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Carbohydrate analysis of fermentation broth by high-performance liquid chromatography utilizing solid-phase extraction

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ABSTRACT

Nutrient information concerning the specific needs in fermentation broth is crucial to the potency and production of an antibiotic. Nutrient analysis of carbohydrate metabolism by HPLC is hindered by an array of fermentation broth interferences. These interferences can be effectively removed by solid-phase extraction. The fermentation broth was diluted with an equal amount of water and vortexed. The sample mixture was eluted through an activated Ace11 QMA solid-phase cartridge. The resultant eluent was diluted with an equal amount of a acetonitrile–ethanol (75:25) mixture, vortexed and filtered. The sample extract was chromatographed on a YMC polyamine column using refractive index detection. The mobile phase consisted of acetonitrilewater (75:25) at a flow-rate of 1 ml/min. Under these conditions, glucose, fructose, sucrose and maltose were separated in less than 13 min with baseline resolution. This method is capable of monitoring the carbohydrate consumption and metabolism throughout the fermentation process.

INTRODUCTION

Nutrient information concerning the specific needs of microorganisms in fermentation broth is crucial to the potency and production of an antibiotic [1]. Two nutritional factors essential to microbial activity are a source of energy for cellular metabolic processes and a source of starting components for cellular synthesis. Carbohydrates are excellent cources of carbon, oxygen and hydrogen, and metabolic energy for many microorganisms. They are available as simple sugars or sugar polymers such as starch, dextrins, cellulose or hemicellulose. Since the biomass is typically 50% carbon on a dry weight basis, carbohydrates are frequently present in the media in concentrations higher than other nutrients and can account for about 20% of the total nutrient media [2].

The microbial environment is largely determined by the composition of the growth media. The preferred media in an industrial fermentation setting is a complex or natural medium [3]. This media uses ingredients of natural origin and is not completely defined chemically. Although all carbohydrates have an empirical formula of $(CH_2O)_n$, they are not equally available to microorganisms. In general, availability may be ranked as hexoses > disaccharides > pentoses > polysaccharides. Many microorganisms can grow well on a variety of carbohydrates, yet the yield of the product may be strongly dependent on the source. This is demonstrated by the microorganism ***Cephalosporium acrimonium*** in which glucose favors cell growth, galactose maximizes antibiotic concentration and sucrose optimizes antibiotic yield per cell [4]. Industrial fermentation broth contains a host of natural ingredients, such as soy and cottonseed flours, peanut and corn meals, molasses, starch, beef extract and lard oil, as well as the starting culture. It is crucial to the production and potency of an antibiotic to determine these nutritional characteristics before selecting a carbohydrate source for the cultivation of a specific

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species. Determination of the carbohydrate needs of a microorganism is accomplished by monitoring the concentration of carbohydrates in the growth media during the microorganism lifetime. Through aerobic respiration, these starting materials undergo enzymatic and chemical degradation which result in numerous metabolic by-products which can interfere with chromatographic analysis. These fermentation broth matrix interferences can be effectively removed by the use of solid-phase extraction (SPE) enabling carbohydrate analysis of the broth by high-performance liquid chromatography (HPLC). SPE is a rapid and reproducible sample preparation technique that allows the SPE eluent to be injected immediatley into the HPLC system without further preparation. The main advantages of SPE include small sample and solvent volumes, extended column life, shorter sample preparation times and better recovery levels [5,6]. This paper will describe the sample preparation technique and HPLC analysis for glucose, fructose, sucrose and maltose in fermentation broth.

EXPERIMENTAL

Materials

HPLC grade acetonitrile, ethanol, isopropyl alcohol and water (Burdick and Jackson, Muskegon, MI, USA) were used for extraction and chromatographic analysis. Biochemical reagent grade glucose, fructose, sucrose and maltose (J. T. Baker, Phillipsburg, NJ, USA) were used for standard and system suitability preparations. SPE cartridges were Ace11 Plus QMA Sep-Paks (Waters Assoc., Milford, MA, USA). Syringes (Becton Dickinson and Company, Rutherford, NJ, USA) were used for sample delivery into the SPE cartridges.

Chromatographic system

The chromatographic system was equippped with a constant flow, low pulse pump (Shimadzu LC-600, Kyoto, Japan) a refractive index detector (Shimadzu RID-6A), an autosampler (Shimadzu SIL-9A) and an integrator (Shimadzu CR-501). The separations were performed on a polyamine column [250 × 4.6 mm, I.D., 5 μm, (YMC, Morris Plains, NJ, USA)]. The detector and column were maintained at 35°C. The mobile phase consisted of acetonitrile-water (75:25, v/v) at a flow-rate of 1 ml/min. The mobile phase was filtered and degassed. The injection volume was 50 μl.

Sample prepuration

SPE cartridges were previously wetted and activated by flushing the cartridge with 2-4 ml of methanol followed by 2-4 ml of water. Two ml of fermentation broth were mixed with an equal amount of water and rapidly agitated (vortex) for 1 min. The mixture was centrifuged for 5 min at 14 000 rpm (1600 g) using an ultracentrifuge (Eppendorf Centrifuge 5415C, Brinkman Instruments, Westbury, NY, USA). The resulting supernatant was transferred into a syringe with an activated SPE cartridge at its tip. The supernatant was slowly forced through the SPE cartridge at an approximate rate of 1 ml/min. The first milliliter that eluted was discarded. A volume of 1 ml of the eluent was accurately pipetted and added to 3 ml of a acetonitrile-ethanol (75:25, v/v) mixture and rapidly agitated (vortex) for 1 min. A precipitate was formed. The mixture was filtered through a 0.45-μm polypropylene filter disc. The resultant eluate was ready for HPLC analysis.

Standurd and system suitability preparation

Standards were prepared from the individual sugars (glucose, fructose, sucrose and maltose) by dissolving an accuratley weighed amount into a 100-ml volumetric flask and diluting with mobile phase. The concentration of the stock solutions of the sugars was 1 mg/ml. Dilutions were made accordingly. The system suitability solution contained the aforementioned sugars at approximate concentrations of 0.1 mg/ml.

Fermentation media

Defined media can be prepared by the procedure outlined by Vogel and Bonner [7] or Davis and Mingioli [8]. The end products of fermentation depends on the type of bacteria used and the nature of the fermentation media *(i.e.,* wine, beer, yogurt, yeast, antibiotics etc.)

Defined media [7] is prepared by dissolving 10 g of magnesium sulfate, 100 g citric acid, 500 g dipotassium hydrogenphosphate, 175 g ammonium sodium phosphate in 670 ml of water. This mixture represents the nutrient concentrate. Final medium is made by aseptically adding 1 ml of nutrient con-

centrate to 49 ml of a sterilized solution of 3% corn steep, 3% glucose, 4% soybean meal and 2% lard oil.

RESULTS AND DISCUSSION

The chromatographic procedure was validated for precision (n = 6) (<2.0%), accuracy (99%), linearity (10^4), recovery (99%) minimum detectable quantity (10^{-8}) and minimum quantitable quantity (10^{-7} g) for glucose, fructose, maltose and sucrose. Fig. la illustrates the ability of the chromatographic system to separate the sugars present in the system suitability mixture. Elution order is fructose (t_R = 7.1 min), glucose (t_R = 8.5 min), sucrose (t_R = 10.9 min) and maltose (t_R = 12.5 min). There is excellent resolution (R_s > 1.25) for the sugars examined. SPE recovery efficiencies were performed with the system suitability solution and spiked fermentation starting broth. Recoveries were greater than 99% (99.87% ± 0.73% system suitability, 99.22% ± 2.13% spiked broth). A calibration curve for the sugars is linear (r > 0.999) in the concentration range 0.001-l mg/ml.

Fig. 1b–e depict fermentation chromatograms at fermentation times of 0, 6, 12 and 18 h. This chromatographic procedure is capable of identifying and quantifying these sugars during the fermentation cycle. The relative concentrations of these sugars can be plotted as a function of fermentation cycle time. The carbohydrate demands of the microorganism undergoing fermentation can be optimized by such plots by noting the metabolic consumption and production patterns of the various carbohydrates in relation to antibiotic production. At specific times during the fermentation process, carbohydrates can be spiked into the broth to enhance the growth or the production phases.

Fig. 2 compares extraction of SPE with an isopropyl alcohol liquid extraction at 5 and 17 h. (Isopropyl alcohol was chosen for its ability to extract the carbohydrates and precipitate the proteinaceous biomass simultaneously. An equal amount of isopropyl alcohol and fermentation broth were mixed and then shaken for 30 min in a wrist shaker. The mixture was centrifuged and filtered.) As illustrated in Fig. 2, SPE provides an excellent means of effectively removing the hydrophobic matrix interferences that exist in fermentation media. Comparisons of the sample preparation techniques clearly demonstrates that SPE will minimize extraneous peaks and interferences and maintain baseline integrity better than solvent extraction. These hydrophobic interferences are due to the chemical and

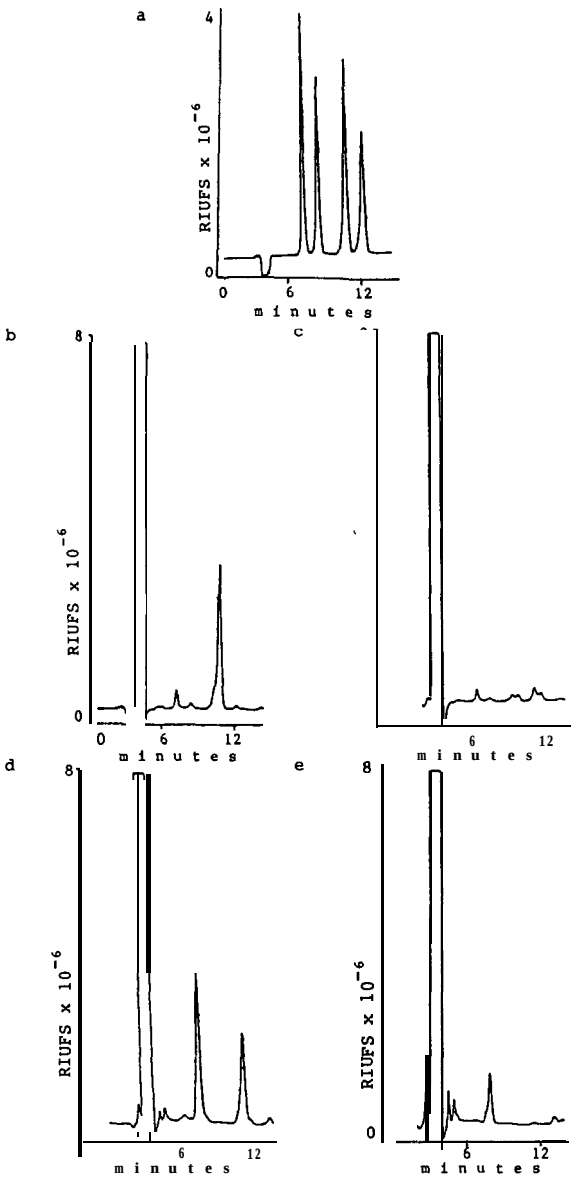

Fig. 1. Chromatograms of carbohydrate system suitability (a) and SPE extracts of fermentation broth (b–e) at 0, 6, 12 and 18 h of a fermentation cycle. Elution order: fructose (t_R = 7.1 min), glucose (t_R = 8.5 min), sucrose (t_R = 10.9 min), maltose (I, = 12.5 min). RIUFS= Refractive index units full scale.

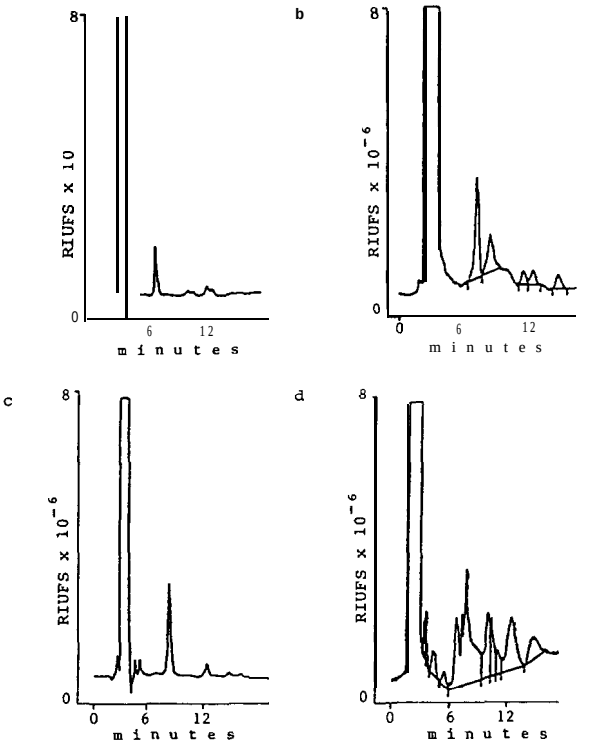

Fig. 2. Comparison chromatograms of SPE and and isopropyl alcohol solvent extraction of fermentation broth. (a) and (c) are broth extracts at 5 and 17 h using SPE. (b) and (d) arc broth extracts at 5 and 17 h using isopropyl alcohol liquid extraction.

enzymatic by-products of fermentation metabolism generally consisting of crude proteins and amino acids, waxes, sterols, phospholipids, pigments, vitamins, minerals, soluble gums and other related nutrients needed for fermentation. Eliminating these analytical interferences becomes increasing important during the fermentation cycle. As aerobic respiration of the microorganism proceed, the concentration of metabolic by-products increase and can interfere with identification and quantitation of the sugars as depicted in Fig. 2d. SPE provides other advantages for the chromatographic system. Column cleanup and maintenance is minimized as well as injector reproducibility and clogging.

Fermentation broth consistencies can vary from a watery consistency to a mud-like sludge. This is usually a function of the extent of fermentation and processing. Accuracy of this procedure is dependent upon proper sampling, dilution and resuspendability of the fermentation broth. Failure to perform these may result in inadequate extraction, clumping and/or SPE overload.

This procedure will provide a rapid and accurate method for assessing carbohydrate needs and consumption patterns of microorganisms in fermentation media. This procedure can also discern other sugars, such as lactose, galactose, xylose, pyranose, inositol, and mannitol that may be required for other types of fermentation processes. Elucidation of these carbohydrate nutrient patterns will enable fermentation engineers to define their complex natural media, allowing adjustments to the broth using scientific judgement rather than inherited art. Identification of the metabolic processes and their nutrient needs will enhance the potency of industrial antibiotics production.

CONCLUSIONS

HPLC analysis of carbohydrates using SPE provides a rapid and reliable means in which to analyze individual sugars in fermentation media. SPE provides an excellent means to remove fermentation broth matrix interferences, allowing a clear interpretation of the chromatographic results. SPE provides several chromatographic advantages over traditional solvent extraction that result in less interferences, simultaneous elution and baseline shifting as well as maintaining column and chromatographic system integrity. The chromatographic assay is rugged and reliable allowing detection of carbohydrates in the fermentation extract throughout the microorganism's lifetime. This information can be used to optimize the microorganism's growth and subsequent antibiotic production in the fermentation broth since the simple sugars are the preferred carbon and energy source.

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