Glucose tests

Assessing the method for glucose determination

To ensure the validity of the glucose measurements the following tests were performed:

1. Determination of the extracellular glucose concentration from the stepwise experiment by the three methods available in place: HPLC, GC-MS and enzymatic.
2. Blank samples (no addition of glucose) with MQ-water were tested by the three methods to check for contamination during the sample processing. The current protocols for sampling and analysis of both, intracellular and extracellular metabolites were followed.

Results

Table S4-1 shows the results from the different methods for the set of samples analyzed. Standards for calibration lines were prepared to be in the range from 0.05 – 1 mM. Samples EC15 and EC16 were negative controls for the three methods used. In these samples no glucose was added but the usual protocol for extracellular sampling was followed with MQ water. As expected, it was not possible to detect glucose by any of the three methods, confirming that the samples did not get contaminated due to the sampling process itself.

Table S4-1. Comparison of three different methods for intracellular samples from experiment SCCCR02

		Enzymatic		GC-MS		HPLC	
Vial code	SampleID	Average	Stdev	Average	Stdev	Average	Stdev
EC15, EC16	Negative control	0.0033	0.0004	7.68×10^{-5}	$< 1 \times 10^{-6}$	missing	NA
EC17, 18,19	SS1 0.3	0.3853	0.0078	0.4223	0.0108	0.4845	0.0030
EC20,21,22	SS1 0.2	0.1852	0.0077	0.1853	0.0062	0.2692	0.0143
EC23,24,25	SS1 0.1	0.1758	0.0110	0.1828	0.0023	0.2982	0.0077
EC26,27,28	SS1 0.05	0.0822	0.0056	0.0669	0.0011	0.1693	0.0184

Note. Glucose concentration in mM. Missing means that not peak was detected by the method; NA: Not applicable

GC-MS method has been assumed as the reference method. Standard mixes were used to check that the method returned the expected concentrations. Results from HPLC were closer to the GC-MS ones for concentrations about 0.5 mM (Table S4-1), while for low concentrations the enzymatic method appeared to be more reliable than HPLC due to smaller relative errors with respect to GC-MS. In case of the lowest concentration, the HPLC method returned a concentration about 2.5-fold higher than the one obtained by GC-MS, while the enzymatic

method about 1.2-fold. In addition, three mock samples underwent the same protocol for intracellular sampling and were processed by GC-MS. No peak was detected for any of the three samples suggesting that there is not contamination by this sampling method either. The glucose that has been observed either as intracellular or extracellular glucose seems to be related to a biological source.

Checking whether the correct data is being retrieved for intracellular glucose

In order to evaluate the effect the method of sample processing may have on intracellular glucose measurements, the filtration method was tested and compared against the centrifugation method. Basically, 12 samples were taken from a steady state culture, 6 of them were processed following the filtration method described in the materials and methods section of the present work; the other 6 samples were processed by centrifugation method. In the latter, the biomass was quenched on methanol 100% at -40 °C but was subsequently centrifugated and washed with methanol at the same conditions. The pellet was added with the internal standard and boiled in ethanol at 95 °C for 3 minutes for metabolite extraction. Following steps in the sample processing were the same for the two methods. From the results shown in table S4-2 we found a difference of less than one percent between the two methods, which confirmed that the presence of glucose in intracellular samples was not influenced by the sample processing method available.

Table S4-2. Intracellular glucose from samples processed by two different methods

Sample code	Method used	Concentration (µmol g_{DW}^{-1})
SCCCFF03_01	Filtration	1.926
SCCCFF03_02	Centrifugation	1.806
SCCCFF03_03	Filtration	1.749
SCCCFF03_04	Centrifugation	1.742
SCCCFF03_05	Filtration	1.791
SCCCFF03_06	Centrifugation	1.730
SCCCFF03_07	Filtration	1.632
SCCCFF03_08	Centrifugation	1.239
SCCCFF03_09	Filtration	1.725
SCCCFF03_10	Centrifugation	1.667
SCCCFF03_11	Filtration	1.748
SCCCFF03_12	Centrifugation	1.520
Average ± std error	Filtration	1.629 ± 0.039
Average ± std error	Centrifugation	1.617 ± 0.085

Checking whether the correct data is being retrieved for extracellular glucose

In order to check whether the observations were not subject to misleading effects the following hypotheses have been worked out:

Cold shock

To test whether the use of cold beads has an impact on the extracellular glucose, a set of samples were taken from a running anaerobic culture of *S. cerevisiae* in a chemostat at D=0.1 h^{-1}. It has been suggested that the shock suffered by the cells when in contact with cold steel beads would cause a leakage/detachment of glucose into the medium. The extracellular samples were taken by two methods, the current protocol for extracellular sampling with cold beads and a method without cold beads (from now on referred to as No-cold beads). The sampling processes are described below:

With cold beads (current protocol in CSE)

Broth samples were withdrawn from the reactor and poured into a syringe containing cold steel beads. Approximately 2 mL of broth were placed in contact with 26 g of cold beads (-20 °C). Immediately after, the sample was filtered through a cellulose filter and the filtrate was recovered into a plastic capped-vial, then placed into an ice-bath until analysis by HPLC.

No-cold beads (modified protocol)

Broth samples were withdrawn from the same reactor at the same steady state, and directly poured into an empty syringe with a cellulose filter on the tip. Thereafter, the same process as described above for cold beds was followed. Figure S4-1 shows the behavior for samples taken at different dates and times. Except for the case of sample taken on 3-11-2011, which was a single sample, the other samples were taken by triplicate.

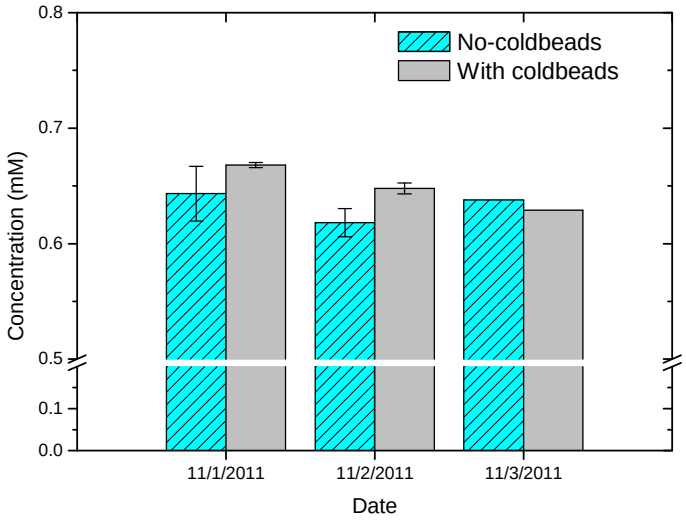

Figure S4-1. Cold shock test on extracellular glucose

Although some differences were found among the different samples, it is not possible to assure that these differences were due to a cold shock phenomenon because the metabolism was not quenched down when no cold beads were used. Therefore, any difference can be the result of any glucose consumption during the time elapsed between taking the sample and the time at which the biomass was separated by filtration. Based on a calculated -qS of 159 mmol C-mol^{-1} h^{-1} (H. Cueto-Rojas, personal communication) and assuming an average processing time of 4 s (sample taking + filtering), the residual glucose would drop by 0.016 mM, which represents about 2.5% of the residual glucose and would account for about 55% of the difference encountered in the samples. The larger relative deviations in samples from the no-cold beads method also indicates that differences were most likely due to the lack of quenching. Even in case of samples taken on 3-11-2011, a higher concentration was obtained with the no-cold beads method. In principle, no real impact may be expected from the cold shock treatment.

2.2 Bound glucose

The hypothesis of glucose bound to the cell wall and detached during the sampling process has been analyzed. In this respect, one would expect to have the same unlabeled glucose in the extracellular space per unit g_{DW} provided that the cell membrane composition is the same, and the same sampling protocol is followed, which is the case here. Assuming that at the different dilution rates tested there was not a significant change in the amount of glucose 'attached' to the cell wall, this is can be calculated as follows:

$$Glucose_{Bound} = \frac{(1 - Maximum_{Enrichment}) * [EC\_Glucose]}{[C_X]}$$

Table S4-3 lists the results for four different dilution rates. It can be seen that the specific unlabeled glucose was not constant. Moreover, it can be said that in general the higher the dilution rate the larger the specific unlabeled glucose. These results suggest that there might not be bound glucose detaching during the sampling process, since the larger the dilution rate the larger the residual glucose concentration and enrichment, but a similar biomass concentration.

Table S4-3. Specific unlabeled extracellular glucose that would cause the observed enrichment drop during the metabolic steady state

Experiment	Dilution rate (h^{-1})	Unlabeled glucose (mmol g_{DW}^{-1})
SCCCR03	0.054	0.0019
SCCCR02	0.101	0.0034
SCCCR02	0.207	0.0026
SCCCR02	0.307	0.0058

For instance, to get an enrichment of 96.41% in the extracellular glucose at a dilution rate of 0.307 h^{-1}, a concentration of unlabeled glucose equal to 10.08 μM would be required. In the scenario that all the cells undergo lysis, the unlabeled glucose released to the extracellular space would be 4.34 μM, which would still not be sufficient to decrease the labeling to the observed level. Furthermore, a cell lysis of more than 25% would be required in all cases in order to explain the observations, which makes this hypothesis very unlikely. Therefore, there should be still a process that continuously produces unlabeled glucose from the intracellular space other than the ones studied here.