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AUTOMATED EVOLUTIONARY ENGINEERING OF MICROORGANISMS IN BIOREACTOR SYSTEMS USING THE SOFTWARE LUCULLUS®

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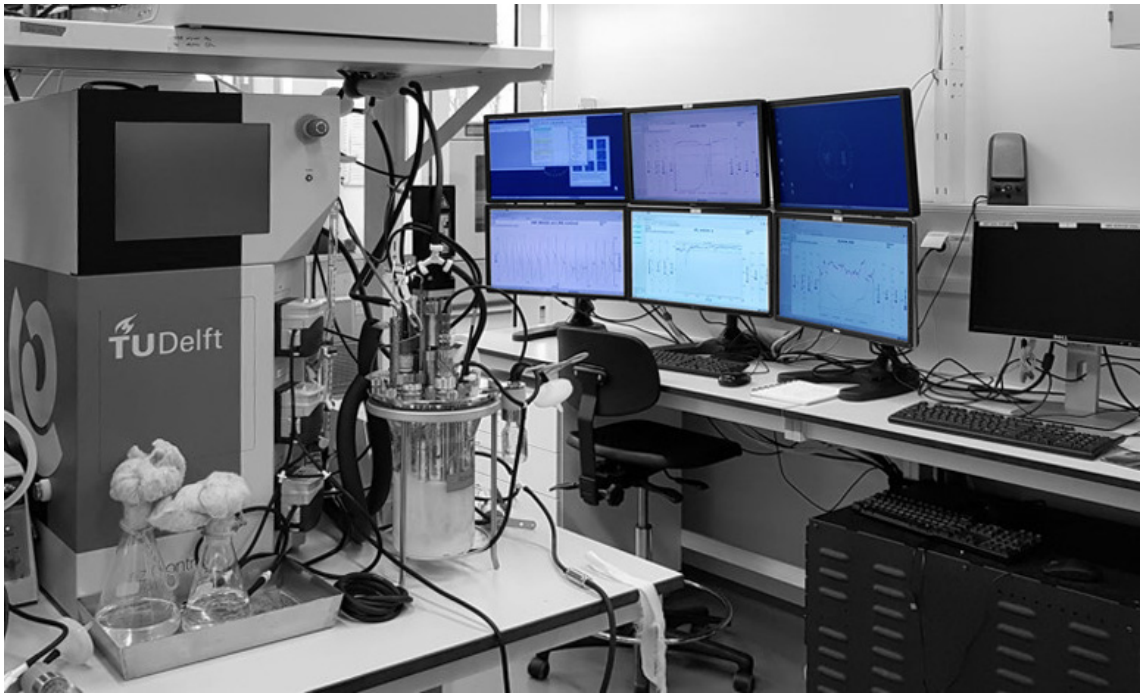
Rowin Timmermans (rowin.timmermans@securecell.ch) Adapted from de Hulster et al., 2022.



Introduction
For economically viable bioprocesses, strain improvements are essential to achieve the desired productivity. Targeted genetic modifications using a combination of bioinformatics and mutagenesis, for example, CRISPR-Cas-mediated genome engineering, enable fast and precise introduction of genetic modifications in the target genome (Stovicek et al., 2017). Evolutionary engineering, also known as adaptive laboratory evolution, is a complementary strain-improvement strategy that exploits the plasticity of microbial genomes. Depending on the selective pressure applied during fermentations, cell populations evolve toward certain desired characteristics, for example, a higher specific growth rate, a lower death rate, or an increased retention in the culture (Dragosits & Mattanovich, 2013). Unlike targeted genetic modifications, evolutionary engineering approaches are commonly used to "generate understanding". This approach becomes valuable when researchers would like to investigate which genes they need to modify to obtain cells with certain characteristics.

This article discusses evolutionary engineering for the industrial production of bioethanol and demonstrates how the efficiency of evolutionary engineering experiments can be drastically increased through automation using the Lucullus® process information management system.

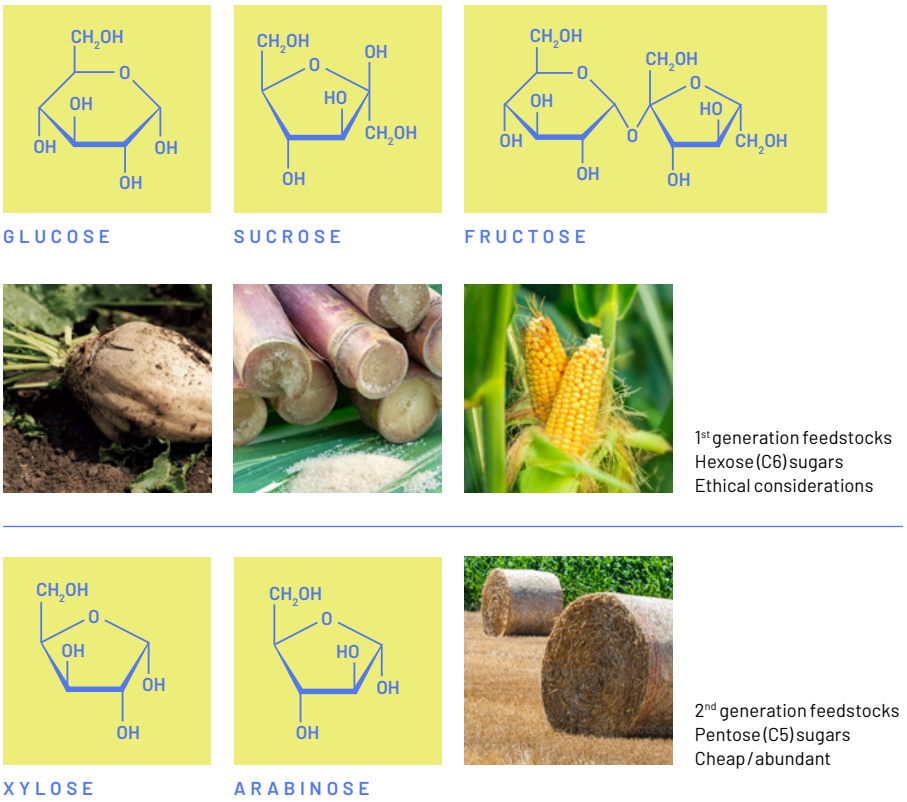


Figure 1: Comparison of first- and second-generation feedstocks for the production of bioethanol.

Production of bioethanol

The growing demand for liquid transport fuels alongside concerns about climate change caused by greenhouse gas emissions from the use of fossil fuels has become one of the greatest challenges for modern society. The generation of biofuels from biomass is a sustainable solution that could substantially decrease the usage of fossil fuels (Herrera, 2006). The yeast, *S. cerevisiae*, is an excellent host microbe and a widely used cell factory for the biomass-based production of fuels and chemicals, in particular ethanol. Common substrates that *S. cerevisiae* uses in the industrial production of ethanol are hexose sugars such as sucrose, glucose, or fructose (Figure 1). These substrates are derived mostly from sugar beets in Europe, sugar cane in South America, or corn in India and the United States (Kang & Lee, 2015). As these so called first-generation feedstocks have the potential to serve as a valuable food source and influence deforestation and biodiversity, ethical considerations arise when utilizing them for the production of ethanol (Paravantis, 2022). There is substantial interest in switching to alternative feedstocks such as waste streams from agricultural production, which are considered second-generation feedstocks. Similar to first-generation feedstocks, second-generation feedstocks are relatively rich in hexose sugars. In addition, they also contain significant amounts of pentose sugars, such as xylose and arabinose. The production of ethanol from these pentose sugars would be interesting from both a social and an economic perspective. Unfortunately, when *S. cerevisiae* is exposed to hexose and pentose sugars during cultivation, the yeast readily uses the hexoses, but only slowly consumes the pentose sugars. In order to cost-effectively produce biofuels from plant biomass, all sugars, including all pentose and hexose sugars present in the raw starting material must be converted efficiently into the final products.

Generating a yeast strain that efficiently uses pentose and hexose sugars has been an object of extensive research for several decades. In a study employing an evolutionary engineering approach, promising results were achieved. An engineered *S. cerevisiae* strain capable of metabolizing pentose sugars was used to explore whether an evolutionary engineering approach could lead to the development of enhanced strains with improved pentose sugar consumption. Before the evolutionary experiment, the strain had a generation time of multiple days. This strain was inocu-

lated in shake flasks containing medium where only pentose sugars were supplied as a carbon source. Whenever the culture reached a high cell density, part of the culture was transferred into a new shake flask. After repeating this growth and transfer process many times, eventually, an evolved strain was obtained with a generation time of less than 1 day. Whole-genome sequencing was performed of the evolved strain and a single amino acid mutation was detected in a hexose transporter. The evolved strain showed improved xylose uptake rates, as well as partial co-utilization of glucose and xylose in mixed sugar cultivations (Reider Apel et al., 2016). The capability of this strain to consume both glucose and xylose at comparable rates is a very relevant characteristic to efficiently and industrially produce ethanol from second-generation feedstocks.

"Using the Lucullus® software, we are able to cultivate microbial cultures for long periods with complex feeding routines. To ensure optimal control of culture parameters and minimize manual labor, it is essential to have reliable and adaptable culture automation software. Our experience with the Lucullus® software has been very positive, and we have received excellent support in customizing its features to fit our needs."

Marcel Vieira-Lara
Assistant Professor at TU Delft

Automated evolutionary engineering experiments
In the previous example, cells were cultivated in shake flasks. For the automation of evolutionary engineering experiments, cultivations in bioreactor systems are better suited, as the local environment can be controlled much more precisely and therefore, the process conditions can be better defined and control tasks automated. As the setup of a bioreactor experiment is more complex than the setup of a shake flask experiment, a protocol was written that outlines a generic setup for automated evolutionary experiments using Getinge Applikon bioreactor systems combined with the process information management software Lucullus® (de Hulster et al., 2022).

The material requirements for evolutionary engineering experiments vary depending on the target strain, desired evolutionary outcome, and selective conditions in the culture. Figure 2 shows a schematic representation of the bioreactor setup that was used for the automated evolutionary engineering experiments with aerobic and anaerobic cultures of *S. cerevisiae* with a variety of carbon sources as growth-limiting nutrients. The setup consisted of (1) a autoclavable bioreactor, (2) an electronic liquid level sensor, (3) a medium inlet, (4) an effluent pipe near the reactor bottom, (5) a medium reservoir connected to the bioreactor with a controllable pump, (6) a waste container connected to the bioreactor with a controllable pump, (7) pressurized gas line with mass flow controller (MFC) and (8) the Lucullus® control software for the automation of the setup during the evolutionary engineering experiment.

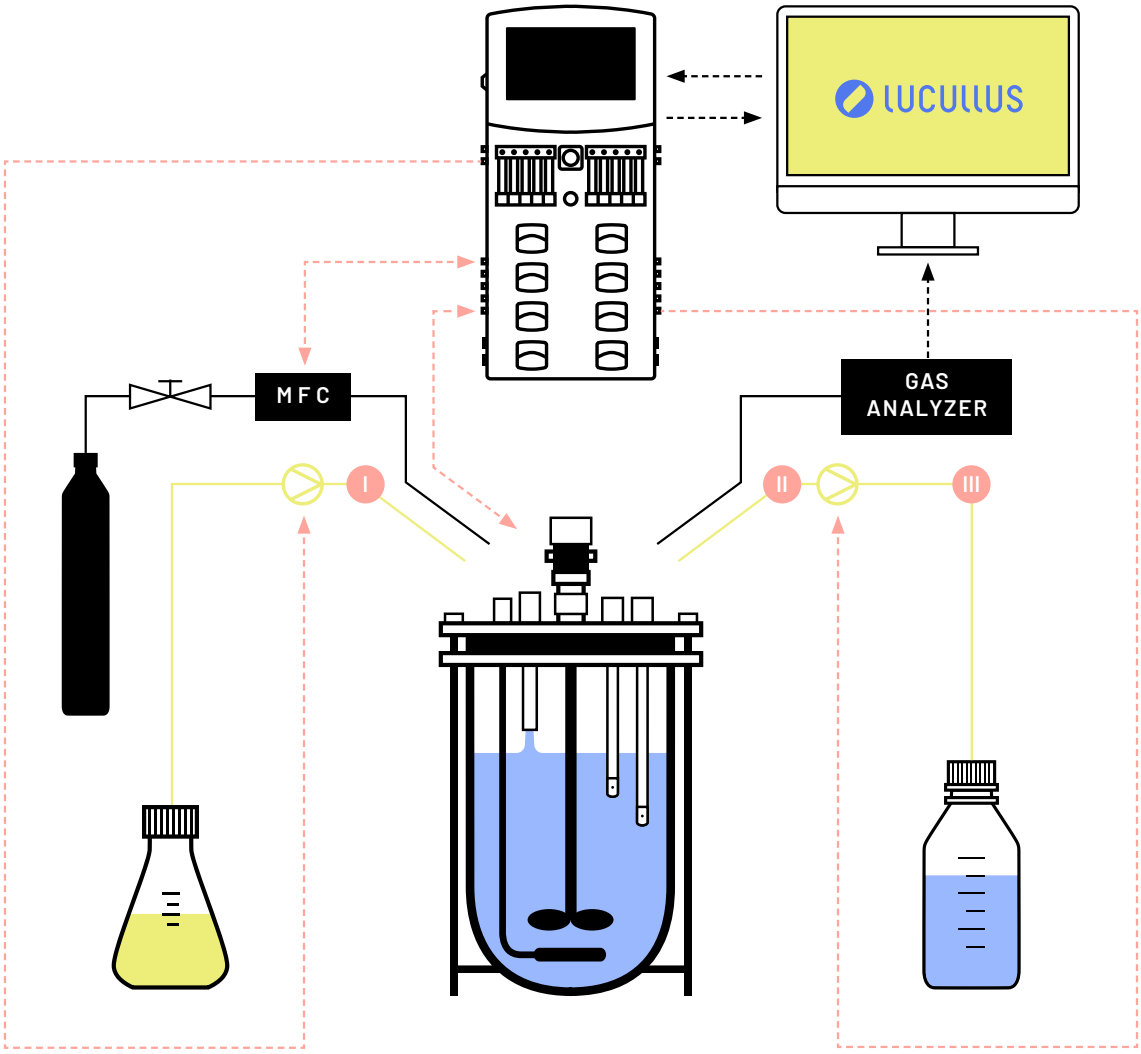


Figure 2: Schematic representation of the bioreactor setup used for automated evolution.

Sequential batch cultures

Different cultivation strategies for evolutionary engineering experiments exist, namely sequential batch cultivation, chemostat cultivation, and accelerostat cultivation. The different cultivation strategies are described in detail in the following paper: *Under pressure – evolutionary engineering of yeast strains for improved performance in fuels and chemicals productions* (Mans et al., 2018). In this article, the concept of evolutionary engineering is described for sequential batch cultivations (repeated batch).

The operator starts the preparation of the materials for the automated evolutionary engineering experiment (Figure 2). The prepared bioreactor is inoculated with the first batch. This first batch is normally not representative in terms of batch time and other process values (Figure 3A). After the first, non-representative batch, the emptying and refilling of the reactor are performed manually. The reactor is then inoculated with the second batch. After the second batch, the batch time is determined (20 h), the highest outflow CO_2 value (blue line; an indicator of the number of cells in the bioreactor) is measured (0.46%) and the cumulative level of CO_2 (orange line; an arbitrary value estimating how much of the carbon substrate was consumed) is calculated (3.1) (Figure 3B). These three process values are used to set the threshold parameters for the automated empty and refill cycles in the Lucullus® software. To safeguard against unintended emptying/refill cycles, check-

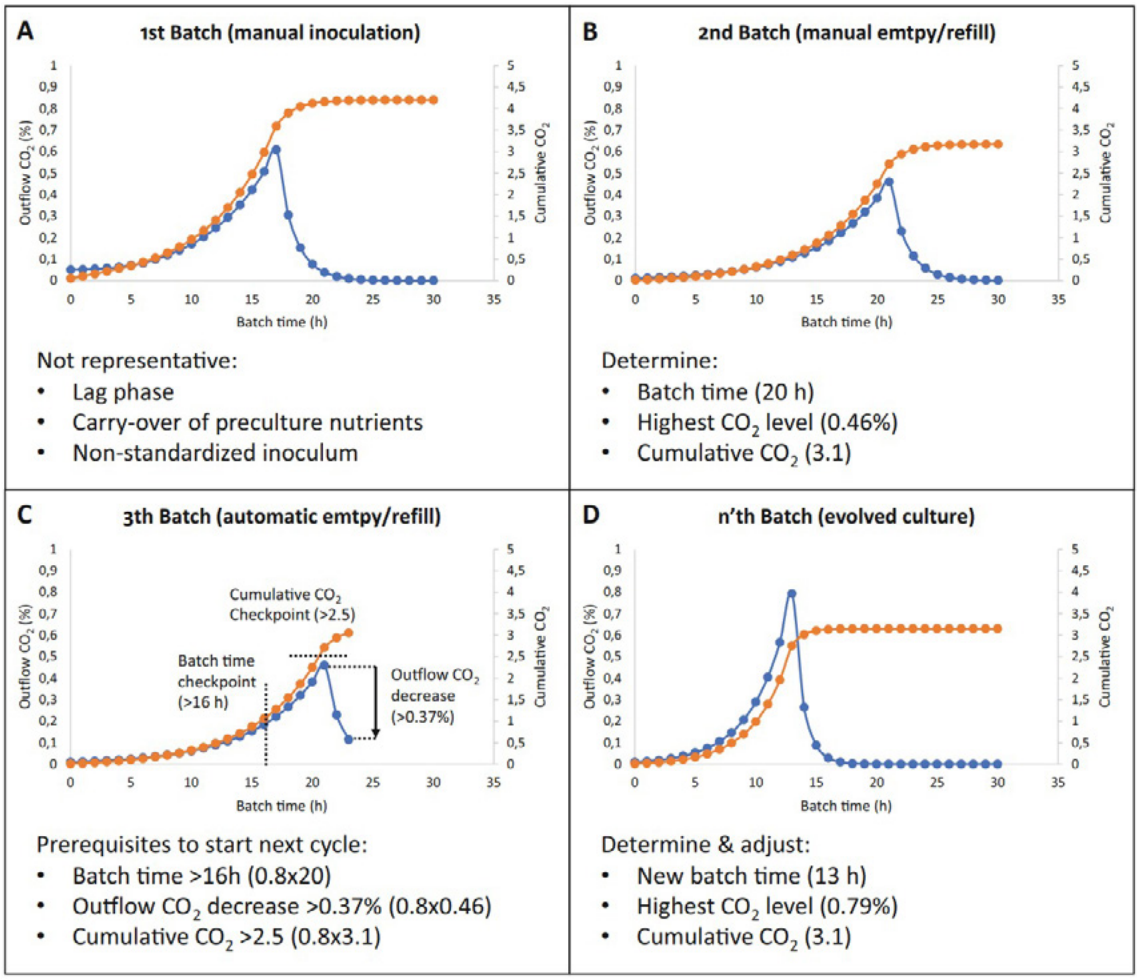


Figure 3: Schematic representation of theoretical CO_2 profiles obtained when setting up automated evolutionary experiments in a sequential batch setup. Blue lines represent the concentration of CO_2 (%) in the outgoing gas, and the orange line represents the (arbitrary) cumulative CO_2 value. From de Hulster et al., 2022.

points of batch time, outflow CO_2 level decrease, and cumulative level of CO_2 are set (Figure 3C). The prevention of unintended empty/refill cycles is important because the loss of a significant part of the population might perturb the automated evolution. After these threshold values are set in the software Lucullus®, the sequential batch process can run on its own. Over time, the culture starts evolving i.e., the batch time, highest outflow CO_2 value, and cumulative level of CO_2 change (Figure 3D). Periodically these threshold parameters need to be adapted to make sure that the empty/refill cycles are still triggered at the right time, which is fundamental to keep the culture evolving.

Software Lucullus®

The Lucullus® process information management system is a comprehensive bioprocess automation software. Besides basic SCADA functionalities that allow for device monitoring and control of over 100 devices found in typical bioprocessing environments, Lucullus® has an inbuilt sample management and raw material management functionality, and interfacing capability with various LIMS and ELN systems, as well as statistical software packages, such as Phyton, MATLAB, and DataHowLab.

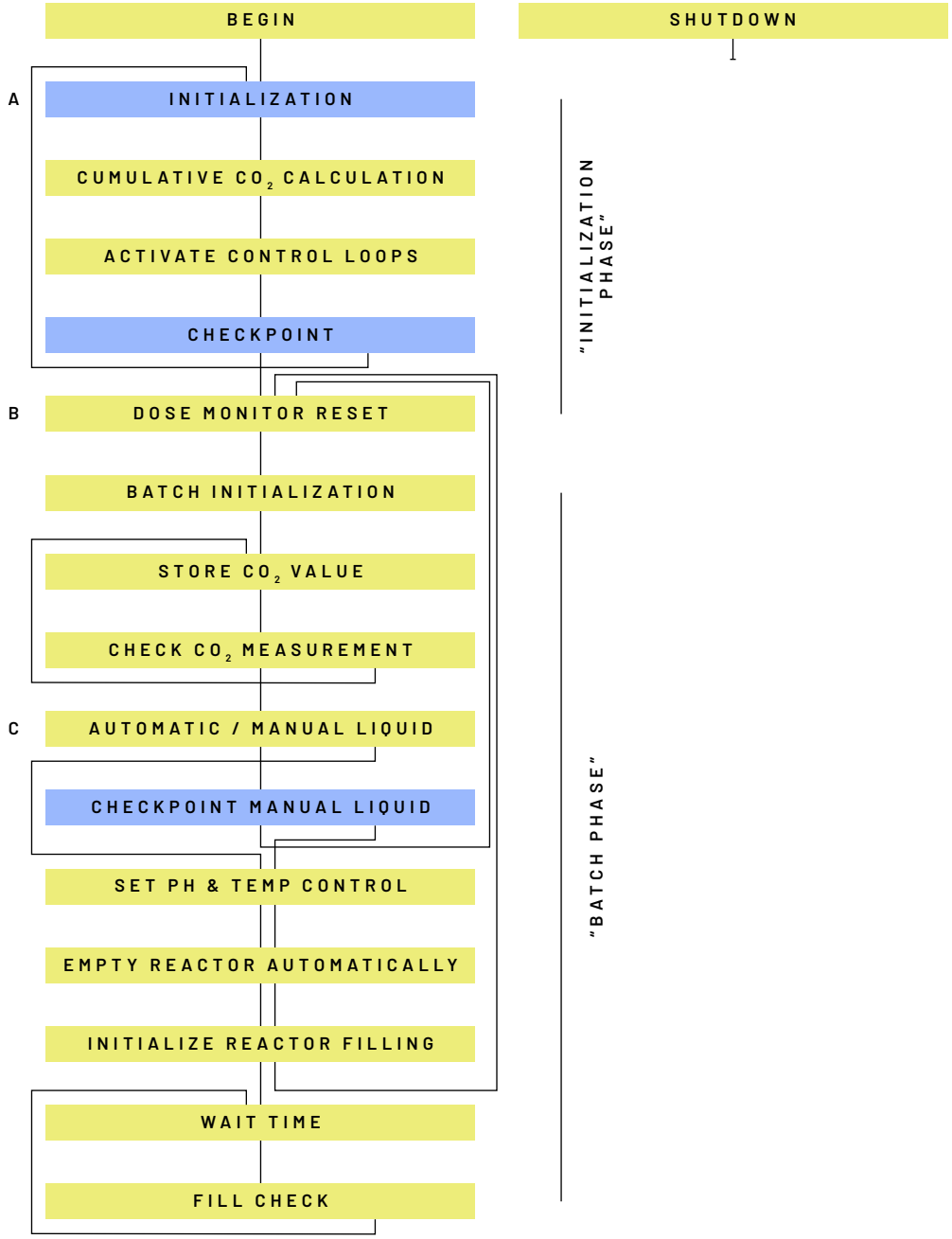


Figure 4: Repeated Batch Operation in the Lucullus® process information management system version 3.11.7. Blue blocks indicate manual steps to prepare the cultivation and process. Yellow blocks indicate automated steps for autonomous process operation. The Step Chain can be divided into three different parts: (A) Initialization phase, (B) a Batch Execution phase, and (C) a Medium Replacement phase.

Sequential batch cultures with Lucullus®
Researchers at the TU Delft used Lucullus® to automate and digitalize their evolutionary engineering experiments. Figure 4 shows a schematic of the Operation or Step Chain/process recipe used to automate the Repeated Batch experiment. The Operation guides the operator through the setup of the process with manual user interaction steps (blue blocks) and thereafter executes the bulk of the process autonomously (yellow blocks).

Explanation of the Repeated Batch Operation
The individual steps of the Step Chain presented in Figure 4 are comprised of the following functions:

- **Begin:** Default step with no commands, executed at the start of every process
- **Initialization:** User interaction step, checking/adjusting critical process parameter values
- **Cumulative CO_2 Calculation:** Formula definition step, calculating the cumulative amount of CO_2 produced in the bioreactor
- **Activate Control Loops:** Controller activation step, starting pH, stirrer, and temperature control
- **Checkpoint**: User interaction step, checking whether to proceed to the Dose Monitor Reset step or return to the Initialization step
- **Dose Monitor Reset:** Step, resetting the totalizer values of the gas flow, acid, and base pumps
- **Batch Initialization:** Step, starting (the first or one of the consecutive) batch cultivations
- **Store CO_2 Value:** Step, storing the value of the CO_2 off-gas measurement for subsequent value comparison (see Check CO_2 Measurements step)
- **Check CO_2 Measurements:** Step, comparing the current CO_2 value with the previously stored value. Loops back repeatedly until the end of the batch is reached
- **Automatic / Manual Liquid Cycling:** Step, directing Lucullus® to an automated or manually executed medium exchange
- **Checkpoint Manual Liquid Cycling:** Step, allowing the user to confirm the completion of a manual medium exchange or to switch to an automated medium exchange
- **pH & Temp Control:** pH and temperature control step, activating or deactivating pH and temperature control loops as selected by the user during the initialization
- **Empty Reactor Automatically:** Harvest step, emptying the reactor automatically by the harvest pump, based on a predefined pump time activity selected by the user during the initialization
- **Initialize Reactor Filling:** Refilling step, adding fresh medium to the reactor by the feed pump
- **Wait Time:** Waiting step of 6 seconds that is required for the Fill Check step
- **Fill Check:** Step, checking the level inside the bioreactor. May loop back to the previous step repeatedly.

An example of a manual user interaction step from the Repeated Batch Operation (Figure 4) is shown in Figure 5. A pop-up window signifies the manual interaction step. Within this window, the operator encounters a list of parameters labeled as „Name“. Each parameter can be associated with a scientific unit (“Unit”). To assist the operator, a description can be provided for each parameter (“Description”), as well as an acceptable range of values (“Range”). The default process values assigned to each parameter can be modified before starting the process (“Default”).

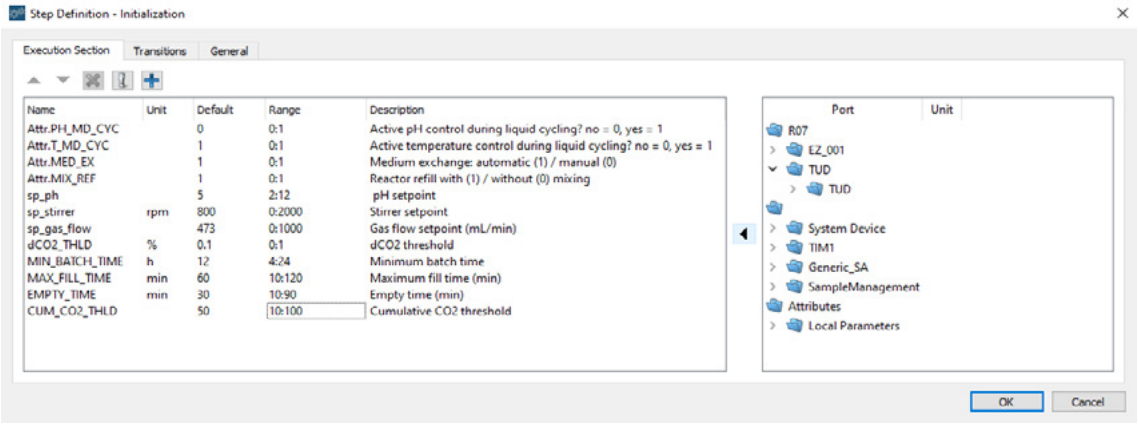


Figure 5: Example of the "Initialization" manual interaction step in the Repeated Batch Operation.

During the process, Lucullus® signals the current execution stage through process indicators known as "Phases". In Figure 6, an excerpt of the "Batch Initialization" step of the Repeated Batch Operation is shown, marking the start of the batch phase. Once the batch phase has reached a point where the criteria for termination are fulfilled (see "Check CO_2 Measurements" step), Lucullus® will then proceed with the "Empty Reactor Automatically" step, provided that the user has not opted before for manual medium exchange (Figure 4). The "Empty Reactor Automatically" step is succeeded by the "Fill Reactor Automatically" step, after which a subsequent batch phase is started.

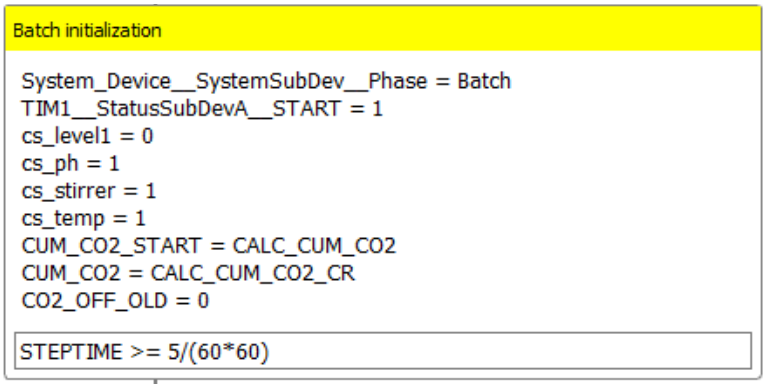


Figure 6: Example of the "Batch Initialization" step of the Repeated Batch Operation marking the start of the batch phase.

A crucial part of the Repeated Batch Operation is the monitoring CO_2 in the off-gas. By programming a loop consisting of two interconnected steps, Lucullus® keeps track of the highest recorded value of CO_2 for a particular batch. The two-step loop is presented in Figure 7.

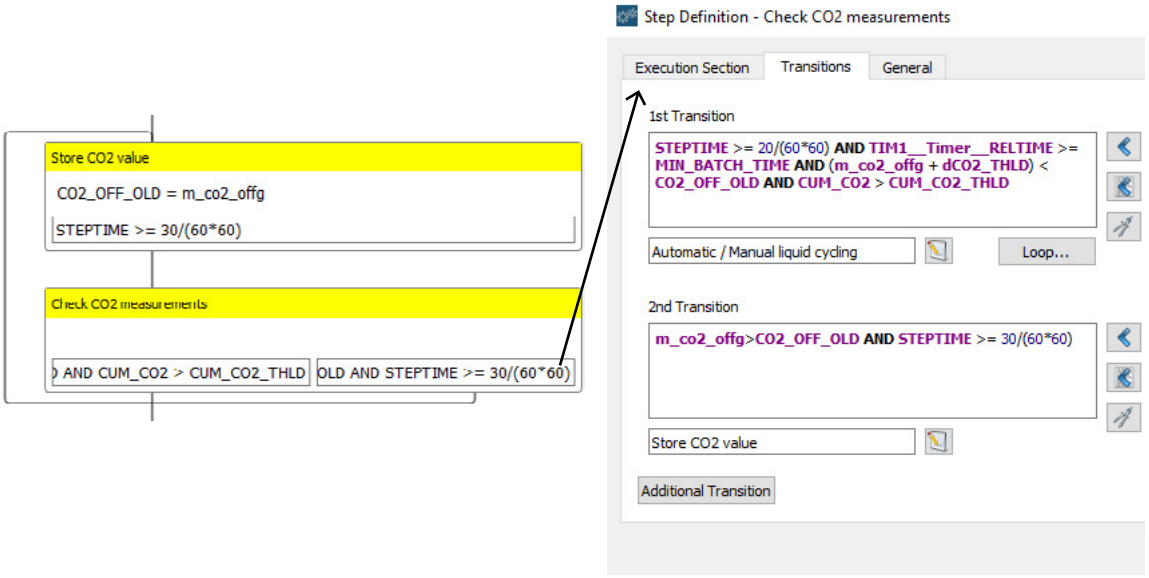


Figure 7: Example of a programmed loop in the Repeated Batch Operation to capture and store the highest measured value of the CO_2 from the off-gas. If the 1st transition criterion is met in the "Check CO_2 Measurements" step, the current batch is terminated; three indicators are used for this: (1) a minimum amount of time spent in the current batch, (2) the current CO_2 value dropping below a calculated threshold and (3) the total amount of CO_2 produced in the current batch exceeding a pre-defined threshold. If the 2nd transition criterion is met in the "Check CO_2 Measurements" step, Lucullus® loops back to the "Store CO_2 Value" step and will overwrite the old CO_2 value (CO2_OFF_OLD) and store the new one (m_co2_offg).

When a batch has been completed, the majority of the fermentation broth needs to be removed (harvested). A small volume of liquid that remains in the bioreactor after harvesting serves as inoculum for the next batch. Operators can select the empty and refill of the bioreactor manually or automatically. In the second case, Lucullus® is programmed to perform the harvest and refill by executing two consecutive steps (Figure 8).

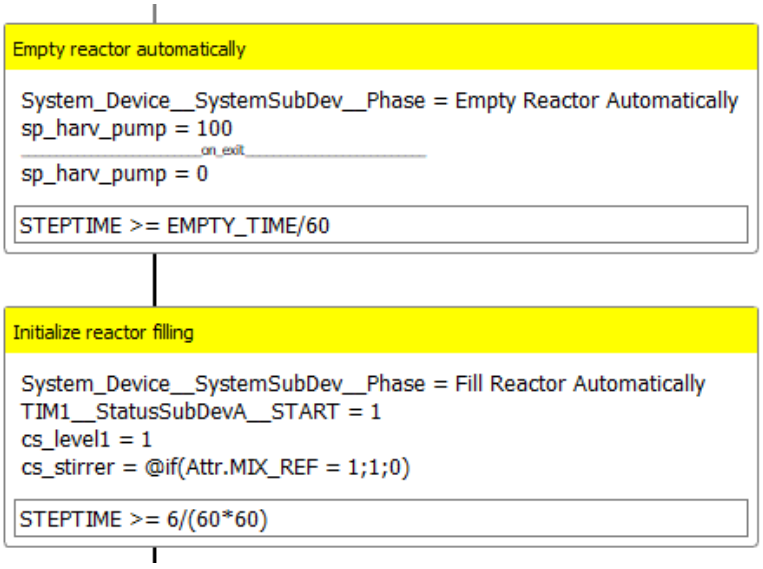


Figure 8: Example of the two steps involved in the harvest and refill of the bioreactor. The harvest pump is responsible for emptying the bioreactor (parameter "sp_harv_pump"). After a pre-defined period (parameter "EMPTY_TIME"), Lucullus® stops the harvest pump and proceeds with refilling the bioreactor. Refilling the bioreactor is achieved by activating the level control loop (cs_level1 = 1). When this control loop becomes active, the feed pump starts adding fresh medium to the bioreactor until the level sensor makes contact with the liquid indicating that the bioreactor has been successfully refilled to the proper cultivation volume.

Lucullus® automatically ceases all active control loops and actuators upon the conclusion of an experiment. This functionality is illustrated in Figure 9.

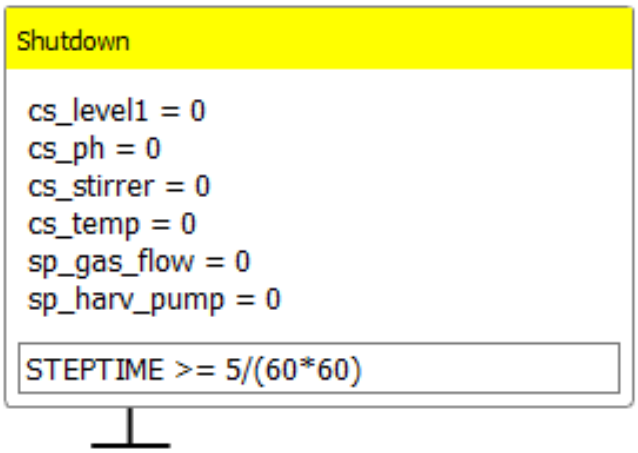


Figure 9: The Shutdown step of the Repeated Batch Operation automatically puts the bioreactor controller into a neutral state by deactivating the level, pH, stirrer, and temperature control loops, as well as stopping gas additions and the harvest pump. Once this has been done, the process is terminated.

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