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Research Article

Using CO_2 level monitoring to adjust the stress conditions of morbidostats

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ABSTRACT

In a conventional morbidostat, cell growth is monitored by measuring OD, and stress conditions are automatically adjusted using OD values. However, phenomena such as biofilm formation, agglomeration, and the presence of opaque substrates or products can result in inaccurate OD measurements of population size, causing morbidostat systems to fail to adjust stress conditions appropriately.

This study offers a solution for circumstances where it is impractical to determine vital activity based on OD by developing a novel morbidostat system that adjusts stress conditions based on measurements of exhaust CO_2. As a proof of concept, the adaptation of *E. coli* ATCC 47076 to 48 °C was performed with two morbidostats using this new strategy. Both populations evolved in the morbidostats were confirmed to grow at 48 °C, a temperature their ancestral strain cannot withstand.

1. Introduction

A morbidostat is a continuous-culture device. Designed for experimental evolution studies, it selects individual cells that can adapt to conditions of increasing stress. It accelerates the process of genetic adaptation with its constant monitoring and automated control capabilities. Although morbidostats were first developed by Toprak et al. (2012) to study bacterial drug resistance [1] and have since been most commonly used in antibiotic resistance studies [2–4], they can also be used to adapt microorganisms to a variety of stress conditions such as pH and temperature extremes, toxic chemicals and nutrient deprivation.

In a conventional morbidostat, cell growth is monitored by measuring optical density (OD), and the stress conditions are automatically adjusted in accordance with the OD values. This method assumes that individual cells are uniformly distributed throughout the bioreactor, and that only cells, and no other culture components, interfere with light transmission. However, there are many real-world situations in which phenomena such as biofilm formation, agglomeration, and the presence of opaque substrates or products invalidate these assumptions, resulting in inaccurate OD measurements of population size and thus causing morbidostat systems to fail to adjust stress conditions appropriately.

Microbial growth in a morbidostat can also be monitored by measuring the release of metabolic carbon dioxide (CO_2). Many microorganisms emit CO_2 during their metabolic processes, and CO_2 has been used as an indicator of life-sustaining activities in a variety of studies [5–9]. The average CO_2 concentration in the atmosphere is about 400 ppm, but microbial cultures can produce exhaust with CO_2 concentrations of higher than 2000 ppm [10,11]. This striking difference makes it possible for us to track microbial populations by analyzing their exhaust gasses.

This study's aim was to develop a morbidostat system that uses exhaust CO_2 measurements to adjust stress conditions in order to function properly in circumstances where determining microbial populations by OD is impractical. As proof of concept, the adaptation of *E. coli* ATCC 47076 to 48 °C was performed with two morbidostats using this new strategy. This temperature was chosen because it is known that *E. coli* must undergo genetic changes to withstand it [12].

2. Materials and methods

2.1. Bacterial strain and growth media

This study used *E. coli* ATCC 47076 (MG 1655). This strain has a well-characterized genome and is widely used in genetic engineering studies. LB growth medium (BD Difco) was used for all the experiments.

2.2. Sensors

An MH-Z19b (Winsen, China) infrared CO_2 sensor was placed in a gas chamber at the exhaust, which can measure CO_2 levels with an accuracy of ±3 % in its range of 0 to 5000 ppm. An MLX90615 (Melexis, Belgium) infrared thermometer with an accuracy of ±0.2 ºC in its range of −20 to 115 ºC was attached to the outer wall of the morbidostat for non-contact

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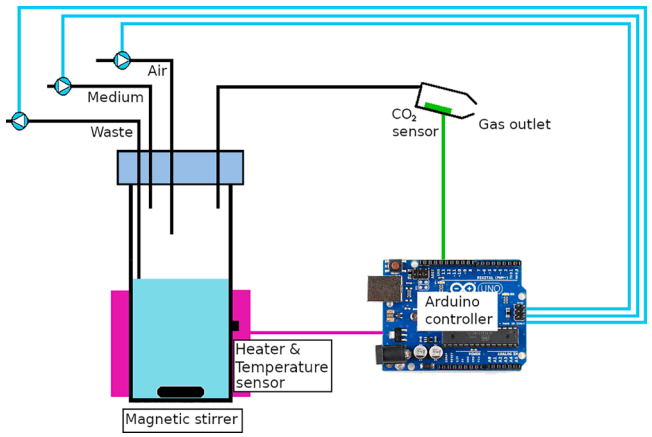

Fig. 1. The morbidostat system that adjusts the stress conditions based on measurements of exhaust CO_2. OD was measured only for monitoring purposes.

temperature measurements. Optical density was measured by shining a 600 nm LED light source through the vials and measuring the light transmitted through them with a 02-LDR1 photoresistor (NTE, USA).

2.3. The morbidostat system

Based on the methods of Toprak et al. [13] and Liu et al. [14], an Arduino UNO driven morbidostat system (Fig. 1) was designed for this study. Arduino UNO microcontrollers (Arduino, Italy) were programmed using the Arduino Integrated Development Environment. The peristaltic pumps (Grothen, China) were controlled by TB6560 motor drivers (Toshiba, Japan), and YYP370 air pumps (Huizhou Yingyi, China) were used for ventilation. The COM-11288 heating pads (Sparkfun, USA) around the bioreactors were controlled using L298n motor drivers (STMicroelectronics, Switzerland). N24 vials (Isolab, Germany) were used as bioreactors. All liquid and gas transfers were done using 2 × 4 mm silicone tubing.

The morbidostat system continuously transfers fresh culture medium to the bioreactor while simultaneously moving an equal volume of microbial culture from the bioreactor to the waste line. The Arduino board periodically measures the temperature and, as necessary, energizes a heating pad to heat the bioreactor. The Arduino controller also measures the amount of CO_2 in the exhaust gas every hour and increases the temperature setpoint by 0.02 ºC when the CO_2 level is above 800 ppm. The OD values are measured at 600 nm (OD600). All electronic components, except for the magnetic stirrer, are controlled by the Arduino board. Two copies of the system were fabricated.

The bioreactors were operated with a working volume of 15 ml, a flow rate of 5 ml/h and a dilution rate (D) of 0.33 h^{-1}. In a continuous culture, the specific growth rate (μ) is equal to the dilution rate [15]. Therefore, the doubling time (t_d) can be calculated using the following equation:

$$t_d = \ln(2)/\mu$$

If $\mu = D = 0.33\ h^{-1}$, a new generation forms every 2.08 h, and the number of generations is the elapsed time (in hours) divided by this number. This figure was used to represent time in the morbidostats as generations in the results section.

2.4. CO_2 measurement

Considering similar chamber designs[16–18], the following design was developed to ensure that the CO_2 readings from the exhaust line accurately reflect conditions inside the bioreactor. The Arduino controller turns on the ventilation once an hour, adding about 120 ml of air (which is about 4x the headspace volume) to the headspace of the bioreactor. CO_2 is measured in the exhaust line ten seconds after each ventilation to allow enough time for the CO_2 molecules inside the bioreactor to travel to the measurement chamber. This chamber is made of a plastic bottle tilted at 30 degrees to ensure that CO_2, which is denser than air, flushes out within a few minutes after ventilation. A flow meter is installed in the exhaust line to confirm that the system's fittings are tight, and that there are no gas leaks.

2.5. Inoculation

A 250 ml seed flask containing 50 ml of LB was inoculated from a glycerol stock and was grown overnight at 37 °C with shaking at 120 rpm. Both morbidostats were inoculated with a 5 % (v/v) culture from the seed flask.

2.6. Validation tests

To evaluate the effectiveness of the CO_2 measurement system, the bioreactors were first operated in chemostat mode at a constant temperature of 37 °C, and two separate tests were performed. In the first setup, designed to compare CO_2 in the bioreactor exhaust and CO_2 in fresh air, 120 ml of fresh air was introduced into the measurement chamber every first hour, while the same volume of bioreactor exhaust was introduced every second hour. Both chemostats were operated for six hours, and their CO_2 levels were measured and recorded. Statistical analysis was performed using one-way ANOVA in R software (version 4.1.2) followed by Tukey's post-hoc test ($n = 6$ replicates) to compare CO_2 levels recorded in the bioreactor exhaust and fresh air. A p-value less than 0.01 was considered statistically significant.

In the second test, to determine the response time of the measurement system, bioreactor exhaust was introduced into the measurement chamber at the beginning of every hour. In this set-up, both chemostats were operated for six hours, and their CO_2 levels were measured every two minutes. The rationale behind taking measurements at such frequent intervals was to make sure that the hourly measurements would not interfere with each other in later morbidostat experiments.

2.7. The morbidostat experiments

After checking the accuracy of the CO_2 measurement system, the bioreactors were switched to morbidostat mode and began to increase the culture temperature automatically based on CO_2 measurements. The temperatures, and CO_2 and OD600 levels were recorded every hour while the replicates were operated until the temperature setpoint target (48 °C) was reached.

2.8. Bacterial growth in flasks

Pre-cultures (50 ml LB medium in 250 ml flasks) were inoculated from 1 ml glycerol stocks and incubated overnight at 37 °C with shaking at 120 rpm. From these pre-cultures, 250 ml flasks containing 50 ml LB medium were inoculated to an OD600 of 0.05. Cultures were incubated at 37 °C and 48 °C with shaking at 120 rpm. OD600 values were measured at 24 h. Statistical analysis was performed using one-way ANOVA in R software followed by Tukey's post-hoc test ($n = 3$ replicates) to compare the population growth of the evolved *E.coli* strains with the ancestral strain. A p-value less than 0.01 was considered statistically significant.

3. Results and discussion

3.1. Accuracy of the CO_2 measurement system

The first validation test showed that the recorded CO_2 levels of the chemostat exhaust gasses were significantly higher ($p < 0.01$) than the CO_2 levels recorded after introducing fresh air into the measurement

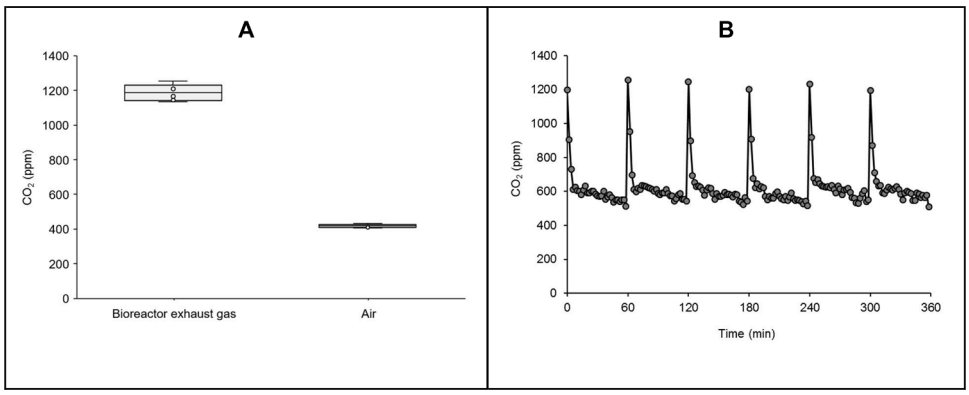

Fig. 2. Accuracy of the CO_2 measurement system. (A) CO_2 levels of bioreactor exhaust and fresh air were compared. (B) Bioreactor exhaust CO_2 levels were monitored over time to assess the response time of the measurement system.

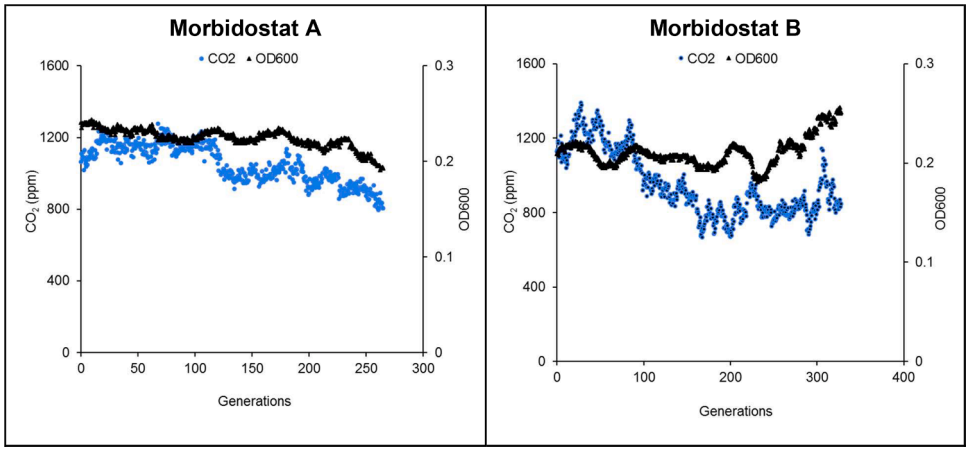

Fig. 3. Change in CO_2 (blue) and OD600 (black) levels in morbidostats.

chamber (Fig. 2a). To be certain of preventing the microbial population from being washed out during the morbidostat experiment, a threshold CO_2 level of less than 800 ppm was chosen based on this difference. The Arduino controllers driving the morbidostats were programmed not to raise the temperature setpoint when the CO_2 level is below the threshold.

In the second validation test, the CO_2 level in the measurement chamber fell by half within a few minutes (Fig. 2b), indicating that the CO_2 transported from the bioreactor quickly left the chamber as designed. Based on this observation, it can be concluded that hourly CO_2 measurements did not interfere with each other in morbidostat experiments.

IR spectroscopy is a non-invasive, low-cost analytical technique that can be used to measure CO_2 concentration in bioreactor off-gas[19]. However, this technique is a more indirect method than determining the microbial population by OD and can be expected to be less accurate. In the first validation test, fluctuations of up to 10 % were observed in the chemostat exhaust measurements, compared to fluctuations of about 4

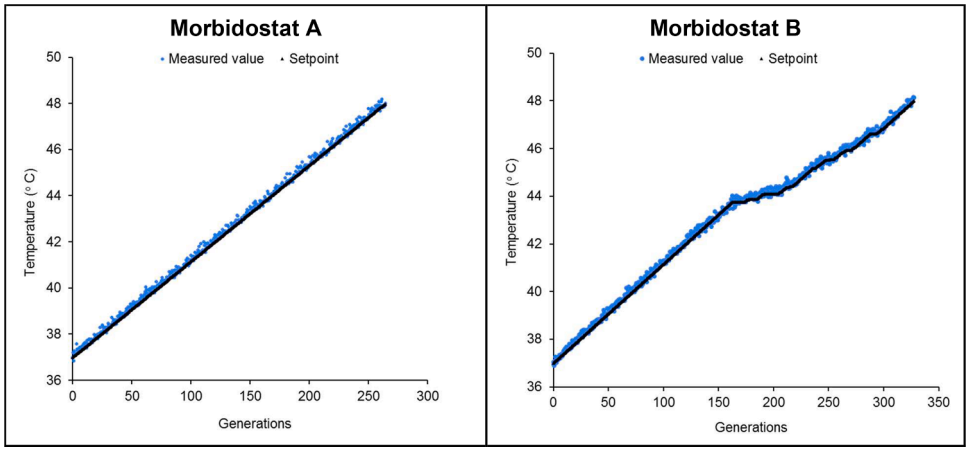

Fig. 4. Temperature change in the morbidostats. The temperature setpoints are shown as solid black lines, and the measured values are shown as blue dots.

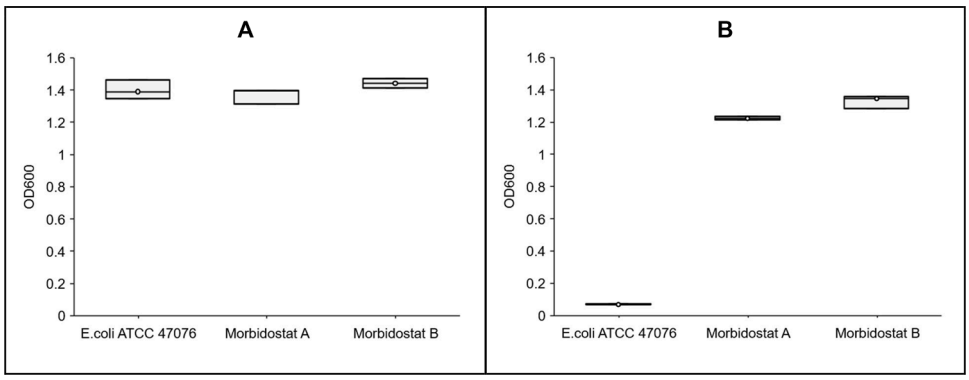

Fig. 5. The population growth of *E. coli* ATCC 47076 and the evolved *E. coli* strains at 37 °C (A) and 48 °C (B).

% in the fresh air CO_2 measurements, indicating the magnitude of the error. In terms of this study, these fluctuations are acceptable for conducting adaptation experiments in morbidostats, since evolutionary processes do not require fast control mechanisms. The key is using an effective approach to controlling the evolution of microorganisms. With the appropriate programming strategy, it is possible to compensate for inaccurate or delayed measurements.

3.2. The morbidostat experiment

In their adaptive laboratory evolution experiment, Rudolph et al. [20]. reported that *E. coli* strain MG1655, which had a maximum growth temperature of 45.5 °C, evolved to grow at 48.5 °C. Considering the above research, 48 C was chosen as the target for the morbidostat experiments in the current study.

One of the cultures in the morbidostats adapted to 48 °C in 265 generations, and the other did so in 328 generations. The CO_2 and OD600 values recorded during the adaptation process are shown in Fig. 3, and the temperature values are in Fig. 4.

The temperature increases were not paused at any time during the adaptation process in morbidostat A, since the CO_2 level remained above the threshold throughout the adaptation process. However, morbidostat B's Arduino controller detected CO_2 levels below the threshold multiple times, indicating a potential risk of washout, so the temperature increases were paused until the CO_2 level rose above the threshold again, at which point they were automatically reactivated. Fig. 3 shows that a similar adaptation process would have been observed, if both morbidostats had been controlled based on OD measurements with an OD600 threshold set at 0.2.

3.3. Comparison of growth of E. coli strains

After the morbidostat experiments, the growth of *E. coli* ATCC 47076 and the two evolved *E. coli* strains were compared at 37 °C and 48 °C according to their OD600 values at 24 h. No significant differences were observed at 37 °C; however, the population growth of the evolved *E. coli* strains were significantly higher ($p < 0.01$) than that of the ancestral strain at 48 °C (Fig. 5).

4. Conclusion

This study's findings show that the stress levels in morbidostat systems can be successfully regulated based on CO_2 measurements. This approach is particularly important from an engineering perspective because it can be used in a variety of challenging environments such as mixed-culture fermentations, solid state fermentations, fermentations with complex substrates, emulsions, activated sludges, etc.

However, there are limitations to adjusting stress conditions in morbidostats based on CO_2 levels. For example, if the microorganisms in them consume CO_2, estimating vital activity from CO_2 concentration will yield inaccurate results. The use of other parameters such as dissolved oxygen concentration, pH or nutrient concentrations as indicators of survival should also be considered in such cases.

An important tool for microbial evolution, the morbidostat forces microorganisms to adapt to harsh environmental conditions. Increasing the flexibility of morbidostat systems will increase their usefulness both for experimental evolution studies and for engineering practices.

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CRediT authorship contribution statement

Kerem Bora: Writing – review & editing, Writing – original draft, Validation, Visualization, Software, Supervision, Methodology, Project administration, Resources, Funding acquisition, Investigation, Conceptualization, Data curation, Formal analysis.

Declaration of competing interest

I declare that I have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2024.e00836.

References

[1] E. Toprak, A. Veres, J.-B. Michel, R. Chait, D.L. Hartl, R. Kishony, Evolutionary paths to antibiotic resistance under dynamically sustained drug selection, Nat. Genet. 44 (2012) 101–105, https://doi.org/10.1038/ng.1034.

[2] B. Dößelmann, M. Willmann, M. Steglich, B. Bunk, U. Nübel, S. Peter, R.A. Neher, Rapid and consistent evolution of colistin resistance in extensively drug-resistant pseudomonas aeruginosa during morbidostat culture, Antimicrob. Agents Chemother. 61 (2017), https://doi.org/10.1128/AAC.00043-17 e00043-17.

[3] S.A. Leyn, J.E. Zlamal, O.V. Kurnasov, X. Li, M. Elane, L. Myjak, M. Godzik, A. de Crecy, F. Garcia-Alcalde, M. Ebeling, A.L. Osterman, Experimental evolution in morbidostat reveals converging genomic trajectories on the path to triclosan resistance, Microb. Genomics 7 (2021) 000553, https://doi.org/10.1099/mgen.0.000553.

[4] M.S. Manna, Y.T. Tamer, I. Gaszek, N. Poulides, A. Ahmed, X. Wang, F.C.R. Toprak, D.R. Woodard, A.Y. Koh, N.S. Williams, D. Borek, A.R. Atilgan, J.D. Hulleman, C. Atilgan, U. Tambar, E. Toprak, A trimethoprim derivative impedes antibiotic resistance evolution, Nat. Commun. 12 (2021) 2949, https://doi.org/10.1038/s41467-021-23191-z.

[5] A.J. Gyawali, B.J. Lester, R.D. Stewart, Talking SMAAC: a new tool to measure soil respiration and microbial activity, Front. Earth Sci. 7 (2019). https://www.frontiersin.org/articles/10.3389/feart.2019.00138 (accessed July 31, 2023).

[6] E. Mutuyemungu, M. Singh, S. Liu, D.J. Rose, Intestinal gas production by the gut microbiota: a review, J. Funct. Foods 100 (2023) 105367, https://doi.org/10.1016/j.jff.2022.105367.

[7] R.C. Smallegange, W.H. Schmied, K.J. van Roey, N.O. Verhulst, J. Spitzen, W. R. Mukabana, W. Takken, Sugar-fermenting yeast as an organic source of carbon dioxide to attract the malaria mosquito Anopheles gambiae, Malar. J. 9 (2010) 292, https://doi.org/10.1186/1475-2875-9-292.

[8] M.J. Surger, L.M. Blank, Assessment of microbial activity by CO2 production during heating oil storage, Eng. Life Sci. 22 (2022) 508–518, https://doi.org/10.1002/elsc.202100144.

[9] M.T. Torres-Mancera, A. Figueroa-Montero, E. Favela-Torres, G. Rosales-Zamora, K.M. Nampoothiri, G. Saucedo-Castañeda, Online monitoring of solid-state fermentation using respirometry. Curr. Dev. Biotechnol. Bioeng., Elsevier, 2018, pp. 97–108, https://doi.org/10.1016/B978-0-444-63990-5.00006-2.

[10] J.F. Harwood, A.G. Richardson, J.A. Wright, P.J. Obenauer, Field assessment of yeast- and oxalic Acid-generated carbon dioxide for mosquito surveillance, J. Am. Mosq. Control Assoc. 30 (2014) 275–283, https://doi.org/10.2987/14-6421R.1.

[11] E. Vaiopoulou, P. Melidis, V. Diamantis, A. Aivasidis, Microbial sensors for monitoring and control of aerobic, anoxic, and anaerobic bioreactors in wastewater treatment, Compr. Biotechnol. (2011) 495–505, https://doi.org/10.1016/B978-0-08-088504-9.00493-1.

[12] B.E. McGuire, F.E. Nano, Whole-genome sequencing analysis of two heat-evolved Escherichia coli strains, BMC Genomics 24 (2023) 154, https://doi.org/10.1186/s12864-023-09266-9.

[13] E. Toprak, A. Veres, S. Yildiz, J.M. Pedraza, R. Chait, J. Paulsson, R. Kishony, Building a morbidostat: an automated continuous-culture device for studying bacterial drug resistance under dynamically sustained drug inhibition, Nat. Protoc. 8 (2013) 555–567, https://doi.org/10.1038/nprot.2013.021.

[14] P.C. Liu, Y.T. Lee, C.Y. Wang, Y.-T. Yang, Design and use of a low cost, automated morbidostat for adaptive evolution of bacteria under antibiotic drug selection, J. Vis. Exp. 54426 (2016), https://doi.org/10.3791/54426.

[15] A. Novick, L. Szilard, Description of the chemostat, Science 112 (1950) 715–716, https://doi.org/10.1126/science.112.2920.715.

[16] A. Yousefi-Darani, M. Babor, O. Paquet-Durand, B. Hitzmann, Model-based calibration of a gas sensor array for on-line monitoring of ethanol concentration in Saccharomyces cerevisiae batch cultivation, Biosyst. Eng. 198 (2020) 198–209, https://doi.org/10.1016/j.biosystemseng.2020.08.004.

[17] M. Koskinen, K. Minkkinen, P. Ojanen, M. Kämäräinen, T. Laurila, A. Lohila, Measurements of CO₂ exchange with an automated chamber system throughout the year: challenges in measuring nighttime respiration on porous peat soil, Biogeochemistry (2013), https://doi.org/10.5194/bgd-10-14195-2013.

[18] M. Krivec, G. Gunnigle, A. Abram, D. Maier, R. Waldner, J. Gostner, F. Überall, R. Leitner, Quantitative ethylene measurements with MOx chemiresistive sensors at different relative air humidities, Sensors 15 (2015) 28088–28098, https://doi.org/10.3390/s151128088.

[19] V. Vojinovic, J.M.S. Cabral, L.R. Fonseca, Ex situ bioprocess monitoring techniques, Chem. Ind. Chem. Eng. Q. 13 (2007) 103–116, https://doi.org/10.2298/CICEQ0702103V.

[20] B. Rudolph, K.M. Gebendorfer, J. Buchner, J. Winter, Evolution of Escherichia coli for growth at high temperatures, J. Biol. Chem. 285 (2010) 19029–19034, https://doi.org/10.1074/jbc.M110.103374.