Microchemical Journal 140 (2018) 232–240

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Rapid detection of hydrogen sulfide produced by pathogenic bacteria in focused growth media using SHS-MCC-GC-IMS

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ARTICLE INFO

Keywords:
Hydrogen sulfide
Pathogenic bacteria
Gas chromatography
Ion mobility spectrometry

ABSTRACT

A new rapid method for the detection of hydrogen sulfide from pathogenic bacteria is reported. The developed method, static headspace – multi-capillary column - gas chromatography - ion mobility spectrometry (SHS-MCC-GC-IMS), has been applied to detect hydrogen sulfide evolution from 61 bacteria. The developed method has been compared against a standard triple sugar iron (TSI) agar approach, and a modified single sugar iron (SSI) agar approach. Hydrogen sulfide detection by SHS-MCC-GC-IMS using an initial inoculum of 1–1.5 × 10^5 CFU/mL can be achieved within 6 h, after incubation at 37 °C, with a limit of detection of 1.6 ng/mL. Data for the standard agar method against the new instrumental approach, and the modified agar method against the new instrumental approach, are compared. The specificity for the new method compared against the standard method and the modified agar approach across all 61 strains was 85.2% and 88.5% respectively, and 86.7% and 91.3% across the 23 Salmonella strains tested.

1. Introduction

Hydrogen sulfide (H_2S) is a volatile compound with a characteristic rotten egg odour at low concentrations, and is commonly associated with bacterial contamination of food and water sources, particularly involving bacteria of the family *Enterobacteriaceae* [1]. It is one of the earliest volatile compounds identified as a product of microbial decomposition. One of the first documented studies of microbial H_2S was published in 1875 [2]. The Doctoral Thesis [2] primarily focused on examining H_2S production from undefined microbes associated with chicken egg spoilage using lead acetate paper as the method of detection. Further work, during the same period, conducted by Orlowski (1895) [3] described H_2S production by *Typhoid bacillus*; according to current taxonomy this strain is likely to be a sub-species of *Salmonella enterica*. Further studies have examined H_2S production by *Salmonella* and other putrefactive organisms isolated from contaminated soil and faeces, and have been instrumental in the improvement of sanitation procedures for public drinking water [4–6]. Production of H_2S is particularly prevalent in members of the Gram-negative *Enterobacteriaceae* family, and is particularly associated with the *enterica* sub-species of the genus *Salmonella* [7]. However, H_2S has also been positively identified in *Citrobacter* spp., *Proteus* spp., *Edwardsiella* spp. [7], as well as in the non-*Enterobacteriaceae* Gram-negative bacterial genus *Shewanella,* which are often involved in marine carrion cycles [8]. The most infamous members of the *Enterobacteriaceae* family, *Escherichia coli* serotypes, are generally accepted as H_2S negative according to current testing methods [9]. However, many studies have shown positive H_2S production from *E. coli* strains isolated from various backgrounds. For example Lautrop et al. isolated 26 different H_2S positive *E. coli* strains from 25 different patients over a period of 9 months [10], a similar situation was also reported by Maker et al. [11] Furthermore, Magalhaes et al. [12] isolated positive H_2S producing *E. coli* strains from swine livestock. Clearly there is much contradiction throughout the literature regarding the H_2S production status of many bacteria, which when combined with a lack of recent studies into bacterial H_2S production, leads to a potentially misrepresented consensus.

Microbial sulfate reduction has been identified as one of the earliest complex biological pathways to develop, with isotopic sedimentary data indicating its emergence as early as 3.47 billion years ago [12]. Production of H_2S by microbes is a by-product of microbial anaerobic respiration, where sulfate is used in place of oxygen as a terminal electron acceptor [13]. Hydrogen sulfide production is highly variable throughout microorganisms at multiple taxonomic ranks, and there are multiple production pathways dependent on the type and concentration of the sulfur source present in their immediate environment. The main sources utilised by microbes for H_2S production are the sulfur containing amino acids cystine and cysteine, and thiosulfate. Hydrogen sulfide production can also be achieved through utilisation of

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https://doi.org/10.1016/j.microc.2018.04.026
Received 26 March 2018; Received in revised form 25 April 2018; Accepted 25 April 2018
Available online 27 April 2018
0026-265X/

tetrathionate, sulfite, and sulfate, however this is less prevalent [14]. Cystine and cysteine are generally acquired through protein decomposition, whereas other sulfur containing compounds such as thiosulfate are generally found in anaerobic environments containing decaying organic matter, primarily soils and sea/river beds [15].

The pathway for cysteine utilisation for H_2S production has been somewhat explored, with cysteine desulfhydrase identified as the enzyme responsible [14], resulting in the formation of pyruvic acid, ammonia, and H_2S, which is then liberated as a gas [16]. However, there is also evidence that H_2S may be induced in response to excess cysteine as a protective mechanism against toxicity [17–19]. The enzyme responsible for thiosulfate utilisation has been identified as thiosulfate reductase, which reduces thiosulfate to sulfite and gaseous H_2S [20,21].

Current tests employed for the detection of bacterial H_2S tend to rely on nutrient rich growth media supplemented with a sulfur source, usually including the addition of sodium thiosulfate, cystine, or cysteine hydrochloride, to induce significant production of H_2S [14]. These media are also combined with a visible colour change following incubation; usually facilitated via metallic salts, such as, ferric ammonium citrate or lead acetate, which forms a black precipitate with H_2S [7,11]. One of the primary drawbacks of these methods is the subjective nature of the visual colour interpretation, which combined with relatively low sensitivity has meant that current methods have little application outside of differential taxonomic testing. This paper proposes a new method for the rapid and sensitive detection of H_2S using static headspace – multi capillary column - gas chromatography - ion mobility spectrometry (SHS-MCC-GC-IMS), with potential future application for detection of bacterial contamination in food and water sources, as well as detection of bacteria within various human clinical samples.

2. Experimental

2.1. Materials and reagents

Meat extract, yeast extract, bacteriological peptone, lactose, tryptone soya agar, sodium sulfide (97%), hydrochloric acid, and Triple Sugar Iron agar were purchased from Sigma-Aldrich (Dorset, UK). Sucrose, dextrose, sodium chloride, and sodium thiosulfate were purchased from Melford Laboratories Ltd. (Ipswich, UK).

2.2. Instrumentation

A static headspace-multi-capillary column-gas chromatography-ion mobility spectrometer (SHS-MCC-GC-IMS) manufactured by G.A.S.-Gesellschaft für Analytische Sensorsysteme mbH (Dortmund, Germany), was used [22,23]. The instrument was fitted with an automatic sampler unit (CTC-PAL; CTC Analytics AG, Zwingen, Switzerland) and a heated gas-tight syringe. A multi-capillary column (MCC) (Multichrom, Novosibirsk, Russia) was used for the chromatographic separation. The MCC comprised a stainless steel tube, 20 cm × 3 mm ID, containing approximately 1000 parallel capillary tubes, 40 µm ID, coated with 0.2 µm film thickness of stationary phase (Carbowax 20 M). Atmospheric pressure ionisation is generated by a Tritium (^3H) solid state bonded source (β-radiation, 100–300 MBq with a half-life of 12.5 years). The IMS has a drift tube length of 50 mm. Separation in the IMS drift tube is achieved by applying an electric field of 2 kV to the ionised volatiles in a pulsed mode using an electronic shutter opening time of 100 µs. The drift gas was N_2 (99.998%) with a drift pressure of 101 kPa (ambient pressure). Samples were run under the following operating conditions: incubation conditions (time, 3 min; and, temperature, 37 °C); MCC-IMS conditions (syringe temperature, 50 °C; injection temperature, 80 °C; injection volume, 2.5 mL; column temperature, 40 °C; and, a column carrier gas flow programme rate, 5 mL/min with IMS conditions (temperature, 50 °C; and, drift gas flow rate, 500 mL/min). The total analysis time was 5 min. All data was acquired in the negative ion mode and each spectrum is formed with the average of 12 scans. All data are processed using the LAV software (version 1.5.1, G.A.S). The experimental procedure has previously been reported for analysis of VOCs from bacteria [22,23].

2.3. Preparation of H_2S standards

Initially, nitrogen gas was continuously bubbled through 0.01 M aqueous hydrochloric acid solution for 30 min to expel any dissolved oxygen within the acid, as oxygen interferes with the generation of gaseous H_2S. Then, H_2S standards were prepared by dissolving 0.01 g (accurately weighed) of sodium sulfide in 100 mL of the previously prepared 0.01 M HCl solution, liberating H_2S gas to a stock concentration of 0.1 mg/mL (100 µg/mL). From this stock solution, 1 mL was added to the previously prepared 0.01 M HCl solution to create a 1 µg/mL working solution. Using the working solution further dilutions were made in the concentration range 5 to 500 ng/mL and analysed via SHS-MCC-GC-IMS. Control samples of the 0.01 M HCl were run during the analyses of the standards, alongside TSI broth samples, to allow for blank subtraction. In addition, H_2S standards of 20, 40 and 60 ng/mL were prepared daily and ran on every test sampling day to compensate for any potential instrumental variance.

2.4. Microbiology

Bacteria used in this study were acquired from numerous sources, and are predominantly strains acquired from the National Collection of Type Cultures (NCTC) (Salisbury, UK) or other culture collections. Further wild and type culture strains were kindly provided by the Freeman Hospital, Newcastle UK, many of which were isolated from routine patient samples. Wild type *Escherichia coli* strains CPE 14/15/20 and ES 17/20 were named so due to their antimicrobial resistance profiles (CPE = carbapenemase producing *enterobacteriaceae,* ES = Extended spectrum β lactamase). All bacterial strains used are shown Table 1, along with their identification number where applicable. The majority of the bacterial strains used in this study were of the family *Enterobacteriaceae,* which were selected due to their high association with human pathogenicity, and also because of their role in food and water contamination [24]. Furthermore, many of the bacteria were selected due to their relevance regarding human pathogenicity and antimicrobial resistance, such as *Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter* spp., organisms which are particularly relevant due to their status as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter species*) pathogens [25,26].

2.5. TSI broth preparation

To prepare the TSI broth, 1 g of dextrose, 10 g of lactose, 10 g of sucrose, 3 g of meat extract, 3 g of yeast extract, 20 g of bacteriological peptone, 5 g of sodium chloride, and 0.3 g of sodium thiosulfate were dissolved in 1 L of distilled water and subsequently sterilized via an autoclave at 121 °C for 15 min. The recipe used for this TSI broth mimics the TSI Agar recipe (Sigma-Aldrich, product code: 92499), with the omission of agar, phenol red, and ferric ammonium citrate.

2.6. Agar slopes preparation & procedure

To prepare the TSI agar slopes, 64.6 g of the TSI agar powder (Oxoid, Basingstoke, UK) was added to 1 L of deionised water (Milli-Q, Integral 3, 18 MΩ cm) and brought to boil using a hot plate with a built-in magnetic stirrer. SSI agar slopes were prepared using an identical composition to that of TSI agar slopes, with the omission of sucrose and lactose. For both agar types, 7 mL of the freshly boiled agar mixture was then aliquoted into 20 mL headspace vials (with lids loosely screwed on) and subsequently autoclaved at 121 °C for 15 min to achieve