Enzymatic Determination Of Glycerol Contents In Biological Samples Using The Biomek 4000 Laboratory Automation Workstation

Abstract

Glycerol is an important platform chemical and also serves as feedstock for a broad range of bioprocesses. Yeast strains are known to produce glycerol under certain conditions. We intended to identify yeast strains with improved capacity to produce glycerol from renewable feedstocks. As even small improvements in glycerol formation have a strong commercial impact, we took special care of precise and reliable methods for glycerol quantification. Due to the high number of yeast strains to be tested (>10000), standard HPLC-methods were ruled out for routine analysis due to time constraints. Colorimetric detection of glycerol by a coupled enzymatic assay is highly sensitive and can be scaled in numbers to meet demands. Automation of the workflows was mandatory, and we employed the Biomek 4000 Laboratory Automation Workstation. A commercial enzymatic kit for the detection of glycerol in complex biological samples was implemented for our screening. We demonstrate successful scale-down of the standard assay from the ml to the µl-scale, thus enabling screening in 96-well microplates.

Table 1. Composition of assay solutions for microscale determination of glycerol contents in biological samples. *, diluted 10x with phosphate buffer
**Materials and Methods**

**Scale Down Of Volumes To Meet Microscale Sampling Demands**

The commercial glycerol-kit is designed for a final assay volume of 3 ml (ENZYTECTM Glycerol detection kit, E1224; R-Biopharm AG, Germany). Basically, glycerol is stoichiometrically converted to pyruvate by the concerted action of glycerol-kinase and pyruvate-kinase. Pyruvate is then reduced to lactate by the consumption of NADH. The oxidation of NADH is equivalent to the amount of glycerol in the sample and determined by light absorption at 340 nm.

To enable microscale sampling in standard microwell plates, the volumes were linearly scaled down to a final volume of 160 µl as shown in Table 1. Furthermore, a master mix containing solution 1 and suspension 2 was freshly prepared to accelerate automatic processing on the Biomek 4000 workstation (master mix M1). Suspensions 2 and suspension 3 provided by the manufacturer were diluted 10-fold with phosphate buffer (100 mM KH2PO4, pH 7.2) before use.

**Biomek 4000 Laboratory Automation Workstation Configuration**

Figure 1 shows the workflow for the determination of glycerol contents in samples from yeast fermentations described in this information bulletin. Figure 2 shows the Biomek 4000 Laboratory Automation Workstation employed for automated use of the commercial ENZYTECTM Glycerol detection kit.

**Workflow**

Yeasts cells were cultivated in complex media at 30°C for several days. At constant time intervals, samples (5 - 1000 µl) were taken from the lab-scale fermentations (1 ml – 10 l), cells were removed by centrifugation (4000xg) and the cell-free supernatant was analyzed. Using the Biomek 4000 Laboratory Automation Workstation, the samples were processed automatically.
the amount of glycerol and absorbance at 340 nm. The mean relative standard error was 2,1% (0,13% – 3 %). As little as 0,63 µg glycerol could be quantified, and the assay showed linearity up to 3,13 µg glycerol in the test. Higher amounts of glycerol gave a non-linear response, most likely due to technical constraints of the microplate spectrophotometer.

Comparing Enzymatic Glycerol Detection With HPLC-Analysis
Accuracy of glycerol determination was a key requirement of the project. Therefore, we compared levels of glycerol in biological samples obtained by the enzymatic assay with those obtained by HPLC-analysis as shown in Figure 4. HPLC-analysis was done using ion-exchange chromatography. The individually obtained values differ by less than 10% (average 9,2%; standard deviation 7,7%). Glycerol levels determined by the automated enzymatic assay routinely gave lower values compared to HPLC determinations. Small molecules produced

Results

Establishing A Calibration Curve For Glycerol
Reference standard solutions of glycerol (125 – 750 mg/l) were generated from an ultrapure stock-solution by serial dilutions using the Biomek 4000 Laboratory Automation Workstation. The enzymatic assay was done as described above. All individual concentrations were tested as triplicates. In Figure 3, the calibration curves showed excellent correlation (R2 = 0,9995) between the amount of glycerol and absorbance at 340 nm. The mean relative standard error was 2,1% (0,13% – 3 %). As little as 0,63 µg glycerol could be quantified, and the assay showed linearity up to 3,13 µg glycerol in the test. Higher amounts of glycerol gave a non-linear response, most likely due to technical constraints of the microplate spectrophotometer.

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during the yeast fermentation might interfere with the enzyme assay and probably account for the observed deviation.

**Screening For Yeast Strains With Improved Glycerol Productivity**

We performed two campaigns and screened more than 10000 individual clones for improved glycerol formation. Using the Biomek 4000 Laboratory Automation Workstation, we routinely processed 25 microplates per day that sum up to 2400 assays. A typical screening result is shown in Figure 5. The corresponding time needed for conventional HPLC-analyses (30 min/run) would sum up to 1200 h, i.e. 50 days. Out of the > 10000 clones screened, 24 clones showed improved glycerol formation, corresponding to a hit-rate of 0,2%, a typical value. Population analysis of 4750 clones revealed two distinct phenotypes as shown in Figure 6. We observed a smaller subpopulation with remarkably lowered capacity to produce glycerol, whereas the majority of cells showed intermediate or slightly improved glycerol formation. Following a typical Gaussian distribution, only a small fraction of clones (1,2%) showed an significant improved phenotype, i.e. a glycerol formation increased by some 30% above average.

**Summary**

We successfully employed the Biomek 4000 Laboratory Automation Workstation for screening yeast variants with improved capacity to produce glycerol. For this purpose, we adopted a commercially available kit for the detection of glycerol in biological matrices and observed excellent robustness of the newly established assay format. Automation enabled time-constrained screening campaigns that could not be accomplished using HPLC-analysis. Given the plethora of readily available commercial detection kits, we expect more enzyme-based assays to be implemented on the Biomek 4000 Laboratory Automation Workstation in the near future.

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