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Metabolic engineering of *Saccharomyces cerevisiae* for production of spermidine under optimal culture conditions

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ABSTRACT

Spermidine is a polyamine compound exhibiting important biological activities, such as increasing lifespan, inflammation reduction, and plant growth control. As such, many applications of spermidine as a bio-modulating agent are anticipated. However, sustainable and scalable production of spermidine has not been achieved yet. Therefore, construction of a spermidine production system using *Saccharomyces cerevisiae* was attempted in this study. In order to secrete spermidine into fermentation broth, *TPO1* coding for the polyamine transporter was overexpressed in an engineered *S. cerevisiae* strain capable of accumulating high concentrations of spermidine. Through optimization of fermentation conditions, the resulting strain (OS123/pTPO1) produced 63.6 mg/l spermidine with a yield of 1.3 mg spermidine/g glucose. However, we observed that spermidine production was repressed in the presence of glucose. To circumvent this problem, the genetic modifications for overproducing spermidine were introduced into an engineered *S. cerevisiae* capable of fermenting xylose. In a fed-batch fermentation using a mixture of glucose and xylose, the resulting strain (SR8 OS123/pTPO1) produced 224 mg/l spermidine with a yield of 2.2 mg spermidine/g sugars. These results suggest that engineered yeast constructed in this study can be employed for the production of spermidine.

1. Introduction

Polyamines including putrescine, spermidine (SPD), and spermine are low molecular weight aliphatic nitrogen compounds that are found ubiquitously in microorganisms, plants, and animals [1]. Many studies have reported the role of SPD in stabilizing chromatin, as well as in biological processes ranging from DNA replication, transcription and translation, to the regulation of cell growth and apoptosis [2–4].

In recent years, SPD has been used to combat skin ageing, stimulate human hair growth, treat type 2 diabetes, and increase fruit shelf life [5–8]. Given the advantages in safety and sustainability aspects as compared to chemical synthesis, biological production of SPD has gained interests from academia and industries. Biological production of putrescine, a precursor of SPD, using recombinant *Escherichia coli* has been reported [9]. However, no attempt to produce SPD via microbial fermentation has been reported yet. We have reported that the intracellular concentration of SPD in native *Saccharomyces cerevisiae* was a 5.5-fold higher than that of putrescine [10]. Therefore, construction of a SPD production system based on *S. cerevisiae*, a GRAS (Generally Recognized as Safe) microorganism, has a potential for economic uses.

In order to produce SPD from glucose by engineered *S. cerevisiae*, the *TPO1* gene coding for polyamine transporter was overexpressed in the *S. cerevisiae* OS123 strain which was previously constructed for improving tolerances against fermentation inhibitors [10]. The OS123 strain was constructed by overexpressing the genes (*SPE1*, *SPE2*, and *SPE3*) coding for the enzymes in the SPD biosynthetic pathway along with disruption of the gene (*OAZ1*) involved in feedback inhibition of the SPD biosynthesis pathway by SPD (Fig. 1). As a result, the OS123 strain exhibited a 15.5-fold higher intracellular SPD level than that of the control strain [10]. TPO1 protein (Tpo1) is a typical H^+ antiporter and the activity of H^+ antiporter is known to be dependent on the presence of H^+ gradient across the plasma membrane [11]. Therefore, pH of culture medium was also optimized in terms of Tpo1 activity and cell growth.

While SPD production was observed by the engineered yeast (OS123/pTPO1), it was hypothesized that the production of

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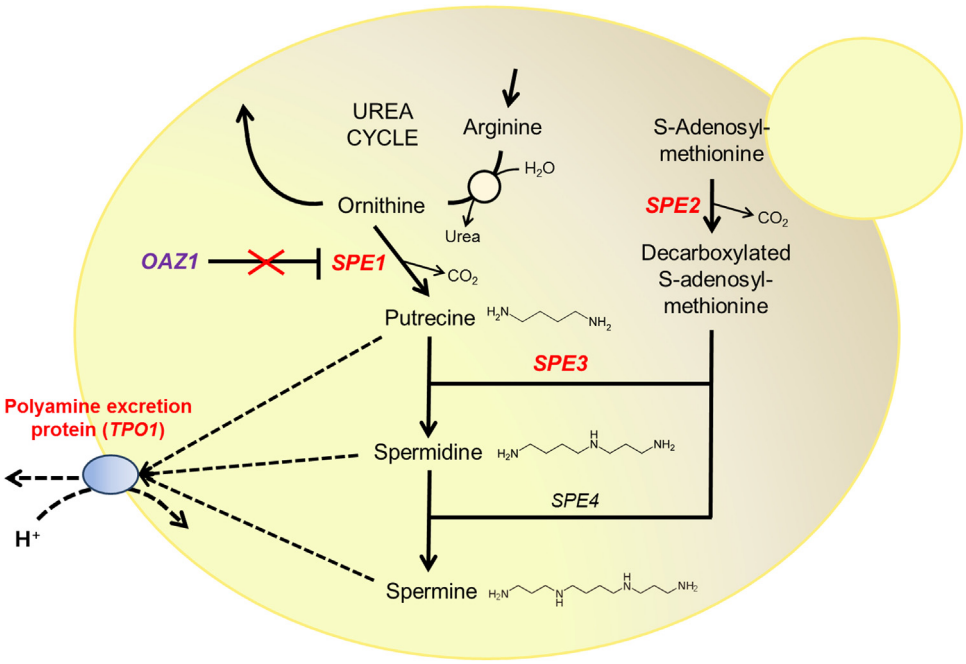

Fig. 1. Strategy for production of spermidine in culture medium by engineered *S. cerevisiae*. Fluxes in the spermidine biosynthetic pathway were amplified by overexpressing ornithine decarboxylase (ODC, *SPE1*), *S*-adenosylmethionine decarboxylase (*SPE2*), and spermidine synthase (*SPE3*). For alleviation of the feedback inhibition on ODC, *OAZ1* coding for ODC antizyme was disrupted. As a polyamine transporter (Tpo1p) located in *S. cerevisiae* plasma membrane was overexpressed in order to excrete intracellular spermidine to the medium.

SPD might be repressed by the presence of glucose as glucose metabolism limits metabolic fluxes in non-ethanol producing pathways. In order to overcome the glucose repression, previous studies constructed engineered strains defective in carbon catabolite repression (CCR) [12], or substituted glucose with other carbon sources [13]. Of two approaches, the approach using other carbon sources for the production of SPD by engineered yeast was adopted. This article reports the development of a SPD overproducing *S. cerevisiae* strain by introducing the genetic modifications eliciting SPD overproduction into an efficient xylose fermenting *S. cerevisiae* strain (SR8-4) [14].

2. Materials and methods

2.1. Strains and plasmids

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for constructing plasmids. *S. cerevisiae* D452-2 (*MATα*, *leu2*, *his3*, *ura3*, and *can1*), OS123 [10], and SR8-4 [14] strains were used for constructing SPD overproducing strains. Strains and plasmids used in this study are described in Table 1.

2.2. Genetic manipulation

The *TPO1* gene coding for polyamine transporter was PCR-amplified from the genomic DNA of *S. cerevisiae* D452-2 using the TPO1[F] (5′-GGACTAGTAAAATGTCGGATCATTCTCCCATTTC-3′) and TPO1[R] (5′-CCGCTCGAGTTAAGCGGCGTAAGCATACTTG-3′) primers. After digestion of the PCR product with *Spe*I and *Xho*I restriction enzymes, the digested DNA fragment was ligated with plasmid p423GPD [15] digested with the same restriction enzymes, resulting in the construction of plasmid p423GPD-TPO1. The *TPO1* expression cassette consisting of GPD_P-*TPO1*-$CYC1_T$ was PCR-amplified from p423GPD-TPO1 plasmid using GPDp[F] and CYC1t_1[R] primers from the previous study [10]. After digestion of the PCR product with *BamH*I and *Not*I restriction enzymes, the GPD_P-*TPO1*-$CYC1_T$ fragment was ligated with plasmid p42K [15] digested with the same restriction enzymes, resulting in the construction of plasmid pTPO1. The mutated *TPO1* (mTPO1) containing a mutation of T52E was synthesized from IDT Inc. and then was cloned into plasmid p42K as described above. The truncated *OAZ1* gene (280–580 bp) was obtained by PCR using the genomic DNA of *S. cerevisiae* D452-2 as a template with the d_OAZ1[F] (5′-TAAGAATGCGGCCGCATCTCAATTCATATTAGATTACAATGTTC-3′) and d_OAZ1[R] (5′-CGCGGATCCCCTACTATTCAGTAAAGATGGTAATAC-3′) primers. The 300 bp-size DNA fragment was cut with *Not*I and *BamH*I, and combined with pRS404 plasmid, resulting in plasmid p404_d_OAZ1. *OAZ1* disruption was confirmed by PCR using two primers of ch_OAZ1[F] (5′-TTTCTACGTATAGTTTGGCTAGTGGGG-3′) and ch_pRS404[R] (5′-CATTCAGGCTG-CGCAACTGTTG-3′). Transformation of the expression cassettes for overexpressing *TPO1*, *mTPO1*, *SPE1*, *SPE2*, and *SPE3* and the *OAZ1* disruption cassettes was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). Transformants were selected on YSC medium (6.7 g/l yeast nitrogen without amino acids and 2.0 g/l synthetic complete supplement mixture) containing 20 g/l glucose. Amino acids and nucleotides were added as necessary. For the selection of *TPO1* and mTPO1 overexpressing strains, YP medium containing 20 g/l glucose and 200 mg/l G418 was used.

2.3. Culture conditions and fermentation experiments

E. coli was grown in LB medium (5 g/l yeast extract, 10 g/l bacto tryptone, and 10 g/l NaCl) with 50 μg/ml of ampicillin for genetic manipulation. *S. cerevisiae* strains were pre-cultured at 30 °C and 250 rpm for 24 h in YPD medium (10 g/l yeast extract, 20 g/l bacto peptone, and 20 g/l glucose) containing 200 mg/l G418. YP medium containing 50 g/l glucose or xylose with 200 or 400 mg/l G418 was used for fermentation experiments, and medium pH was adjusted to target pH values by adding 5 N HCl or 2 N NaOH. For SPD production by engineered yeast, pre-cultured yeast cells from YP medium with 20 g/l glucose were harvested and inoculated into