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Growth of Methanotrophic Bacteria in Chemostat Mode in Eppendorf BioBLU® f Single-Use Vessels

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Abstract

To investigate the methanol metabolism of *Methylomicrobium alcaliphilum* researchers at the San Diego State University analyzed bacterial growth and substrate utilization in chemostat mode. They used a parallel DASbox® Mini Bioreactor System equipped with BioBLU 0.3f Single-Use Vessels. Parallel experimentation using single-use equipment saved time and reduced possible sources of fluctuation between replicates.

Introduction

Microbial utilization of C1 substrates like methane and methanol is becoming an attractive platform for the production of chemicals and fuels. Though our understanding of its whole cell metabolic network is not complete, it is important to increase efficiency by optimizing process conditions and applying metabolic engineering.

Researchers use the aerobic methanotroph *Methylomicrobium alcaliphilum 20Z*^{R} as a model system for investigating C1 utilization networks. Trace elements are important environmental factors that can influence metabolic networks, either as cofactors of enzymes in the pathway or because they alter gene expression levels. Copper, lanthanum, and calcium – among others – were previously predicted to influence *M. alcaliphilum* growth [1]. Lanthanum and calcium are cofactors of the methanol dehydrogenases XoxF and MxaF1, respectively, which catalyze the formation of formaldehyde in the methanol metabolic pathway [1]. Copper influences the activity of the methane monooxygenase (pMmo), which converts methane to methanol.

In the project presented here, the team of Marina Kalyuzhnaya, from San Diego State University, cultivated *M. alcaliphilum 20Z*^{R} in chemostat mode, using methanol as a substrate. In chemostat mode the bacteria stay in the exponential growth phase, when the growth rate is

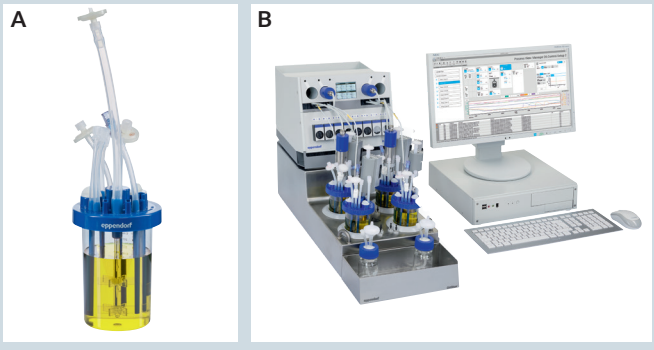

Fig. 1:
A: BioBLU 0.3f Single-Use Vessel **B:** DASbox Mini Bioreactor System

maximum. Due to the continuous addition of fresh medium there are no limiting nutrients. This allows the researchers to better understand the culture's metabolism, because the culture is not strained for any resources. The researchers analyzed the influence of calcium, lanthanum, and copper on bacterial growth, substrate utilization, oxygen utilization, and pH.

To be able to compare biological replicates and perform side-by-side comparisons, it was essential to perform each step of the experimental procedure the same way. The researchers achieved this by parallel processing using an Eppendorf DASbox Mini Bioreactor System. They equipped the bioreactor system with BioBLU 0.3f Single-Use Vessels for fermentation. Single-use equipment eliminated the risks deriving from improper cleaning and sterilization of vessels, hence reducing the risk of contamination, which is especially likely in long-lasting processes at low bacterial densities.

Material and Methods

Bacterial strain

In this study the researchers used *Methylomicrobium alcaliphilum 20Z*^{R}. This gram-negative, gamma proteobacterium was isolated from a saline soda lake [2]. The strain can be grown on methanol as its main carbon source. It is gaining momentum as a promising industrial catalyst and could become the microbial platform of choice for production of fuels and chemicals from C1 substrates [3].

Culture media

The researchers grew *M. alcaliphilum* in the growth medium described in [4], supplemented with 3 % sodium chloride. The pH of the media was 8.6 - 9.0. Four variants of the medium were used. They contained 0.5 mM calcium/no copper, 0.5 calcium/0.19 mM copper, 0.5 mM lanthanum/no copper, and 0.5 mM lanthanum/0.19 mM copper, respectively. The researchers grew the cultures on 2 % methanol throughout the experiment.

Bioprocess system and cultivation conditions

The researchers cultivated *M. alcaliphilum* in a DASbox Mini Bioreactor System equipped with BioBLU 0.3f Single-Use Vessels (Figure 1).

The medium was sterilized by autoclaving, cooled, supplemented with phosphate and carbonate, and transferred to the fermentors (200 mL per unit). The researchers calibrated pH and DO sensors according to standard operating procedures. They calibrated the DO sensor with air flowing and agitation running to ensure accurate results. The gas inflow was set at 0.3 sL/h. The cultures were agitated at 500 rpm. The researchers controlled the temperature at 30 °C. To start the fermentation process, the researchers added a preculture (5 % of total volume), which was grown overnight.

Operation in chemostat mode

The researchers switched the processes to chemostat mode when the cell density reached an optical density at 600 nm (OD_{600}) of 2. This corresponds to a dry cell weight of 0.6 g/L. Their aim was to keep the optical density constant. To achieve this, medium was pumped into and out of the bioreactors using the system's integrated pumps. The researchers calculated the required dilution rate based on the optical density of the culture. If the optical density was increasing, they increased the flow rate until the optical density stabilized. The dilution rate was 14 mL/h in the lanthanum environment and 7 mL/h in the calcium environment.

To investigate the influence of copper on bacterial growth and metabolism, the researchers exchanged the copper-free culture medium with medium containing 0.19 mM copper in the course of the chemostat process.

The cultures were grown in chemostat mode for up to 14 days. Four processes were run in parallel.

Analytics

To monitor bacterial growth, the researchers hourly measured the optical density of each culture offline. They quantified the concentration of methanol in the culture medium spectrophotometrically, using a catalytic test. The samples were diluted by 1000 and then averaged out to an OD of 1. DO and pH were measured online.

Results

The experiments aimed to identify the key physiological factors that control methanol-carbon assimilation and respiration. By controlling the availability of calcium and lanthanum, the researchers investigated differences in the methanol metabolism of the bacteria due to having to use the La- and Ca-dependent methanol dehydrogenase, respectively. By controlling the availability of copper, they tested whether the activity of the copper-dependent methane monooxygenase pMMO changed the bacterial metabolism.

The researchers analyzed bacterial growth and substrate utilization.

To investigate differences in methanol consumption due to the two different dehydrogenases, the researchers analyzed the concentration of the substrate in the culture media. They cultivated the bacteria in medium with calcium or lanthanum, in presence or absence of copper. For each tested environment, a sample was taken during the initial growth phase. The culture consumed more substrate in the presence than in the absence of copper, indicating differences in the methanol metabolism (Figure 2). Differences in the metabolism of the cultures from the different conditions were confirmed by metabolomic analysis of bacteria samples (data not shown).

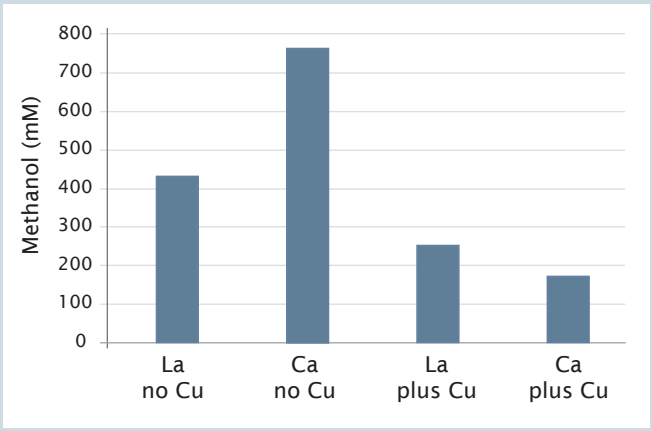

Fig. 2: Methanol concentration in the medium, measured during the initial growht phase.

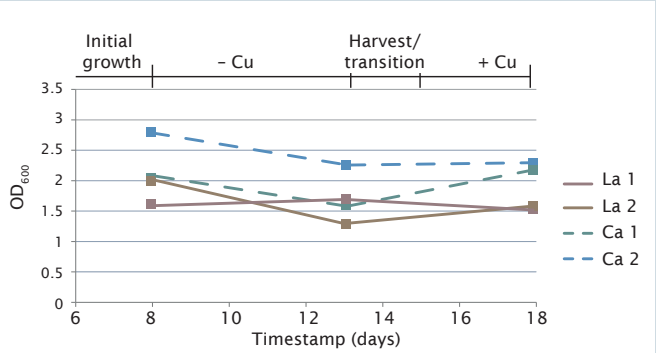

Fig. 3: Bacterial growth curves in chemostat mode.

Then the researchers analyzed processes in chemostat mode. Figure 3 displays bacterial growth in presence of calcium and lanthanum, respectively. Eight to nine hours after inoculation the cultures reached an optical density of approximately 2, which stayed largely constant when the process was switched to chemostat mode. The dilution rate was 14 mL/h in the lanthanum environment while it was 7 mL/h in the calcium environment, indicating that the bacteria grew faster in presence of lanthanum. However, in the calcium environment the bacteria used the substrate more efficiently (data not shown).

Figures 4 and 5 show the DO and pH of the cultures growing in presence of calcium or lanthanum. In both cases the DO concentration and the pH of the culture medium decreased after the addition of copper, again indicating changes in the bacterial metabolism.

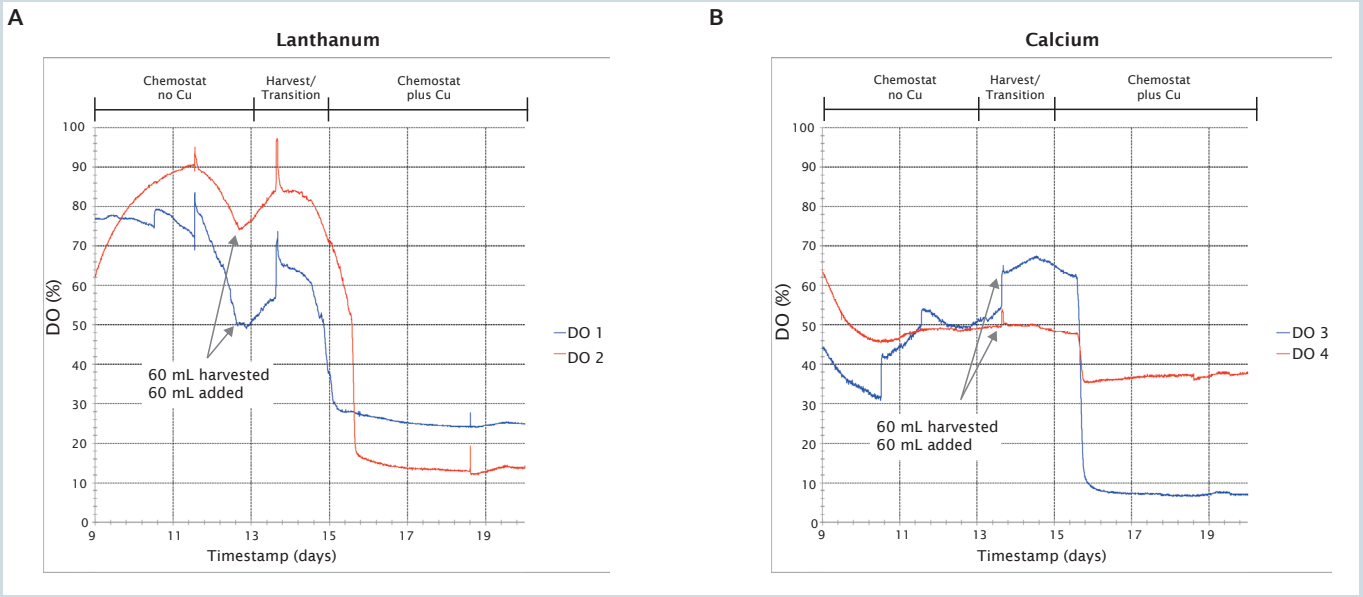

Fig. 4: Dissolved oxygen concentration. Four processes were run in parallel, two in presence of lanthanum and two in the presence of calcium. **A:** Bacterial cultures were grown in presence of lanthanum. **B:** Bacterial cultures were grown in presence of calcium.

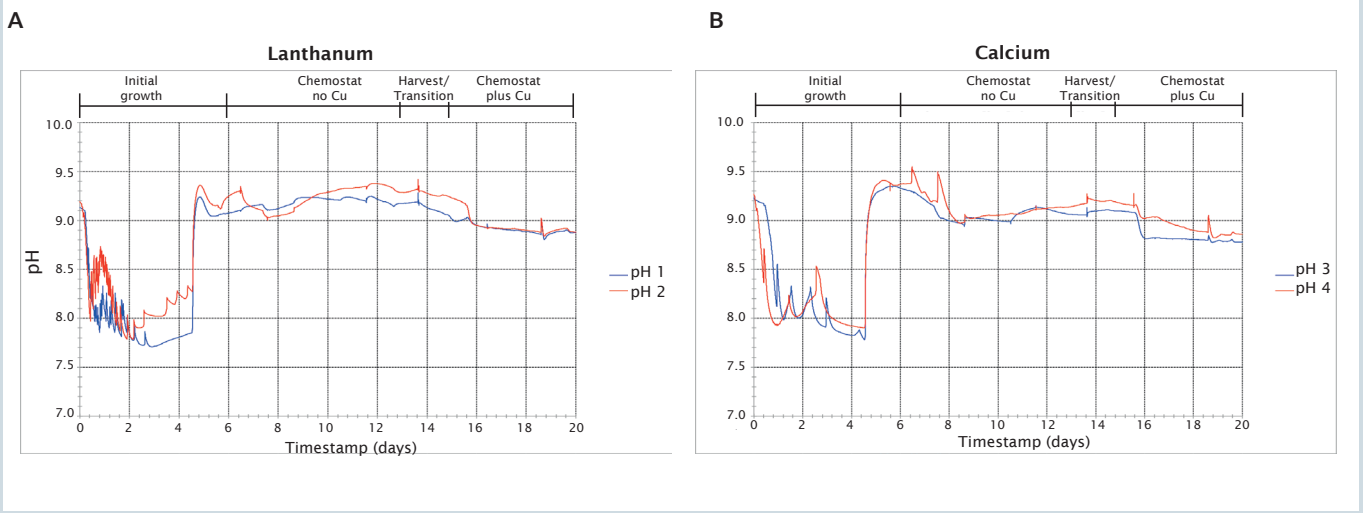

Fig. 5: pH of the culture medium. Four processes were run in parallel, two in presence of lanthanum and two in the presence of calcium. **A:** Bacterial cultures were grown in presence of lanthanum. **B:** Bacterial cultures were grown in presence of calcium.

Conclusion

In the study, the researchers used a DASbox Mini Bioreactor system to cultivate *M. alcaliphilum* in chemostat mode. The experimental system gave insights into the methanol metabolism of the methanotroph. "The equipment allowed us to save time, because we could run four bioreactors in parallel. The average cultivation time for these experiments was two weeks. With other available bioreactors not running in parallel, it would have taken us about two months to obtain the same amount of data.", said Richard Hamilton, who performed the experiments. "Furthermore, the DASbox proved to be essential for side-by-side comparisons."

The experimental setup demonstrates the suitability of BioBLU 0.3f Single-Use Vessels as drop-in replacement for conventional glass vessels. They offered comparable functionality in terms of process control and connectivity. Operating the vessels with a DASbox Mini Bioreactor System facilitated the adjustment of process parameters, like temperature and agitation, and allowed them to regulate medium inflow and outflow for operation in chemostat mode. The single-use vessels performed robustly in processes lasting as long as three weeks.

Literature

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Ordering information

Description	Order no.
DASbox® Mini Bioreactor System for Microbial Applications, max. 25 sL/h gassing, 4-fold system for single-use vessels	76DX04MBSU
DASbox® Feed Line Set PTFE, for DASGIP® MP8, including addition bottles, L 1.0/0.07/1.0 m, I.D. 0.25/0.8 mm	76DXFL025P11
DASbox® Feed Line Set PTFE, for DASGIP® MP8, including addition bottles, L 1.0/0.07/1.0 m, I.D. 0.5/0.8 mm	76DXFL05P11
BioBLU® 0.3f Single-Use Vessel, fermentation, open pipe, 2 Rushton-type impeller, no pH, sterile, 4 pieces	78903509

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