High-Performance Liquid Chromatography Determination of the Effect of Rumen Methanogen Coexistence on Organic Acid Production Characteristics from Glucose Metabolism by Anaerobic Fungi (Postprint)

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Abstract

This study aimed to investigate the effect of coexisting rumen methanogens on the characteristics of organic acid production from glucose metabolism by anaerobic fungi using an established high-performance liquid chromatography (HPLC) method. HPLC conditions including UV detection wavelength, buffer concentration, pH, flow rate, column temperature, and injection volume were determined based on the chemical properties of six organic acids; the established HPLC method was used to detect the concentrations of six organic acids in the supernatants of pure anaerobic fungal cultures and co-cultures of anaerobic fungi with methanogens. The results showed that the HPLC conditions were: 5 mmol/L potassium dihydrogen phosphate-phosphoric acid buffer (pH=2.4) as the mobile phase, flow rate of 0.5 mL/min, column temperature of 25 °C, injection volume of 20 L, and detection at 214 nm wavelength. The six organic acids could be well separated within 30 min. The linear correlation coefficients for all organic acids were greater than 0.999, with detection limits of 0.20–1.00 mol/L, quantification limits of 0.667–3.333 mol/L, and recovery rates of 92.17%–101.61%. The coexistence of methanogens affected the metabolic products of anaerobic fungi. The main water-soluble metabolic products of anaerobic fungi utilizing glucose were formic acid, lactic acid, and acetic acid, along with trace amounts of succinic acid, citric acid, -ketoglutaric acid, and ethanol; the coexistence of methanogens significantly reduced the contents of formic acid and lactic acid ($P<0.05$) and significantly increased the content of acetic acid ($P<0.05$) in the supernatant. In summary, this study used the established HPLC method to rapidly, sensitively, and effectively detect the contents of six organic acids produced from glucose metabolism by anaerobic fungi, and found

that the coexistence of methanogens significantly promoted the metabolism of carbohydrates by anaerobic fungal hydrogenosomes.

Full Text

Effects of Associated Methanogen on Organic Acid Profile of Glucose Metabolism by Anaerobic Fungus Revealed by High Performance Liquid Chromatography

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Abstract

This study aimed to investigate the effect of associated methanogen on the organic acid profile of glucose metabolism by anaerobic fungus using an established high-performance liquid chromatography (HPLC) method. Based on the chemical properties of six organic acids, the HPLC conditions including detection wavelength, buffer concentration, pH, flow rate, column temperature, and injection volume were optimized. The established method was then applied to quantify six organic acids in the supernatants of both pure anaerobic fungal cultures and co-cultures of anaerobic fungi with methanogens. The results demonstrated that optimal HPLC conditions were: 5 mmol/L KH PO -H PO buffer (pH=2.4) as mobile phase, flow rate of 0.5 mL/min, column temperature of 25 °C, injection volume of 20 L, and detection wavelength of 214 nm. All six organic acids were well separated within 30 minutes. The linear correlation coefficients for all acids exceeded 0.999, with detection limits ranging from 0.20 to 1.00 mol/L, quantification limits from 0.667 to 3.333 mol/L, and recovery rates from 92.17% to 101.61%. The presence of methanogens significantly altered the metabolic profile of anaerobic fungi. The primary water-soluble metabolites from glucose utilization by anaerobic fungi were formate, lactate, and acetate, along with trace amounts of succinate, citrate, -ketoglutarate, and ethanol. Co-culture with methanogens significantly decreased formate and lactate concentrations ($P<0.05$) while significantly increasing acetate concentration ($P<0.05$). In conclusion, the established HPLC method enables rapid, sensitive, and effective detection of six organic acids produced by anaerobic fungal metabolism, revealing that methanogen co-culture significantly enhances carbohydrate metabolism in the hydrogenosomes of anaerobic fungi.

Keywords: high performance liquid chromatography; organic acids; anaerobic fungi; methanogens; co-culture

Introduction

Anaerobic fungi are among the first microorganisms to colonize plant fiber tissue in the rumen, efficiently degrading plant fiber through their robust rhizoid system and secretion of lignocellulolytic enzymes. These fungi perform mixed-acid fermentation, producing primarily formate, acetate, lactate, succinate, ethanol, carbon dioxide (CO), and hydrogen (H) [2-4]. Methanogens are strictly anaerobic archaea that cannot utilize complex organic compounds, relying instead on simple substrates such as H , CO , formate, acetate, and methanol for energy. Anaerobic fungi and methanogens can stably coexist, with methanogens significantly enhancing substrate degradation and utilization by anaerobic fungi [5-6]. In the presence of methanogens, the major metabolites of anaerobic fungi shift from formate, acetate, H , and lactate to acetate, methane, and lactate [7-8]. Therefore, establishing a reliable method for detecting organic acids in anaerobic fungal metabolites is essential for understanding how methanogen co-culture influences fungal metabolism.

Currently, few studies have reported methods for detecting organic acids from anaerobic fungi, and existing methods typically focus on major metabolites such as formate, acetate, and lactate. These are often measured using separate techniques, which is time-consuming, labor-intensive, and uneconomical. Moreover, no method has been available for simultaneous detection of these organic acids from anaerobic fungal metabolism. Recent research suggests that anaerobic fungi may produce certain trace metabolites, such as -ketoglutarate and citrate [9-10]. However, no quantitative method has been established for detecting these two trace metabolites from anaerobic fungal fermentation. Therefore, this study aimed to develop a method for simultaneous detection of six organic acids produced by anaerobic fungi (formate, acetate, lactate, -ketoglutarate, citrate, and succinate) and to apply this method to investigate the characteristics of organic acid production by anaerobic fungi and the influence of methanogen co-culture.

Materials and Methods

1.1 HPLC Instrumentation and Reagents

The HPLC system consisted of a Waters 2489 HPLC unit (Waters, USA) equipped with a Waters 515 pump, 2489 UV detector, HT-330 column oven (Waters, USA), Agilent SB-Aq column (26 mm × 250 mm, 5 m; Agilent, USA), 7725i manual injector (Rheodyne, USA), and UV2450 UV-Vis spectrophotometer (HITACHI, Japan). HPLC-grade formate, acetate, lactate, -ketoglutarate, citrate, and succinate were purchased from Sigma (St. Louis, MO, USA). Analytical-grade potassium dihydrogen phosphate (KH PO) was obtained from Beijing Chemical Reagent Company. Ultrapure water (18.2 MΩ/cm) was prepared using a Milli-Q ultrapure water system (Millipore, USA).

1.2 Preparation of Standard Solutions and Calibration Curves

Individual stock solutions of formate, lactate, acetate, -ketoglutarate, citrate, and succinate were prepared at concentrations of 50–100 mmol/L. These were serially diluted 1, 5, 10, 100, 200, 500, and 1000-fold to prepare standard solutions of various concentrations. All solutions were filtered through 0.22 m disposable syringe filters before use. Individual organic acid standards were used to determine retention times, while mixed standards were used to construct calibration curves.

1.3 Establishment of the Analytical Method

Standard solutions of each organic acid were scanned across 200–600 nm. At 214 nm, all organic acids exhibited strong absorbance while glucose showed virtually no absorption, making this wavelength optimal for detection. Various concentrations of KH PO -H PO buffer (5, 10, 15, 20 mmol/L) were tested, with 5 mmol/L providing the best separation. At this concentration, buffer pH was adjusted to 1.6, 2.0, 2.4, and 2.7 using H PO , with pH 2.4 yielding optimal separation. Therefore, 5 mmol/L KH PO -H PO buffer (pH=2.4) was selected as the mobile phase with detection at 214 nm. After comprehensive optimization, the final HPLC conditions were: mobile phase of 5 mmol/L KH PO -H PO buffer (pH=2.4), flow rate 0.5 mL/min, detection wavelength 214 nm, column temperature 25 °C, and injection volume 20 L. Under these conditions, retention times were determined, calibration curves established, and detection/quantification limits calculated.

1.4 Determination of Precision and Recovery Rate

Mixed standard solutions of appropriate concentrations were injected five consecutive times under the established HPLC conditions. Peak areas were recorded to calculate precision. For recovery determination, a fermentation broth sample was divided into two portions: one analyzed directly for organic acid content, and the other spiked with mixed standards before analysis. Peak areas were recorded with three parallel measurements, and the experiment was repeated five times to calculate recovery rates.

1.5 Effect of Methanogen Co-culture on Anaerobic Fungal Metabolism

Anaerobic fungus (*Piromyces* sp.) and methanogen (*Methanobrevibacter thaueri*) co-cultures isolated from goat rumen fluid [5] were used as test strains. Chloramphenicol was added to eliminate methanogens for pure fungal culture. Culture medium was prepared according to Barichievich et al. [11]. Ten milliliters of 3-day-old culture was inoculated into 90 mL pre-warmed (39 °C) medium containing 4 g/L glucose as substrate and incubated statically at 39 °C for 3 days. Supernatant was collected according to Marin-Sikkema et

al. [12], aliquoted into 2 mL cryovials, and stored at -80 °C for substrate and metabolite analysis.

1.6 Analysis of Substrate and Metabolites

Glucose concentration in supernatants was determined using the glucose oxidase/peroxidase method (Nanjing Jiancheng Bioengineering Institute). For organic acid analysis, supernatants were centrifuged (12,000 r/min, 4 °C, 10 min), mixed with KH PO -H PO buffer (5 mmol/L, pH=2.4) at a 1:2 ratio, filtered through 0.22 m membranes, and analyzed using the established HPLC method. Ethanol concentration was determined according to Edgardo et al. [13] by gas chromatography using an Agilent 7890B system (Agilent, USA) with high-purity nitrogen as carrier gas (column head pressure 0.04 MPa), split ratio 100:1, KR-9 column (0.32 m × 30 m), FID detector, injector temperature 150 °C, column temperature 105 °C, and detector temperature 220 °C.

1.7 Data Processing

Data were initially processed using Excel 2007. Independent samples t-test in SPSS 16.0 was used to compare substrate and metabolite concentrations between pure and co-cultures. Results are expressed as mean ± standard error, with significance set at $P<0.05$.

Results

2.1.1 Determination of Retention Time, Standard Curve, LOD and LOQ

Retention times for each organic acid were determined using individual standards (Table 1). Analysis of mixed standards showed that all six organic acids were well separated within 30 minutes (Figure 1 [Figure 1: see original paper]). Calibration curves were constructed by plotting injection concentration (mmol/L) against peak area (mV · min). Linear regression analysis yielded correlation coefficients exceeding 0.999 for all six acids (Table 1). Detection limits (3× signal-to-noise ratio) and quantification limits (10× S/N) were calculated. -ketoglutarate showed the lowest detection limit (0.20 mol/L) and quantification limit (0.667 mol/L), while acetate showed the highest detection limit (1.00 mol/L) and quantification limit (3.333 mol/L) (Table 1).

2.1.2 Precision and Recovery Tests

Table 2 presents the precision test results. The relative standard deviation (RSD) of peak areas for all organic acids was below 6%, indicating good precision. Table 3 shows the recovery test results. Lactate exhibited the highest recovery rate (101.61%), while citrate showed the lowest (92.17%). These results demonstrate satisfactory recovery for all organic acids.

2.2 Effect of Methanogen Co-culture on Glucose Metabolism by Anaerobic Fungi

After 72 hours of incubation, glucose was completely utilized in both pure fungal cultures and fungal-methanogen co-cultures, with no significant difference between them (P>0.05). HPLC analysis revealed that methanogen co-culture significantly altered the metabolic profile of anaerobic fungi (Figure 2 [Figure 2: see original paper], Table 4). The primary water-soluble metabolites from glucose fermentation by anaerobic fungi were formate, lactate, and acetate. Co-culture with methanogens significantly decreased formate and lactate concentrations (P<0.05) while significantly increasing acetate concentration (P<0.05). No significant effects were observed on trace metabolites including succinate, citrate, -ketoglutarate, and ethanol (P>0.05).

Discussion

3.1 Establishment of Method for Detecting Six Organic Acids

High-performance liquid chromatography offers high column efficiency, good reproducibility, and convenient operation for simultaneous quantification of multiple compounds [15]. However, the complex composition of anaerobic fungal culture medium and metabolites can interfere with organic acid determination. During method development, numerous interfering peaks were observed near the formate target peak, compromising accurate quantification. Various parameters were systematically optimized to eliminate this interference, including buffer pH (1.6–2.7), buffer concentration (5–20 mmol/L), column temperature (15–30 °C), methanol proportion in mobile phase, and flow rate. The optimal conditions were identified as: buffer concentration 5 mmol/L, pH 2.0–2.4, column temperature 30 °C, and lower flow rates (which improved separation but excessively increased analysis time). This optimized combination maximally eliminated interference with formate detection.

Previous studies typically measured organic acids from anaerobic fungi using separate methods, which was time-consuming, labor-intensive, and exhibited low sensitivity and high error. Moreover, these methods focused on major metabolites like formate, acetate, and lactate, with less attention to trace metabolites such as succinate, citrate, and -ketoglutarate. No previous method has been reported for simultaneous detection of multiple organic acids from rumen anaerobic fungi. Formate has traditionally been measured using formate dehydrogenase assays [3,8], which involve expensive and unstable reagents, while lactate determination by gas chromatography requires derivatization [3], reducing accuracy. The present method achieved good separation of all six organic acids within 30 minutes, with recovery rates of 92.17%–101.61% and relative standard deviations below 10%. Notably, the mobile phase concentration (KH PO - H PO buffer) was lower than that reported in other studies [14-15], minimizing adverse effects of high salt on the chromatographic system. The method also features simple sample preparation without toxic reagents, making it fully suitable

for organic acid determination in anaerobic fungal fermentation broth.

3.2 Metabolites and Metabolic Pathways of Anaerobic Fungal Glucose Fermentation

Anaerobic fungi perform mixed-acid fermentation of glucose, producing primarily formate, acetate, ethanol, lactate, CO , and H [2-3]. Unlike aerobic fungi, anaerobic fungi lack mitochondria but possess hydrogenosomes—membrane-bound organelles approximately 1 m in diameter that generate ATP through substrate-level phosphorylation under anaerobic conditions [12]. Carbohydrate metabolism occurs in both the cytoplasm and hydrogenosomes: the cytoplasmic pathway produces lactate, ethanol, and formate, while the hydrogenosomal pathway generates H , CO , acetate, and formate [16]. In this study, the dominant metabolites in pure fungal culture were formate, lactate, and acetate, with lactate showing the highest concentration, indicating that a substantial portion of carbohydrate was metabolized in the cytoplasm.

Cheng et al. [10] reported that anaerobic fungi possess an incomplete tricarboxylic acid (TCA) cycle comprising both oxidative and reductive branches, with -ketoglutarate and succinate as respective end products. The detection of -ketoglutarate and succinate in the fungal supernatant in this study confirms the findings of Kwon et al. [9] and Cheng et al. [10].

3.3 Effect of Methanogen Co-culture on Anaerobic Fungal Glucose Metabolism

In co-culture with methanogens, the major metabolites of anaerobic fungi were lactate and acetate, with acetate being the most abundant. Our previous research demonstrated negligible hydrogen accumulation in fungal-methanogen co-cultures, whereas significant H accumulated in pure fungal cultures [6]. *Methanobrevibacter* species are hydrogenotrophic methanogens that utilize H or formate to reduce CO to methane, with minimal acetate utilization [17]. Therefore, this study demonstrates interspecies hydrogen transfer between anaerobic fungi and methanogens. In the presence of *Methanobrevibacter*, H and formate produced by anaerobic fungi are rapidly consumed by methanogens, relieving inhibition of hydrogenase by H [18] and pyruvate:formate lyase by formate [19]. This enhances hydrogenosomal metabolic flux and increases ATP production, which is accompanied by acetate generation [20-21]. The significantly higher acetate concentration in co-culture supernatant indicates that more carbohydrate was metabolized through the hydrogenosomal pathway in co-culture. Hydrogenosomes contain hydrogenases that catalyze NADPH oxidation to NADH and H , while lactate production requires NADH [4]. Reduced NADH availability consequently decreases lactate production, explaining the significantly lower lactate concentration in co-culture. No significant differences were observed in succinate, citrate, and -ketoglutarate concentrations between co-culture and pure culture, likely because carbon flux through the TCA reductive and oxidative branches was minimal and the

substrate (glucose) was completely consumed by both systems. Future work will utilize the established method to determine organic acid concentrations during dynamic fermentation processes to further investigate the effects of methanogen co-culture on anaerobic fungal metabolism.

- The primary organic acids produced by anaerobic fungi from glucose metabolism were formate, lactate, and acetate, with trace amounts of succinate, citrate, and -ketoglutarate.
- Methanogen co-culture had no significant effect on succinate, citrate, -ketoglutarate, and ethanol concentrations, but significantly reduced formate and lactate concentrations while significantly increasing acetate concentration.

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