Model-Based Design and Analysis of a Reconfigurable Continuous-Culture Bioreactor (Work in Progress)

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ABSTRACT

In this paper, we present a model-based design and analysis of prototype laboratory equipment used for growing bacteria under precisely controlled conditions for systems biology experiments. Continuous-culture bioreactors grow microorganisms continuously over periods as long as several months. Depending on the particular experiment, the reconfigurable continuous-culture bioreactor we model and analyze may operate as: (a) a chemostat with constant volume, (b) a turbidostat with constant bacterial concentration as observed through turbidity (optical density), or (c) a morbidostat with constant death-rate of bacteria. Such systems have interesting safety specifications such as not overflowing beakers, maintaining bacterial concentrations within ranges, etc., that must be maintained over long experimental periods. We develop preliminary controller and plant models and analyze them through simulation in Simulink/Stateflow (SLSF), and using reachability analysis in SpaceEx by translating the SLSF models to hybrid automata. The analysis indicates that the proposed design satisfies its regulation specifications for microorganism concentration may avoid error scenarios encountered in experiments with a prior design.

Keywords

model-based design, verification, simulation, hybrid systems

1. INTRODUCTION

A variety of fields in both the basic and applied sciences have come to rely upon the microbe as a fundamental tool. Organisms such as bacteria and yeast are of great interest due to the diverse and efficient chemistries they choreograph and, perhaps more importantly, to the direct and often complex roles they play in the context of human health.

The recent and dramatic rise of antibiotic-resistant bacteria and fungi has placed new emphasis on the need to better understand the evolutionary processes leading to resistance in populations exposed to different classes of drugs [1]. Given the immense cost of microbial infection in terms of both economic output and human lives, as well as the lack of development of new antibiotics by pharmaceutical companies, laboratory experiments designed to understand the mechanisms and dynamics underlying the evolution of resistance are essential. These studies require methods to carefully manipulate microbial environments and to sensitively measure any meaningful, albeit subtle, changes in the growth rates of the microorganisms that may result [5]. Such experiments are typically performed in a bioreactor, with the microorganisms in question suspended in a liquid culture with the proper nutrient delivery to maintain growth.

This configuration is referred to as a continuous-culture apparatus, and a number of such devices have have been developed. The chemostat is one such manifestation; it was developed in the 1950s to study growth dynamics of bacteria [10] and was subsequently adopted by Saldanha et al. for studying the growth of these organisms close to a steady state in which the rate of growth of the culture equals the dilution rate of media [11]. This permitted the investigators to measure the effects of environmental perturbations independent of confounding growth effects observed in less sophisticated experiments where bacterial cultures were allowed to experience more dramatic growth rate fluctuations. Also of importance, the method of continuous-culture in the laboratory is deeply analogous to growth in natural environments [5]. Recent experiments have exploited this similarity to design evolution experiments where bacteria are grown in the presence of increasing concentrations of antibiotics as they gradually evolve resistance to them [13,14]. By keeping the evolutionary pressure constant, these efforts not only led to the identification of mutations in genes responsible for the underlying resistance, but also allowed accurate comparisons between patterns of genetic changes observed in other iden-

CYPHY '14 Berlin, Germany
 978-1-4503-2871-5/14/04 ...$15.00.

tical cultures and cultures with different antibiotics. The ultimate aim is to predict genetic changes that confer resistance and design strategies to prevent them in the clinic.

The implementation of a basic continuous-culture device is dependent on a controller that can monitor changes in culture density and, depending on set threshold parameters, turn on pumps that deliver new media or antibiotic drug and remove excess culture. Experiments using such devices are usually carried out for long periods of time—greater than a week, or in one instantiation, several decades [3]—and are executed in independent vessels (beakers) to permit assessment of reproducibility or to simultaneously measure many different systems at once. In practice, however, construction and use of these devices has proven challenging, in part due to technical issues surrounding the design of a reliable and robust hardware and software platform capable of performing monitoring and feedback in a modular fashion over an array of beakers. Among the specific problems encountered are: crashing of or unaccounted delays in the command execution and data acquisition and storage systems (which also puts into question the reliability of data dependent on precise timing of events); failure of a feedback signal to execute, resulting in the overflow of liquid in beakers; and lack of independent control of beakers, hindering the investigator's ability to sample many experimental conditions.

Of these issues, the first is perhaps the most insidious. As discussed above, the primary parameter of interest throughout the continuous-culture experiment is the growth rate of the microbial population. It is not unusual in the case of an automated system to measure population growth rate over a period of several minutes; as such, even timing errors on the order of tenths of seconds would certainly appear to be a significant and therefore actionable. Such errors can lead to controller errors—the addition of too much or too little drug, for example—or, even worse, to incorrect conclusions about microbial growth rates. Furthermore, if one envisions more advanced experiments with complex control systems requiring finer time slicing, these errors will only increase as the magnitudes of the error become more significant relative to the sampling intervals in question.

Current Prototype and Proposed Redesign. A morbidostat is an automated continuous-culture device that aims to achieve a better understanding of evolved drug resistance [14]. It measures the growth rate of some microorganism, and automatically adjusts the concentration of microorganism by injecting the different concentrations of a dissolved drug. The goal is to keep the drug-induced inhibition constant. The concentration of the microorganism can be measured optically by a densitometer. Moreover, the microorganisms grow in the fixed volume of a culture medium, and the system is continuously stirred using magnetic stirring bars. The current prototype can be configured to grow microorganisms under a variety of conditions, such as constant volume (chemostat), turbidity (turbidostat), and/or morbidity (morbidostat). The microorganisms are grown in parallel in N separate beakers, and depending upon the configuration and experiment, the reaction undertaken in each beaker is controlled by a number of peristaltic pumps to input media, output beaker waste contents, input antibiotics, etc. In this paper, we focus on the controller implemented for each of the N beakers.

The current prototype has several architectural and implementation shortcomings that prevent it from meeting researchers' needs, and to cause them to question the validity of results generated using it. The research use is for microorganism growth experiments studying evolution, with experiment periods on the order of months. The current prototype cannot generally execute longer than about a week at a time, in part due to architectural choices. In particular, the prototype uses Matlab executing on a Windows desktop computer for both data recording and pump control to ensure constant growth conditions. The current prototype's control and sampling tasks are executed periodically using Matlab's `timer` class [14, Supplemental Matlab Code], in spite of warnings in documentation: "The timer object is subject to the limitations of your hardware, operating system, and software. Avoid using timer objects for real-time applications" [9]. The `timer` class is not suitable for real-time tasks since (a) Matlab is implemented in Java and may experience garbage collection events that are non-deterministically long, that is, some computations may execute indefinitely long, and (b) standard versions of operating systems (Windows) have no real-time guarantees. This is unrelated to the fact that the time constants of growth are quite large—on the order of minutes to hours—as real-time corresponds to repeatability (deterministic execution) [7].

To enhance confidence in data validity, the system is being redesigned with a focus on reliability and repeatability of experiments using a real-time operating system (RTOS) for sampling and control. In this paper, we present an analysis of the controller being implemented in the redesigned prototype and show that the control design meets several specifications, such as the microorganism concentration remains within a threshold of a desired value and that the beaker does not overflow. We develop hybrid automata models of the pump controller and bacterial growth dynamics [12]. We implement this model in Simulink/Stateflow (SLSF) and simulate its behavior to illustrate these specifications are met by the controller. Due to unsoundness of simulation alone, we then use an extension of HyLink [8] to translate from the SLSF model to a SpaceEx [4] model to analyze the controller through reachability analysis.

2. BIOLOGY AND SYSTEM MODELING

We model the continuous-culture apparatus as the composition of two hybrid automata, where $\mathcal{A}_M$ models the media pump's controller and $\mathcal{A}_D$ models a drug pump's controller, shown respectively in Figures 1 and 2. We model two versions of the system $\mathcal{A}$. First, $\mathcal{A}$ may operate as a *turbidostat*, in which case $\mathcal{A}_M$ operates alone ($\mathcal{A} \triangleq \mathcal{A}_M$), and second, $\mathcal{A}$ may operate as a *morbidostat*, in which case $\mathcal{A}_M$ and $\mathcal{A}_D$ operate in parallel ($\mathcal{A} \triangleq \mathcal{A}_M \| \mathcal{A}_D$). Due to space, we do not define hybrid automata, but refer to technical definitions of syntax and semantics in [2, 4]. $\mathcal{A}$ has the following continuous variables: (a) g: the microorganism's (varying) growth rate, (b) η: the microorganism's (varying) mutation rate, (c) τ_μ: the mutation time, (d) τ_m: the operating time of media pump, (e) τ_d: the operating time of drug pump, and (f) OD: the microorganism's concentration χ measured by the beaker and media's optical density (OD). The concentration of microorganisms changing over time can be determined the optical density of the culture suspension using a densitometer. As the principle of this technique, light which remains unscattered after passing through a suspension of particles is detected by photoelectric cells and converted to

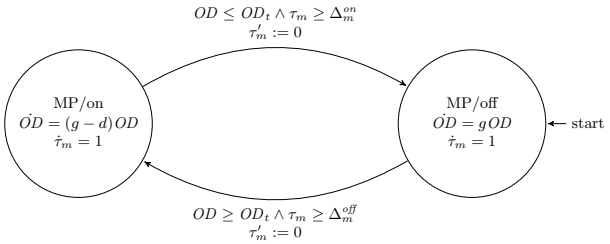

Figure 1: Hybrid automaton model $\mathcal{A}_M$ of the morbidostat's media peristaltic pump.

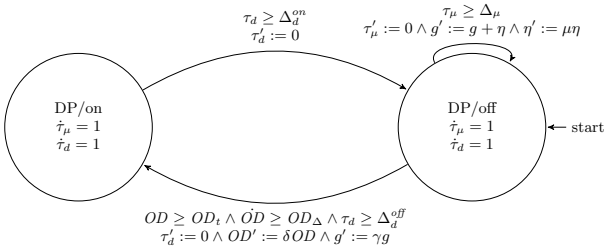

Figure 2: Hybrid automaton model $\mathcal{A}_D$ of the morbidostat's drug peristaltic pump.

an electric response. In the case of microbes growing in liquid culture, less light transmitted through the culture suspension thus indicates a higher concentration of microorganisms and is manifest in the recorded OD value. Within a certain range, the OD is proportional to the microorganism's concentration, allowing quantification of the microbial population size and, if measured over time, growth rate. In general, the growth rate of a microorganism is higher than the dilution rate of its culture media, thus the concentration of the microorganism tends to increase over time. When the microorganism concentration reaches a threshold as measured by the densitometer, the growth rate can be inhibited by adding media containing dissolved drugs.

Microorganism growth is expressed as [15]:

$$N = N_0 2^{t/t_d} = N_0 (e^{\ln 2})^{t/t_d} = N_0 e^{gt}, \quad (1)$$

where N is the total number of cells, N_0 is the initial number of cells, $1/t_d$ is the number of divisions per unit time, t is the time, and $g = \ln 2/t_d$ is the specific growth rate. In addition, the cell density is defined as:

$$\chi = \frac{N}{V}, \text{ where} \quad (2)$$

V is the culture volume. OD is proportional to cell density, $OD \sim \frac{N}{V}$, and thus, OD can be written as:

$$OD = OD_0 e^{gt}, \quad (3)$$

where OD_0 is the optical density at $t = 0$. The OD is unitless (n/a in Table 1) and only proportional to cell density as long as its value is approximately less than 0.4 [15]. If the system is diluted at a constant dilution rate d, then Equation 3 can be rewritten as:

$$OD = OD_0 e^{(g-d)t}, \text{ and thus:} \quad (4)$$

$$\dot{OD} = (g-d) OD_0 e^{(g-d)t} = (g-d) OD. \quad (5)$$

The morbidostat actuators are three distinct peristaltic pumps: a washout pump, media pump (MP), and drug pump (DP). The pumps are driven by AC motors and are controlled using solid-state switches to connect or disconnect them from AC power to turn them on or off. While on, the pumps have a constant flow-rate. The washout pump dilutes the culture media of morbidostat at a constant dilution rate, which is lower than the maximum growth rate of the microorganism. Depending on the microorganism's concentration χ measured via OD values, the same fixed volume of either media or media containing dissolved drugs is added from the media pump or the drug pump, respectively.

Table 1: Morbidostat Parameters and Variables.

Name	Description	Value (Init. Cond.)	Unit
OD	Optical density of microorganism concentration χ	vary (0.05)	n/a
V	Total volume of beaker	vary (100)	mL
g	Specific growth rate	vary (0.01)	s^{-1}
η	Microorganism mutation rate	vary (0.0004)	s^{-1}
τ_μ	Mutation time	vary (0)	s
$\tau_{\mathrm{m,p}}$	Operating time of media and drug pump, respectively	vary (0)	s
OD_t	Optical density threshold	0.15	n/a
OD_Δ	Optical density derivative threshold	0.01	n/a
$\Delta_m^{on,off}$	On/Off operating period of media pump	15, 5	s
$\Delta_d^{on,off}$	On/Off operating period of drug pump	5, 5	s
Δ_μ	Mutation time threshold	30	s
d	Dilution rate	0.0225	s^{-1}
γ	Growth rate gain by drug addition	25	%
μ	Microorganism mutation rate gain	101	%
δ	Microorganism concentration gain by drug addition	10	%

The morbidostat controller includes four operating modes: MP/on, MP/off, DP/on and DP/off, which respectively correspond to the discrete states where the media pump is on or off and the drug pump is on or off. When the continuous-culture system functions as a turbidosatat, the media pump's controller operates alone. If the OD value exceeds threshold (OD_t), the control of the media pump switches to the MP/on operating mode. Then, the OD value is updated by the differential equation $\dot{OD} = (g-d)OD$, where g is the specific growth rate of the microorganism and d is the dilution rate of the media pump [12, 14]. The variable τ_m denotes the operating time of the media pump, and the constant $\Delta_m^{on}, \Delta_m^{off}$ denote on and off operating periods of the media pump, respectively. When the OD value decreases below threshold (OD_t), the media pump is automatically turned off after Δ_m^{on}, and the system changes its state back to MP/off operating mode.

When configured as a morbidostat, the media pump and the drug pump operate synchronously. The media pump operates the same as when configured as a turbidostat. When the OD value exceeds a threshold (OD_t) and its rate of change ($\dot{OD}$) is greater than a derivative threshold (OD_Δ), the drug pump may switch states to the DP/on operating mode. As a result, the high concentration of drug dissolved in fresh media is added to the morbidostat to inhibit the growth rate of the microorganism. Thus, the values of OD and microorganism's specific growth rate are updated by two following equations: $OD' := \delta OD$ and $g' := \gamma g$, where δ, γ are the gains of microorganism's concentration and specific growth rate due to drug addition, respectively. The control of drug pump switches its state to DP/off operating mode after Δ_d^{on}. Additionally, the self-loop in Figure 2 presents the effect of mutations on growth rate and the inhibitory effect of one unit of drug. The microorganism's specific growth rate

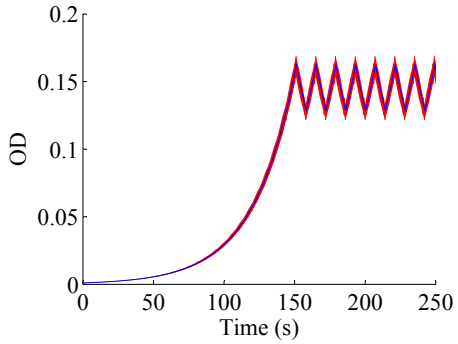

Figure 3: SLSF simulation (blue) and SpaceEx reachability (red) of turbidostat model $\mathcal{A}_M$ showing OD versus time. The simulation trace is contained in the reachable states computed using the STC algorithm in SpaceEx, and validates the translation.

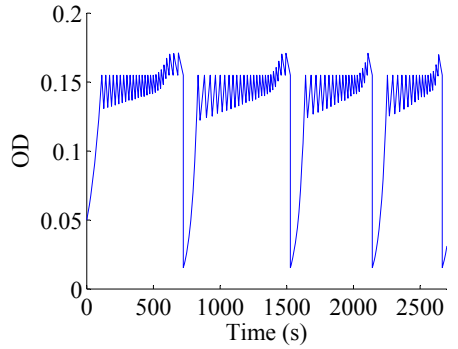

Figure 4: Simulation of morbidostat model $\mathcal{A}_M \| \mathcal{A}_D$ showing OD versus time. Here, the accumulation of mutations imparting antibiotic resistance leads to increased growth rate in the presence of drug. To maintain control over growth here, increasing amounts of drug volume must be delivered over time in order to compensate for increasing resistance.

and mutation rate are updated discretely as $g' := g + \eta$, and $\eta' := \mu\eta$ after each mutation period (Δ_μ), where η and μ are the microorganism's mutation rate and mutation rate gain. The microorganism should grow in a fixed culture volume, so the controller ensures the flow rates of the input fluids (media or dissolved drug) and the waste fluid are equal.

3. MODEL-BASED ANALYSIS

We analyze the turbidostat and morbidostat controllers with Simulink/Stateflow (SLSF) simulation and SpaceEx [4]. We utilize a HyLink [8] extension to translate the SLSF model to input for SpaceEx. Figure 3 shows the turbidostat's OD versus time from a SLSF simulation trace and the reachable states computed by SpaceEx. The OD increases exponentially until reaching the threshold $OD_t = 0.15$, then remains within a tolerance band of it. If t_α denotes the settling time, $\forall t \geq t_\alpha$, $OD(t) = OD_t \pm \epsilon$, where ϵ denotes a tolerance value. Figure 3 shows that $t_\alpha = 150s$ and $\epsilon \approx 0.03$. Thus, the controller model meets the specification that the concentration remains nearly constant. However, the simulation of the morbidostat mode does not meet this specification when drug is added since ϵ becomes very large. In this case, the specification is that the growth rate remains bounded. In addition to this specification, the controller ensures the beaker volume remains constant (in spite of adding media/drug and removing fluid through washout). Figure 4 shows the morbidostat's OD versus time from a SLSF simulation trace which incorporates the acquisition of antibiotic resistance through mutation. Using SpaceEx to analyze the morbidostat controller with mutation rates is the subject of ongoing work and is challenging due to nonlinearities ($\dot{OD} = gOD$ where both g and OD are variables), so we are investigating piecewise-linear models.

4. CONCLUSION AND FUTURE WORK

In this paper, we present a model-based design and analysis of a continuous-culture apparatus. We model the system in Stateflow and show it meets specifications like ensuring a microorganism's concentration remains within bounds of a threshold. Additionally, we utilize HyLink to translate the SLSF model to a SpaceEx model and analyze the system and the same specifications through reachability analysis. For future work, we are using code-generation to implement the high-level control algorithm in a redesigned prototype with the BeagleBone Black development kit. The complete experimental setup utilizes N morbidostats operating in parallel, each of which may mutually exclusively access shared/multiplexed peristaltic pumps, so we plan to utilize the *Passel* verification tool [6] to analyze a specification that only one morbidostat has access to shared pumps simultaneously.

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