A Simple High-Performance Liquid Chromatography Method for the Analysis of Glucose, Glycerol, and Methanol in a Bioprocess

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Abstract

A reliable high-performance liquid chromatography–refractive index detection method for the simultaneous analysis of glucose, methanol, and glycerol in a bioprocess fermentation media using direct injection is presented. The validation studies show a satisfactory selectivity, linearity, accuracy, and recovery of the method. The lowest concentration detectable for glucose and methanol is 3.5 and 6.7 mg/100 mL, respectively. This method could be an attractive choice for the analysis of these compounds not only in fermentation media but also in biomedical and environmental samples.

Introduction

Monitoring bioprocesses is fundamental in characterizing cellular metabolism and controlling microbial performance and productivity. In particular, the screening step for the characterization of microbial performance is time consuming and requires fast, reliable, and inexpensive analysis. For that reason, different chromatographic methods have recently been successfully applied for the analysis of microbial substrates and end-products. Gas chromatography is suitable for the analysis of carbohydrates and sugar alcohols; however, derivatization is always necessary (1). High-performance liquid chromatography (HPLC) is a well established tool for the analysis of these compounds without derivatization (2,3). In particular, cation-exchange resins are suitable for the analysis of carbohydrates and products of carbohydrate metabolism in fermentation mixtures (4).

In the present study, a fast HPLC method with refractive index detection (RI) was developed for the simultaneous analysis of glucose, methanol, and glycerol in complex media during the growth of *Hansenula polymorpha*.

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Experimental

Standards and sample preparation

Glucose, glycerol, and methanol were used as standards (Sigma Chemical, St. Louis, MO). Peak identification was based on HPLC retention times compared with those of selected standards and confirmed by a spiking technique. Quantitation was based on the external standard method using calibration curves fitted by linear regression analysis using Statistica 5.0 software (StatSoft, Tulsa, OK). The calibration curves were obtained by plotting peak area (mV/min) versus amount injected (range covered, 0.2–2 g/100 mL).

Samples were centrifuged at 3000 × *g* for 15 min, and the supernatant was filtered through a 0.22-µm cellulose–acetate filter (Sigma F-0139); then, the filtrate was diluted two times before direct injection into the HPLC.

Instrumentation

The HPLC system was a Jasco (Easton, MD) LC-800 series equipped with an 880-PU pump and 830-RI refractive index detector. Data were acquired using the chromatographic integrator DP-700 (Carlo Erba, Milano, Italy). Samples were injected using a 7125 valve equipped with a 20-µL loop (Rheodyne, Cotati, CA) on an Aminex FOA (Bio-Rad, Richmond, CA) cation-exchange (H^+) resin-based column (100 × 7.8-mm i.d.; 9 µm) protected with a guard column of the same material. The analysis was performed at 60°C with a flow rate of 0.6 mL/min using isocratic elution with 0.01M H_2SO_4 as a mobile phase. The eluent was filtered prior to the analysis using 0.22-µm nylon GV membrane (Millipore, Bedford, MA).

Cultures and media

Mutant strain AG2 and wild type strain L1 (control) were used for this comparative study. Minimum medium contained 0.2% Yeast Nitrogen Base (YNB) without amino acids (Difco Lab, Detroit, MI), 2% glucose, 1% methanol, 0.002% methionine, and 0.006% leucine. Cultures were grown in 250-mL flasks with 100 mL of medium using an orbital incubator set at 37°C and 220 rpm.

Results and Discussion

Specificity and separation

Method specificity can be achieved using a selective detection and/or a more capable separation system. The HPLC chromatogram demonstrates the satisfactory result achieved for the analysis of glucose, glycerol, and methanol (Figure 1). The compounds of interest eluted in less than 15 min. YNB and amino acids saturated the detector without interfering with the elution of the compounds of interest. This result allowed the direct injection of the sample without pretreatment. Despite glycerol partially overlapping with closely eluting methanol, the precision of the method was not affected. A late eluting small peak, tentatively identified as ethanol, was also present in some samples (data not shown). The capacity of the cation-exchange column to separate sugars and alcohols makes it an excellent column for monitoring the bioprocess. RI detection is an excellent choice for the simultaneous determination of sugars and alcohols. In particular, the RI detector provides less background interference from organic acids eventually present in the sample (5).

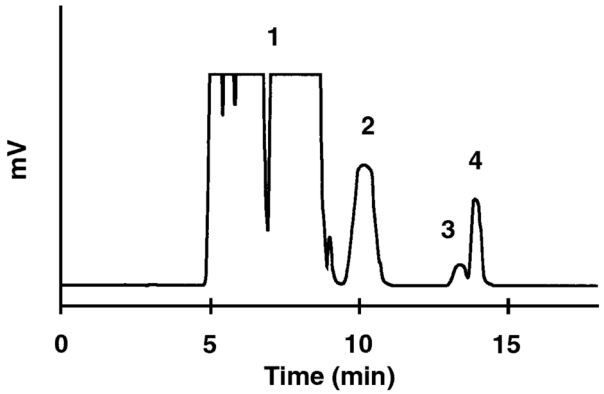

Figure 1. HPLC–RI chromatogram of medium components determined in the *Hansenula polymorpha* culture. Peak identification: YNB, methionine, and leucine, 1; glucose, 2; glycerol, 3; methanol, 4.

Table I. Glucose and Methanol Concentration in Growth Medium Determined with Time*

Time (h)	Glucose (g/100 mL)		Methanol (mL/100 mL)	
	L1	AG2	L1	AG2
0.0	2.00	2.00	1.00	1.00
3.0	2.00	2.00	–*	–
5.5	1.98	–	1.00	1.00
7.5	1.91	2.00	–	–
9.5	1.80	–	1.00	0.98
11.0	1.78	2.00	–	–
14.0	1.64	–	1.00	0.96
24.0	1.45	2.00	0.97	0.88
26.5	1.41	2.00	–	0.85
32.0	1.34	2.00	0.92	0.74
34.0	1.28	–	0.91	–
45.0	1.04	2.00	0.74	0.58
68.0	0.77	1.78	0.49	0.32
76.0	0.57	1.35	–	–
92.0	0.38	0.95	–	0.11

* –, not determined.

Calibration and linearity

The response, expressed as coefficient of determination (r^2), showed good linearity ($r^2 \geq 0.999$) for all standards prepared in the HPLC mobile phase. There was no significant difference between each intercept and the origin ($P \leq 0.05$), and the slopes were 129565 and 55745 for glucose and methanol, respectively.

Precision and accuracy

The precision of the method was calculated over six replicate injections of each standard at 500 mg/100 mL. Peak retention times (t_R) and areas were both expressed in terms of relative standard deviation (RSD). Peak t_R showed an RSD ≤ 0.4%, and area repeatability was 1.2, 2.5, and 1.9% for glucose, glycerol, and methanol, respectively. These values were similar for actual samples.

Accuracy was evaluated by spiking the minimum media with glucose and methanol at different levels. Recovery values (mean ± SD) were 98.4 ± 1.61% and 99.2 ± 2.01% for glucose and methanol, respectively. The slopes of these regression lines did not differ from the calibration curves prepared in HPLC mobile phase.

Limit of detection

The limits of detection (signal-to-noise ratio = 3) were 3.5 and 6.7 mg/100 mL for glucose and methanol, respectively. The value found for glucose is six times lower in comparison with Fourier-transform infrared detection (6). Sensitivity for glucose, however, could be further increased with the use of a capillary electrophoresis system (7), whereas methanol could be detected at trace level using a GC–FID technique (8). The simultaneous analysis of glucose, methanol, and glycerol is still difficult to achieve.

Microbial metabolism

Hansenula polymorpha, a methylotrophic yeast able to grow on methanol as the carbon and energy source, plays an important role in biopharmaceutical (9) and biosurfactant production (10). *Hansenula polymorpha* was grown in a medium containing both glucose and methanol. As demonstrated by HPLC analysis, the mutant strain AG2 used methanol as the favored carbon source (Table I). On the other hand, the wild type L1 strain metabolized glucose first. The late decrease in methanol concentration that occurred for the wild type could be attributed to evaporation instead of microbial metabolism. Traces of glycerol were detected in some samples of L1. The unexpected presence of ethanol, a product of fermentation metabolism, needs to be confirmed by further analysis to clarify its role in this yeast characterized by respiratory metabolism.

Conclusion

The HPLC–RI method allows the simultaneous separation and quantitation of glucose, glycerol, and methanol in culture media.

Selectivity, accuracy, linearity, and sensitivity were satisfactory and demonstrated the reliability of this method. Due to its rapidity and simplicity, this method is useful and convenient for the routine control of bioprocesses including pharmaceutical biotechnology and compositional assay.

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