Journal of Biotechnology 150 (2010) 404–407

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Short communication

Repeated-batch fermentations of xylose and glucose–xylose mixtures using a respiration-deficient *Saccharomyces cerevisiae* engineered for xylose metabolism

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ARTICLE INFO

Article history:
Received 27 July 2010
Received in revised form
24 September 2010
Accepted 30 September 2010

Keywords:
Saccharomyces
Yeast
Respiration deficient
Xylose
Repeated-batch fermentation
Bioethanol

ABSTRACT

Xylose-fermenting *Saccharomyces* strains are needed for commercialization of ethanol production from lignocellulosic biomass. Engineered *Saccharomyces cerevisiae* strains expressing *XYL1*, *XYL2* and *XYL3* from *Pichia stipitis*, however, utilize xylose in an oxidative manner, which results in significantly lower ethanol yields from xylose as compared to glucose. As such, we hypothesized that reconfiguration of xylose metabolism from oxidative into fermentative manner might lead to efficient ethanol production from xylose. To this end, we generated a respiration-deficient (RD) mutant in order to enforce engineered *S. cerevisiae* to utilize xylose only through fermentative metabolic routes. Three different repeated-batch fermentations were performed to characterize characteristics of the respiration-deficient mutant. When fermenting glucose as a sole carbon source, the RD mutant exhibited near theoretical ethanol yields ($0.46\,g\,g^{-1}$) during repeated-batch fermentations by recycling the cells. As the repeated-batch fermentation progressed, the volumetric ethanol productivity increased (from 7.5 to $8.3\,g\,L^{-1}\,h^{-1}$) because of the increased biomass from previous cultures. On the contrary, the mutant showed decreasing volumetric ethanol productivities during the repeated-batch fermentations using xylose as sole carbon source (from 0.4 to $0.3\,g\,L^{-1}\,h^{-1}$). The mutant did not grow on xylose and lost fermenting ability gradually, indicating that the RD mutant cannot maintain a good fermenting ability on xylose as a sole carbon source. However, the RD mutant was capable of fermenting a mixture of glucose and xylose with stable yields ($0.35\,g\,g^{-1}$) and productivities ($0.52\,g\,L^{-1}\,h^{-1}$) during the repeated-batch fermentation. In addition, ethanol yields from xylose during the mixed sugar fermentation ($0.30\,g\,g^{-1}$) were higher than ethanol yields from xylose as a sole carbon source ($0.21\,g\,g^{-1}$). These results suggest that a strategy for increasing ethanol yield through respiration-deficiency can be applied for the fermentation of lignocellulosic hydrolyzates containing glucose and xylose.

Biofuel production from crops such as corn and sugarcane has been considered as a solution for weaning the world off fossil fuels. However, the competition between using these crops as raw materials for fuel versus food production has created many social dilemmas and criticisms (Cassman and Liska, 2007; Hill et al., 2006; Muller et al., 2008). As an alternative, abundant non-food feedstock which is exemplified by lignocellulosic biomass, such as corn stover and hardwood wastes has been presented as a sustainable source of ethanol production (Gray et al., 2006; Kim and Dale, 2004; McMillan, 1997; Wyman, 2007; Zaldivar et al., 2001). Unlike the hydrolyzates of edible parts of crops, the hydrolyzates of the lignocellulosic biomass contain glucose and xylose as major carbon sources. Unfortunately, native *Saccharomyces cerevisiae*, which has been industrially used for ethanol production from glucose, is not capable of fermenting xylose. Many genetic and metabolic engineering approaches have been attempted for developing an engineered *S. cerevisiae* strain which can ferment xylose with comparable yields and productivities observed in glucose fermentation. As wild-type *S. cerevisiae* can metabolize xylulose using pentose phosphate pathway (PPP), the introduction of genes (*XYL1* and *XYL2*) coding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* facilitated xylose assimilation in this

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doi:10.1016/j.jbiotec.2010.09.962

yeast (Jeppsson et al., 2003; Jin et al., 2000; Karhumaa et al., 2007; Meinander et al., 1999; Walfridsson et al., 1997). Above that, either overexpression of *XKS1* coding for endogenous xylulokinase or introduction of *XYL3* coding for *P. stipitis* xylulokinase significantly improved rate and yield of xylose fermentation in *S. cerevisiae* (Eliasson et al., 2000; Ho et al., 1998; Jin et al., 2003; Johansson et al., 2001; Katahira et al., 2006; Matsushika and Sawayama, 2008; Shen et al., 2004; Toivari et al., 2001). In spite of these endeavors, ethanol production from xylose by engineered *S. cerevisiae* is not as efficient as glucose fermentation. Several hypotheses related with slow xylose fermentation have been proposed: First, XR and XDH introduced from *P. stipitis* require different types of cofactors, NAD(P)H and NAD^+, respectively, which may cause unbalances in redox state of cellular environment (Kuyper et al., 2004; Watanabe et al., 2007). Second, *in vivo* enzyme activities of XR and XDH are not optimized in engineered *S. cerevisiae* so that significant amounts of xylitol are accumulated and overall xylose uptake rate decrease (Walfridsson et al., 1997). Third, *S. cerevisiae* lacks efficient xylose transporters which may exist in xylose-fermenting yeasts, such as *P. stipitis*. Therefore, the xylose fermentation rate by engineered *S. cerevisiae* might be limited by transport reactions rather than subsequent metabolic reactions (Gárdonyi et al., 2003; Sedlak and Ho, 2004).

Xylose metabolism by the engineered *S. cerevisiae* was reported to be highly dependent on aeration. First of all, it only grows on xylose in the presence of oxygen even though it can produce ethanol from xylose (Eliasson et al., 2000; Jin et al., 2004). Also, it favors respiration on xylose rather than fermentation as on glucose. Transcriptome analysis of the engineered *S. cerevisiae* revealed that expression levels of the genes coding for the tricarboxylic acid cycle, respiration enzymes (*HXK1*, *ADH2*, *COX13*, *NDI1*, and *NDE1*), and transcriptional activators of respiration (*HAP1/2/3/4*), increased significantly when cells are grown on xylose (Jin et al., 2004). Another report mentioned that the engineered *S. cerevisiae* growing on xylose showed significant expression of glucose-repressing genes through a Snf1p/Mig1p pathway (Salusjarvi et al., 2008). These results suggest that the engineered *S. cerevisiae* does not respond to xylose as a fermentable carbon source, resulting in the xylose metabolism in an oxidative manner. Although the other possibilities are still valid, oxidative response is probably one of limiting factors contributing to the poor xylose fermentations of the engineered *S. cerevisiae*. Maintaining low dissolved oxygen level of the culture on xylose is a generally accepted way to optimize an ethanol production as well as to limit respiration.

As a practical solution to the problem, the induction of respiration deficiency in the engineered *S. cerevisiae* was proposed before (Jin et al., 2004). Fermenting glucose, a respiration-deficient mutant of *S. cerevisiae* has already known for showing higher ethanol productivity and/or yield than that of the respiration-sufficient parental strain (Dikicioglu et al., 2008; Hutter and Oliver, 1998; Oner et al., 2005; Panoutsopoulou et al., 2001). A respiration-deficient mutant of engineered stain also produced more ethanol (0.25 g ethanol/g xylose) from xylose as compared to the parental strain (0.15 g g^{-1}) (Jin et al., 2004). However the respiration-deficient mutant could not grow on xylose as a sole carbon source, which is agreed with previous observation by Wang and Schneider (1980). Moreover, some studies pointed that functional mitochondria are responsible for ethanol tolerance (Aguilera and Benitez, 1985; Costa et al., 1997). These concerns immediately brought up the question: How long can the respiration-deficient mutant maintain fermentation activity without cell growth using xylose as a sole carbon source? In order to address this question, we investigated fermentation activities of recycled cells during repeated-batch fermentation using xylose, glucose, and a mixture of xylose and glucose, respectively.

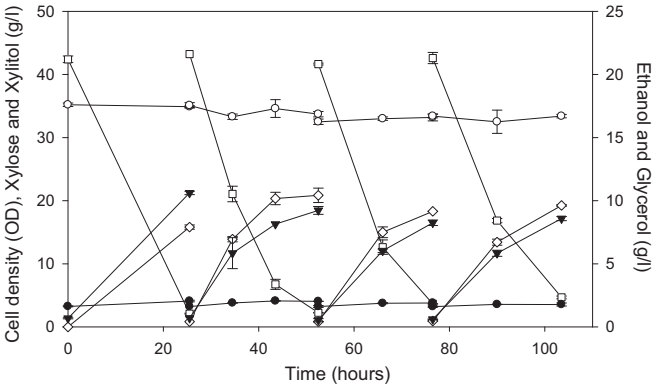

Fig. 1. Profiles of cell optical density (open circle) and xylose (open square), xylitol (open diamond), ethanol (closed triangle down) and glycerol (closed circle) concentrations during the repeated-batch fermentation using the YPX (40 g L^{-1} of xylose) media by the respiration-deficient mutant of *Saccharomyces cerevisiae* YSX3 strain.

The respiratory-deficient mutant of *S. cerevisiae* YSX3 (*MATα leu2::LEU2-XYL1 ura3::URA3-XYL2 Ty3::NEO-XYL3*) made by using an ethidium bromide treatment as described previously (Jin et al., 2004) was cultivated using YPD (10 g L^{-1} of Bacto yeast extract, 20 g L^{-1} of Bacto peptone, and 20 g L^{-1} of D-glucose) medium. The concentrated cells were inoculated into 50 ml of YP medium with glucose, xylose, and a mixture of glucose and xylose in a 250-ml flask, and then incubated at 30 °C and 200 rpm for fermentation experiments (all experiments were performed in triplicate). Fig. 1 shows the fermentation profiles by the respiration deficient mutant using xylose as a sole carbon source. The respiration-deficient mutant utilized xylose and produced both ethanol and xylitol without cell growth. During the four times of repeated-batch fermentations by recycling cells from the previous fermentation, the cell densities of the culture slightly decreased due to some losses of the cells caused by sampling for fermentation product assays and incomplete rescue of yeast cells during the transfers between repeated-batch fermentations. Except for the first fermentation, the fermentation profiles of the second, third, and forth fermentation were almost identical: about 20 g L^{-1} of xylitol and 9 g L^{-1} of ethanol were produced from 40 g L^{-1} of xylose. Significant amounts (about 20 g L^{-1}) of xylitol were accumulated during the repeated-batch fermentation. In case of a respiration deficient mutant, the conversion of xylose to xylitol might serve as an electron sinker which regenerates NAD^+ from NADH. Therefore, we speculate that excess amounts of xylitol accumulation might result from maintaining redox balances without respiration.

We observed more ethanol production (12 g L^{-1} of ethanol) and less xylitol production (8 g L^{-1} of xylitol) from the first fermentation. It was likely that the cells transferred from glucose showed a better fermenting ability on xylose. As the repeated-batch fermentations preceded, the remaining xylose after each fermentation slightly increased from 2 to 5 g L^{-1}, which suggested the fermentation activity of the respiration-deficient mutant decreased gradually by the repeated-batch xylose fermentation. On the other hand, the respiration-deficient mutant showed almost identical fermentation activities during the repeated-batch fermentation using glucose as a sole carbon source (Fig. 2). Because the biomass of the respiration-deficient mutant increased on glucose as the fermentation proceeded, the glucose consumption rates increased following the fermentations, which also led to the improvement of overall volumetric ethanol productivity. Surprisingly, the fermentation time required for consuming 40 g L^{-1} of glucose was just 2 h. It is only 1/20th of those required for consuming the same amount of xylose under the same conditions. Moreover, the amounts of produced ethanol from glucose were more than double the amount

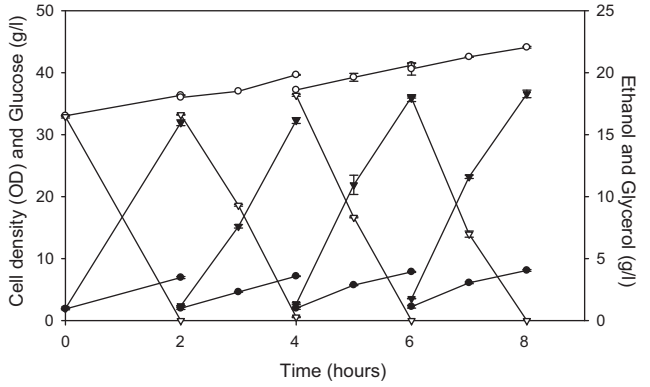

Fig. 2. Profiles of cell optical density (open circle) and glucose (open triangle down), ethanol (closed triangle down) and glycerol (closed circle) concentrations during the repeated-batch fermentation experiments using the YPD (40 g L^{-1} of glucose) media by the respiration-deficient mutant of *S. cerevisiae* YSX3 strain.

produced from xylose. Although it is not clearly known, there might be limiting factors which constrain fermentation rate of xylose fermentation.

In order to take the advantage of fast fermentation on glucose, the respiration-deficient mutant was applied to repeated-batch fermentation with a mixture of glucose and xylose (Fig. 3). As we expected, the cell density increased during the fermentation despite the loss of cells caused by sampling and incomplete rescue. Interestingly, we did not notice the reduction of xylose fermentation activity, which was observed previously in repeated-batch fermentations using xylose as a sole carbon, during the fermentation with a mixture of glucose and xylose. It is likely that this co-substrate system offers the regeneration of xylose fermenting activity of the respiration-deficient mutant which results in high ethanol yield and volumetric productivity. The calculated ethanol yield from xylose after assuming that the consumed glucose was converted into ethanol with a theoretical maximum yield (0.51 g g^{-1}) was about 0.30 g g^{-1}. This yield is much higher than the calculated yields (0.20–0.25 g g^{-1}) from the repeated-batch fermentation with xylose as a sole carbon source. This result is also consistent with the better ethanol yield of the first fermentation on xylose using cells grown on glucose. The details of three different types of fermentations are summarized in Table 1. The RD mutant showed stable ethanol yield (0.21, 0.46 and 0.35) during the repeated-batch fermentation on xylose, glucose and

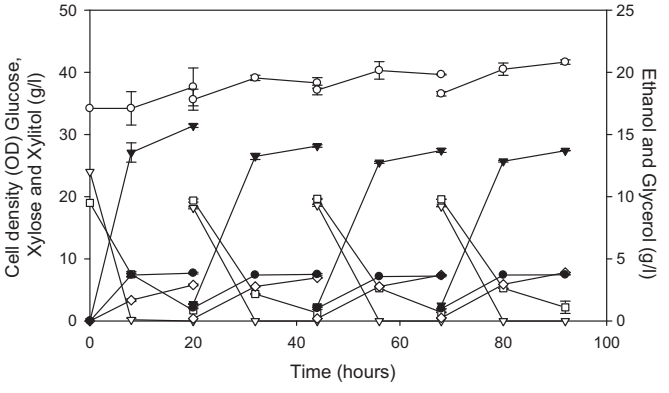

Fig. 3. Profiles of cell optical density (open circle) and xylose (open square), xylitol (open diamond), glucose (open triangle down), ethanol (closed triangle down) and glycerol (closed circle) concentrations during the repeated-batch fermentation experiments using the YPDX (20 g L^{-1} of glucose and 20 g L^{-1} of xylose) media by the respiration-deficient mutant of *S. cerevisiae* YSX3 strain.

Table 1
The metabolite production yields (g produced metabolite/g consumed sugar) and volumetric ethanol productivity (g L^{-1} h^{-1}) from repeated-batch fermentation experiments.

Batch	Xylose 40 g L^{-1}				Glucose 40 g L^{-1}				Glucose 20 g L^{-1} + xylose 20 g L^{-1}			
	Y_{EtOH}	$Y_{Xylitol}$	$Y_{Glycerol}$	P_{EtOH}	Y_{EtOH}	$Y_{Xylitol}$	$Y_{Glycerol}$	P_{EtOH}	Y_{EtOH}	$Y_{Xylitol}$	$Y_{Glycerol}$	P_{EtOH}
1	0.25 ± 0.00	0.39 ± 0.01	0.01 ± 0.00	0.39 ± 0.01	0.46 ± 0.01	–	0.08 ± 0.00	7.50 ± 0.13	0.38 ± 0.00	0.14 ± 0.00	0.09 ± 0.00	0.79 ± 0.01
2	0.21 ± 0.01	0.49 ± 0.03	0.01 ± 0.00	0.32 ± 0.01	0.46 ± 0.01	–	0.08 ± 0.00	7.47 ± 0.12	0.35 ± 0.01	0.18 ± 0.00	0.07 ± 0.00	0.53 ± 0.00
3	0.20 ± 0.00	0.46 ± 0.00	0.01 ± 0.00	0.32 ± 0.00	0.46 ± 0.00	–	0.08 ± 0.00	8.35 ± 0.01	0.34 ± 0.00	0.19 ± 0.00	0.07 ± 0.00	0.52 ± 0.00
4	0.21 ± 0.00	0.48 ± 0.01	0.00 ± 0.00	0.30 ± 0.01	0.46 ± 0.00	–	0.08 ± 0.01	8.26 ± 0.16	0.35 ± 0.01	0.20 ± 0.00	0.08 ± 0.00	0.52 ± 0.00

a mixture of glucose and xylose, respectively. The volumetric ethanol productivity was also maintained at the similar levels during the repeated-batch fermentation on both xylose and a mixture of xylose and glucose, except for glucose which showed the improvement due to the increased biomass: the volumetric ethanol productivities were 0.30, 8.26, and 0.52 g L^{-1} h^{-1} on xylose, glucose and a mixture of xylose and glucose, respectively.

Although the respiration-deficient mutant of the engineered *S. cerevisiae* could not maintain xylose fermenting activities during repeated-batch fermentation using xylose only, the mutant exhibited good yield (0.35 g g^{-1}) and productivity (0.52 g L^{-1} h^{-1}) when a mixture of glucose and xylose was used as carbon sources. These results suggest that the respiration-deficient mutant can be applied for the industrial ethanol fermentation of lignocellulosic hydrolyzates because most of the hydrolyzates contain both glucose and xylose.

Acknowledgments

This work was supported by funding from Energy Biosciences Institute to Y.-S. Jin. The authors thank to Dr. Thomas Jeffries for providing the YSX3 strain. K.-S. Lee is grateful for financial support from the International Research & Development Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) of Korea. This work was also supported by a grant from the BioGreen 21 Program (No. PJ007073201005), Rural Development Administration, Republic of Korea.

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