Bioresource Technology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Challenges of non-flocculating *Saccharomyces cerevisiae* haploid strain against inhibitory chemical complex for ethanol production

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HIGHLIGHTS

- Non-flocculating *Saccharomyces cerevisae* NBRC849 was robust haploid yeast.
- Upregulations of ZWF1 and ALD6 were required for yeast robustness against inhibitors.
- NADPH and NADH were used for *in situ* detoxification process of inhibitors.
- ABC and polyamine transporters play an important role in the inhibitor efflux.
- The enhancement of metabolic flux to the shikimic pathway is important for cell survival.

ARTICLE INFO

Article history:
Received 31 March 2017
Received in revised form 1 June 2017
Accepted 3 June 2017
Available online xxxx

Keywords:
Fermentation
Inhibitory chemical complex
Inhibitor tolerance
Saccharomyces cerevisiae

ABSTRACT

This study provides insight observation based on the gene expression and the metabolomic analysis of the natural robust yeast *Saccharomyces cerevisiae* NBRC849 during the fermentation in the medium containing inhibitory chemical complexes (ICC) at different concentrations. The tolerance mechanisms involved in the strain might have existed through the upregulation of genes involved in NAD(H)/NADP(H) cofactors generations (ALD6, ZWF1, GND1), membrane robustness for efflux pump (YOR1, PDR5, TPO3) and cation/polyamine transport (TPO3). The alteration of metabolic flux to the shikimic pathway was also found in this strain, resulted in the enhanced formation of aromatic amino acid required for cell survival. Enhanced expression of these genes as well as the increase of metabolic flux to shikimic pathway were suggested to result in the robustness of non-flocculating *S. cerevisiae* haploid strain.

1. Introduction

One major barrier of biomass conversion to ethanol is the presence of inhibitory chemical complexes (ICC) generated as byproducts during biomass pretreatment, which interfering microbial growth during fermentation process. Practically, it is difficult to estimate varieties of ICC, since the species of byproducts released not only depend on the kind of biomass processed but also the kind of the pretreatment process implemented. The potent inhibitors in ICC released upon the pretreatment are 2-furaldehyde (furfural), 5-hydroxymethylfurfural (HMF), syringaldehyde and vanillin coupled with some cell membrane-permeative acids such as acetic acid, formic acid and a small fraction of levulinic acid. These inhibitors are released mainly due to the over-acid hydrolytic degradation of cellulose and hemicellulose. Among them, furfural and 5-HMF are major constituent parts of inhibitors released from the

Abbreviations: ACD, acetaldehyde; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; F16P, frucotose-1,6-bisphosphate; G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; GC–MS, gas chromatography–mass spectrometry; G3P, glycerol-3-phosphate; 5-HMF, 5-hydroxymethylfurfural; HPLC, high-performance liquid chromatography; LC-QqQ-MS, liquid chromatography-tandem quadrupole mass spectrometry; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; $NADP^+$ and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; 6PG, 6-phosphoglycerate; 6PGL, 6-phosphogluconate; PEP, phosphoenolpyruvate; PGA, 2- or 3-phosphoglycerate; PPP, pentose phosphate pathway; PYR, pyruvate; R5P, ribose-5-phosphate; Ru5P, rubulose-5-phosphate; S7P, sedoheptulose-7-phosphate; X5P, xylulose-5-phosphate.

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http://dx.doi.org/10.1016/j.biortech.2017.06.009
0960-8524/

biomass. The furfural and 5-HMF are known to cause specific biological effects. Furfural induces the accumulation of reactive oxigen species (ROS) in *Saccharomyces cerevisiae* to cause cellular damage includes damage to mitochondria and vacuole membranes, the actin cytoskeleton and nuclear chromatin (Allen et al., 2010) also causes DNA damage, induce DNA mutations in many organisms. The inhibitory effect on various microorganisms including budding yeasts (Almeida et al., 2007; Delgenes et al., 1996) and bacteria (Zaldivar et al., 2000) such as reducing fermentation rate and/or stop growing and enter an extended lag phase (Branberg et al., 2004; Heer and Sauer, 2008). For some tolerance *S. cerevisiae* yeasts, they could convert furfural and 5-HMF to their less reactive alcohol derivatives using NAD(P)H-dependent reduction reactions mostly during the lag phase (Almeida et al., 2007).

The pentose phosphate pathway (PPP) plays an essential role in furfural tolerance (Liu et al., 2009). When single PPP genes (ZWF1, GND1, TKL1 or RPE1) are absent, yeast, that would normally allow growth after a 24-h lag, are unable to grow when concentration of furfural (25 mM) is present (Gorsich et al., 2006). The greatest growth defect is seen when the ZWF1 gene is disrupted. ZWF1 encodes glucose-6-phosphate dehydrogenase, which catalyzes the rate-limiting step of the PPP and produces NADPH. This growth defect is probably not due to an inability to reduce furfural, as furfural can be reduced using NADH. However, NADPH produced in PPP is also an important co-factor used to protect cells against cellular stress caused by ROS.

Different approaches have been used to solve the inhibitory effects including chemically, biology and physical approaches (Ludwig et al., 2013). The chemically treatment in the production process for example, treatment with reducing agents (Alriksson et al., 2011), addition of activated charcoal (Kim et al., 2013), overliming (Prasertwasu et al., 2014), anion exchanger (Ludwig et al., 2013), evaporation, enzymatic treatment with peroxidase and laccase (Zhu et al., 2011), *in situ* detoxification by fermenting microbes (Tomas et al., 2013), solvent extraction (Zhu et al., 2011), and membrane extraction (Grzenia et al., 2012) have been investigated to reduce their inhibitory effects in ethanol fermentation. Physical methods have also been investigated such as evaporation and membrane separation which it requires a lot of energy and this might make it uneconomical at industry level (Prasertwasu et al., 2014). However, all of the procedures for inhibitors removal makes the production process more complex and causes a higher cost. Therefore, utilizing inhibitors-tolerant microorganisms for inhibior detoxification during the fermentation is a more favorable method.

S. cerevisiae is superior to bacteria, other yeasts, and filamentous fungi in various physiological characteristics regarding ethanol production in industrial manner. It tolerates a wide range of pH with acidic optimum (Ortiz-Muñiz et al., 2010), which makes its fermentation less susceptible to infection than bacteria. It also tolerates ethanol better than other ethanol producing microorganisms (Prasertwasu et al., 2014). *S. cerevisiae* is GRAS (generally regarded as safe) for human consumption which enhances its advantageous utilization more than other yeasts and microorganisms. In fact, only few yeast strains tolerant to inhibitors are available. The flocculating yeast strain CCUG53310, for an example, has been reported to ferment toxic hydrolysate in both batch and continuous mode (Purwadi et al., 2007; Dehkhoda et al., 2009) and performed better than other investigated strains regarding various environmental stresses (Albers and Larsson, 2009). PE-2 diploid strain isolated from harsh industrial environments have been also shown to be more efficient in detoxifying and fermenting lignocellulosic hydrolysates than other industrial and laboratory background strains (Pereira et al., 2014; Cunha et al., 2015). The studies suggested that the flocculation and the diploidity of the strains caused the robustness against the inhibitors in *S. cerevisiae*.

The development of stress-tolerant ethanologenic yeast is one of the significant challenges for cost-competitive bioethanol production. Many yeasts reported could grow on the medium containing inhibitor by adaptation (Heer and Sauer, 2008; Liu et al., 2004; Parawira and Tekere, 2011). Some genetic traits also proposed for the improvement of the yeast strain mainly through gene overexpression in laboratory background strains (Klein et al., 1999). However, most of the studies investigated the strain tolerances in the medium containing single inhibitor or simple mixtures of furfural, 5-HMF and acetic acid (Gorsich et al., 2006; Park et al., 2011).

It is important to get the knowledge regarding the bottle neck of growth limitation upon the stress challenge in the presence of ICC containing furfural, 5-HMF, acetic acid and other weak acids such as formic acid and levulinic acid. Also, how to improve the inhibitor tolerance without alteration in the flocculation phenotype. Therefore, the presence of robust non-flocculating yeast is indespensable in this purpose. Yeast *S. cerevisiae* NBRC849 is a wild type haploid strain, which is naturally tolerant to ICC. This strain is non-flocculating yeast, completely different with CCUG53310, and is a haploid, completely different with the genetic background of PE-2. However, this strain could rapidly grow in real biomass hydrolysate as well as in the medium containing ICC at a typical strength, makes it interested as a platform model. To evaluate the effect of ICC on this strain, the discussion in this study was focused on the cell growth, the expression of some essensial genes of interest (particularly related to glycolysis, pentose phosphate pathway, regulator and ABC-transporter), and also the changes of metabolomic pathway during the stress challenge under the presence of ICC at varied concentrations.

2. Methods

2.1. Yeast strain and seed preparation

S. cerevisiae NBRC849 strain was obtained from NITE Biological Resource Center (NBRC, Chiba, Japan) and routinely maintained on YPD plate containing Yeast Peptone Dextrose (YPD: 10 g/L yeast extract; 20 g/L bacto peptone; 20 g/L glucose) and 15 g/L agar. For seed preparation, one single colony of the strain was transferred from two days-old YPD plate to 12 ml YPD liquid medium prepared in 100 ml Erlenmeyer flask and then incubated overnight at 30 °C and 150 rpm. In this study, the initial OD_{600} was at around 0.2, to ensure that the fermentation was followed by the cell growth.

2.2. Chemicals, medium preparation and fermentation condition

Furfural and HMF were purchased from Sigma-Aldrich (San Louis, MO). The purity of both furaldehydes was 95.0%, determined by GC-analysis. Acetic acid, formic acid and levulinic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan).

The batch cultivations were carried out in 100 ml erlenmeyer flask at 30 °C and 150 rpm. In this study, YNB medium containing 50 g/L glucose, 50 g/L xylose, 6.7 g/L yeast nitrogen base (without amino acids), 40 mg/L adenine was used as an inhibitor-free medium. YNB medium supplemented with 60 mM acetic acids, 30 mM formic acid, 60 mM furfural, 10 mM 5-HMF, 5 mM leuvinic acid was used as an inhibitory medium (YNBi). YNBi medium was diluted with YNB medium at 0.6-fold (0.4YNB+0.6YNBi), 0.5-fold (0.5YNB+0.5YNBi), 0.4-fold (0.6YNB+0.4YNBi). The concentration of inhibitors in the YNBi medium were set at representative levels as such reported elsewhere for completely reducing the growth of most yeast cells (Purwadi et al., 2007).

Seed culture at 1.2 ml was used to inoculate 12 ml fermentation medium in 100 ml Erlenmeyer to give 10% inoculation size. The

Please cite this article in press as: Kahar, P., et al. Challenges of non-flocculating *Saccharomyces cerevisiae* haploid strain against inhibitory chemical complex for ethanol production. Bioresour. Technol. (2017), http://dx.doi.org/10.1016/j.biortech.2017.06.009