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Analysis of mixtures of fatty acids and fatty alcohols in fermentation broth

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ABSTRACT

Microbial production of fatty acids and fatty alcohols has attracted increasing concerns because of energy crisis and environmental impact of fossil fuels. Therefore, simple and efficient methods for the extraction and quantification of these compounds become necessary. In this study, a high-performance liquid chromatography-refractive index detection (HPLC-RID) method was developed for the simultaneous quantification of fatty acids and fatty alcohols in these samples. The optimum chromatographic conditions are C18 column eluted with methanol:water:acetic acid (90:9.9:0.1, v/v/v); column temperature, 26 °C; flow rate, 1.0 mL/min. Calibration curves of all selected analytes showed good linearity ($r^2 \geq 0.9989$). The intra-day and inter-day relative standard deviations (RSDs) of the 10 compounds were less than 4.46% and 5.38%, respectively, which indicated that the method had good repeatability and precision. Besides, a method for simultaneous extraction of fatty acids and fatty alcohols from fermentation broth was optimized by orthogonal design. The optimal extraction conditions were as follows: solvent, ethyl acetate; solvent to sample ratio, 0.5:1; rotation speed, 2 min at 260 rpm; extraction temperature, 10 °C. This study provides simple and fast methods to simultaneously extract and quantify fatty acids and fatty alcohols for the first time. It will be useful for the study of microbial production of these products.

1. Introduction

Fatty acids, fatty alcohols and their derivatives can be used not only as alternative sources of transportation fuels [1,2], but also as precursors for agrochemicals, textile processing agents, soaps, surfactants, and polymer additives [3,4]. Microbial production of fatty acids and fatty alcohols has attracted much interest due to energy crisis and environmental impact of fossil fuels [5,6]. Genetical modifications of microbes have been performed [7–9]. To support these studies, analytical methods capable of real time monitoring of production of bioreactors are required. However, until now, fast and accurate methods for simultaneous extraction and quantification of fatty acids and fatty alcohols from fermentation broth have not been developed.

Fatty acids and fatty alcohols are generally analyzed separately by gas chromatography (GC). However, due to their limited volatility, both fatty alcohols and fatty acids with more than 10 carbon atoms should be derivatized before GC analysis [7,10–16]. Yet, fatty acids and fatty alcohols are derivatized using different methods. Further, the characterization of mixtures of alcohols and acids is not possible without hindering ester formation between the analytes during derivatization. This largely complicates sample preparation, also increases the analysis time. Other drawbacks are incompleteness of derivatization reactions, risk of degradation of the analytes or their derivatives during GC separation and high analysis cost.

Recently high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) has been developed for the determination of 9 fatty acids [12]. However, this method is only applicable for the determination of chemicals with high boiling points. It is not suitable for the quantification of fatty alcohols with low boiling points. As far as we know, methods for simultaneous quantification of fatty alcohols and fatty acids have not been reported.

HPLC-RID method has been successfully applied to fatty acid analysis [17]. Compared with GC and HPLC-ELSD methods, HPLC-RID method has two advantages. First, it is able to quantify compounds with high or low boiling points without derivatization.

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Table 1
Strains and plasmids in this study.

Plasmids	Relevant characteristics	Source
pMD™ 18-T Vector	T-easy vector	Takara
pTrcHis B	Expression vector	Invitrogen
pTrcHis B *FadM*	pTrcHis B containing *E. coli FadM* gene	In laboratory
pTrcHis B *AccBC*	pTrcHis B containing *E. coli AccBC* gene	In laboratory
Strains		
P. aeruginosa	Wild-type strain	In laboratory
E. coli K-12 MG1655	F-lambda-*ilvG-rfb*-50 *rph*-1	In laboratory
MGKL	*E. coli* K-12 MG1655 Δ*ldhA*	In laboratory
MGKS	*E. coli* K-12 MG1655 Δ*sucCD*	In laboratory
MGKL *FadM*	MGKL containing pTrcHis B *FadM*	In laboratory
MGKS *FadM*	MGKS containing pTrcHis B *FadM*	In laboratory
E. coli DH5α *AccBC*	*E. coli* DH5α containing pTrcHis B *AccBC*	In laboratory

Note: *FadM*, *E. coli* acyl-CoA thioesterase; *ldhA*, *E. coli* Lactate dehydrogenase; *sucCD*, *E. coli* succinyl-CoA synthetase; *AccB*, *E. coli* biotin carboxyl carrier protein; *AccC*, *E. coli* biotin carboxylase.

Secondly, all compounds analyzed with HPLC-RID will not be destroyed during the process of analysis and could be recovered in the original state. However, to the best of our knowledge, there are no reports detailing the use of HPLC-RID for the analysis of fatty alcohols.

Although the separation of fatty acids and fatty alcohols from fermentation broth is necessary before analysis, there is no literature available that describes the extraction of fatty acids and fatty alcohols from media. Indeed, previous studies have only focused on the extraction of fatty acids and fatty alcohols from plant seeds, plant leaves and microalgae [16,18,19]. However, these methods are not suitable for biofuel extraction from fermentation broth.

In this work, a protocol for the simultaneous quantification and extraction of fatty acids and fatty alcohols from fermentation broth was established. The parameters for the analysis of fatty acids and fatty alcohols by HPLC-RID were optimized based on system suitability parameters, signal-to-noise ratio (S/N) and analysis time. The extraction approach was optimized based on orthogonal design. In the end, the optimal extraction and quantification methods were successfully applied in the extraction and quantification of fatty acids and fatty alcohols from *Escherichia coli* and *Pseudomonas aeruginosa* fermentation broth.

2. Experimental

2.1. Chemicals and materials

Five fatty acid standards (decanoic acid, lauric acid, tetradecanoic acid, hexadecanoic acid, and octadecanoic acid) were purchased from Nu-Check-Prep (Elysian, MN, USA). Five fatty alcohol standards (1-decanol, 1-dodecanol, 1-tetradecanol, 1-hexadecanol, and 1-octadecanol) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The other chemicals used were HPLC grade.

2.2. Bacterial strains and media

P. aeruginosa and *E. coli* were selected for fermentation, because they have been used for the production of fatty acids and fatty alcohols previously [2,20]. The strains used in this study are listed in Table 1. Seed culture of *P. aeruginosa* was cultivated in LB medium. Seed culture of *E. coli* MGKL *FadM* and *E. coli* MGKS *FadM* were cultivated in LB medium containing 100 μg/mL ampicillin and 30 μg/mL kanamycin, respectively. Seed culture of *E. coli* DH5α *AccBC* was cultivated in LB medium containing 100 μg/mL ampicillin. M9 medium supplemented with 20 g/L glucose and 20 g/L sodium succinate was used for biofuel production by *P. aeruginosa*. LB medium supplemented with 20 g/L glycerol and 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used for biofuel production by *E. coli* strains.

2.3. Preparation of standard solutions and method validation

Ten standard compounds were accurately weighed, dissolved in MeOH respectively to make the stock solutions of 10 mg/mL. The stock solutions were diluted to six appropriate concentrations and analyzed in triplicate to construct the calibration curves. In accordance with previous reports, the limit of detection (LOD) and the limit of quantification (LOQ) are defined as the signal 3 times and 10 times the baseline noise, respectively [21]. S/N was calculated with Agilent ChemStation software. Other parameters including linearity, correlation coefficients, and recovery, were assessed for the analytes.

2.4. HPLC analysis of fatty acids and fatty alcohols

The analyses of fatty acids and fatty alcohols were performed by HPLC with an Agilent 1200 (Agilent, Co. Ltd. USA) equipped with a quaternary pump, an auto sampler and a temperature-controlled RI detector. The injection volume was 10 μL. A SilGreen ODS C18 column (4.6 mm × 250 mm, 5 μm) was used for sample detection. Methanol was chosen as the mobile phase in this study. The separation of fatty acids and fatty alcohols was optimized by varying chromatographic parameters such as the methanol concentration, flow rate and column temperature.

2.5. Optimization of fatty acid and fatty alcohol extraction

M9 medium was supplemented with 0.2 g/L of each standard for subsequent extraction. The extraction of fatty acids and fatty alcohols from spiked samples was optimized via an orthogonal experimental design by changing the solvent, solvent-to-sample ratio, and rotation speed and extraction temperature. Each extraction was performed 3 times with 5 mL cultures (acidified with 500 μL 10 N HCL) in a rotary shaker incubator for 2 min. The mixture was shaken vigorously for a few seconds before placement in a rotary shaker incubator. After extraction, the cultures were left static for 10 min. The organic phase was filtered through a 0.45 μm millipore filter before analysis with HPLC-RID.

2.6. Analysis of fatty acids and fatty alcohols from fermentation broth

The fermentation broth (5 mL) was acidified with 500 μL 10 N HCL. Fatty acids and fatty alcohols were then extracted from acidified cultures with 2.5 mL ethyl acetate at 10 °C, 260 rpm for 2 min. The mixture was shaken vigorously for a few seconds before placement in a rotary shaker incubator. After extraction, the mixtures were left static for 10 min. The organic layer was transferred to a new centrifuge tube. After centrifugation at 12,000 rpm for 5 min, the clear supernatant was collected and filtered through a 0.45 μm millipore filter and injected into the HPLC-RID system for analysis. In some samples, there were some byproducts with retention time of approximately 4–5 min close to that of ethyl acetate, which has a retention time of 4 min. Thus, high concentrations of these byproducts, together with ethyl acetate, could affect the quantification of decanoic acid and 1-decanol. Therefore, the organic layer was evaporated with nitrogen, and re-dissolved in methanol before HPLC analysis.