Construction of various mutants of xylose metabolizing enzymes for efficient conversion of biomass to ethanol

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ABSTRACT

We applied protein engineering to construct an efficient biomass-ethanol conversion system using Saccharomyces cerevisiae. Intercellular redox imbalance caused by the different coenzyme specificity of xylose reductase (XR) and xyitol dehydrogenase (XDH) has been thought to be one of the main factors of xyitol excretion. Introduction of NADH-dependant XR generated in this study reduced the xyitol excretion probably because of maintaining the intercellular redox balance. Ethanol fermentation was measured in batch culture under anaerobic conditions. The best strain R276H produced a maximum of 5.94 g/l ethanol with yield of 0.43 g/g from 5 g glucose/l plus 15 g xylose/l.

RESULTS AND DISCUSSION

Specific enzyme activity – The XR activity was measured by monitoring the oxidation of NADPH/NADH at 340 nm and 35°C using a V-550 model spectrophotometer (JASCO Corporation, Japan) in a reaction mixture with the following composition: 60 mM sodium phosphate buffer (pH 7.0), 133 mM xylose, and 1.5 mM NADPH/NADH. One unit of xylose reductase activity was defined as the amount of enzyme that oxidized 1 μmol NADPH/NADH per minute. Figure 1 showed the specific activities of XR mutants in recombinant S. cerevisiae. A recombinant yeast strain, named K270R, improved 6 fold XR enzyme activity with NADH compared with the wild type. K270R/N272D strain showed more than 5 fold higher XR activity with NADH. R276H strain improved almost 6 fold XR activity with NADH compared with the wild type. All strains (used in Fig. 1) showed similar specific XDH activities in S. cerevisiae (data not shown).

INTRODUCTION

Xylose is one of the major fermentable sugars present in lignocellulosic biomass, the second most abundant carbohydrate polymer in nature. Saccharomyces cerevisiae is used widely for industrial ethanol production because of the ability to produce high concentrations of ethanol and high inherent tolerance. Since the native S. cerevisiae cannot ferment xylose, molecular engineering of S. cerevisiae for xylose utilization has focused on adapting the xylose metabolic pathway from the xylose-utilizing yeast Pichia stipitis. S. cerevisiae transformed with the native genes encoding XR, XDH from P. stipitis and the endogenous gene xyulokinase (XK) ferments xylose to ethanol but has not yet been applied to the industrial bio-process due to the unfavorable excretion of xyitol. Intercellular redox imbalance caused by the different coenzyme specificity of XR (with NADPH) and XDH (with NAD⁺) has been thought to be one of the main factors of xyitol excretion¹,². In the present study, we constructed recombinant yeasts that were transformed with the genes encoding the protein engineered XR (with NADH) and a wild type XDH of P. stipitis. These recombinant yeasts were characterized based on the enzyme activity and fermentation ability of xylose to ethanol. Introduction of NADH-dependent XR generated by site-directed mutagenesis reduced the xyitol excretion probably because of maintaining the intercellular redox balance.

![Enzyme activities of protein engineered XR in the recombinant S. cerevisiae](https://academic.oup.com/nass/article-abstract/50/1/279/1342115/fig1.png)

**Fig. 1** Enzyme activities of protein engineered XR in the recombinant S. cerevisiae were assayed in the direction of polyol reduction by measuring the reduction of NADPH or NADH. Values are the mean ± S.D., n=3.

**Cell growth of the recombinant yeasts** – Cell growth was monitored by measuring the absorbance at 600 nm. Cells were cultured under O₂-limited conditions in a yeast minimal medium with 10 g of glucose/l plus 10 g of xylose/l. Similar growth rate was observed in all recombinant strains (data was not shown). Almost the same amount of cells of each recombinant yeast strain was used in fermentation experiments.
**Anaerobic batch fermentation in shake flask** - Anaerobic batch fermentation was measured in an incubator with shaking at 30°C. One set of fermentation was started with 5 g of glucose/l plus 15 g of xylose/l. The R276H recombinant yeast strain produced maximum ethanol 2.30 g/l with a yield of 0.12 g of ethanol/g of initial total fermentable sugars and excreted minimum amount of xylitol (0.97 g/l). The mutants K270R/N272D and K270R produced ethanol 2.10 g/l with yield of 0.10 g/g and 2.26 g/l with yield of 0.11 g/g respectively. Glucose was completely fermented in 25 to 35 h. Of the xylose 37% to 46% was fermented in 72 h.

**Anaerobic batch fermentation in bioreactor** - Ethanol fermentation was measured in batch culture under anaerobic conditions in an incubator with shaking for recombinant yeast strains (that contain a wild type XDH and NADH-dependent XR) and one of the strains, named R276H produced maximum ethanol. Therefore, product formation by the recombinant yeast strain R276H was measured in a high performance bioreactor under anaerobic conditions. R276H produced ethanol 5.94 g/l with a yield of 0.43 g of ethanol/g of total consumed sugars (Fig. 2). The yield of ethanol from consumed sugars reached 84% of the theoretical yield and xylitol excretion was below 1.29 g/l. Glucose was completely fermented in 15 h. Of the xylose 59% was consumed in 72 h. Little amount of glycerol (0.42 g/l) and acetic acid (0.36 g/l) were produced mainly during glucose consumption phase.

**CONCLUSION**

We applied the protein engineering to generate enzymes with reversed coenzyme specificity and developed recombinant yeasts containing those engineered enzymes for construction of an efficient biomass-ethanol conversion system. In this study, we introduced a wild type XDH and the protein engineered NADH dependent XR and measured the efficiency of ethanol fermentation from xylose and the excretion of metabolites including xylitol. Eliasson et al. were the first to report the anaerobic production of ethanol from xylose by recombinant S. cerevisiae TMB3001 carrying XR, XDH genes from P. stititis and the endogenous gene XK in mineral medium. Xylose was co-utilized with glucose under anaerobic conditions. Xylose uptake varied inversely with its concentration, but even at the highest xylose concentration, (15 g xylose/l plus 5 g glucose/l) only 12% of the xylose was consumed. Ho et al. developed their recombinant strain S. cerevisiae pLNH32 that can ferment a mixture of 45 g xylose/l plus 90 g glucose/l to give an ethanol yield 0.46 g of ethanol/g of total consumed sugars. This is the highest ethanol yield and fermentation rate from xylose/glucose mixtures reported for a recombinant S. cerevisiae to date. Our results are comparatively better than Ho et al. results albeit we used different medium and high concentrations of xylose. We did not overexpress xylulokinase gene in this study. If xylulokinase gene is overexpressed in our recombinant strain R276H, the efficiency of ethanol fermentation will hopefully be more improved.

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**REFERENCES**