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Exploring the potential of the glycerol-3-phosphate dehydrogenase 2 (GPD2) promoter for recombinant gene expression in *Saccharomyces cerevisiae*

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ABSTRACT

A control point for keeping redox homeostasis in *Saccharomyces cerevisiae* during fermentative growth is the dynamic regulation of transcription for the glycerol-3-phosphate dehydrogenase 2 (*GPD2*) gene. In this study, the possibility to steer the activity of the *GPD2* promoter was investigated by placing it in strains with different ability to reoxidise NADH, and applying different environmental conditions. Flow cytometric analysis of reporter strains expressing green fluorescent protein (*GFP*) under the control of the *GPD2* promoter was used to determine the promoter activity at the single-cell level. When placed in a *gpd1Δgpd2Δ* strain background, the *GPD2* promoter displayed a 2-fold higher activity as compared to the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (*TDH3*). In contrast, the *GPD2* promoter was found to be inactive when cells were cultivated in continuous mode at a growth rate of 0.3 h^{-1} and in conditions with excess oxygen (i.e. with an aeration of 2.5 vvm, and a stirring of 800 rpm). In addition, a clear window of operation where the *gpd1Δgpd2Δ* strain can be grown with the same efficiency as wild type yeast was identified. In conclusion, the flow cytometry mapping revealed conditions where the *GPD2* promoter was either completely inactive or hyperactive, which has implications for its implementation in future biotechnological applications such as for process control of heterologous gene expression.

1. Introduction

Baker's yeast, *Saccharomyces cerevisiae,* can be used for production of a range of different commodity chemicals [1,44], biofuels [20,25,41], bioflavours [8], fine chemicals [45,46,54] and pharmaceuticals [17,53]. It is used as production host for approximately 20% of biopharmaceuticals approved by the Food and Drug Administration (FDA) or by the European Medicines Agency (EMA) [15]. In all yeast-based processes, a carbon source, which is normally sugar, is converted into biomass, and depending on the specific process, also into a spectrum of products and by-products. To reach optimal productivity and yield of biomass and products, it is often crucial to control the central metabolic pathways for efficient carbon assimilation and reduction of by-products. Not least control of NADH/NAD$^+$-ratios during processing can be important since it determines the thermodynamic feasibility of a large number of metabolic reactions [16], whereof some are more beneficial than others from a process perspective.

Glycerol is a preferred metabolic end-product during wine-making where it contributes favourably to the organoleptic characteristics of the product [10]. However, in most other cases, glycerol is a yield-lowering by-product that negatively influences process economy [55]. From an applied perspective, there is thus a need to monitor and control the requirement for glycerol formation, or perhaps more importantly the need for NADH oxidation in the production strain and during different process conditions. Production of glycerol by yeast under fermentative conditions occurs as a consequence of a need for recycling of NADH formed from anabolic reactions, such as amino acid biosynthesis [32,51]. The gene products of glycerol-3-phosphate dehydrogenase (GPD) catalyse the NADH-dependent conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3 phosphate, which is further converted to glycerol by DL-glycerol-3-phosphatase [2,11,33]. The GPD activity resulting from the two isoenzymes Gpd1p and Gpd2p

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is essential for growth under anaerobic conditions; however, removal of one of the isoenzymes by gene deletion can be compensated by the activity of the other [3]. Under aerobic conditions the double gene knockout has previously been reported to be able to grow, but at a reduced rate compared to wild-type yeast [6].

The *GPD2* promoter activity is known to be up-regulated during growth under conditions with limited supply of oxygen, i.e. under conditions with elevated need for fermentative oxidation of NADH [3]. However, a thorough investigation of its relative activity in strains with different capacity for anaerobic NADH oxidation, and during growth at different dilution rates in continuous cultivation mode and under different levels of aeration is still lacking. Furthermore, the potential of *GPD2*p for controlling recombinant gene expression by varying environmental conditions has previously not been addressed. The controlling of promoter activity is a useful tool to optimize activities of heterologous biosynthetic pathways. Recombinant gene expression in *S. cerevisiae* is often made from constitutive native promoters of different strengths [36,47]. There are also several inducible promotors, such as *CUP1*p (copper-inducible promoter)[12], that previously have been employed to steer recombinant gene expression. In recent years, screening of synthetic promoter libraries has been used to identify promoters with varying activity under different applied conditions (see for example review by Redden et al. [39].

Flow cytometry (FC) analysis of green fluorescent protein (GFP) based biosensors is a well-established and rapid methodology for determining promoter activity in microbial populations (reviewed by Fernandes et al. [14]. The usefulness of FC in combination with fluorescent proteins has previously been demonstrated for measuring the activity of a number of different yeast expression systems [24,47]. Also, FC have been used to measure fluorescent proteins correlated to other user-specified properties, for example for measuring intracellular pH [49] or for monitoring cell growth and cell membrane robustness [7].

In this study, a set of previously developed reporter strains [26] with or without *GPD* activity, where expression of green fluorescent protein (GFP) is under control of the *GPD2* promoter, were cultivated under different conditions in batch, accelerostat, and chemostat mode and at different levels of aeration. Flow cytometric analysis was employed to determine a blueprint of the *GPD2* promoter activity within the cell population with single-cell resolution. Our results suggest that if sufficient oxygen is supplied the *GPD2* promoter is fully inactive in both glycerol-positive (wild-type background) and glycerol-negative (*gpd1Δgpd2Δ* background) yeast as long as the growth rate is controlled at approximately 0.3 h^{-1}. However, for a *gpd1Δgpd2Δ* strain grown at maximum growth rate in batch cultivation mode with limited oxygen supply, the activity of the *GPD2* promoter was higher than the well-known strong constitutive glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) promoter. The potential of using the *GPD2* promoter for recombinant gene expression is discussed, including the possibility to induce its activity by simply controlling process parameters such as level of aeration and substrate feed-rate.

2. Material and methods

2.1. Strains

The *S. cerevisiae* strains used in the study are summarized in Table 1. *Escherichia coli* NEB 5α (New England BioLabs, Ipswich, MA, USA) was used for subcloning. All strains were stored in 20% glycerol at −80 °C. Yeast cells from freshly streaked yeast nitrogen base (YNB) plates (6.7 g/l yeast nitrogen base without amino acids, 20 g/l glucose, and 2% agar) were used for all cultivations.

2.2. Molecular biology methods

Plasmid DNA was prepared using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA). Restriction and modification enzymes as well as T4 DNA ligase were obtained from the same manufacturer. The QIAquick gel extraction kit (QIagen, Hilden, Germany) was used for DNA extractions from agarose. All nucleotides were ordered at Eurofins (Germany). All genetic constructs were checked by sequencing (Eurofins, Germany). The primers used in the study are listed in Table 2.

Competent cells of *E. coli* NEB 5α were prepared and transformed by the method of [22] and yeast transformations were performed using the lithium acetate method [18]. *E. coli* transformants were selected on lysogeny broth (LB) plates [43] with 50 μg/ml ampicillin (IBI Shelton Scientific, Shelton, USA). *S. cerevisiae* transformants were selected on YNB.

2.3. Plasmid construction

Plasmid YEpJK03 was constructed by ligation of two DNA fragments using T4 DNA ligase: (1) The *yEGFP3* fragment that was PCR amplified from pYGFP3, using the primers SpeI_yEGFP3_f and XhoI_yEGFP3_r, digested with *Spe*I and *Xho*I and finally purified, and (2) 426GPD digested with *Spe*I and *Xho*I. The ligation mix was then used to transform *E. coli*.

Table 1
Strains and plasmids used in this study

Strains and plasmids	Relevant features	Reference
Plasmids		
p426GPD	*URA3 Mumberg vector*	[29]
pYGFP3	*URA3 ADH1p-yEGFP3-ADH1t*	[9]
YIpJK01	*HIS3 GPD2p-yEGFP3-PGK1t*	[26]
YEpJK01	*URA3 GPD2p-yEGFP3-PGK1t*	[26]
YEpJK03	*URA3 TDH3- yEGFP3-ADH1t*	This study
S. cerevisiae strains		
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ 1 MAL2-8C SUC2*	EUROSCARF
TMB4114	CEN.PK2-1C *trp1::TRP1 leu2::LEU2 his3⁻ ura3⁻*	This study
TMB4112	CEN.PK2-1C *gpd1::TRP1 gpd2::LEU2 his3⁻ ura3⁻*	This study
TMB4122	CEN.PK2-1C YEpJK01 *trp1::TRP1 gpd2::LEU2 his3::HIS3*	[26]
TMB4140	CEN.PK2-1C *trp1::TRP1 leu2::LEU2 his3::*YIpJK01 *ura3*::YIplac211	[26]
TMB4144	CEN.PK2-1C *gpd1::TRP1 gpd2::LEU2 his3::*YIpJK01 *ura3*::YIplac211	[26]
TMB4145	TMB4114 *his3::HIS3* YEpJK03	This study
TMB4146	TMB4112 *his3::HIS3* YEpJK03	This study