Determination of Dihydroxyacetone and Glycerol in Fermentation Process by GC after *n*-Methylimidazole Catalyzed Acetylation

Jian Wu[1,2], Ming-Hua Li[1], Jin-Ping Lin[1,*], and **Dong-Zhi Wei[1,*]**

[1]New Word Institute of Biotechnology, State Key Lab of Bioreactor Engineering, East China University of Science and Technology, 200237 Shanghai, China; [2]Institute of Biomass Energy, School of the Environment, Jiansu University, 212013 Jiangsu, China

Abstract

A gas chromatographic method that accurately measures glycerol and dihydroxyacetone from a fermentation broth is described in this paper. The method incorporates a sample derivatization reaction using *n*-methylimidazole as catalyst in the presence of acetic anhydride. Resulting derivatives are separated on a DB-5 capillary column and flame ionization detector. Results show that 10 µL *n*-methylimidazole and 75 µL acetic anhydride are sufficient to complete the acetylation for glycerol and dihydroxyacetone at room temperature for 5 min. The present method exhibits good linearity at a concentration range of 1–100 g/L with excellent regression (R^2 > 0.9997). The limits of detection are 0.025 and 0.013 g/L for dihydroxyacetone and glycerol, respectively. The method has been successfully applied to the monitoring and control of the fermentation process, and recoveries are in the range of 95.5–98.8% with relative standard deviations below 1%.

Introduction

Dihydroxyacetone (DHA) is the simplest ketose. It is used commercially in cosmetics as the main active ingredient in all sunless tanning agents, and serves as a versatile building block for the organic synthesis of various fine chemicals (1). Moreover, DHA is the oxidation product of glycerol and is industrially produced via Gluconobacter oxydans (2). Monitoring simplest DHA and glycerol is necessary for understanding, optimizing, and controlling microbial processes. Traditional techniques such as layer chromatography have low reliability (3), and enzymatic methods are difficult to implement (4,5). Advanced techniques such as high-performance liquid chromatography are considered limited due to the use of refractive index detection and special chromatographic columns (6,7). In a recent study (8), DHA and glycerol in a fermentation broth was estimated by gas chromatograph (GC) with flame ionization detector (FID) after pyrolitic methylation. A single derivative was not obtained and the procedure required an additional accessory to the standard GC apparatus. In practice, conversion of sugars into volatile alditol acetates is the most widely used method for GC analysis of sugars (9). Traditional alditol acetate derivatization using acetic anhydride ideally requires high temperature, long periods, and anhydrous conditions. However, this method is not suitable for determining DHA in a fermentation broth because DHA is unstable above 40°C and its content can be dramatically reduced by processing at elevated temperatures (10). The method is improved by using n-methylimidazole (NMIM) as catalyst instead of pyridine (11,12). Using NMIM could enable the reaction to complete quickly at room temperature and may solve DHA instability problem at high temperatures. Currently, no study has reported on the universal suitability of this catalysis or used it for simultaneous quantitative analysis of glycerol and DHA, especially in the composition of a complicated fermentation broth. In the present study, therefore, the use of the NMIM catalyzed alditol acetate derivatization procedure is investigated for simultaneous DHA and glycerol analysis in the fermentation process by GC. *n*-Hexadecane was used as the internal standard (IS) to improve the reliability of the method. Relevant parameters are estimated in the actual fermentation broth sample to obtain optimized response and reproducibility.

*Author to whom correspondence should be addressed: email dzhwei@ecust.edu.cn and jplin@ecust.edu.cn.

Experimental

Chemicals and reagents

DHA, NMIM, and *n*-hexadecane were purchased from Sigma–Aldrich (Dorset, UK). Glycerol, acetic anhydride and others reagents were supplied by Sinopharm Medicine Chemical Reagent (Shanghai, China), analytical-grade. Yeast powder and D-sorbitol were obtained from local suppliers and were biochemical reagent grade.

Standards solution

DHA and glycerol were dissolved in deionized water as standard stock solutions at a concentration of 100 g/L, respectively. Hexadecane was dissolved in dichloromethane at 10 g/L as IS. The stock solutions were all stored in glass-stopped bottles at 4°C. Working solutions were prepared freshly by serial dilution of the stock solutions. The calibration curves (linearity) were

achieved for both DHA and glycerol by using serial solutions with concentrations range from 1 to 100 g/L. The optimum of derivatization and GC conditions was established by using a standard solution containing a mixture of 50 g/L DHA and glycerol.

Preparation of fermentation broth samples

The microorganism Gluconobacter oxydans DSM 2003 was used in this work, the strain was conserved at 4°C in the slant medium containing the following components (per liter): 80 g glycerol, 10 g sorbitol, 2 g yeast powder, 1 g KH2PO4, 0.5 g MgSO4·7H2O, and 20 g agar. The same composition except agar was used as seeds medium. Seeds were inoculated into 500 mL flasks with 100 mL sterile medium in a rotary shaker (200 rpm) at 30°C for 20 h. The fermentation was conducted in a 3.7 L stirred bioreactor (KLF2000, Company, City, Switzerland) containing 2 L glycerol medium, the inoculation volumes were 10% (v/v), the pH was controlled at 6 by automatic addition of 2M NaOH. Agitation (800 rpm) and aeration (300 ln/h) ensured the rich oxygen in the biotransformation process. Samples were obtained from the bioreactor every 4 h, and were centrifuged at 10,000 rpm for 5 min in order to remove the biomass. The supernatants were collected as the sample for derivatization.

Derivatization procedure

NMIM was added to 10 µL of the standard solution or fermentation broth containing glycerol and DHA in 1.5 mL eppendorf tubes. Acetic anhydride was added, and the tube was allowed to stand at room temperature. To eliminate the excess derivatization reagent, 100 µL of deionized water was added and the tube was vortex-mixed. Then, 10 µL of hexadecane IS solution and 100 µL of dichloromethane were added. The organic phase was separated and dried with anhydrous Na_2SO_4, the resulting derivatives of 0.2 µL were injected into the GC system.

Chromatographic condition

The GC analysis was performed on an Agilent 6890 GC system equipped with flame ionization detector (FID) (Agilent, Santa Clara, CA). A DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm, Agilent) was used to separate the DHA and glycerol derivatives with a split ratio of 50:l. Nitrogen gas was used as a carrier gas at flow rate of 1.0 mL/min with constant flow mode. The temperature programme was set as follows: the initial column temperature of 140°C was held for 2 min, then to 240°C at a rate of 20°C/min, held for 2 min. The inlet temperature was 210°C and the detector was 250°C. The results were processed using the Agilent ChemStation A.08.03.

Results and Discussion

Optimization of derivatization

In order to obtain a highest derivatization yields, several key parameters was investigated, such as the volume of acetic anhydride, the amount of NMIM and reaction time. In this study, the derivatization yields of glycerol and DHA, which were evaluated by peak area ratio between glycerol triacetate or DHA diacetate and the IS of hexadecane.

Effect of volume of acetic anhydride

The different volume of acetic anhydride was added to the standard solution containing 50 µL NMIM, the reaction was performed at room temperature for 5 min. As shown in Figure 1A, the peak area ratio markedly increased at the volume of acetic anhydride ranging from 25 to 75 µL. When the volume was beyond 75 µL, the yields did not improve further. Thus, 75 µL of acetic anhydride was considered as the optimum amount for the following experiments.

Effect of NMIM amounts

The amounts of NMIM were also optimized. Figure 1B shows the results, in which the ratio of peak area did not vary when the amount of NMIM was increased to more than 10 µL. Obviously, the most suitable NMIN was concluded to be 10 µL.

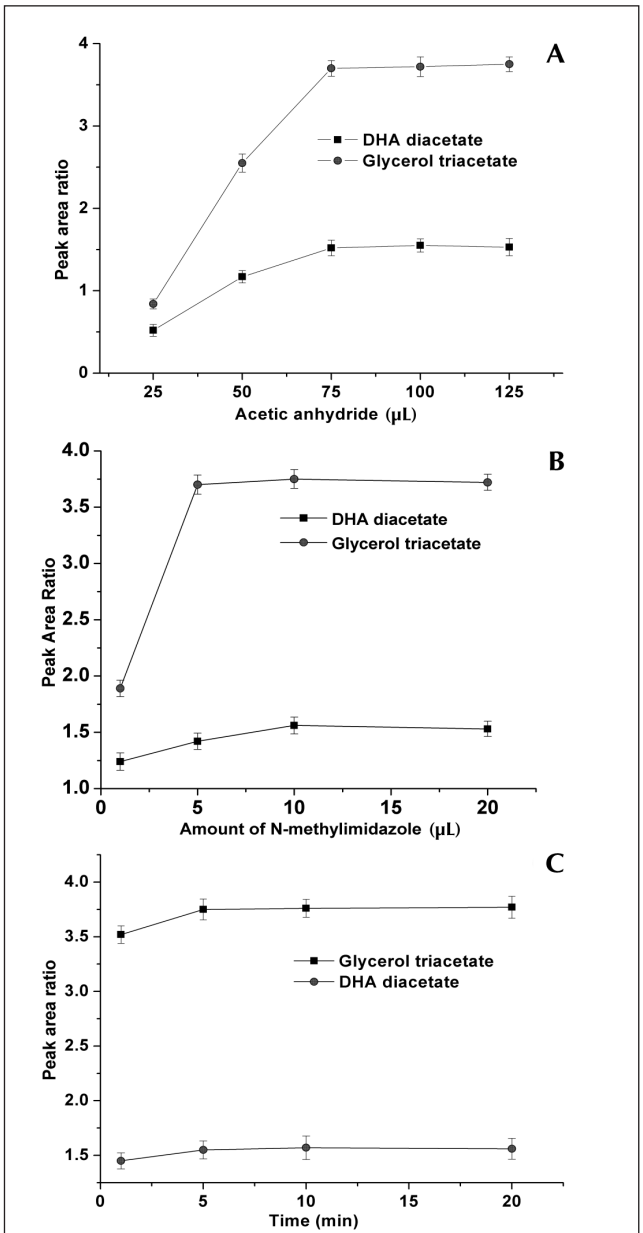

Figure 1. Effects of acetylation conditions on the peak area ratio between derivatives of glycerol or DHA and IS. (A) Effect of volume of acetic anhydride, (B) effect of NMIM amounts, and (C) effect of reaction time.

Effect of reaction time

The effect of reaction time at room temperature on the acetylation was further studied. The results from Figure 1C reveal that 5 min was needed for the reaction to reach the plateau; therefore, the optimal time was set at 5 min.

Chromatography

Figure 2 shows typical chromatograms obtained from the fermentation broth at 16 h. Both DHA and glycerol derivatives had a single chromatographic peak, and the peaks were sharp and symmetrical. Retention times of DHA, glycerol derivatives, and hexadecane were 2.94, 3.93, and 5.57 min, respectively. Obviously, interfering compounds of the fermentation broth had no influence on the separation of DHA and glycerol, and the impurity peak was not presented until the hexadecane eluted. These results show that this method had excellent selectivity for glycerol and DHA in complicated fermentation broths. The hexahydric alcohol Sorbitol, which was another carbon source for G. oxydans growth, could also be analyzed; the peak time was 7.85 min. All derivates were identified with GC-mass spectrometry (data not shown). In other words, a fast GC analysis could be obtained within 6 min for glycerol and DHA in a fermentation broth.

Linearity and limit of detection

Calibration curves were obtained by plotting the peak area ratio between standard sample derivatives and the IS against their concentrations. The limits of detection (LODs) under the present chromatographic conditions were calculated at a signal to noise ratio (S/N) of 3. Linear regression equations, correlation coefficients (r), and detection limits are summarized in Table I. Correlation coefficients were > 0.9997 and LODs were 0.025 g/L for DHA and 0.013 g/L for glycerol.

Table I. Regression Equation, Correlation Coefficients, and Detection Limits

Compound	Regression equation	Linear range (g/L)	Correlation coefficient	LOD (g/L)
DHA	$y = -0.021 + 0.032x$	1–100	0.9997	0.025
Glycerol	$y = -0.0072 + 0.076x$	1–100	0.9998	0.013

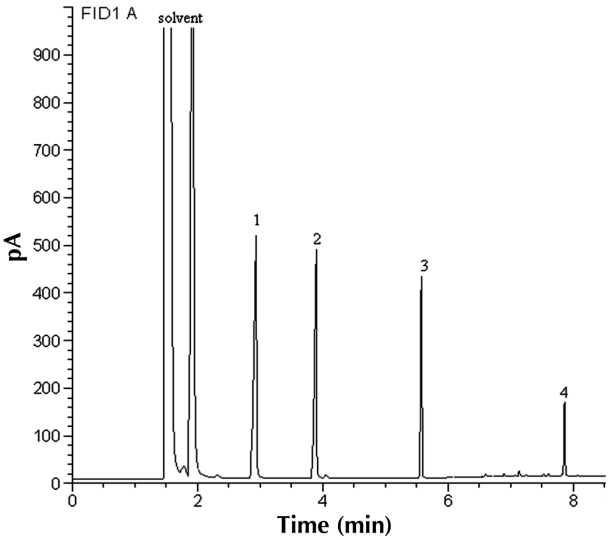

Figure 2. Typical chromatogram of 16 h fermentation broth sample. Peaks: 1, DHA diacetate; 2, glycerol triacetate; 3, *n*-hexadecane; 4, sorbitol derivate.

Precision and recovery

To test the accuracy and precision of the analytical technique, recovery and relative standard deviations (RSD) were assessed by the analysis of actual fermentation broth samples at three concentrations. Table II shows that the average recoveries obtained from five runs ranged from 95.37–98.76%. Relative standard deviations were between 0.43–0.82%, which indicates that the method was accurate and reproducible for the simultaneous determination of DHA and glycerol.

Application

The developed method was applied to analyze authentic fermentation broth samples. Figure 3 illustrates the time course of producing DHA from glycerol by G. oxydans in a 3.7 L stirred bioreactor. The initial concentration of glycerol was 80 g/L. Obviously, DHA concentration increased with the consumption of glycerol, and reached its maximum of 67.2 g/L at 28 h with

Table II. Recovery and Precision for the Analysis of DHA and Glycerol in the Real Fermentation Sample (n = 5)

Compound	Contained (g/L)	Spiked (g/L)	Found (g/L) (Mean ± SD)	Recovery* (%)	Precision† (RSD%)
DHA	4.42	4.54	8.75 ± 0.072	95.37	0.82
	30.15	15.43	45.14 ± 0.28	97.15	0.62
	62.56	30.08	92.07 ± 0.47	98.11	0.51
Glycerol	72.20	23.32	95.23 ± 0.41	98.76	0.43
	41.45	20.75	61.7 ± 0.30	97.59	0.48
	5.76	2.89	8.55 ± 0.06	96.54	0.48

* = [(amount found – amount contained) / amount spiked] × 100%
† 100 × SD / mean.

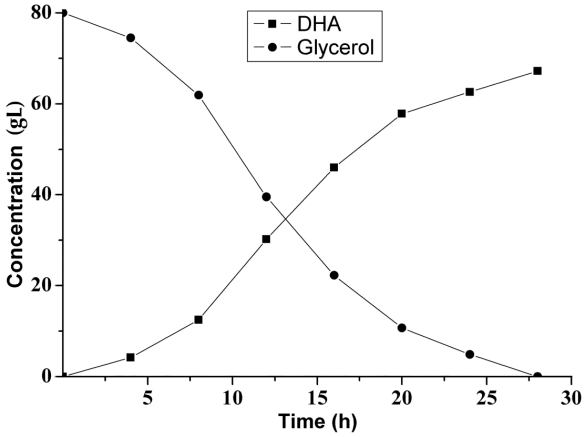

Figure 3. The time course of the glycerol fermentation for producing DHA in 3.7 L stirred bioreactor.

glycerol converted completely. It is interesting to note that the yield is below the initial glycerol concentration, because a part of the glycerol was used as the carbon source for biomass growth during the fermentation process.

With further studies, this method can be developed for the simultaneous determination of more sugar alcohols and ketoses in the fermentation process, especially for DHA determination in cosmetic samples.

Conclusion

A simple, rapid, and accurate GC method was developed for the simultaneous analysis of DHA and glycerol in the fermentation process. Prior to GC analysis, the sample was handled by NMIM catalyzed acetylation. Reaction was completed quickly at room temperature with only minimal sample manipulation compared with that in anhydrous and high temperature circumstances. The proposed method was performed by a normal GC-FID rather than by special GC apparatus. It has high sensitivity and selectivity in the fermentation media and has been successfully applied to monitor the biotransformation process of DHA production from glycerol.

Acknowledgements

This work was financially supported by the National Key Basic Research Development Program of China ("973" Program, No.2009CB724703), and the National Special Fund for State Key Laboratory of Bioreactor Engineering, Grant No. 2060204.

References

1. M. Ruchi, R.J. Seema, and K. Ashok. Microbial production of dihydroxyacetone. *Biotech. Adv.* **26:** 293–303 (2008).
2. D. Hekmat, R. Bauer, and V. Neff. Optimization of the microbial synthesis of dihydroxyacetone in a semi-continuous repeated-fed-batch process by in situ immobilization of Gluconobacter oxydans. *Process. Biochem.* **42:** 71–76 (2007).
3. S. Yamada, K. Nabe, N. Izuo, and I. Chibata. Enzymic producrion of dihydroxyacetone by Acetobacter suboxydans ATCC 621. *J. Ferment. Technol.* **57:** 221–226 (1979).
4. N. Marian, T. Jan, S. Juraj, D. Bengt, and S. Ernest. Monitoring of the bioconversion of glycerol to dihydroxyacetone with immobilized Gluconobacter oxydans cell using thermometric flow injection analysis. *Process. Biochem.* **36:** 1045–1052 (2001).
5. S. Ueda, M. Takahashi, and H. Misaki. High precision determination of glycerol, dihydroxyacetone, D-glyceraldehyde and composition therefore. WO. Patent. No: 9220818 (1992).
6. H.W. Chen, S.F. Bai, and Z.D. Hu. Simultaneous HPLC determination of four Key Metabolites in the Metabolic Pathway for Production of 1, 3-Propanediol from *Glycerol. Chromatogra.* **65:** 629–632 (2007).
7. J. Chen, J.H. Chen, and C.L. Zhou. HPLC methods for determination of dihydroxyacetone and glycerol in fementation broth and comparison with a visible spectrophotometric method to determine dihydroxyacetone. *J. Chromatogr. Sci.* **46:** 912–916 (2008).
8. L.L. Wang, J. Qian, Z.C. Hu, Y.G. Zheng, and W. Hu. Determination of dihydroxyacetone and glycerol in fermentation broth by pyrolytic methylation/gas chromatography. *Analytica. Chimica. Acta.* **557:** 262–266 (2006).
9. N.P. Brunton, T.R. Gormley, and B. Murray. Use of the alditol acetate derivatisation for the analysis of reducing sugars in potato tubers. *Food. Chem.* **104:** 398–402 (2007).
10. Y. Zhu, D. Youssef, C. Porte, and A. Rannou. Study of the solubility and the metastable zone of 1,3-dihydroxyacetone for the drowning-out process. *J. Cryst. Growth.* **257:** 370–377 (2003).
11. R. Wachowiak and K.A. Connors. N-Methylimidazole-catalyzed acetylation of hydroxy compounds prior to gas chromatographic separation and determination. *Anal. Chem.* **51:** 27–30 (1979).
12. B.B. Anthony, J.H. Philip, J.H. Robert, and A.S. Bruce. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohyd. Res.* **133:** 291–299 (1983).

Manuscript received March 28, 2010;
revision received June 9, 2010.