Standardization and '*In Vivo*'-Like Enzyme Activity Measurements in Yeast

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Abstract

The aim of this study was to standardize the cultivation conditions of *Saccharomyces cerevisiae* and the *in-vitro* enzyme activity assays between different laboratories. Furthermore, the conditions under which the enzyme activity measurements were carried out were adapted such that they would be as close as possible to *in vivo* conditions, thus yielding results which are relevant for Systems Biology. This approach is different from the classical enzymologists' approach which is to optimize for the highest catalytic activity.

Saccharomyces cerevisiae strain CEN.PK113–7D was cultured in aerobic, glucose- limited chemostats under standardized conditions. It was shown that, in accordance with earlier interlab comparisons, the main culture characteristics, including biomass, dry weight, glucose flux, and mRNA levels of glycolytic enzymes were comparable between five different laboratories.

As could be expected, the V_{max} values of the glycolytic enzymes were lower when measured under *in vivo*-like conditions than in optimized assays, but still sufficient to account for the glycolytic capacity of the cells. The addition of a crowding agent (polyethylene glycol) hardly affected the measured enzyme activities.

Introduction

In Systems Biology the question is addressed as to how biological functions emerge from the interactions between the molecular components of the cell. In a project with 6 groups from three different universities we attempt to understand what regulates changes in glycolytic flux in bakers' yeast as a function of time and upon a number of different perturbations. To this end mRNA concentrations, protein concentrations, V_{max} values, metabolite concentrations and fluxes are experimentally determined and the extent to which various processes contribute to the regulation of metabolic flux are quantified with Regulation Analysis [1–3].

To be able to integrate the results from different laboratories into a coherent picture, we have standardized the cultivation and all assay protocols. We have chosen to examine the regulation of glycolysis in yeast, as it is one of the few pathways for which the kinetic properties of the enzymes are known sufficiently to calculate the flux from the enzyme activities. Furthermore, yeast can be cultured under well-defined steady-state and transient conditions. In this article we describe the results of the standardization process, with an emphasis on the enzyme activity assays.

In the project yeast cells are grown in chemostat cultures under well-defined steady-state conditions in terms of pH, temperature, dissolved oxygen concentration, and substrate and product concentrations. The CEN.PK113 – 7D yeast strain was used since its physiology has been well-characterized and it was used successfully in earlier attempts at standardization [4,5].

All groups started from the same CEN.PK113 – 7D stock, freshly obtained from the Euroscarf collection of yeast strains. Cells were grown at a dilution rate of 0.1 h^{-1} in the mineral medium described by Verduyn [6] supplemented with 7.5 g/L glucose as the sole carbon source, because this medium does not contain sodium, which is toxic for CEN.PK113 – 7D. Samples were taken after at least 5 residence times of chemostat cultivation, to ensure that steady-state conditions are satisfied, and not later than 20 residence times, to prevent physiological changes associated with prolonged chemostat cultivation [7,8].

To estimate the *in vivo* enzyme activities in the cell, assays were developed that mimicked the intracellular conditions as closely as possible. Recent data obtained within one of the contributing labs (Orij, R. and Smits, G. J.) show that the cytosolic pH is approximately 7 if the external pH is 5.0. Therefore, the *in vivo*-like assays were performed at pH 7.0. Intracellular potassium concentrations between 50 mM and 200 mM have been reported for yeast [9]. Therefore, the potassium concentration was fixed at 200 mM. Sulfate was added, since it is the main anion in our medium. Therefore, if magnesium was needed for the assay, it was added in the form of magnesium sulfate instead of magnesium chloride. An intracellular phosphate concentration of 7 mM was reported at a cytosolic pH of 7.5 [10]. Therefore, phosphate was added to a concentration of 10 mM. Finally, it was tested as to whether the enzyme activities were affected by a crowding agent (polyethylene glycol (PEG)). Since macromolecular crowding promotes the binding of macromolecules to each other, the activity of enzymes composed of several subunits could be affected by the addition of PEG.

Materials And Methods

Growth conditions

The growth procedures have been described in detail in Van Hoek *et al.* [11]. Shortly, *S. cerevisiae* strain CEN.PK113 – 7D was grown in aerobic glucose-limited chemostat cultures at a dilution rate of 0.1 h^{-1} at 30 °C in defined mineral medium [6] kept at pH 5.0 with 2 M of KOH. The feed medium contained 42 mM of glucose (7.5 g l^{-1}). The chemostats were stirred at a rate of 800 rpm, aerated at 0.5vvm and most equipment was acquired from Applikon (Schiedam, NL).

qPCR

Oligonucleotide primers were designed to amplify an 80 – 120bp amplicon. PDI1 was chosen as an internal standard. Primers were designed using Primer Express software 1.0 (PE Applied Biosystems, Foster City, CA, U.S.A). PCR reactions (20 µl) were set up and run as described by the manufacturer. Briefly, the reactions contained 10 µl SYBR Green

PCR Core Kit (PE Applied Biosystems, Foster City, CA, U.S.A), 20 pmol of each primer (Sigma or Eurogentec, Seraing, Belgium); and 3 µl of cDNA template (equivalent to 1 ng RNA). Amplification, data acquisition, and data analysis were carried out in the ABI 7900 Prism Sequence Detector (once at 2 min, 50 °C; 10 min, 95 °C; and 40 cycles at 95 °C, 15 s; 59 °C, 1 min). The calculated cycle threshold values (Ct) were exported to Microsoft Excel for analysis using the ΔΔCt method [12]. Dissociation curves (Dissociation Curves 1.0 f. software, PE Applied Biosystems, Foster City, CA, U.S.A) of PCR products were run to verify by amplification of the correct product.

Fermentative capacity assay

Steady-state fluxes were measured for 30 min in a cell suspension kept anaerobic at 30 °C in a setup described by Van Hoek *et al.* [11] for the determination of fermentative capacity, with the modification that the headspace was flushed with N_2 instead of CO_2. Ethanol, glucose, glycerol, succinate, acetate, and trehalose were measured by HPLC (300 mm x 7.8 mm ion exchange column Rezex ROA-organic acid (Phenomenex), with 22.5 mM H_2SO_4 kept at 55 °C as eluent at the flow rate of 0.5 ml min^{-1}).

Enzyme activity measurements

Cell free extracts were prepared by sonication (6 times for 30 s.) with glass beads (250 – 500 µm) on ice water as described by Van Hoek *et al.* [11]. The total protein content of the cell free extract was measured using the Lowry method [13]. The absorbance (750nm) was measured in a Novostar plate reader (BMG Labtech, Germany).

The enzyme assays were carried out on four dilutions of freshly prepared extracts through NAD(P)H-linked assays as described by Van Hoek *et al.* [11], with a Cobas Bio automated analyser for spectroscopic measurements (Roche, Switzerland). Enzyme assays were performed in three different buffers. The first set of activities was measured according to Van Hoek *et al*. [11], in which the buffer content was different for the different enzymes. The second set of enzyme assays was performed in 100 mM K_2SO_4, 10 mM KH_2PO_4, and at a pH of 7.0. In every assay essential components were added if required (NADH, ATP, EDTA, $MgSO_4$, coupling enzymes and substrates). The third set of assays was done in the same conditions as the second assay, but with the addition of 10 % PEG (3350).

Table 1. Enzyme activity protocols according to Van Hoek *et al*., [11]. *In the '*in vivo*'-like enzyme activities the buffer concentrations were for all enzyme assays 100 mM K_2SO_4, 10 mM KH_2PO_4, 5mM $MgSO_4$ (if needed), and the pH was 7.0. Furthermore, in the '*in vivo*'-like enzyme activities all concentrations were the same as in the Van Hoek protocol.

	ADH	ALD	GAPDH	HXK	TPI	PDC	PFK	PGI	PGK	GPM	PYK	G6PDH
Salt1* **conc**	Glycine 50mM	Tris-HCl 50mM	Triethanolamine 100mM	Imidazol-HCl 50mM	Triethanolamine 100mM	Imidazol-HCl 40mM	Imidazol-HCl 50mM	Tris-HCl 50mM	Triethanolamine 100mM	Triethanolamine 100mM	Cacodylic Acid 100mM	Triethanolamine 100mM
Salt 2* **conc**		KCl 100mM	EDTA 1mM			TPP 0.2mM			EDTA 1mM	2,3dPGA	KCl 100mM	
Salt 3* **conc**			MgSO4 1.5mM	MgCl2 5mM		MgCl2 5mM	MgCl2 5mM	MgCl2 5mM	MgSO4 1.5mM	MgSO4 1.5mM	MgCl2 25mM	
pH*	9	7.5	7.6	7.6	7.6	6.5	7.0	8.0	7.6	7.6	6.2	7.6
Substrate1 conc			ATP 1mM	ATP 1mM			F2,6bP 0.1mM		ATP 1mM	ADP 10mM	ADP 10mM	
Substrate2 conc	NAD 1mM	NADH 0.15mM	NADH 0.15mM	NADP 1mM	NADH 0.15mM	NADH 0.15mM	NADH 0.15mM	NADP 0.4mM	NADH 0.15mM	NADH 0.15mM	NADH 0.15mM	NADH 0.15mM
Startreagent1 conc	Ethanol 100mM	F1,6bP 2mM	3-PGA 5mM	Glucose 10mM	GAP 5.8MM	Pyruvate 50mM	F6P 0.25mM	F6P 2mM	3-PGA 5mM	3-PGA 5mM	F1,6bP 1mM	F1,6bP 1mM
Startreagent2 conc							ATP 0.5mM					TPI 75U/ml
Startreagent3 conc												ALD 1U/ml
Enzyme1 conc		G3PDH 0.6U/ml	PGK 22.5U/ml	G6PDH 1.8U/ml	G3PDH 8.5U/ml	ADH 88U/ml	ALD 0.45U/ml	G6PDH 1.75U/ml	G3PDH 8.0U/ml	PYK 13U/ml	LDH 11.3U/ml	
Enzyme2 conc		TPI 1.8U/ml					G3PDH 0.6U/ml			LDH 11.3U/ml		
Enzyme3 conc							TPI 1.8U/ml			ENO 2U/ml		

RESULTS

Interlab comparison of culture characteristics

To be able to later integrate all project data in one model, the cultivation conditions should be the same in the different laboratories. We compared the steady-state properties of the standard chemostat cultures, which were performed in four different labs (Table 2). The glucose flux and biomass yield on glucose were similar in all groups. The specific consumed oxygen and produced carbon dioxide however, deviated from the published standard (reference) in group 2 and group 4. This is probably a matter of calibration of our gas-analysing systems, which can be improved. The respiratory quotient (i.e. the ratio of the specific CO_2 production over the specific oxygen consumption) was close to 1 in all cultures, implying that they were all fully respiratory. Accordingly, no ethanol was detected in any of the cultures.

In addition, the levels of a number of glycolytic transcripts were compared between two labs (Fig. 1). For this we subtracted the cycle threshold of the control gene PDI1 and the transcript of interest. This gives a relative estimate of the amount of transcript present in the cell. The cycle threshold represents the number of cycles after which a certain sample exceeds the threshold signal intensity. Thus, when two samples differ by one cycle, the one with the lowest cycle threshold has a two-fold higher mRNA concentration than the other sample. The transcript levels from two different laboratories show a similar pattern. This is

in agreement with a more extensive inter-laboratory comparison of transcript levels in samples that were obtained from the same culture conditions and analysed using micro-arrays [4].

These data show that if we standardize the conditions in different laboratories, we end up with a similar culture.

Table 2. Steady-state properties of the aerobic glucose-limited chemostat cultivation in the different laboratories, compared to a published reference [14] (N.D. is not detectable).

	RQ	**qCO2 Produced** (mmol/gDW.h)	**qOJ Consumed** (mmol/gDW.h)	**qglucose Consumed** (mmol/gDW.h)	**qethanol Produced** (mmol/gDW.h)	**Yglucose** (gDW/gglu)	**C-recovery** (%)
Literature	1.0	2.3 ± 0.3	2.8 ± 0.3	1.1 ± 0.0	N.D.	0.49 ± 0.0	98
Group 1	1.1	3.0 ± 0.1	2.3 ± 0.0	1.0 ± 0.1	ND.	0.56 ± 0.0	98
Group2	1.0	2.5 ± 0.0	2.4 ± 0.0	1.0	ND.	0.51	100
Group3	1.0	2.8 ± 0.1	2.7 ± 0.1	1.1 ± 0.1	ND.	0.48 ± 0.0	101
Group4	1.0	2.3 ± 0.2	2.3 ± 0.4	1.1 ± 0.1	ND.	0.48 ± 0.0	94
Group5	1.0	2.3 ± 0.1	2.7 ± 0.1	1.1 ± 0.0	ND.	0.50 ± 0.0	103

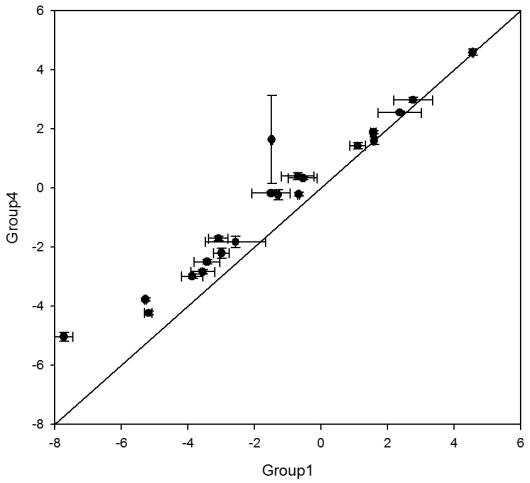

Figure 1. qPCR signal relative to our control gene (PDI1). We subtracted the cycle threshold signal of the control gene (PDI1) from the cycle threshold of our transcript of interest. Comparing these data from different laboratories gives an estimate of the similarity of our fermentors at the transcriptional level.

Enzyme activity assays under in vivo conditions

We analysed the effect of the different buffers on the enzyme activities. For most enzymes the new *in vivo* protocol resulted in lower enzyme activities than the existing optimized protocol (Fig. 2), as should have been expected. Aldolase was the only enzyme for which the enzyme activity increased using the new assays. The activity of TPI was in large excess according to the Van Hoek assay, but the under the *in vivo*-like assay conditions it is of the same order of magnitude as the other enzyme activities. This was probably caused by the addition of phosphate, which is known to be an inhibitor of TPI [15]. Even in the *in vivo*-like assay the activity of TPI was among the highest measured activities, in agreement with the fact that it is close to equilibrium in most cases [16].

Addition of a crowding agent (10% PEG) had hardly any effect on the enzyme activities (Fig 2).

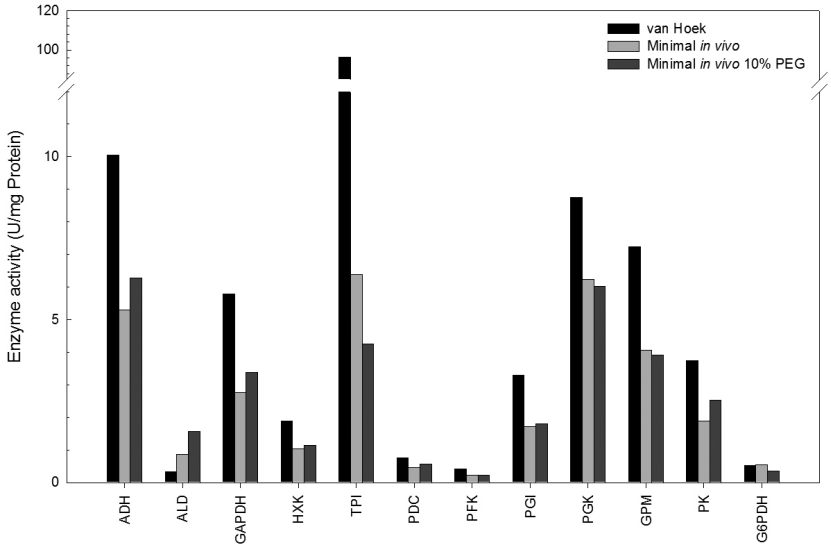

Figure 2. Enzyme activities relative to the amount of total soluble protein. The V_{max} values analysed with the different buffers are shown: Van Hoek (buffer according to [11]) (black columns), minimal '*in vivo*'-like (light grey columns), and minimal '*in vivo*'-like with 10% PEG (grey columns). The V_{max} was measured in the catabolic direction (from glucose to ethanol) except for the enzymes ADH, GAPDH, PGI, and PGK, which were measured in the reverse direction.

The measured V_{max} values should be enough to account for the fluxes measured both under the steady-state conditions and in the fermentative capacity assay. The glucose flux under the steady-state conditions has been shown to be 4.0 mmol h^{-1} $gProt^{-1}$. The ethanol flux in the fermentative capacity measurements was 29.6 mmol h^{-1} $gProt^{-1}$. Taking into account the direction in which the enzyme activities were measured and the branching of glycolysis

(roughly the flux through the lower part of glycolysis should be twice as high as the flux through the upper part), the measured enzyme activities in all assays are large enough to account for the calculated fluxes (Table 3).

Table 3. V_{max} values (mmol h^{-1} $gProt^{-1}$) from Fig. 2 were recalculated in the direction of the flux from glucose to ethanol of the in vivo assay (V_{max} values were obtained in the catabolic direction making use of the equilibrium constants, Michaelis-Menten constants and forward and reverse V_{max} values from literature (ADH [17]), (GAPDH [18]), (PGI [19]), (PGK [20])).

	ADH	ALD	GAPDH	HXK	PDC	PFK	PGI	PGK	GPM	PK
mmol h^{-1} $gProt^{-1}$	*318.3*	*51.4*	*29.7*	*62.6*	*27.1*	*13.1*	*150.8*	*29.9*	*235*	*113*

Conclusions

This paper describes the standardization of yeast cultivation and analysis for Systems Biology research. A single laboratory will never be able to obtain the large amount of data that are required to understand the cell in terms of the interactions between its molecular components. In view of the quantitative nature of this type of research, it is of vital importance to standardize cultivation conditions and analytical procedures between laboratories.

In this study the same yeast strain and cultivation conditions were used that were applied earlier [14] and obtained comparable results in four different laboratories. We optimized the enzyme activity assays to mimic the *in vivo* conditions as closely as possible. The measured activities could account for the calculated flux in the chemostat cultures and in an off-line fermentative capacity assay.

For these assays the ion content of the cell was estimated from literature. In the near future, we will further improve the method by analysing the intracellular ion concentrations using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) [21] and apply the results in the *in vivo*-like assay conditions.

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