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Chapter 15

Automated Evolutionary Engineering of Yeasts

Erik de Hulster, Christiaan Mooiman, Rowin Timmermans,
and Robert Mans

Abstract

Evolutionary engineering of microbes provides a powerful tool for untargeted optimization of (engineered) cell factories and identification of genetic targets for further research. Directed evolution is an intrinsically time-intensive effort, and automated methods can significantly reduce manual labor. Here, design considerations for various evolutionary engineering methods are described, and generic workflows for batch-, chemostat-, and accelerostat-based evolution in automated bioreactors are provided. These methods can be used to evolve yeast cultures for >1000 generations and are designed to require minimal manual intervention.

Key words Evolution, Bioreactor, Batch, Chemostat, Accelerostat, Automation, Control software

1 Introduction

Evolutionary engineering of microorganisms is a powerful approach to obtain mutants with improved phenotypes for desired traits. Experimental procedures range from simple serial passaging of cultures to prolonged cultivation in tightly controlled bioreactors [1]. Especially when the desired phenotype is the result of multiple genetic mutations with incremental beneficial effects, several hundreds to thousands of generations may be required to accumulate each mutation in the final dominant population. Albeit the short generation times of popular microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, these evolutions are often time intensive, and automated systems provide an opportunity to greatly reduce manual labor. Here, methods are described for evolution of yeasts in bioreactors where a combination of online measurements and control software is applied to enable automated progression of the culture. The experimental setup of the reactor

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system is designed such that it can be used for evolution in repeated batch cultivation, chemostat cultivation, and accelerated chemostat (accelerostat) cultivation. Compared to conventional methods, little manual intervention is required, and cultures can be maintained for >1000 generations.

2 Materials

The material requirements for evolutionary engineering experiments vary depending on the target strain, desired evolutionary outcome, and selective conditions in the culture. This protocol describes a minimal set of equipment required to run the automated evolution in batch, chemostat, and accelerostat mode and has been validated with aerobic and anaerobic cultures of *Saccharomyces cerevisiae* with a variety of carbon sources as growth-limiting nutrient.

Depending on the target organism and desired evolutionary outcome, the described methods should be further modified to achieve the desired selective pressure in the culture. Figure 1 provides a schematic representation of the minimal equipment requirements, which are comprised of as follows:

2.1 Hardware: Bioreactor and Related Equipment

A sterilizable bioreactor must be equipped with the following:

1. Electronic liquid level sensor: when the top of the turbulent culture broth connects to the sensor, it activates the controllable waste pump, removing the medium via the effluent pipe
2. Medium inlet: a point of entry into the bioreactor for fresh culture medium behind the controllable medium pump
3. Effluent pipe near the reactor bottom: a point for well-mixed broth removal that allows for near-complete emptying of the bioreactor
4. Gas in- and outlet: a point of entry for gasses, preferably using a sparger at the bottom of the reactor and a point for exhaust gas (“off-gas”) to leave the reactor
5. Optional: thermostat and pH control, for maintaining constant temperature (active or passive control) and pH via a pH probe coupled to controllable pumps for acid/base addition
6. Medium reservoir, which is connected to the bioreactor with a controllable pump
7. Waste container, which is connected to the bioreactor with a controllable pump
8. Pressurized gas line with (mass) flow controller (MFC)

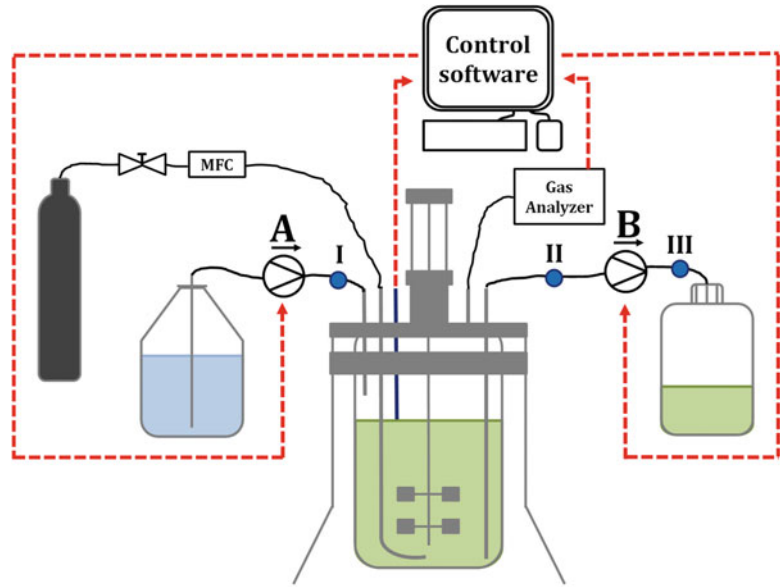


Fig. 1 Schematic representation of the bioreactor setup used for automated evolution. The medium reservoir (blue) and waste vessel (green) are connected to the bioreactor via peristaltic pumps (a, b, respectively), which are controlled via the control software (red arrows). The medium in- and outlet lines can contain various sample ports (I, II, and III), which are used to check the state of the cultivation. The inflow of gas is regulated by a mass flow controller (MFC), and the outgoing gas is analyzed for CO₂ content by a gas analyzer, which provides online measurements of the off-gas composition to the control software. The bioreactor is equipped with an electronic-level sensor, which provides a binary signal to the control software based on the level of the liquid in the bioreactor

9. Bio controller, which is connected to the relevant sensors (level, pH, and temperature) and actuators (stirrer, pumps, and MFCs), as well as the computer with a SCADA (Supervisory Control and Data Acquisition) software
10. Off-gas analyzer with CO₂ detection

2.2 Software

1. Computer with a SCADA software. For this protocol, Lucillus PIMS (Process Information Management System) Standard edition with *Input* from liquid-level sensor and off-gas analyzer and *Output* to controllable liquid pumps

2.3 Accessories

1. Optional, sample ports for (i) sampling reservoir culture medium (Fig. 1I), (ii) direct sampling of culture broth (Fig. 1II), and (iii) sampling culture effluent (Fig. 1III)
2. Cultivation medium used for the selection regime

3 Methods

3.1 Setting Up the Lucullus PIMS Control Software

The methodologies for evolutionary engineering, described in Sub-headings 3.2, 3.3, and 3.4 and in the notes, can be used to set up automated workflows with different SCADA software packages. The steps described in this protocol are based on the use of the Lucullus PIMS software, which has to be properly set up before operational scripts can be executed.

1. In the supplementary materials, the operational scripts for control of the bioreactor cultivations (Operations) using the Lucullus PIMS (Standard edition) software (version 3.8.3) are available (Supplementary file Repeated_Batch.xml and Chemostat_Accelerostat.xml). To enable successful import and execution of these scripts, the parameters in the Lucullus destination installation must be made compatible to those that have been defined in the source Operations. Similarly, the process phases and online calculations used in the source Operations have to be configured in the Lucullus destination installation. The file “Import_Ports.xlsx” (supplementary materials) contains data for all relevant parameters (indicated as “ports” in Lucullus) used in the operations and can be imported separately for each biocontroller to configure the ports in Lucullus using the “Import Channels” function in the System Admin tool. When importing ports into Lucullus, select the correct file with “Open File...,” and then select “Validate Data,” and after clicking “Apply Changes,” the ports are configured.
2. All process phases and online calculation formulas are described in the file “Formulas_Phases.pdf” (*see* supplementary materials and **Note 1**), and their configuration in Lucullus is done using the “Process phase configuration” function in the System Admin tool. Formulas are defined using the “Evaluations > Calculate...” function in the Graphic tool and are made individually in the “Calculation” window. When all the formulas are incorporated and saved, they should be added to the online calculator using the “Online Calc...” function found in the bottom left of the “Calculation” window. To make the formulas available for active use during running processes, they are moved from the left to the right window in the “Configuration of Online Calculations” screen. After configuration of the port settings, process phases, and online calculations, Lucullus can import the provided operations using the “File > Import from file...” functionality in the “Operation” tool. In case there are mismatches between the port names used in the Operation that is imported and the names of the ports already defined in the System Admin tool, Lucullus provides a port mapping functionality to make a valid link between these ports. Using the “Verify” functionality, any ports that are left undefined can be found and corrected accordingly.

3.2 Repeated Batch

Repeated batch cultivation is often used for selection of mutants with increased specific growth rates under the chosen conditions. Repeated batch experiments have, for example, resulted in mutants with improved rates of substrate consumption [2] and product formation or better stress tolerance to high inhibitor concentrations [3], extreme temperatures [4], or extreme pH [5] conditions.

1. The bioreactor is prepared, sterilized, and assembled according to [6] (*see Note 2*).
2. Fill the bioreactor with culture medium or sterile water to the precise intended working volume.
3. Turn on the gas analyzer and all equipment used to control the culture parameters of the bioreactor.
4. Launch the Lucullus PIMS software, and load the *Repeated Batch* operation (Supplementary file *Repeated_Batch.xml*).
5. In the “Initialization” step of the *Repeated Batch* operation, enter the desired set points for gas flow, stirring, pH, and temperature in the “Input Console.” Ensure “Medium exchange” is set to manual.
6. Proceed to the first “Checkpoint” step of the *Repeated Batch* operation to start active control of culture parameters, and wait for the system to stabilize at the respective set points (*see Note 3*).
7. Aseptically adjust the tip of the level sensor to the top of the turbulent liquid level. This step will determine the working volume of the culture (*see Note 4*).
8. Turn on the outflow pump (Fig. 1b) at maximum speed, and determine the time (in minutes) required to empty the reactor to the bottom of the effluent pipe.
9. Select the option to return to the initialization screen, and enter 110% of the time required to empty the reactor (determined at **step 8** in Subheading 3.2) in Lucullus Input Console under “Empty time (min)” (*see Note 5*).
10. If necessary, adjust the bottom of the effluent pipe to ensure that the volume remaining in the reactor after draining the culture is 0.2–1% of the working volume (determined at **step 2** in Subheading 3.2). This remaining culture volume will determine the number of generations per batch (*see Note 6*).
11. Refill the reactor using the inflow pump (Fig. 1a) at maximum speed until contact with the electronic level sensor is made and the intended working volume is reached, and determine the time (in minutes) required to completely fill the reactor.
12. Inoculate with the desired yeast strain, and select the option to proceed with the “Batch initialization” step.

13. Wait until the culture has depleted the limiting medium component, which is often indicated by a drop in the CO₂ concentration in the exhaust gas (*see* **Note 7**).
14. Repeat **steps 8** and **11** in Subheading **3.2** to completely empty and refill the reactor. The culture volume that remains after emptying the reactor (determined at **step 10** in Subheading **3.2**) acts as inoculum for the next batch phase (*see* **Note 8**).
15. In the second “Checkpoint” step of the Repeated Batch operation, choose the first option to start a new batch.
16. Wait until the culture has depleted the limiting medium component, which is often indicated by a drop in CO₂ in the exhaust gas (if the limiting component is the carbon source), and determine (1) the time (in hours) required to complete a single batch phase, (2) the highest concentration of exhaust gas CO₂ that is reached within the batch phase, and (3) the final calculated cumulative CO₂ (CUM_CO2) value (Fig. **2b**). The cumulative CO₂ value can be obtained by plotting CUM_CO2 in the “Trend” tab of the “Execution Monitor” tool.
17. Open the Input Console using the “Monitor/Console” menu (Online Tool) or the “Console” button (Execution Monitor), and enter 80% of the highest CO₂ level reached in the field “dCO2 threshold (%)” enter 80% of the time required to complete the batch in the field “Minimum batch time (h),” enter 80% of the cumulative CO₂ value in the field “Cumulative CO2 threshold,” enter 120% of the time required to fill the reactor (determined at **step 11** in Subheading **3.2**) in the field “Maximum fill time (min),” and finally set “Medium exchange” to automatic (*see* **Note 9**). These set points will determine the prerequisites for initiating the empty/refill cycle to start the next batch and act as safeguard to prevent washout of the culture (*see* Fig. **2c**).
18. In the second “Checkpoint” step of the Repeated Batch operation, now with all parameters set, choose the second option to start the automated evolution (*see* **Note 10**).
19. When the growth rate of the evolving culture increases, repeat **steps 16** and **17** in Subheading **3.2** to update the threshold settings accordingly (*see* Fig. **2d** and **Note 11**).
20. When the growth rate has increased to a satisfactory level, the evolution can be stopped, and an aliquot of the evolved population stored for further analysis.

3.3 Chemostat

Evolutionary engineering in nutrient-limited chemostat cultivations has been extensively used to improve the affinity of (engineered) yeasts for the growth-limiting nutrient (*see* **Note 12**). Examples include strains with improved affinity for the carbon substrates xylose [7], sucrose [8], or sugar mixtures [9].

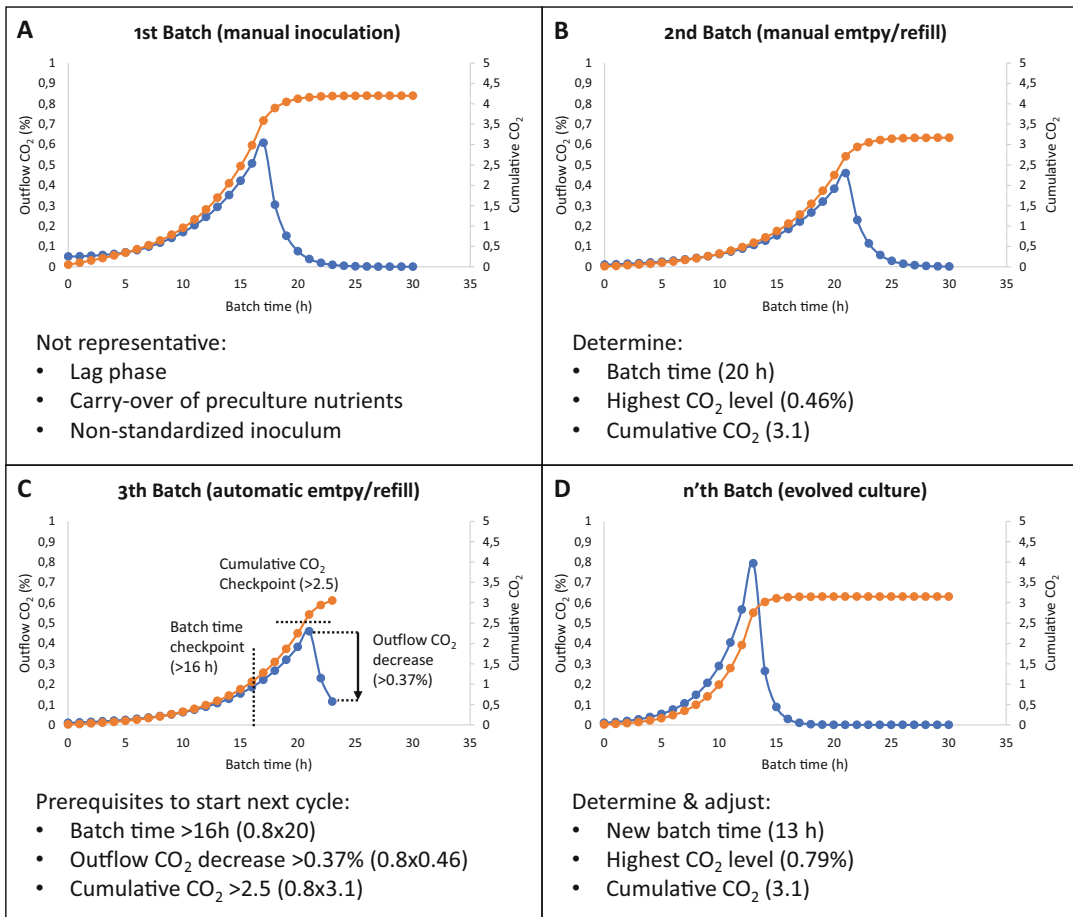


Fig. 2 Schematic representation of theoretical CO₂ profiles obtained when setting up automated evolution in a sequential batch setup. Blue lines represent the concentration of CO₂ (%) in the outgoing gas, and the orange line represents the (arbitrary) cumulative CO₂ value. Panel (a) represents a typical profile obtained in the batch phase after the bioreactor is inoculated with an external preculture. This profile is usually not representative of the profiles obtained during the automated evolution because of possible changes in growth conditions and variations in inoculum size. Panel (b) represents the profile obtained after the first manual empty/refill of the reactor. The obtained profile resembles the evolution profiles and is therefore used to determine the expected batch time, maximum CO₂ concentration, and cumulative CO₂ value (indicated by the values between brackets for this specific example). Panel (c) represents a single batch during evolution, where checkpoints for batch time, a drop in CO₂ concentration, and the cumulative CO₂ value are used to prevent an early trigger of the next empty/refill cycle and washout of the culture (indicated by the calculated values between brackets for this specific example). Panel (d) represents a batch cycle of the evolved population. As a result of the gradually increasing maximum specific growth rate, the checkpoints (Panel c) have to be adjusted at regular intervals (indicated by the values between brackets for this specific example)

1. The bioreactor is prepared, sterilized, and assembled according to [6] (*see Note 2*).
2. Fill the bioreactor with culture medium to the precise intended working volume. This step can be used to calibrate the feed pump and determine its set point to provide the preferred liquid flow rate for the chemostat phase (*see Note 13*).

3. Turn on the gas analyzer and all equipment used to control the culture parameters of the bioreactor (bio controller).
4. Launch the Lucullus PIMS control software, and load the *Chemostat & Accelerostat* operation (Supplementary file *Chemostat_Accelerostat.xml*).
5. In the “Initialization” step, enter the desired set points for aeration, stirring, pH, temperature, and the feed rate (set point feed pump) in the “Input Console.”
6. Proceed to the first “Checkpoint” step of the Repeated Batch operation to start active control of culture parameters, and wait for the system to stabilize at the respective set points (*see Note 3*).
7. Aseptically adjust the tip of the level sensor to the top of the turbulent liquid level. This step will determine the working volume of the culture (*see Note 4*).
8. Inoculate the reactor with the desired yeast strain, and select the first option to proceed to the “Batch initialization” step.
9. Wait until the culture has depleted the limiting medium component, which is often indicated by a drop in the CO₂ concentration in the exhaust gas (*see Note 7*), and proceed to the “Chemostat initialization” phase. By default, the chemostat phase is automatically initiated when a drop in CO₂ in the exhaust gas of >50% compared to the maximum value is detected (*see Note 14*).
10. Wait until the culture has reached steady-state conditions, indicated by a constant metabolite composition in the culture broth and a constant CO₂ content in the exhaust gas.
11. Over time, evolution of the culture is expected to lead to a lower concentration of the growth-limiting nutrient (usually the carbon source) in the culture broth and consequently a higher concentration of biomass and/or higher formation rate of extracellular catabolic products (such as ethanol and/or CO₂; Fig. 3). When the culture has evolved to a satisfactory level, the evolution can be stopped, and an aliquot of the evolved population stored for further analysis. Alternatively, the evolution can be continued in accelerostat mode (*see Note 15*).

3.4 Accelerostat

Evolution in accelerostat cultivation, after a chemostat phase, can be applied to maintain selective pressure on affinity for the growth-limiting nutrient while simultaneously increasing the growth rate. The triggers for increasing or decreasing the dilution rate in this protocol are based on the ratio between the CO₂ concentration in the outgoing gas and the inflow rate of fresh medium (*see Fig. 3 and Note 16*). This method has been successfully applied to select for yeast mutants with biotin prototrophy [10] and improved sucrose uptake [11].

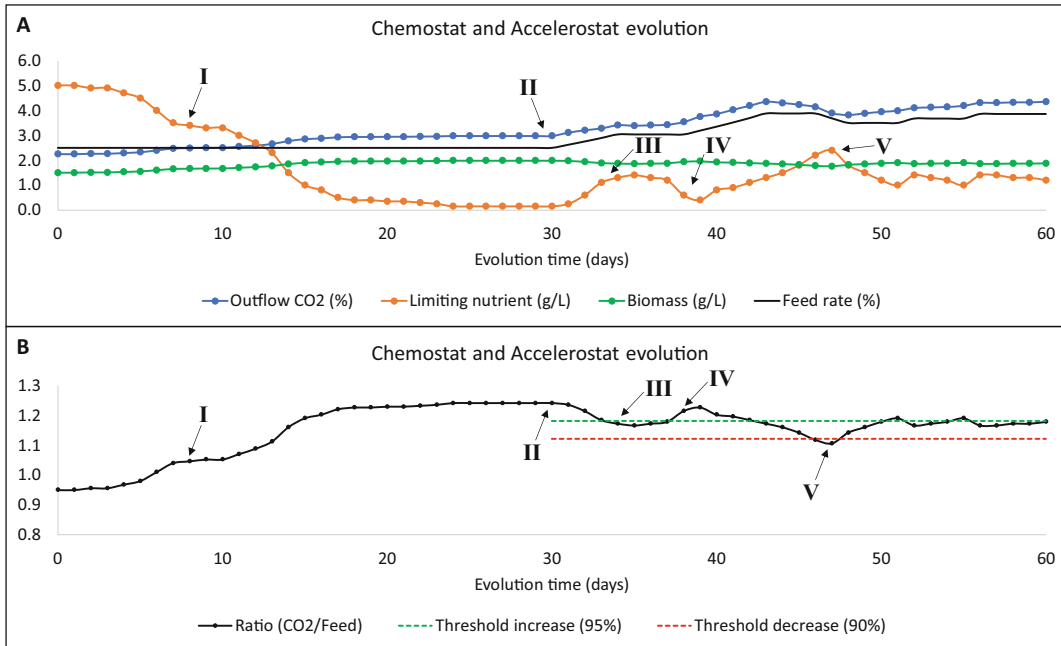


Fig. 3 Schematic representation of theoretical nutrient and biomass concentrations, CO₂ profiles, and the ratio between the CO₂ output and feed rate obtained when performing automated evolution in a chemostat (day 1–30) and accelerostat (day 30–60). Blue lines represent the concentration of CO₂ (%) in the outgoing gas, and the green and orange lines represent the concentrations of the limiting nutrient and biomass, respectively. The black line in Panel (a) represents the feed rate, and the black line in Panel (b) represents the calculated ratio between the exhaust CO₂ and feed rate. The green and red dotted lines in Panel (b) represent the threshold values for increasing and decreasing the dilution rate, respectively. (I) During chemostat evolution, the feed rate remains constant, and there is a selective pressure for increased substrate affinity. As a result, a decrease in the residual concentration (the concentration inside the bioreactor) of the limiting nutrient is observed, and as a consequence, more CO₂ is produced, increasing the CO₂/feed ratio. (II) When the accelerostat phase is started, the final CO₂/feed ratio from the chemostat phase is determined as a reference to calculate the threshold values to increase the dilution rate (>95% of the reference) or decrease the dilution rate (<90% of the reference). At the start of the accelerostat phase, the CO₂/feed ratio is always above the >95% threshold value, and every 24 h, the dilution rate is increased. (III) After increasing the dilution rate, the residual concentration of the limiting nutrient increases, and as a consequence, (relatively) less CO₂ is produced, decreasing the CO₂/feed ratio below the >95% threshold, and the culture is maintained at a constant dilution rate. (IV) After evolution at the higher dilution rate, the residual nutrient concentration drops, increasing the CO₂/feed ratio above the >95% threshold, and the dilution rate is further increased. (V) A high dilution rate again results in an increase of the residual concentration of the limiting nutrient and partial washout of the culture. The CO₂/feed ratio drops below the <90% threshold, and as a result, the dilution rate is decreased to allow the biomass to accumulate and maintain the evolution culture

1. Open the Input Console using the “Monitor/Console” menu (Online Tool) or the “Console” button (Execution Monitor), and select the desired inputs for the “Feed increment threshold (%)”, “Feed decrement threshold (%)”, “Feed time period (h)”, and “Increment/decrement degree (%)” (*see Note 16*).

2. Start the accelerostat by selecting the first option in the “Checkpoint manual start accelerostat.” Throughout the evolution, the feed rate adjustments are automatically done according to the parameters set in the Input Console (Fig. 3).
3. When the growth rate has increased to a satisfactory level, the evolution can be stopped, and an aliquot of the evolved population stored for further analysis.

4 Notes

1. In Formulas_Phases.pdf file, “CALC_CUM_CO2” is the formula for integrating the CO₂ curve, which calculates the value for the cumulative production of CO₂ during the experiment. “CALC_CUM_CO2_CR” calculates the cumulative CO₂ value for every batch by taking the total cumulatively produced CO₂ up to that point (CALC_CUM_CO2) and then subtracts the cumulatively produced CO₂ already formed there at start of the batch, which ensures that the CO₂-based triggers for automated evolution can be the same throughout the experiment or until significant strain improvement occurs (*see* Fig. 1). “CALC_DECR_FEED” and “CALC_INCR_FEED” are the formulas used to decrease or increase the feed flow by a value defined by the user for automated accelerostat experiments. CALC_CO2_PUMP is the formula that determines the ratio between the amount of CO₂ in the off-gas and the current set point of the feed (see **Note 16**).
2. An elaborate description on how we set up cultivations in bioreactors is provided in [6] and can be viewed using the Riffyn Nexus user interface. Riffyn supports open science and has made all data, methods, code, and supporting information freely available for viewing or downloading via the Riffyn Nexus user interface (<http://app.riffyn.com>) using a Web browser and programmatically accessible via an open application program interface (API) using any software supporting secure http requests (api.riffyn.com). Readers may request (via <https://riffyn.com>) an open-access online account in the Riffyn Nexus to view these experiments. Riffyn provides a copy of its open-access policy at <https://riffyn.com/riffyn-open-access-policy>.
3. Adjusting the level sensor to the top of the turbulent liquid level will ensure the reactor is filled to the correct volume while the process parameters that might affect culture volume (usually pH, gassing, mixing, and temperature) are actively controlled at their respective set points. For the repeated batch operation, the level sensor can also be set to the top of the liquid level when mixing is turned off. In this case, the option

“Reactor refill without mixing” (input 0) is selected in the Input Console menu. Selecting this option will briefly turn off the stirrer when contact with the level sensor is made, and subsequently, the remaining volume is added.

4. Adjustment of the level sensor should not compromise sterile conditions in the bioreactor. If possible, avoid at all times lowering the level sensor after the bioreactor has been autoclaved. When the level sensor must be moved down into the reactor, use 70% ethanol to sterilize the top of the level sensor prior to adjustment. As an alternative to the liquid level sensor, the bioreactor can be placed on a scale that provides continuous weight measurements to the control software. Triggers for the in- and outflow pumps can be based on the weight of the culture broth reaching desired set points. For chemo- and accelerostats, a popular strategy to maintain a constant culture volume is to install an effluent pipe at the desired liquid level and keep the connected pump continuously on. While effective at maintaining a constant working volume, this strategy selectively removes cells in the upper foam layer of the broth and therefore can select for undesired mutants that are less prone to accumulate in the foam layer. Cells will sediment while in the effluent pipe, which can cause an undesired selection for fast-sedimenting (flocculent) phenotypes. Therefore, it is essential that the diameter of the effluent pipe is small enough to ensure a high liquid flow speed, and the effluent pump should always be operated at maximum speed for short time intervals. The (absence of) biomass retention in the reactor can be determined by comparing the biomass concentrations between a culture sample taken directly from the bioreactor (Fig. 1II) and a culture sample automatically drawn by the outflow pump (Fig. 1III). During evolution in chemo- and accelerostats, the formation of foam can trigger the level sensor and disrupt a steady state due to variation in culture volume of broth. Depending on the target strain and organism, the concentration of foam-forming medium supplements such as unsaturated fatty acids can be reduced or a second feed bottle with antifoam agent can be installed to periodically add antifoam and keep the level of foam to a minimum.
5. For draining the reactor, 110% of the minimal empty time is used to ensure the reactor is completely emptied with each empty/refill cycle. For example, if it takes 12 min to empty the reactor, 13 or 14 min is entered as “Empty time (min).”
6. Usually, the effluent pipe is positioned such that the bioreactor is almost completely emptied with each empty/refill cycle to maximize the number of generations per batch. For example, 1% culture retention corresponds to ~6.5 generations per batch, whereas 0.1% retention corresponds to ~10 generations

per batch. In repeated batch cultures, an undesired selection for fast-sedimenting (flocculent) phenotypes can occur when the last part of the “emptying” phase occurs when the liquid level has dropped below the lower level of the stirrer. A possible solution is to empty and refill the reactor twice between batches while leaving a higher volume (~10%) in each cycle. This will result in the same overall dilution between batches, at the expense of using more culture medium. In evolving cultures, an undesired selection for mutants that grow on the walls or instruments inside the reactor can occur. To limit the selective advantage of these mutants in repeated batch evolutions, the stirring speed can be set to the highest setting before and during the empty/refill cycle. The increased turbulence usually breaks down excessive buildup of biofilms in the reactor and allows removal of these mutants via the effluent pipe.

7. In most microbial cultures, CO₂ is produced in catabolic reactions that provide the energy (ATP) for biomass assimilation and cellular maintenance. Therefore, when a nutrient becomes growth limiting and the biomass specific growth rate decreases, the rate of these dissimilatory reactions will decrease as well. Therefore, nutrient limitation is often indicated by a (steep) drop in the concentration of CO₂ in the exhaust gas, which is used to initiate the next phase in the evolution methods of this protocol. When the carbon/energy substrate is not the limiting nutrient, there will still be CO₂ formed in the absence of cellular growth by dissimilatory reactions to provide ATP for cellular maintenance, but at a much lower rate than in a growing culture. However, when depletion of the rate-limiting nutrient does not result in a significant drop in exhaust CO₂, a protocol with a different trigger mechanism (e.g., based on the addition of acid/base) is required.
8. After inoculation and completion of the first batch phase, the bioreactor is emptied and refilled once manually to mimic batch performance after automated progression of the culture is initiated. It is important to time this manual empty/refill cycle such that the new batch is initiated with a representative inoculum. For example, the empty/refill should not be initiated too early or late as this could result in an inoculum with a lower cell density or viability, respectively, which extends the batch time of the “representative batch” (Fig. 2b).
9. The maximal fill time is determined and used as a precaution to prevent complete drainage of the medium container in case of, for example, a malfunction by the level sensor. Additional options in the operation can be enabled if desired. “pH control during liquid cycling” (empty/refill) is turned off by default to prevent excessive addition of acid/base when the liquid level drops below the pH sensor but can be turned on. Similarly,

“temperature control during liquid cycling” is turned on by default and can be switched off. When large batch-to-batch variation is expected, the values set for “dCO₂ threshold (%)” “Minimum batch time (h),” and “Cumulative CO₂ threshold” should be set to values lower (50–70%) than the default 80% of the observed values. This is to ensure that even batches with large (>20%) variations in performance will be able to trigger the next batch phase.

10. This automated evolution process is set up such that inconsistent culture and/or sensor behavior does not result in washout of the culture by incorporating a minimal time required for each batch phase. Triggers based on a maximal CO₂ concentration in the outflow and a calculated cumulative CO₂ level are used to stop the automated progression of the culture when an abnormal batch occurs and allows for investigation of the culture to detect the cause. An abnormal batch can, for example, occur as a result of incomplete emptying or filling of the reactor or when an unintended fluctuation in culture pH or temperature happened. We advise the user to closely monitor progression of the first automated batches and to regularly check the readings of the online measurements throughout the evolution to ensure culture progression works as intended. In long evolution experiments, deterioration of the growth characteristics can sometimes be observed as a consequence of deterioration of one or multiple components (such as vitamins) in the ingoing medium. To prolong the evolution experiment, the ingoing medium can be replaced or supplemented with additional fresh media components.
11. If during evolution in the repeated batch setup the parameters of the strain change, specifically the specific CO₂ production and growth rate, the values discussed and set in **step 17** in Subheading 3.2 should be changed accordingly since the initial values might not be able to act as triggers/safeguards for the evolved strain (Fig. 2).
12. Since cells are grown under substrate limitation, chemostat evolution selects for mutants with improved affinity (μ^{\max}/K_s) for the growth-limiting substrate. The dilution rate, and therefore the growth rate, is fixed, which allows mutants with a lower K_s to take over the population. The dilution rate should be set such that it does not exceed the maximum specific growth rate of the starting strain, and a higher dilution rate results in a lower generation time that accelerates the rate at which spontaneous mutants arise.
13. The flow rate of the ingoing medium is determined by providing a set point for the feed pump as a percentage of its maximal velocity. For commonly used peristaltic pumps, the

correlation between pump and flow rate is dependent on the type of tubing used and could vary over time as the quality of (in-use) tubing deteriorates. When an accurate dilution rate of the chemostat phase is required, we suggest making a new calibration each time the bioreactor is set up or carefully setting the exact intended flow rate while filling the reactor. Alternatively, the rate of feed addition can be accurately controlled by placing the feed container on a digital scale, which is connected to the control software.

14. If the user prefers to start the chemostat phase manually, open the Input Console using the “Monitor/Console” menu (Online Tool) or the “Console” button (Execution Monitor), and switch “Start chemostat” to manual. When an automatic start of the chemostat phase is selected, the user can determine the required drop in the exhaust CO₂ concentration, which is 50% by default, by changing the “dCO₂ threshold (%)” value.
15. Evolution at fixed growth rates in chemostat cultures can select for adaptations that result into a loss of metabolic capacity and lower the maximum specific growth rate of the strain. To prevent the occurrence of such mutations, an accelerostat approach can be used or the system can combine periods of sequential batch evolution with chemostat evolution to maintain selective pressure on high metabolic capacity. Alternatively, the mutations underlying the desired phenotype (such as a transporter with improved affinity) can be reverse engineered back into a healthy, unevolved background strain. Lucullus will ask the user at regular intervals (defined by “Feed time period (h)”) if progression to accelerostat cultivation is desired. Automatic progression to accelerostat cultivation can be achieved by setting “Feed time period (h)” to the desired time in chemostat mode and switching “Start accelerostat” to automatic in the Input Console. This is an advanced option, and in most cases, we recommend sampling and analysis of the chemostat culture before deciding on a manual switch to accelerostat mode.
16. Triggers for increasing and decreasing the dilution rate are based on the ratio between CO₂ in the exhaust gas and the ingoing feed rate, for which a reference value is automatically stored when the culture is switched from chemostat to accelerostat. It is therefore important that a chemostat culture with a satisfactory residual concentration of the rate-limiting nutrient is obtained before switching to accelerostat mode. By default, the feed rate is increased or decreased by 5% after the trigger conditions are met, but this value can be changed via the “Increment/decrement degree (%)” The reasoning behind the use of the ratio between CO₂ in the exhaust gas and the ingoing feed rate is that when an identical proportion of the ingoing limiting nutrient is consumed at a higher

dilution rate, the formation of biomass, CO₂, and other catabolic products is expected to increase accordingly. For example, if 99% of the ingoing glucose (as limiting nutrient) is consumed and results in an exhaust CO₂ concentration of 0.5% at a dilution rate of 0.1 h⁻¹, we expect that the exhaust CO₂ concentration becomes 1.0% at a double dilution rate of 0.2 h⁻¹ and 99% conversion of substrate. In case the higher dilution rate results in an increased residual glucose concentration (<99% conversion rate), the ratio between the exhaust CO₂ and feed rate goes down, and thresholds are set to determine which ratios allow acceleration of the culture (feed increment), maintenance of the current feed rate (between “feed increment” and “feed decrement”), or deceleration of the culture (below feed decrement). The “Feed increment threshold (%)” dictates the minimal ratio between CO₂ and feed rate (as compared to the ratio obtained in the chemostat preceding the accelerostat) that triggers an increase in the feed rate. Similarly, the “Feed decrement threshold (%)” dictates minimal ratio to maintain the current feed rate, and when the ratio drops below this threshold, the feed rate is decreased again. By default, the thresholds are set to 95% and 90%, respectively, and can be adjusted in the Input Console. By default, an increment/decrement or no change in the feed rate can occur every 24 h, and this can be adjusted in the “Feed time period (h)” box. Since a change in the feed rate requires some time for the culture to establish a new steady state, we suggest not lowering the “Feed time period” too much as this could result in an overshoot of the ramping or deceleration of the feed rate (Fig. 3).

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