DEVELOPMENT OF A LOW COST OPENSOURCE AUTOMATED WORKFLOW FOR MINIATURIZED FERMENTATION

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This study presents the development and validation of a low-cost, open-source automated workflow for miniaturized fermentation. The workflow aims to empower laborious large sample experiments by providing an accessible alternative to low access automation and miniaturization equipment and consumables. Using the OT-2 liquid handler to automate and a three-layer system to reduce evaporation, the study compared bacterial growth rates in deepwell plates and Erlenmeyer flasks. Results showed similar growth rates across different volumes, with the 400µL working volume and 20µL sampling volume being optimal. The system demonstrated effective automation and gas transfer, though future work is needed to reduce measurement errors and enhance growth rates. This method holds potential for applications in adaptive laboratory evolution and bioprocess optimization.

Keywords: Automation. Miniaturization. Fermentation.

1 INTRODUCTION

Automation can significantly enhance the number of experiments and conditions to be tested in a round of experiments. One of the key requirements for effective automation is the miniaturization of experiments. Miniaturization ensures that the experimental conditions are similar to those at the flask or bioreactor scale, thereby maintaining the relevance and applicability of the results. When used properly, automation aided miniaturized methods hold the potential to unlock new possibilities in biotechnology field by empowering time consuming and laborious techniques such as bioprocess optimization and strain engineering by adaptive laboratory evolution.

Despite de numerous advantages, miniaturization and automation often requires expansive equipment and specialized materials, such as Biolector (Microbioreactor system), Hamilton Star, Tecan Evo etc. Low-cost liquid handlers for automation such as OT-2 from Opentrons emerged as democratic alternatives to the expensive existing options. Furthermore, miniaturization imposes some challenges, such as high evaporation rates - that causes changes in the fermentation process – and low oxygen transfer rates, due to the difficulty of reproducing the turbulence of higher volume shaking. The solutions available in the market require high-cost equipment and consumables, such as the flower-shaped microtiter plates, gas permeable sealing foil and Microbioreactor system from Beckman.

The objective of this work is to present a more accessible automated system, that once validated may empower repetitive, large sample size experiments through medium-high throughput fermentation in microscale. For that, we introduce the creation and validation of a semi-automated workflow that comprises sample preparation and analysis and the miniaturization system for cultivation of microorganisms. An adapted three-layer system was developed and evaluated for the prevention excessive evaporation while providing proper aeration. The best fermentation and sampling volume were also evaluated to reach most favorable conditions for this method.

MATERIAL & METHODS

Liquid handler OT-2 (Opentrons) was used for automated inoculation and sampling. All scripts containing the commands for automation were written in Python using Opentrons API. For the miniaturized fermentation, squared-shaped deepwell 96 wells were used. Inspired by Duetz (2007) work, an adapted three-layer system was using deepwell plates equipped with elastic handles to hold a silicon seal, a cotton wool and a tiprack lid (fig 1). Multitron shakers (Sartorius) were used to culture the selected bacteria - *C. necator* H16 PH4^{-} (DSM 542).

For method validation, the following workflow was executed: (i) Pre inoculum; (ii) Sterilization of the Liquid Handlers deck; (iii) Preparation of the deck labware and reagents; (iv) Automated inoculum; (v) Cultivation and (vi) Automated sampling.
The pre inoculum (i) was performed manually, using TSB 5 mL in a sterile 50 mL falcon tube and streaked glycerol preserved *C. necator* H16 PH4^{-} (DSM 542) and incubated at 30ªC, 200 rpm overnight. Prior to all experiments (ii), liquid handlers' deck and pipettes were cleaned with isopropanol (70%), silicon seal was cleaned to ethanol (70%) and both sides were exposed to 20 minutes UV radiation. To prevent any contamination, gentamicin (10ug/mL) – antibiotic which the host is naturally resistant – was added at every broth. For deck preparation (iii), a 12 well reagent reservoir was filled with fresh medium (TSB with gentamicin), pre-inoculated cells and put on the deck with tipracks and other labware. Liquid handler OT-2 performed (iv) the automated inoculation in all wells of the deepwell plate that was then incubated at 30ºC, 400 rpm. Plates were collected hour by hour for automated sampling (v). For sample analysis, 384 plates (figure 1) were used, and optical density was measured at 600 nm in Varioskan plate reader.

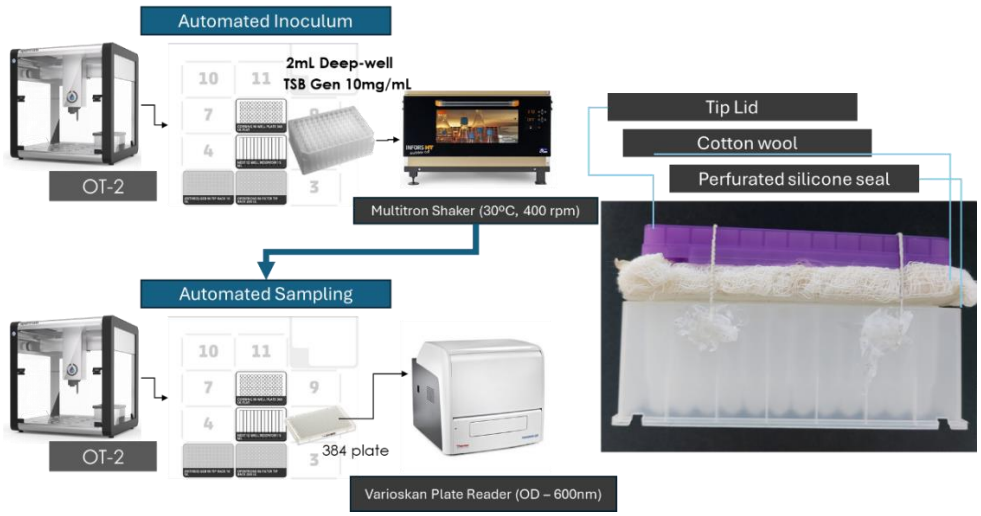

Figure 1 – Simplified workflow for automated inoculation and sampling used at this project (left). Adapted three-layer system inspired by Duetz (2007).

To evaluate the best fermentation volume and sampling, a destructive assay was performed. To analyze the influence of the working volume in the microbial growth, different volumes were tested (200 uL, 300 uL, 400 uL and 500 uL). To compare the growth at different scales, a round of fermentation (n=3) was also conducted in Erlenmeyer (working volume = 25 mL, total volume of 250mL) with the same organism and culture medium and had its growth analyzed by CGQ (cell growth quantifier) coupled Multitron Shaker. Sampling volume may have 2-fold influence in the method design - it determines total number of samples permitted in a well (as 10% of total volume is the total sampling volume to not influence the system) and the standard deviation on the OD readings (as cell growth is analyzed in plate reader, every pipetting error results in variation of the optical path length).

RESULTS & DISCUSSION

An automated workflow for innoculum and sampling was successfully developed in OT-2 liquid handlers.The adapted three layer system proposed for reducing evaporation exihibited a evaporation rate of 30 ± 9 µL/day, considered low compared to 56 ± 8 µL/day of the control plates. The 3 layer system also exhibited better performance for cell growth and lower standard deviations cell quantification (**Figure 2**).

Cell growth profile in 3 layer system deepwell was similar to the erlenmeyer scale (**Figure 2**), while conventional deepwell cultivation was uncomparable to both conditions. To further evaluate the best conditions, growth rate in every condition (total and sampling volume) was calculated by linearizing the equation that related Optical density and time.

$$\ln(OD_{600nm}) = \mu * time(h) + b$$

Table 1 – Microbial growth rate and coefficient of determination from the linearized form of acquired data from every sample and working volume.

Scale	**Deepwell - Sampling 20 uL**				**Deepwell - Sampling 40 uL**				**Erlen meyer**
Sample Volume	200 uL	300 uL	400 uL	500 uL	200 uL	300 uL	400 uL	500 uL	-
R^2	0,99	0,99	0,98	0,78	0,77	0,97	0,6	0,76	0,99
µ (growth rate)	0,39	0,455	0,48	0,18	0,52	0,27	0,25	0,39	0,65

It is noticible that data acquired from 40 uL sampling presented low coefficient of determination and thus low fitness to the used model. The highest volume (500 uL) also presented the lowest growth rate in both sampling volume, though presenting low R^2 in

both cases. The three layer system presented comparable growth rates to erlenmeyer flask despite presenting slower growth rate (30% lower).

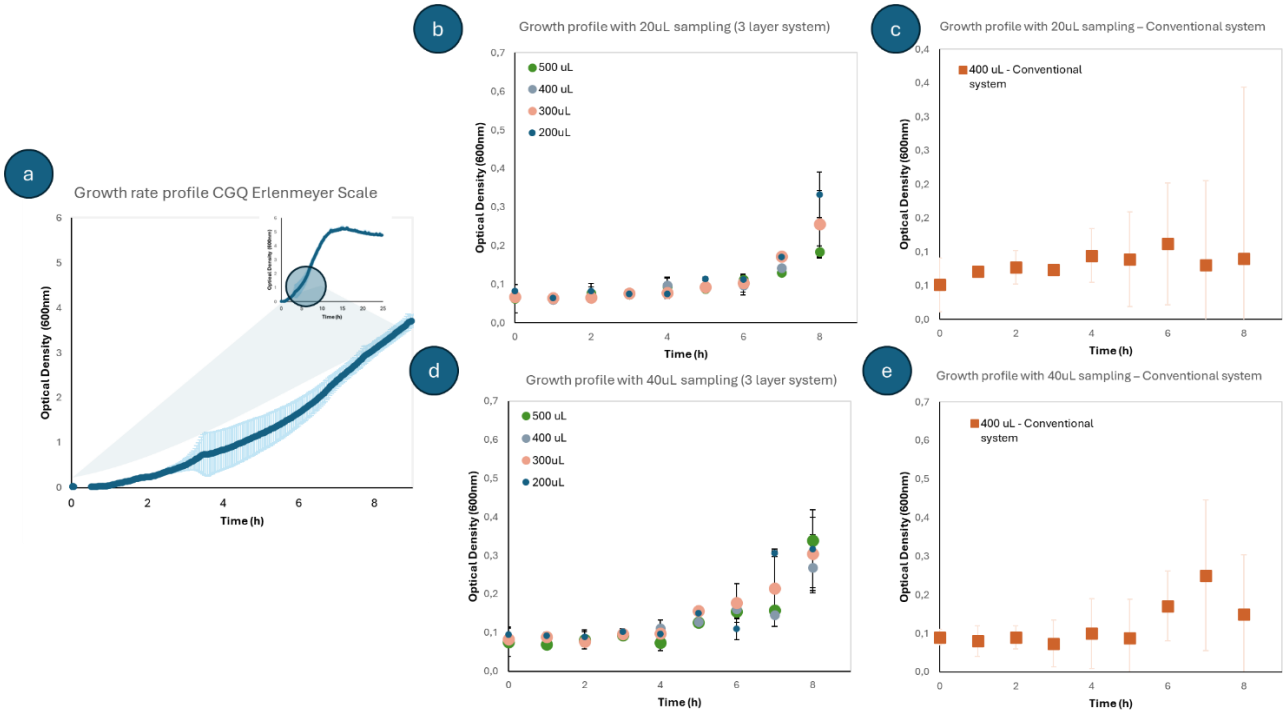

Figure 2 - Growth profiles in different scales and conditions – (a) Erlenmeyer flasks, (b) deepwell plates with 3 layer system (4 different volumes) with 20 uL sampling volume, (c) conventional deepwell plated sealed with gas permeable parafilm with 20 uL sampling volume, (d) deepwell plates with 3 layer system (4 different volumes) with 40 uL sampling volume, conventional deepwell plated sealed with gas permeable parafilm with 40 uL sampling volume.

The presented results indicate that the best sampling volume is 20 uL and the more indicated working volume is 400 uL. That allows the maximum analysis per well without interfering with the fermentation, while fitting in the growth rate linearization.

CONCLUSION

An adapted system for microscale fermentation was validated and compared to the Erlenmeyer scale and to the conventional deepwell cultivation system. The developed method presented comparable growth to the flask scale and presented better performance for cell growth and evaporation rates than the conventional method. Faster growth rates may be achieved by using higher shaking or using equipment with higher shaking throw diameter. The best working volume was 400 uL with 20 uL of sampling volume. This combination allows 3 cell population analysis per well - 2 during fermentation (not exceeding 10% of total volume) and as the final sample.

In summary, this is a first validation of a low cost opensource system for the miniaturization and automation of fermentation and sampling. In the future, this workflow can be applied to ALE for reducing lag phase in fermentation or increasing product tolerance. It is expected that different medium compositions, inoculum load and time, etc. can be simultaneously tested in a single round of experiments. Further selection and new cultures would be determined by two OD (600nm) readings: one supposedly in the middle of the process and other by the end, that could be fit into a previously fit population model.

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