

LUCULLUS® AS THE CONTROL SOFTWARE IN A NOVEL METHOD FOR ANAEROBIC SINGLE-CELL PROTEIN PRODUCTION

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Abstract

Microbial biomass is a promising alternative protein source due to its high nutritional value and lower environmental impact compared to conventional food production. However, submerged high cell density cultivations (HCDCs) are limited by oxygen transfer constraints. To overcome this, researchers at NMBU developed an oxygen-independent HCDC process based on denitrification (Patent GB2602727), using *Paracoccus pantotrophus* in a pH-stat fed-batch system. The process relies on denitrification-driven pH increases to regulate substrate provision, with real-time control implemented by Lucullus® software. By integrating a dynamic pH setpoint with controlled N₂ sparging, CO₂-induced acidification was minimized. This enabled consistent biomass densities exceeding 60 g dry weight L⁻¹ with an average protein content of 75%, without by-product accumulation. Although growth rates remain lower than expected due to a CO₂-mediated pH-lag, the results demonstrate the feasibility of anaerobic HCDC and highlight its potential for single-cell protein production.

Introduction

Microorganisms have been used for thousands of years to produce beer, bread, and fermented foods (Arranz-Otaegui et al., 2018; Liu et al., 2018). More recently, advances in biotechnology and the environmental impact of conventional agriculture have spurred interest in single-cell protein as a new way to utilize microorganisms in food. Single-cell protein is highly nutritious, containing up to 80% protein (Ravindra, 2000), and offers a more sustainable alternative to conventional food production with lower land and water use, reduced greenhouse gas emissions, and the potential to grow on waste substrates (Matassa et al., 2016; Spalvins et al., 2018).

Single-cell protein is typically produced by submerged high cell density cultivations to densities ≥ 20 g dry weight L^{-1} , maximizing volumetric productivity while reducing volume, water consumption, and production cost (Subramaniam et al., 2018). However, current processes are constrained by the low solubility and transport rate of oxygen in water, often resulting in reduced yield and accumulation of byproducts (Garcia-Ochoa & Gomez, 2009). Researchers at the Norwegian University of Life Sciences (NMBU) have addressed this issue by developing a method for anaerobic high cell density cultivation (Bakken et al., 2023; Maråk et al., 2024) relying on denitrification, the dissimilatory reduction of nitrate (NO_3^-) to dinitrogen gas (N_2) via nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (Figure 1). This form of anaerobic respiration is second only to aerobic respiration in terms of energy yield (Chen & Strous, 2013). The anaerobic HCDC process is based on a pH-stat principle where the provision of electron acceptor (HNO_3) is directly linked to denitrification-driven increases in pH. Here, the provision of electron acceptor, carbon source/electron donor, and minerals must be carefully balanced to avoid limitations, toxic effects, or imbalances that could promote the accumulation of byproducts such as polyhydroxyalkanoates (PHAs) (Bedade et al., 2021; Shiloach & Fass, 2005). Another challenge is the accumulation of CO_2 , which reacts with water to form HCO_3^- and CO_3^{2-} , lowering the pH, thereby masking the pH-increase associated with denitrification that would otherwise trigger substrate provision.



Figure 1: Overview of denitrification. Denitrification is a microbial process in which nitrate (NO_3^-) is reduced stepwise to nitrogen gas (N_2), typically under oxygen-limited or anaerobic conditions. It is carried out by a range of microorganisms and plays a key role in the global nitrogen cycle by returning fixed nitrogen to the atmosphere.

With this in mind, the aim was to develop an anaerobic bioprocess for high cell density cultivation by denitrification with balanced, pH-triggered substrate delivery, minimally impacted by CO_2 . A process-specific operation in the Lucullus® software enabled pH-controlled provision of HNO_3 (and minerals) that was balanced with the provision of the carbon and nitrogen sources. Additionally, a dynamic pH setpoint and sparging flow rate were implemented to mitigate CO_2 -driven acidification of the medium.

Materials and Methods

Cultivation conditions

All bioreactor cultivations were performed using *Paracoccus pantotrophus* GB17, adapted to anaerobic conditions in a mineral basal medium containing 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.99 g L^{-1} KH_2PO_4 , 7.45 g L^{-1} K_2HPO_4 , and a mixture of trace elements. The medium was supplemented with glucose as the carbon source and electron donor, NO_3^- as the electron acceptor, and NH_4^+ as the nitrogen source.

Bioreactor setup and sampling

High cell density cultivations were conducted in a Bionet® F1-3 bioreactor with a working volume of 3.0 L and a water circulation jacket for temperature control. The bioreactor setup was equipped with two process gas supplies (both connected to a central N_2 -line), cooling water, an agitator sys-

tem, three fixed-speed pumps (acid, base, and antifoam reservoirs), and two variable-speed pumps (one of which was connected to the feed reservoir). The process was controlled by the Lucillus® 3.11.5 software from Securecell. The vessel was continuously sparged with N₂ to maintain anoxic conditions throughout the cultivations.

The mineral basal medium is added to the vessel before sterilization. The acid (5 M HNO₃ + minerals), base (1 M KOH), feed (2.97 M glucose, 1.42 M NH₄NO₃), and antifoam (Glanapon DB 870 from Busetti, diluted to 200x concentration) reservoirs were filled and connected to the bioreactor. An anaerobic pre-culture was used as inoculum for starting each cultivation.

pH was continuously monitored using an external pH sensor (Hamilton EasyFerm Bio PHI Arc 325). The off-gas was directed to the Bionet® bBreath-1 for CO₂ (nondispersive infrared sensor) and O₂ (zirconia dioxide ceramic electrolyte with gas permeable platinum electrodes) measurements, as well as a robotized incubation system (Molstad et al., 2007) for monitoring NO and N₂O. Throughout the cultivations, liquid samples were extracted for offline measurements of glucose (HPLC), cell density (OD₆₆₀), and NO₂⁻ (LCK342), NO₃⁻ (LCK340), and NH₄⁺ (LCK340) using Hach Lange DR3900.



Results and Discussion

Customized Lucillus® operation to facilitate anaerobic pH-stat

The Lucillus® 3.11.5 software from Securecell was used for data logging and advanced process control of the bioreactor processes. A Lucillus Operation was specifically designed to facilitate the pH-stat fed-batch culturing approach (Figure 2).

Preparation phase

The Operation is initiated with a user-input block, where the setpoints for temperature and stirring are assigned, in addition to several critical process variables used in various calculations throughout the process, including initial pH, k-value (ratio between provided feed versus provided acid), initial volume, and parameters used for temperature, CO₂, and sparging control. Then a preparation phase follows, where the temperature control, stirring, and sparging with N₂ are initiated. When the reactor is anoxic and the temperature has been stabilized (both checked by Lucillus), the user is asked to inoculate the bioreactor.

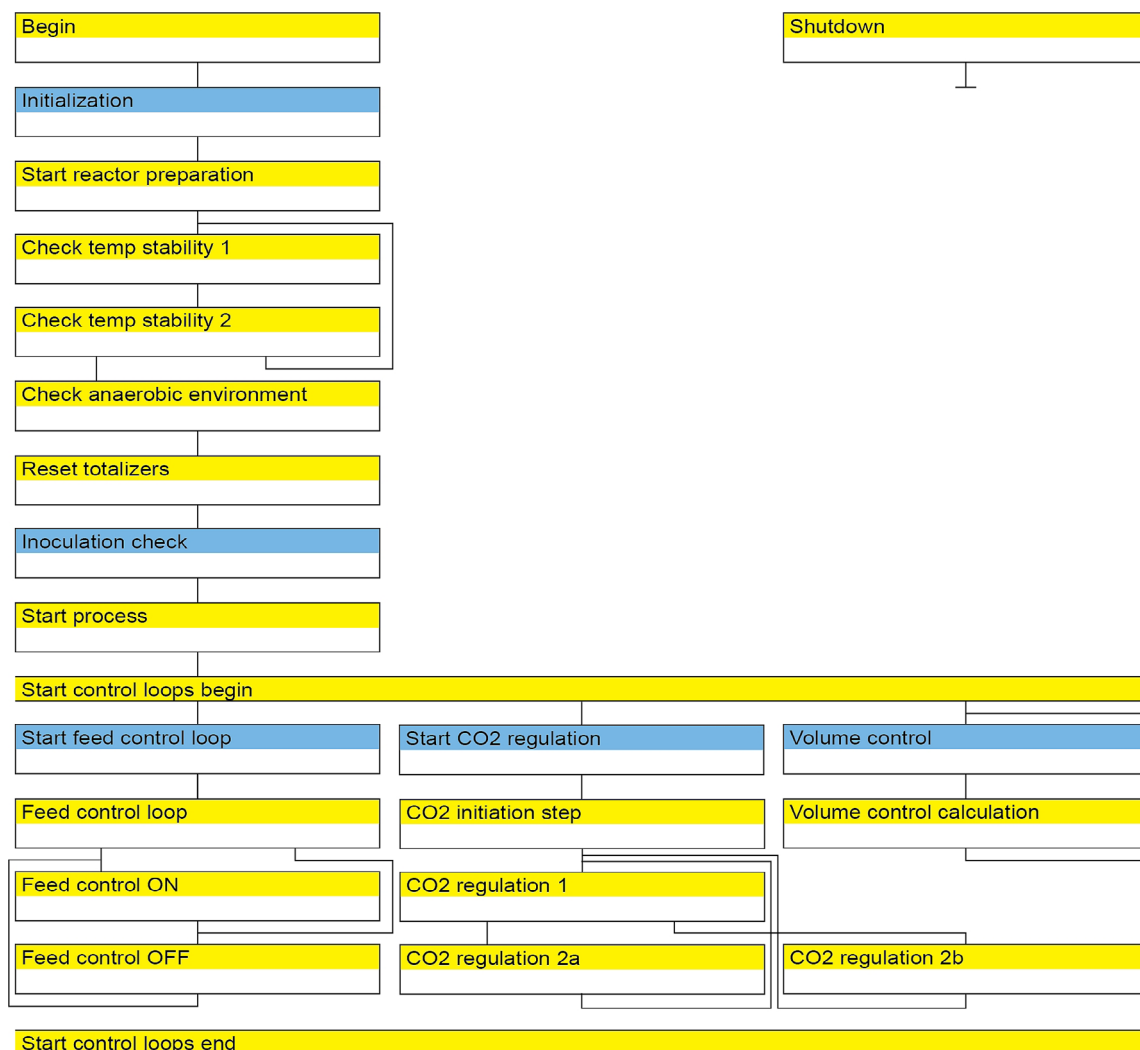


Figure 2 – The Operation created in Lucillus® to support anaerobic high cell density cultivation based on a pH-stat principle. Yellow blocks are automated steps, while the blue blocks require manual user interactions to proceed. The process starts with a preparation phase, where critical process variables are defined and the temperature control and gas sparging are turned on. After inoculation, the advanced control of the process is initiated by defining the equation used to control the pH setpoint and keep track of the pH. The first control loop is used to control the provision of feed from the variable-speed pump. The second control loop regulates the sparging flow rate in response to off-gas CO₂ levels, and a third loop is used to log the total volume in the vessel.

pH-controlled provision of substrate

Equation 1 is defined in the Operation so that the pH setpoint is adjusted based on the off-gas concentration of CO₂ dynamically during the process (Figure 3). Here, ph_sp is the pH setpoint, PH_0 is the intrinsic pH of the medium, $offg_co2_pv$ is the measured concentration of CO₂ in the off-gas, D_CO_2 and D_HNO_3 are empirically determined values for the pH depression per unit of CO₂ and HNO₃, respectively, and SP_HNO_3 is the target HNO₃ concentration in the medium.

$$ph_sp = PH_0 - offg_co2_pv * D_CO_2 - SP_HNO_3 * D_HNO_3$$

Equation 1

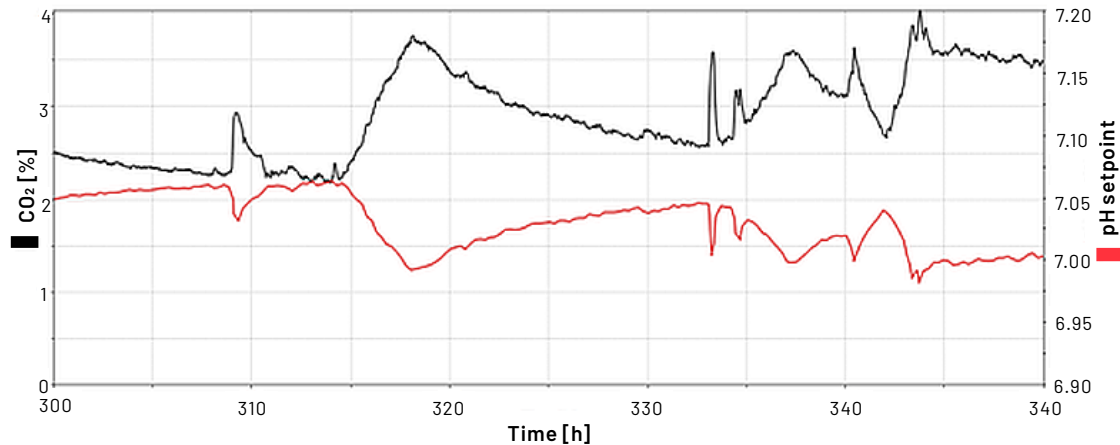


Figure 3 – pH setpoint adjustments based on CO₂ in the off-gas. The pH setpoint (red) is reduced in response to high levels of CO₂ in the off-gas (black) to account for the CO₂-induced acidification of the medium, which masks the denitrification-driven increases in pH that would otherwise trigger substrate injections.

The delivery of feed must be balanced with the electron acceptor provided via the acid to prevent accumulation of storage polymers or denitrification intermediates, of which several are toxic at high concentrations. This is achieved through a control loop (Figure 4, left regulation loop) that turns on the feed pump when the total volume of feed injected is less than the volume of acid injected times the k-value (the ratio between the two).

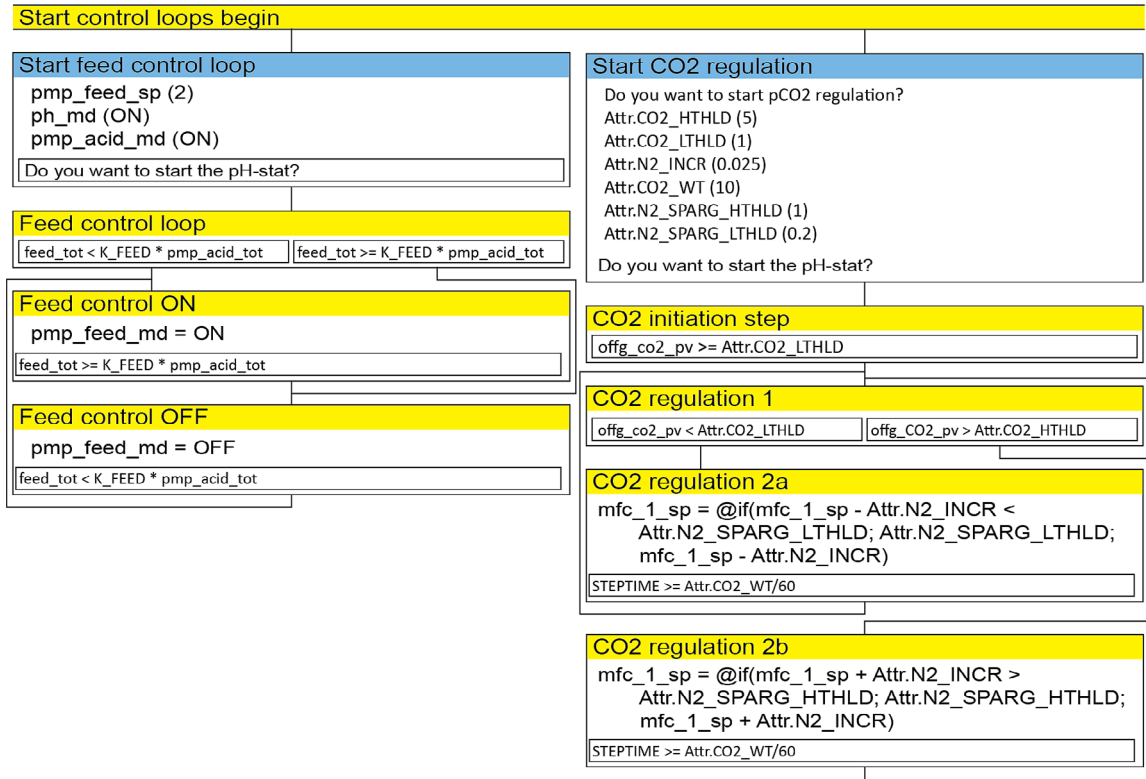


Figure 4 – Regulation loops to control feed provision and CO₂ regulation. The left regulation loop shows the feed control loop. Injections of feed are triggered based on the k-value, representing the ratio between acid injected and feed injected (mL mL⁻¹). The right regulation loop controls the sparging flow relative to the concentration of CO₂ in the off-gas, to maintain it within a defined target range.

Dynamic pH setpoint and sparging flow rate to mitigate CO₂ effects

The sparging flow rate is controlled by a control loop to keep the CO₂ in the off-gas within an acceptable range (Figure 4, right control loop). When the CO₂ concentration in the off-gas is higher than the user-defined high threshold, the sparging flow rate is increased in small increments until the CO₂ concentration in the off-gas is reduced. Similarly, the sparging flow rate is reduced if the CO₂ concentration is below the low threshold.

Volume control

An accurate estimate of the total liquid volume in the bioreactor is required constantly throughout the process, as other calculations depend on it. This is achieved by implementing Equation 2, which calculates the total volume (V_TOT) continuously, based on the initial volume in the vessel (V_INI), the injected volume from each of the reservoirs (pmp_acid_tot, pmp_af_tot, pmp_base_tot, pmp_feed2_tot, and pmp_feed_tot), and the cumulative user injections (V_ADD_CUM) and withdrawals (V_SMPL_CUM) from the vessel.

$$V_{TOT} = V_{INI} + pmp_acid_tot + pmp_af_tot + pmp_base_tot + pmp_feed2_tot + pmp_feed_tot - V_SMPL_CUM + V_ADD_CUM$$

Equation 2

High cell density cultivations by denitrification

Using the Operation described above, several high cell density cultivations with *P. pantotrophus* have been performed without requiring manual intervention (Figure 5). Concentrations exceeding 60 g dry weight L⁻¹ have been consistently achieved, both through repeated cycles of dilution with fresh medium and by maintaining high cell density for over 300 hours. The resulting biomass has an average protein content of 75% with a favourable amino acid profile. No fermentation products or toxic compounds accumulate in the reactor liquid, as verified through HPLC, HS-GC, and bioassays assessing the growth of fresh cells in spent reactor liquid (Marák, 2025).

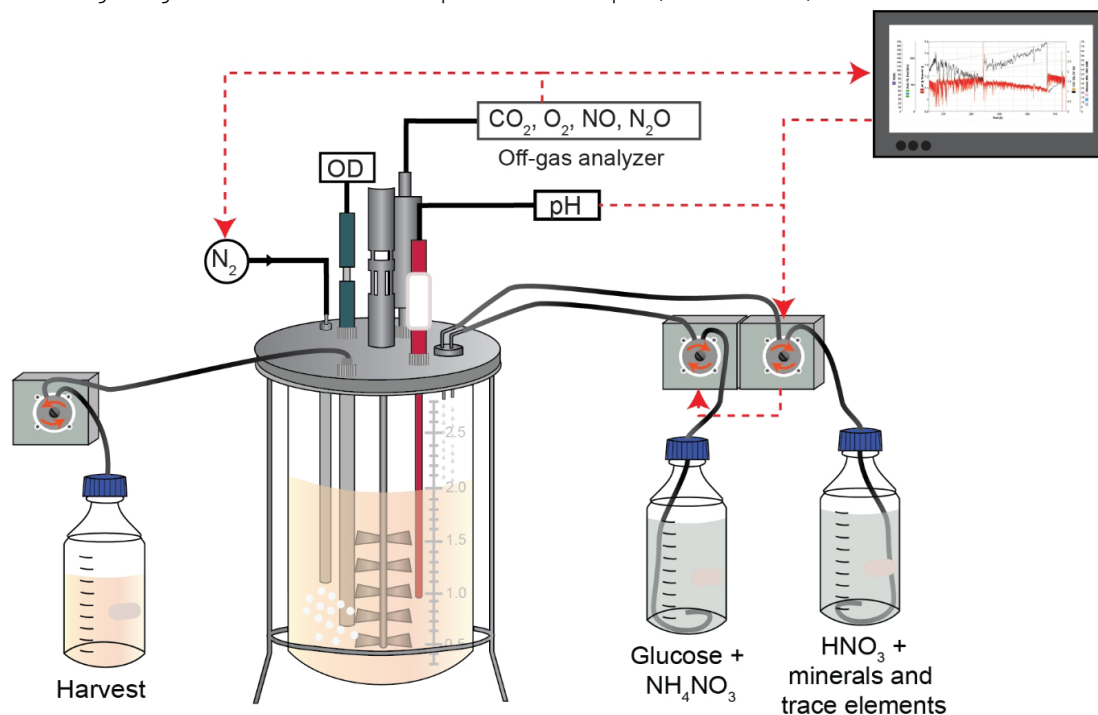


Figure 5 – Overview of the automated bioreactor process. The reactor is kept at 30°C, and anoxic conditions are maintained by continuous sparging with N₂. HNO₃ and feed injections are triggered when the pH is increased above the setpoint. The pH setpoint and the sparging flow rate are changed dynamically, based on the measured concentration of CO₂ in the off-gas.

Although this has demonstrated the feasibility of anaerobic high cell density cultivations with good yields, the growth rate is consistently lower than expected based on low-density cultures. It is suspected that cells experience periodic starvation of electron acceptors due to a CO₂-mediated pH lag, which delays the provision of HNO₃ despite the implementation of a CO₂-dependent dynamic pH set-point and N₂-sparging. This hypothesis is supported by in silico modelling of the pH, CO₂, and NO₃-kinetics.

Current efforts focus on improving production rates through changes in organism selection, medium composition and supply, and optimization of process parameters. In addition, adjustments to the Operation are being explored to further mitigate the CO₂-mediated pH lag and prevent periods of starvation.

Overall, these results demonstrate that anaerobic high cell density cultivation is feasible, routinely achieving high biomass concentrations with yields consistent with substrate input. The biomass has a high protein content, with no observed accumulation of byproducts or fermentation intermediates. Furthermore, it has shown potential as a protein source in fish feed trials with Atlantic salmon and for in vitro meat cultivation (Maråk, 2025).

The process currently relies on glucose as the carbon source. However, the great metabolic diversity of denitrifying organisms facilitates the use of more sustainable carbon sources, such as green methanol. With further optimization, this process has the potential to become a scalable, oxygen-independent platform for sustainable protein production. Its flexibility in feedstock and applicability across food and feed sectors make it a compelling candidate for future biomanufacturing efforts.

KEY TAKEAWAYS

- The Norwegian University of Life Sciences (NMBU) developed a novel oxygen-independent cultivation process using *Paracoccus pantotrophus*, overcoming oxygen transfer limitations typical of aerobic HDC systems.
- A customized Lucillus® Operation enabled real-time, pH-stat-based regulation of substrate and acid addition, incorporating dynamic pH setpoints and automated N₂ sparging to counteract CO₂-induced acidification.
- The system consistently achieved biomass densities above 60 g dry weight L⁻¹ with an average protein content of 75%, while preventing accumulation of toxic intermediates or fermentation by-products.
- The process demonstrates feasibility for large-scale, oxygen-independent protein production, with future opportunities to use sustainable carbon sources (e.g., green methanol) for food and feed applications.

Contributor list

The gravimetric feed control regulation was developed and optimized by Lucillus® Application Specialist Rowin Timmermans from Securecell, based on the requirements and feedback from Marte Mølsæter Maråk, Ph. D. student at the Microbial Ecology and Physiology group (MEP) at NMBU.

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