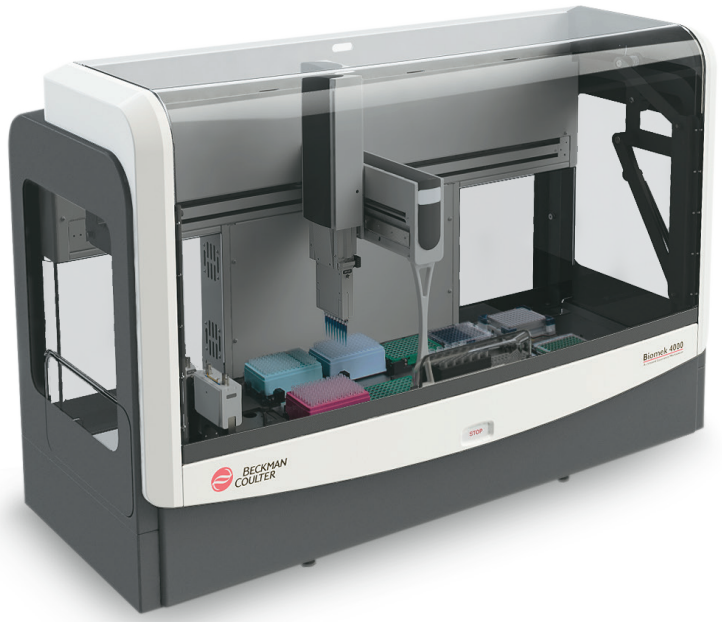
Enzymatic Determination Of Glycerol Contents In Biological Samples Using The Biomek 4000 Laboratory Automation Workstation



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.

Workflow step	Master mix		per single sample	per 96 well plate
			volume [µl]	
1	M1	solution 1	50	5500
		supension 2*	5	550
		H_2O	95	10450
			150	
2	-	**sample**	**5**	**5**
3	M2	suspension 3*	5	550
			160	

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

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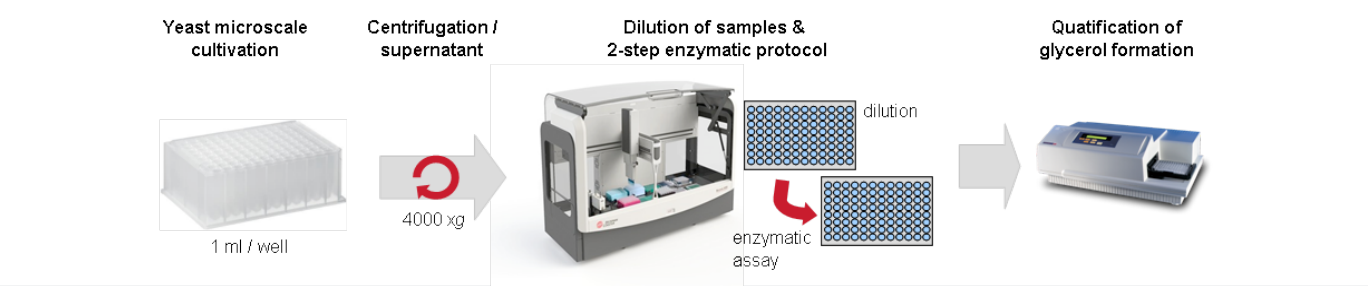

Fig. 1. Integrated workflow of automated determination of glycerol contents in fermentation broth.

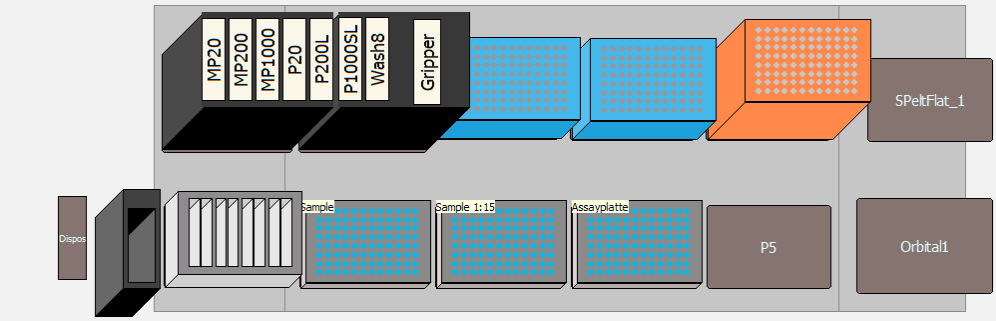

Fig. 2. Screen shot showing the Deck-Layout of the Biomek 4000 Laboratory Automation Workstation used to determine glycerol concentrations in liquid samples.

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 10fold 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

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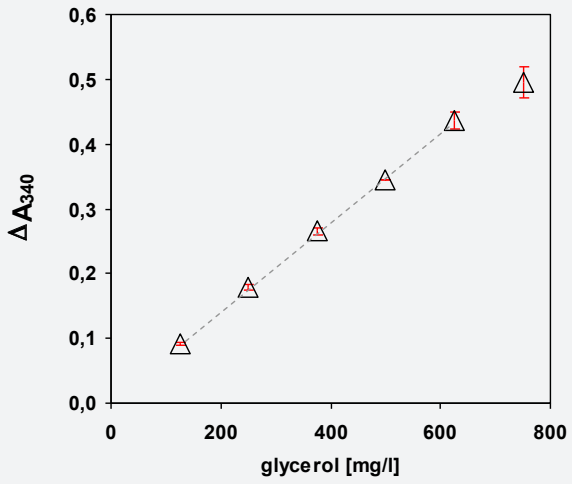

Fig. 3. Calibration curve for glycerol generated with the Biomek 4000 Laboratory Automation Workstation using a modification of the R-Biopharm ENZYTECTM glycerol detection kit. Assay was done in microwell plates (total volume 160 µl; 5 µl sample volume tested). Assays were done in triplicates. Error bars shown in red. y = 0,0007x +0,0063, R2 = 0,9995

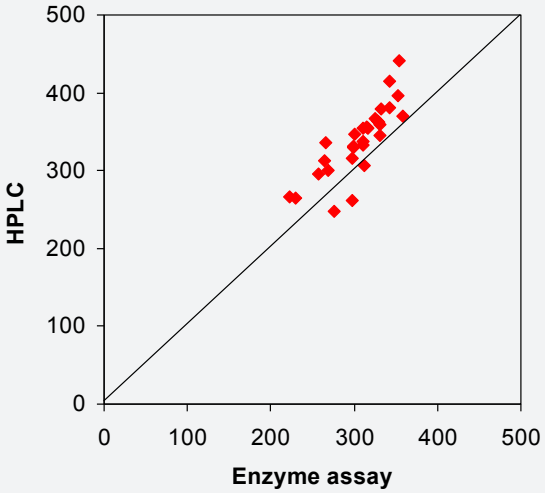

Fig. 4. Determination of glycerol contents in biological samples using the enzymatic assay or HPLC-analysis. The plot gives the correlation between values for glycerol (mg/l) obtained by the enzymatic assay (X-axis) or by HPLC-analysis (Y-axis). Congruent values obtained by both methods plot along the indicated diagonal line.

Automation Workstation, 1:15 dilutions of the samples were prepared in distilled water prior to analysis. Master mix M1 was prepared manually and 145,5 µl were distributed automatically to the single wells of the micro-test plate (Cat-No. 651201, Greiner, Germany). Next, 5 µl of the pre-diluted sample were added and mixed using the Biomek 4000 Workstation. After 7 min, the absorbance (A1) at 340 nm of the solutions was read using a SpectraMax® 190 UV-Vis microplate spectrophotometer. Subsequently, 5 µl of master mix M2 was added automatically, mixed and incubated for another 10 min before the final absorbance (A2) at 340 nm was recorded. The difference (A1 – A2) directly correlates to the amount of glycerol in the sample as shown in Figure 3.

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.

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

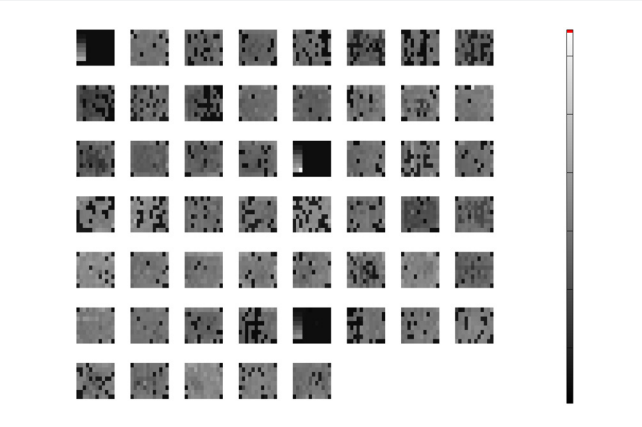

Fig. 5. Typical screening results. Each box represents one microwell plate containing 96 samples. Glycerol contents are indicated by a heat map (black dots, no glycerol detected; white dots, high amounts of glycerol detected).

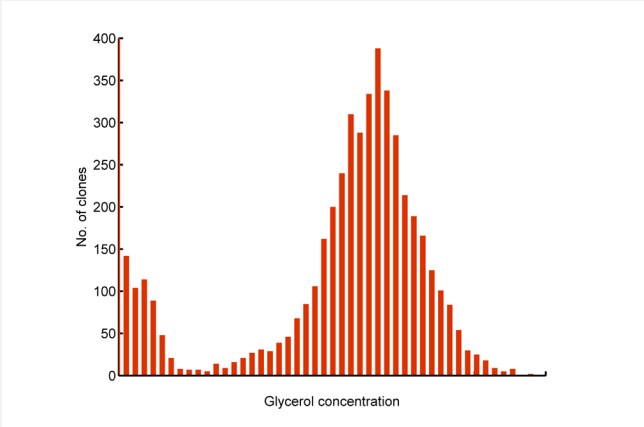

Fig. 6. Population analysis. Histogram of 4750 clones analyzed for glycerol formation. The number of clones (Y-axis) is given per interval of glycerol formation (X-axis; increasing glycerol-formation from left to right).

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 some 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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