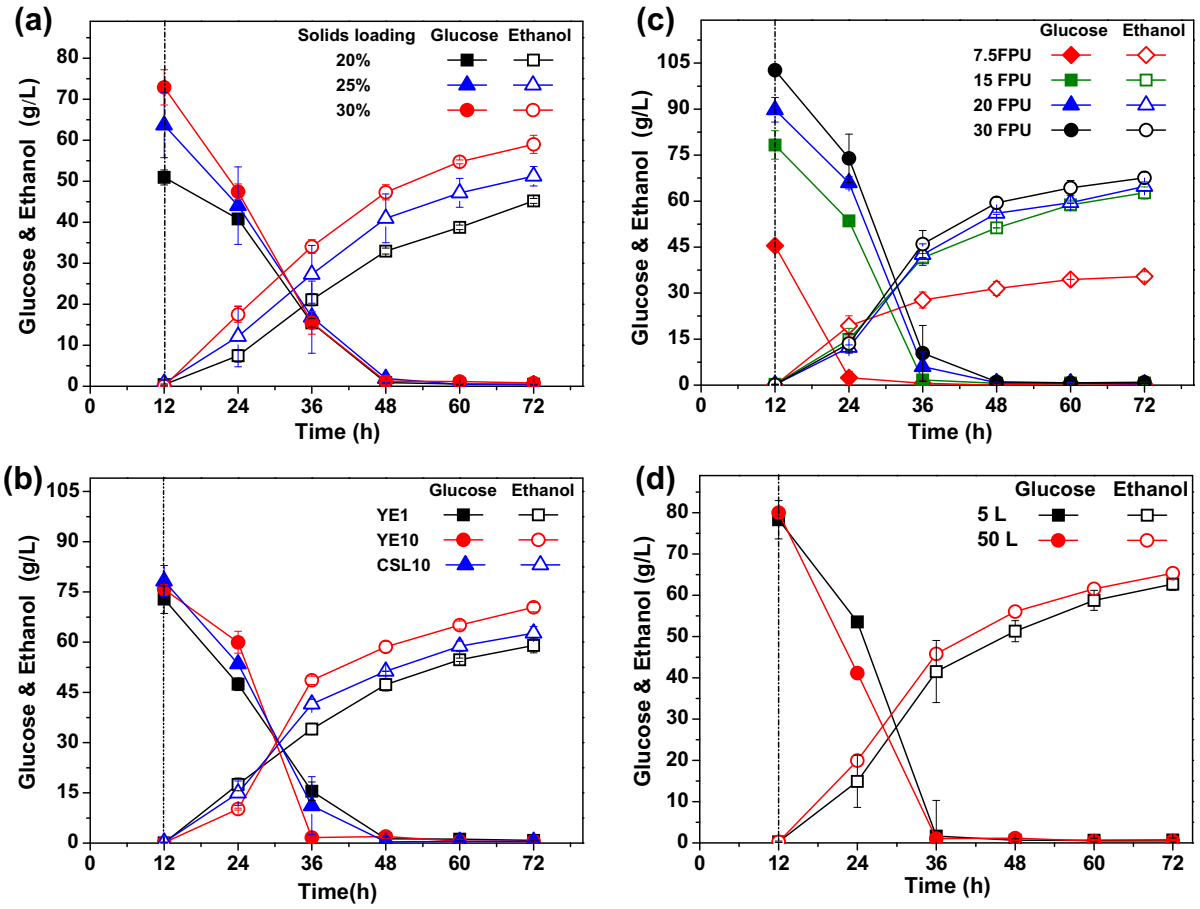


Fig. 5. SSF of CCR at different fermentation parameters. Conditions: Prehydrolysis stage: 50 °C and pH 4.8 for 12 h. SSF stage: the three-step adapted yeast seeds were inoculated after prehydrolysis stage and cultured at 37 °C and pH 5.5. (a) Effect of solids loading on SSF, condition: cellulase dosage of 15 FPU/g DM, yeast extract of 1 g/L; (b) Effect of nutrients on SSF, condition: solids loading of 30% (w/w), cellulase dosage of 15 FPU/g DM, YE1 and YE10 mean yeast extract of 1 and 10 g/L, CSL10 means corn steep liquor of 10 g/L; (c) Effect of cellulase dosage on SSF, condition: solids loading of 30% (w/w), corn steep liquor of 10 g/L; (d) SSF at high solids loading in 5 and 50 L bioreactor, condition: solids loading of 30% (w/w), corn steep liquor of 10 g/L, cellulase dosage of 15 FPU/g DM.

and ferulic acid as shown in Table 1. It is noteworthy that most of these five compounds were lignin derivatives in the form of phenolic acid with the low water solubility (2.71, 1.70, 0.50, 0.10, and 0.59 g/100 ml of water for 2-furoic acid, guaiacol, *p*-hydroxybenzoic acid, *p*-coumaric acid, and ferulic acid, respectively).

In the next step, the inhibition of the five phenolic compounds on the cell growth and fermentability of *S. cerevisiae* DQ1 was investigated by adding each of the compounds to the synthetic medium at the concentration below their solubility in water. As shown in Fig. 3, 2-furoic acid at the lowest level of 0.14 g/L could cause an obvious decrease in the growth and productivity of ethanol fermentation; *p*-coumaric acid and ferulic acid also affected the growth and fermentability of *S. cerevisiae* DQ1 at low concentrations (0.25 g/L). The results indicate that the cell growth and ethanol fermentation of *S. cerevisiae* DQ1 were more sensitive to the presence of phenolic acids, compared with the previous report about the inhibition of phenolic compounds on *S. cerevisiae* (Klinke et al., 2004). All these concentration levels affecting the yeast cell growth and fermentability were in their solubility range or below this range.

Then the total phenolic compounds content in the whole CCR slurry (25%, w/w), including both the solid and liquid portions, was measured. The result shows that a very high total phenolic content of 5.59 g/L in the whole slurry of CCR hydrolysate, which was significantly greater than the phenolic concentrations in the liquid CCR hydrolysate. The origin of the high phenolics with low water solubility in CCR might come from the accumulation of the multiple washing steps following the acid hydrolysis of corncob for xylose extraction (Liu et al., 2010). Since phenolic compounds were able to gradually release from the solid phase and enter into the liquid phase during the hydrolysis and SSF (Thomsen et al., 2009), the phenolics concentration might be maintained at a low but constant level to perform an inhibition on *S. cerevisiae* DQ1. These phenolic acid compounds might be responsible for the poor growth and fermentability behaviors of *S. cerevisiae* DQ1, even at the low concentrations.

3.2. Adaptation strategy for improving fermentation performance

Evolutionary adaption of yeast cells to lignocellulosic inhibitors is an effective method for improving the inhibitor tolerance and fermentation performance (Heer and Sauer, 2008; Landaeta et al., 2013). In this study, a stepwise adaptation strategy by successively transferred the strains to the adaptation medium with increased CCR content was investigated. Four different adaptation methods were tested and the adapted yeast seeds were finally inoculated into the SSF of CCR at the high solids loading of 25% (w/w). As shown in Fig. 4, the glucose consumption rate and ethanol productivity of the one-step adapted strain (by Method A) were 0.27 and 0.35 g/L/h in 12 h after inoculation, respectively, and the fermentation residual glucose still could be detected until the end of the SSF process. The two-step adaptation (Method B) improved the glucose

Table 2
Comparison of SSF for ethanol production from CCR.

Strains	Solids loading (%)	De-lignin step	Lignin content (%, w/w)	Fermentation time (h)	Ethanol titer (g/L)	Ethanol yield (%)	Ethanol productivity (g/L/h)	References
S. cerevisiae CICC 31014	19.5	Yes (Sulfite)	12.6	72	60.8	72.2	0.84	Cheng et al. (2011)
S. cerevisiae (dry yeast)	15	Yes (Alkali)	3.2	142	57.2	85.2	0.40	Liu et al. (2010)
S. cerevisiae (dry yeast)	5	Yes (Alkaline/H_2O_2)	13.2	36	16.9	44.0	0.47	Wang et al. (2013)
S. cerevisiae DQ1	30	No	22.1	60	62.7	55.7	1.04	This study

consumption rate and ethanol productivity to 1.28 and 0.75 g/L/h, respectively, and the very similar fermentation performance was achieved by another two-step adaptation Method C (1.27 g/L/h of the glucose consumption rate and 0.68 g/L/h of the ethanol productivity). The three-step adaptations of Methods D and E further improved the ethanol productivity to 1.23 and 1.50 g/L/h, respectively, and no residual glucose was detected after 36 h of SSF. The highest ethanol concentration and yield of 53.62 g/L and 69.9% were obtained by the SSF after the three-step adaptation (Method E). The present results demonstrated that this three-step adaptation method efficiently shortened lag phase and improved fermentation performance of the yeast strain *S. cerevisiae* DQ1.

3.3. SSF at high CCR loading for ethanol production by adapted *S. cerevisiae* DQ1

The three-step adaptation method (Method E) was applied for SSF experiments at various fermentation conditions as shown in Fig. 5. Fig. 5(a) indicates that the ethanol titer increased from 44.22 to 59.02 g/L with the increasing solids loading from 20% to 30% (w/w), but the ethanol yield decreased from 65.8% to 52.3% when the solids were greater than 30% (w/w). Fig. 5(b) indicates that the ethanol titer and yield increased with increasing yeast extract addition concentration, perhaps due to the elevated viability of yeast cells in the inhibitor containing environment by the rich nutrient ingredients in the yeast extract. However, when the expensive yeast extract ($9.2 per kg) was replaced the corn steep liquor ($0.18 per kg) (Maddipati et al., 2011) at the same dosage (10 g/L), the ethanol concentration and yield were still pretty satisfactory. Fig. 5(c) indicates that with the cellulase dosage increased from 7.5 to 30 FPU/g DM, there was an increase in rate of glucan hydrolysis during prehydrolysis; the ethanol titer significantly increased from 35.43 to 62.68 g/L when the cellulase dosages increased from 7.5 to 15 FPU/g DM, but further increase of cellulase dosage only led to a slight increase in ethanol titer. Fig. 5(d) shows a scale-up case from the 5 to 50 L bioreactors for the SSF of CCR using the fermentation conditions obtained above. The ethanol titer and yield of the SSF in 50 L bioreactor were 65.33 g/L and 58.2%, respectively, which were very similar or even slightly better than that in 5 L bioreactor, indicating that the operation might not cause obvious change on the mass and heat transfer in the enlarged bioreactor. Therefore, SSF for ethanol production from CCR at high solids loading using adapted *S. cerevisiae* DQ1 could be scaled up safely in a certain range.

Several SSF results using CCR as the feedstocks were summarized in Table 2. Although the feedstock options were on the uniform basis, the major difference between the previous studies and the present one is that the de-lignin step was carried out by various methods, such as alkali (Cheng et al., 2011), alkaline H_2O_2 (Liu et al., 2010), and sulfite treatment (Wang et al., 2013), to reduce the lignin content and increase the cellulose content. This step increased the bioconversion efficiency of CCR, with the price of increased processing cost and waste water generation. However, even the lignin levels were reduced below 15% (w/w) in these three cases, the poor fermentation performance such as the long fermentation time, low ethanol titer and productivity still presented in such SSF processes because of phenolics in the CCR (Cheng et al., 2011; Liu et al., 2010; Wang et al., 2013). Compared with above results in Table 2, the ethanol fermentation in this study using the adapted *S. cerevisiae* DQ1 showed a satisfactory result with the higher ethanol concentration of 62.68 g/L (7.7%, v/v) and the fast productivity of 1.04 g/L/h, although the CCR feedstock was the original CCR feedstock without de-lignin step. The results proved the evolutionary adaption method of *S. cerevisiae* strain for improving ethanol fermentation from the industrial CCR feedstock was feasible and efficient.

4. Conclusion

A high level of water-insoluble phenolic compounds such as 2-furoic acid, ferulic acid, *p*-coumaric acid, guaiacol, and *p*-hydroxybenzoic acid were detected in CCR and these phenolics were found to be responsible for the poor growth and metabolism of *S. cerevisiae* DQ1. An evolutionary adaptation strategy was developed by culturing the strain *S. cerevisiae* DQ1 in a series of media with the gradual increase of CCR hydrolysate. The high ethanol concentration (62.68 g/L) and the yield (55.7%) were achieved in the SSF of CCR using the adapted *S. cerevisiae* DQ1.

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