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Expression of *Azotobacter vinelandii* soluble transhydrogenase perturbs xylose reductase-mediated conversion of xylose to xylitol by recombinant *Saccharomyces cerevisiae*

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Abstract

Pyridine nucleotide transhydrogenase is a metabolic enzyme transferring the reducing equivalent between two nucleotide acceptors such as NAD^+ and $NADP^+$ for balancing the intracellular redox potential. Soluble transhydrogenase (STH) of *Azotobacter vinelandii* was expressed in a recombinant *Saccharomyces cerevisiae* strain harboring the *Pichia stipitis* xylose reductase (XR) gene to study effects of redox potential change on cell growth and sugar metabolism including xylitol and ethanol formation. Remarkable changes were not observed by expression of the STH gene in batch cultures. However, expression of STH accelerated the formation of ethanol in glucose-limited fed-batch cultures, but reduced xylitol productivity to 71% compared with its counterpart strain expressing xylose reductase gene alone. The experimental results suggested that *A. vinelandii* STH directed the reaction toward the formation of NADH and $NADP^+$ from NAD^+ and NADPH, which concomitantly reduced the availability of NADPH for xylose conversion to xylitol catalyzed by NADPH-preferable xylose reductase in the recombinant *S. cerevisiae*.

Keywords: NAD(P)H; *Saccharomyces cerevisiae*; Transhydrogenase; Xylitol; Xylose reductase

1. Introduction

Pyridine nucleotide transhydrogenase (E.C. 1.6.1.1) is the enzyme catalyzing transfer of reducing equivalent between the two pyridine nucleotide systems described as.

$$NADH + NADP^+ \leftrightarrows NAD^+ + NADPH$$

Transhydrogenase was found in the cytoplasmic membrane of several microorganisms such as *Escherichia coli*, *Rhodospirillum rubrum*, *Eimeria tenella* and *Entamoeba histolyticabut*, and in the inner membrane of higher animal mitochondria and plant mitochondria (*Arabidopsis thaliana*) [1]. Additionally, soluble transhydrogenase (STH) present in the cytoplasm was identified from *E. coli* [2] and *Azotobacter vinelandii* [3]. But there is no report to exploit this enzyme in yeast successfully [4]. The absence of the pyridine nucleotide transhydrogenation system has considerable consequences for the redox balances of both cofactor systems NAD(H) and NAD(P)H and hence

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for sugar and amino acid metabolism in yeast [5]. Heterogeneous expression of the membrane-bound transhydrogenase from *E. coli* was tried to diminish the amount of glycerol produced by *S. cerevisiae* incidentally, resulting that this protein was not embeded in the plasma membrane and amount of other byproducts increased [6]. The *A. vinelandii* STH gene encodes a 464 amino acid polypeptide with a molecular weight of 54 kDa [3]. When a *Saccharomyces cerevisiae* strain expressing STH gene of *A. vinelandii* was grown in anaerobic conditions using glucose as sole carbon source, an increase in the formation of 2-oxoglutarate, glycerol, and acetic acid was observed, indicating that perturbation of the cellular pyridine nucleotide pool occurred due to expression of the bacterial transhydrogenase gene in yeast. Accumulation of 2-oxoglutarate also pointed out that the transhydrogenase transferred reducing equivalents from NADPH to NAD^+ [3].

Xylitol, a five-carbon sugar alcohol, has the same order of sweetness as sucrose and fructose [7]. In addition, it has substantially low viscosity and positive enthalpy of solution compared with sucrose. Thus, the use of xylitol has been increasing in the food industry [8]. Microbial production of xylitol has been developed using xylose-utilizing yeasts and recombinant *S. cerevisiae* [9–13]. *S. cerevisiae* cannot metabolize xylose as carbon source as this yeast does not possess a metabolic activity for conversion of xylose to xylitol. Expression of the *P. stipitis* xylose reductase (XR) gene in *S. cerevisiae* conferred the ability to produce xylitol from xylose with almost theoretical yield as the xylitol cannot be metabolized further [13–15]. Since xylose reductase of *P. stipitis* requires NAD(P)H as cofactor for its enzymatic action [16], cosubstrate such as ethanol or glucose must be supplied for cofactor regeneration and maintenance of cellular activity. Intracellular redox state generally defined as the concentration ratios of the two pyridine nucleotide systems [NADH]/[NAD^+ + NADH] and [NADPH]/[$NADP^+$ + NADPH] may make a crucial effect on xylitol biosynthesis from xylose in recombinant *S. cerevisiae*.

In this study, modulation of the pyridine nucleotide pools by expression of the *A. vinelandii* STH gene in xylitol-producing recombinant *S. cerevisiae* was tried to investigate its effect on cell growth and xylose metabolism.

2. Experimental

2.1. Strains, plasmid, and culture conditions

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for the preparation of plasmid DNA. *S. cerevisiae* BJ3505 (MATα, *trp1*, *ura3*) and BJ3505/δXR harboring multiple copies of the *P. stipitis* xylose reductase gene in its chromosome [16] were used as host for the episomal expression of the STH gene. Plasmid YEp24-pPGK-STH (2μ, *URA3*) in which the expression of the *A. vinelandii* STH gene is controlled by the constitutive PGK promoter was kindly donated by Dr. Kielland-Brandt at Carlsberg Laboratory (Copenhagen, Denmark).

LB medium (10 g NaCl, 10 g tryptone, and 5 g yeast extract per liter) was used for *E. coli* cultivation. YNB plate (6.7 g yeast nitrogen base without amino acid, 20 g agar, and 20 g glucose per liter) with yeast synthetic dropout supplement (Sigma, St. Louis, MO, USA) was used for the selection of yeast transformants.

Batch and fed-batch cultures were carried out in a 2.5 l bench-top fermentor (KoBiotech, Incheon, Korea) with a 1.0 l working volume. YPD medium (10 g yeast extract, 20 g peptone, and 20 g glucose per liter) supplemented with 20 g/l xylose was used for batch cultures. For fed-batch cultures, initial xylose concentration of 100 g/l was used and 600 g/l glucose solution was fed at a rate of 1.8 g glucose/l h after the depletion of initially added glucose. Medium acidity of pH 5.0 and temperature of 30 °C were maintained throughout the cultivation. Agitation speed and aeration rate were set at 400 rpm and 1 air volume per medium volume per minute (vvm), respectively.

2.2. Analytical methods

Dry cell mass was measured with a spectrophotometer (UltroSpec 2000, Pharmacia Biotech, Piscataway, NJ, USA) at 600 nm. Optical density was converted into dry cell mass using the predetermined conversion factor of 0.30. Concentrations of glucose, xylose, xylitol, and ethanol were measured by an HPLC (Younglin, Seoul, Korea) equipped with the aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) of which temperature was controlled at 60 °C. Mobile phase consisted of 5 mM H_2SO_4 solution

and detection was carried out with a reflective index detertor (Knauer, Berlin, Germany).

2.3. Measurement of enzyme activities

Preparation of cell extract and measurement of xylose reductase activity were done using the method described by Jin et al. [12] with some modifications. The cells were harvested by centrifugation at $8000 \times g$ for 10 min, washed twice with ice-cold water, and then incubated in Y-PER solution (Pierce, Rockford, IL, USA) for 20 min. Cell debris was removed by centrifugation at $10{,}000 \times g$ for 10 min at 4 °C to obtain crude extract. Xylose reductase activity was determined by measuring the oxidation of NADPH at 340 nm with a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA). One unit of xylose reductase activity was defined as the amount of enzyme that can oxidize one micromole of NADPH per minute. Transhydrogenase activity was measured by method described by Voordouw et al. [17]. Specific enzyme activity (U/mg) was estimated by dividing enzyme activity by the cellular protein concentration. Protein concentration of the crude cell extract was measured using a protein assay kit (Bio-Rad).

3. Results and discussion

Batch fermentations were carried out to examine and compare the patterns of cell growth and sugar

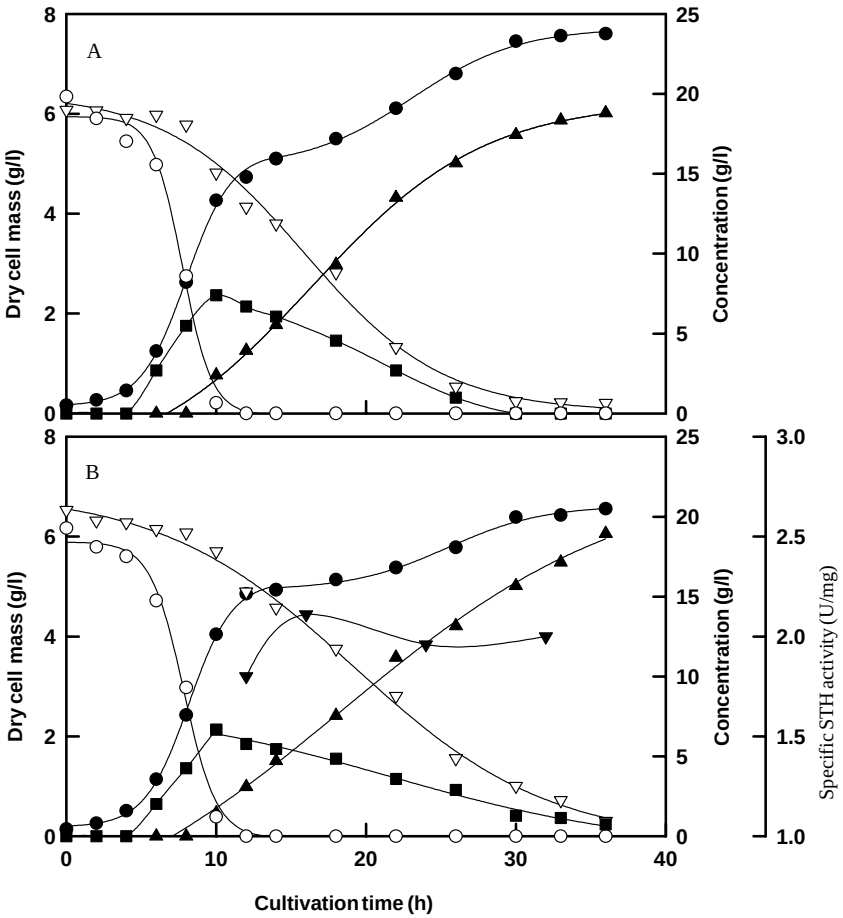

Fig. 1. Batch fermentation profiles of *S. cerevisiae* BJ3505/δXR (A) and BJ3505/δXR/STH (B) strains in YPD medium supplemented with 2% (w/v) xylose at 30 °C and pH 5.0. Dry cell mass (●), ethanol (■), glucose (○), xylitol (▲), xylose (▽), and specific STH activity (▼).

metabolism for the two recombinant *S. cerevisiae* BJ3505 strains, δXR and δXR/STH. As shown in Fig. 1, the expression of the STH gene did not make significant changes in glucose utilization, cell growth, ethanol accumulation, and xylitol production for the two recombinant yeast strains in batch cultures. In both strains, as expected, xylitol was produced only after the complete depletion of initially added glucose with an approximate yield of 1.0 g xylitol (produced per g xylose consumed) using produced ethanol as cosubstrate. The *S. cerevisiae* BJ3505 host strain transformed with YEp24-pPGK-STH did not show remarkable changes in cell growth and sugar metabolic patterns compared with *S. cerevisiae* BJ3505 strain when grown aerobically under the same cultivation conditions as above (data not shown).

Effects of STH expression in a recombinant *S. cerevisiae* strain were further studied in fed-batch cultures (Fig. 2). Unlike the batch cultures, it was observed significant changes in cell mass concentration, xylitol production and ethanol accumulation in glucose-limited fed-batch cultures. The *S. cerevisiae* BJ3505/δXR/STH strain resulted in 8.7 g/l dry cell mass and 60.0 g/l final xylitol concentration, which corresponded to 36% decrease in cell growth and 29% reduction in xylitol production compared with its counterpart recombinant strain expressing xylose reductase alone. However, expression of the

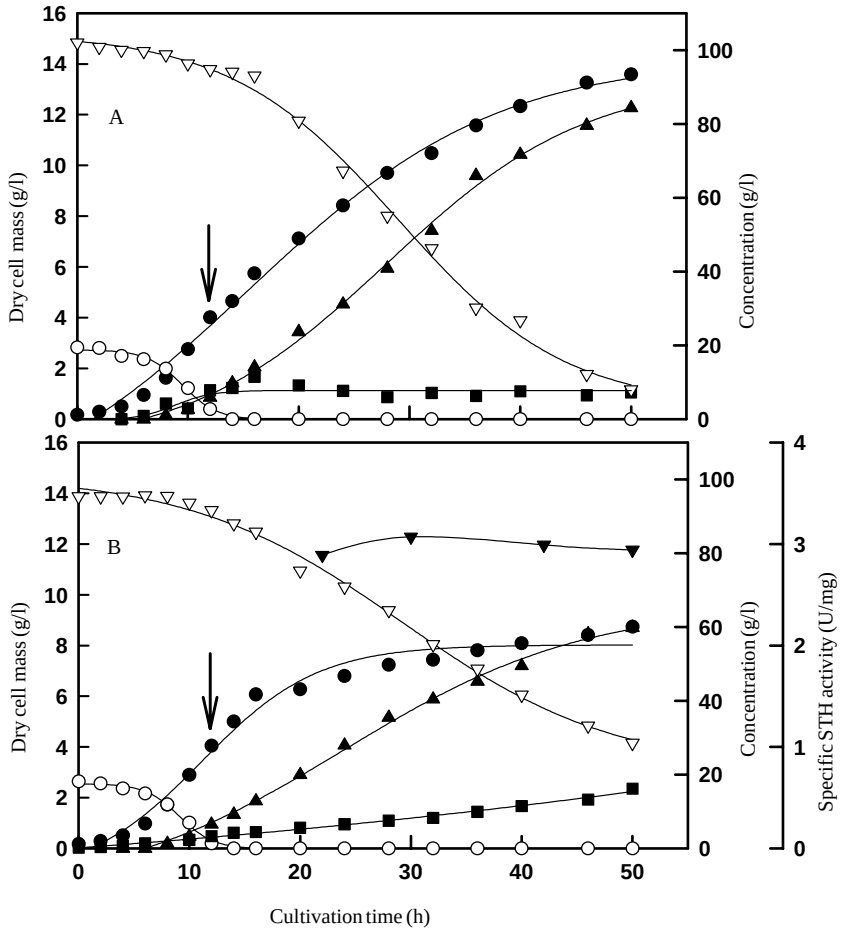

Fig. 2. Fed-batch fermentation profiles of *S. cerevisiae* BJ3505/δXR (A) and BJ3505/δXR/STH (B) strains in YPD medium supplemented with 2% (w/v) xylose at 30 °C and pH 5.0. Arrow indicates initiation of the fed-batch mode. Dry cell mass (●), ethanol (■), glucose (○), xylitol (▲), xylose (▽), and specific STH activity (▼).

Table 1
Comparison of fed-batch fermentation results of recombinant *S. cerevisiae* BJ3505 strains harboring the xylose reductase gene of *P. stipitis* (δXR) and/or the soluble transhydrogenase (STH) gene of *A. vinelandii*

	Host	Recombinant *S. cerevisiae* BJ3505 strains		
	BJ3505	BJ3505/STH	BJ3505/δXR	BJ3505/δXR/STH
Final dry cell mass (g/l)	21.8	20.1	13.6	8.70
Final xylitol concentration (g/l)	7.50	6.40	84.2	60.0
Xylitol productivity (g/l h)[a]	0.20	0.17	2.22	1.58
Final ethanol concentration (g/l)	10.1	14.8	7.20	16.2

[a] Xylitol productivity was estimated during the xylitol-producing period of the fed-batch culture.

STH and XR genes accelerated accumulation of ethanol and made a notable elevation in final ethanol concentration for the STH expressing *S. cerevisiae* BJ3505/δXR/STH strain. The experimental results of fed-batch cultures are summarized in Table 1. In the expression of STH, specific STH activity of 2–3 U/mg was maintained in the *S. cerevisiae* BJ3505/STH and BJ3505/δXR/STH strains throughout the fed-batch cultivation. *S. cerevisiae* BJ3505 and BJ3505/δXR strains showed a negligible level of specific STH activity.

Two interesting experimental observations were found in fed-batch cultures. First, introduction of the *P. stipitis XYL1* gene encoding a NADPH-preferable xylose reductase was detrimental to cell growth of the host *S. cerevisiae* strain, which was supported by the well known fact that NADPH generated in the pentose phosphate pathway act as reducing power required for cellular anabolism [18]. As mentioned above, xylose conversion into xylitol in recombinant *S. cerevisiae* requires a simultaneous metabolism of a cosubstrate such as glucose or ethanol for the continuous regeneration of NAD(P)H and supply of the maintenance energy [15,16,19]. Thus, xylose reductase and other anabolic enzymes have to compete for NAD(P)H within a given amount of intracellular pyridine nucleotides, which inevitably results in a retardation of host cell growth in the presence of an additional NADPH-consuming reaction.

The other is that expression of the STH gene exerted profound effects on cell growth, ethanol accumulation and xylitol production of *S. cerevisiae* BJ3505/δXR harboring multiple copies of xylose reductase gene. Maximum concentrations of dry cell mass and xylitol decreased in *S. cerevisiae* BJ3505/δXR/STH to 64 and 71%, respectively, compared with the recombinant δXR strain without the STH gene. Based upon the above experimental results of fed-batch cultures, it seemed that cellular content of NADPH required both for cell growth and xylose reductase-mediated xylitol production in recombinant *S. cerevisiae* was reduced by expression of the bacterial transhydrogenase. Reduced cellular content of NADPH by expression of the cytoplasmic transhydrogenase in *S. cerevisiae* was also reported elsewhere in which the depletion of NADPH pool was accompanied by accumulation of 2-oxoglutarate from glucose metabolism [3]. Accordingly, it could be estimated that the bacterial STH expressed in the recombinant *S. cerevisiae* strain directed the reaction toward the formation of NADH and $NADP^+$ from NAD^+ and NADPH, which was also supported by higher ethanol concentration for the *S. cerevisiae* BJ3505/δXR/STH strain than the *S. cerevisiae* BJ3505/δXR strain grown under the same fed-batch cultivation conditions.

Xylose reductase from *P. stipitis* prefers NADPH to NADH [20]. Alcohol dehydrogenase I (ADH1) which mediates ethanol formation from acetaldehyde utilizes only NADH as a cofactor and is produced over 1% of cellular protein [21]. For these reasons, most of NADH produced in glycolysis might be used for ethanol formation and therefore, xylose reductase may not efficiently utilize NADH for xylitol production in glucose-limited fed-batch cultures. This hypothesis was supported by the report that the metabolic flux to ethanol formation was not affected by the consumption of cofactors in the xylose reductase-catalyzed reaction [22].

In the above experiments, surplus NADH generated by enzymatic action of the *A. vinelandii* STH might accelerate ethanol formation mediated by ADH1 enzyme which has high affinity for NADH. Further-

more, it became evident that NADH is not as efficient as NADPH for xylose conversion in recombinant *S. cerevisiae* harboring the xylose reductase gene of *P. stipitis*. More research is in progress to characterize the intracellular composition of reducing cofactors at a cell level.

4. Conclusions

The soluble transhydrogenase gene of *A. vinelandii* was expressed in recombinant *S. cerevisiae* strains harboring the xylose reductase gene of *Pichia stipitis*. The reduced xylitol production mediated by NADPH-preferable xylose reductase and increased ethanol formation in the recombinant *S. cerevisiae* allowed to speculate that soluble transhydrogenase of *A. vinelandii* transferred the reducing equivalent from NADPH to NAD^+.

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