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Tunable diode laser absorption spectroscopy as method of choice for non-invasive and automated detection of microbial growth in media fills

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ABSTRACT

Tunable diode laser absorption spectroscopy (TDLAS) was evaluated on its potential to detect bacterial growth of contaminated media fill vials. The target was a replacement/ automation of the traditional visual media fill inspection. TDLAS was used to determine non-invasively O_2 and/or CO_2 changes in headspaces of such vials being induced by metabolically active microorganisms. Four different vial formats, 34 microorganisms (inoculation volume < 10 cells) and two different media (TSB/FTM) were tested. Applying parallel CO_2 and O_2 headspace measurements all format-organism combinations were detected within < 11 days reliably with reproducible results. False negatives were exclusively observed for samples that were intentionally breached with syringes of 0.3 mm in diameter. Overall it was shown that TDLAS functionality for a replacement of the visual media fill inspection is given and that investing in further validation and implementation studies is valuable. Nevertheless, some small but vincible challenges remain to have this technology in practical use.

1. Introduction

Aseptic manufacturing is periodically evaluated by aseptic process simulations using microbiological culture media substituting the sterile product bulk-solution (i.e. typically tryptic soy broth [TSB] is used for aerobic micro-organisms, less frequently fluid Thioglycollate [FTM] for (facultative) anaerobic strains and finally vegetable peptone broth [VPB] where there is concern about prions). This simulation is designated as “media fill” and provides supportive data on the capability and reliability of aseptic processing activities. It is also used to qualify sterile working-techniques of aseptic cleanroom operators. In addition, media fills may identify potential weaknesses with regard to microbiological contamination during production line modification and qualification or during investigations of microbiological deviations (e.g. positive sterility test). The industrial standard in analyzing media filled units is the visual inspection (VI) performed by qualified human operators who check for turbidity increase and abnormality in the aspect of media [1,2]. Regulations and guidelines [3,4] specify that such inspections must be performed at least once after no less than 14 days with media filled units that have been incubated for minimally 7 days at 20–25 °C and another 7 days at 30–35 °C. Several manufacturers perform an intermediate VI after the first incubation period lasting 7 days since fast growing microorganisms would have grown and become visible by then. VI ensures good manufacturing practices (GMP) and contributes to a high standard of patient safety but is associated with high workload related to manual vial and data handling. Furthermore, the detection of medium turbidity by human eye may be prone to error, and requires regular qualification of operators. All this results in unnecessary cost.

Alternative non-invasive methods with the potential for media fill inspection automation could reduce cost, increase process efficiency, and decrease the risk of false negative media fill units (i.e. not detecting microbial growth) by eliminating the human factor. First advancements and proposals in this field were recently made [5,6]. Tunable Diode Laser Absorption Spectroscopy (TDLAS) was evaluated in determining non-invasively microbial growth related O_2 and CO_2 concentration changes in sealed (non-breached) media fill vial headspaces. Microorganisms in TSB were tested on their CO_2 emission and O_2 consumption. In addition, TDLAS data was compared to VI, optical density measurements and isothermal micro-calorimetry (IMC) to

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conclude on the sensibility of TDLAS and assure that gathered O_2/CO_2 data was growth related. Tested microorganisms were detected without observing false negatives and results were reproducible when using a combined O_2 and CO_2 TDLAS measurement. Method development studies for TDLAS including the analysis of a wider range of microorganisms in 2 different media, stressed microbial cells (having normally a prolonged lag phase), additional format sizes and breached containers (headspace equilibration with environment leading to false negative results) are outlined in this work. The findings shall provide the basis for method implementation and validation in sterile drug product manufacturing.

2. Materials and methods

2.1. Basic study design

Two production sites dealing daily with aseptic production and media fill inspection were involved in the study (site X: *F. Hoffmann-La Roche* (Wurmisweg, 4303 Kaiseraugst, Switzerland), and site Y: *Novartis* (Schaffhauserstrasse 101, 4332 Stein, Switzerland)). Media fill incubation times were set to 7 days at 20–25 °C and 7 days at 30–35 °C (overall incubation time > 336 h) and kept during the experiment to allow a comparison of growth detection by VI and TDLAS.

Next to the standard pharmacopoeia (PhEur) reference strains, several plant isolates (i.e., local isolates) from site X and Y (selection based on relative occurrence during routine monitoring in cleanrooms, unpublished internal sources), worst-case organisms (i.e., organisms with little metabolic activity, known long lag phase) and microorganisms with homofermenting characteristics (reduced O_2/CO_2 consumption/release) were included in this study (see Table 1 for strain details).

Both sites used benchtop TDLAS analyzers (Lighthouse Instruments, Charlottesville) with integrated single pass laser beams able to determine O_2 (λ=762 nm) and CO_2 (λ=2000 nm) concentrations respectively. Prior to use analyzers were warmed-up for at least 30 min and then calibrated with attested standards (20%, 0% of the respective format) supplied by the manufacturer. Standards were made of the same glass as experimental containers to keep measurement noise minimal. The experiments started as soon as the measurement chamber was flushed with nitrogen (4 l/min) and the analysis of O_2/CO_2 reference standards (4%, 8%) was inside limits of defined specification (± 0.25%).

3. Preparation of working suspensions

Despite small site specific differences in preparing the microbial working suspension, the main steps were identical. Both sites acquired the PhEur organisms from ThermoFisher Scientific in form of lyophilized cultures adjusted to an approximate cell count of < 100 colony forming units (CFU) per 0.1 ml. Fungi (yeast and mold), homofermenting, aerobic and anaerobic microorganisms were either obtained from the site internal microbial strain collection (plant isolates) or from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). Plant isolates and DSMZ strains were cultured twice at 30–37 °C in tryptic soy broth (or blood agar) to obtain suspensions with unstressed and viable organisms. By using 0.2 ml of these suspensions their cell-density was determined on appropriate media (tryptic soy agar, angular agar or blood agar). After cultivation the suspensions were diluted in sodium chloride-peptone buffer and enriched with 15% glycerin to obtain an appropriate inoculum size (working suspension: site X < 10 CFU in 0.1 ml; site Y < 20 CFU in 0.1 ml) and stored at less than −80 °C. The working suspensions of the eight PhEur organisms were created by dissolving the lyophilisates in Remel buffer being followed by a dilution (1:10) in sterile water according the desired CFU count. In addition site Y created suspensions of heat stressed microorganisms (see Tables 1, 2). The suspensions were exposed to a water bath (T=50–70 °C) during maximally 1–2 min, depending on the test microorganism. An additional experiment for *Corynebacterium afermentans* was executed by adding 0.1% of the detergent Tween80 to TSB media, acting as growth stimulant by increasing the lipophilicity of TSB [7].

3.1. Sample preparation and testing

At *site X* experimental media filled containers were either prepared manually (manual filling, stoppering and capping under aseptic conditions), or directly taken from inspected media fill batches. As gas concentrations in vial headspaces were known to change over time due to media related oxidation processes [5], the headspace of each sample unit was flushed prior inoculation with sterile compressed air until CO_2/O_2 concentrations were comparable to those in atmospheric air. This “headspace flushing” was intended to simulate the conditions prevailing in newly produced media fill units. Experimental containers of site Y were prepared comparably. Filling volumes amounted for FTM (fluid thioglycollate media) filled units to 20% and for TSB filled units to 35–60% of the respective format capacity. Filling levels were defined according internal standards following the regulatory requirement that all inner vial surfaces get in contact with media when units are moved [8].

Samples were then inoculated by injecting 0.1 ml of bacterial suspension through the self-sealing rubber stopper using a syringe (Microlance BD, 0.8 mm in diameter). Site X preparations were incubated for 7 days at 20–25 °C and for another 7 days at 30–35 °C. CO_2 and O_2 measurements were performed every 12 ± 2 h until changes in headspace concentrations remained minimal. After this time-point the measurement interval was increased to 24–48 h. Each organism and format was evaluated by 10 inoculated replicates. At site Y samples were incubated for 7 days at 20–25 °C and 10 days at 30–35 °C and mainly tested on CO_2 production. Besides start- and end-point measurements at least 3 additional CO_2 measurements were performed per week. The sample size per organism consisted of a total of 2 independent test runs with minimally 3 independent replicates. At every time point of a TDLAS measurement the visual inspection was performed in parallel to check for an increase in media turbidity.

Detection thresholds for each format and media combination were needed to identify the time point of growth detection. Medium induces changes in vial headspaces which are related to TSB auto-oxidation and CO_2 degassing effects [5,9]. Site X evaluated 42 blank 2 ml, 20 ml and 2×50 ml (TSB media from different producers) TSB vials for CO_2 and O_2 concentration change in the vial headspace every day (see Fig. 1). Their filling volumes were identical to those mentioned before. At site Y, 50 blank 10 ml vials filled with 2 ml TSB and 20 blank 10 ml vials filled with 6 ml FTM were used to determine every 3–4 days the respective media induced variation in CO_2 concentration (see Fig. 2). The age of media was between 1 and 120 days and originated either from Merck Millipore (50 ml vials), Sifin (10 ml vials), Becton Dickinson & Company (2 ml and 20 ml vials) or Oxoid (10 ml FTM vials) (see Table 3).

4. Design of leakage study

CO_2 and O_2 headspace concentrations were analyzed in intentionally breached vials to evaluate if growth detection remained possible over 14 days. The aim was to assess if leakage related O_2 inflow or CO_2 escape altered headspace concentrations exposed to microbial activity strong enough to produce false negative results. Samples were prepared and tested as described above but punctured through the gum stopper using a stainless steel needle of ~0.25 mm inner diameter imitating a worst case leakage. Site X used ten TSB filled 2 ml, 20 ml and 50 ml vials inoculated with the respective organism and tested their CO_2 and O_2 concentrations over time. Site Y used per (stressed) organism at least 15 replicates and investigated their CO_2 profiles in 10 ml TSB/FTM filled vials (Table 2). Microorganisms in this study

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