Biotech Beer Brewing
Final report

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***Abstract*—Bacterial contamination in alcoholic fermentation affects the performance of yeast, can lower the alcohol production by up to 30% and has a negative effects on product quality. Developing new ways to enhance process control is essential to compete with other suppliers on the market but experimental techniques are very costly. Simulation methods as dynamic flux balance analyses (DFBA) using genome-scale models (GEM) has proven their capabilities in predicting bacteria growth and metabolic behavior in batch processes and competitive bacteria cultures. *Dynamic Multispecies Metabolic Modeling* (DMMM) is a successful application of DFBA in a simulation framework using Matlab. In this work the implementation of the DMMM is analysed and implemented in python using the COBRApy package. As the contamination of Lactobacillus plantarum is a major issue in alcoholic fermentation in food industry, the simulator is demonstrated in a co-simulation of L. plantarum and S. cerevisiae. The results verify proper performance of the simulator and confirm important rule of thumb measures usually employed by brewers like aeration prior to fermentation.**

I. Introduction

Alcoholic fermentation is applied in industrial scale in different markets like food industry e.g. for wine, beer or whisky production but also other market segments like bio fuel production. Reducing production costs while increasing the process efficiency and product quality is a major goal in industry. In the fermentation process contamination from tanks, pipes, centrifuges, heat exchangers or ingredients can have a great impact on process parameters and must be controlled. Contamination can lead to a loss in process productivity of up to 30%. The main contaminants are lactic acid bacteria and wild yeasts, lowering the performance of the applied yeast. Lactobacillus bacteria contribute to induce yeast flocculation what in turn reduces the viability of the cells, the fermentation rate and finally the process yield. Competition between yeasts and microbial contaminants affects the process additionally [6] [26].

In beer, wine or whisky production additional product quality aspects like color, viscosity and flavor plays a big role and has a great influence on its price. On the other hand the process itself underlies strict laws to guarantee transparency for the customers [2] [3]. Additionally the growing organic food market calls for products with even more strict processing standards [4].

Chemicals like acids, antibiotics and antifoam agents as they are used in other industries can not be applied here. These constraints increases the effort of finding optimal ways for controlling product quality dramatically. As experimental development in laboratories is very costly and not affordable by smaller breweries, simulation methods are gaining popularity.

A well established group of methods uses Genome-Scale Models (GEM) to model metabolic fluxes in bacteria [38]. GEMs are created by analysing the information in the genome of a bacteria about its enzymatical processes and extrapolating a network of metabolites and metabolite reactions which represents the internal fluxes of this bacteria. Based on this model possible flux distributions can be predicted using linear optimization methods, known as flux balance analysis (FBA) [29].

In its basic form describes FBA the bahavior of only one bacteria in a constant environment. Zomorri et al. summarizes some approaches to extend FBA to multispecies simulations (*steady-state models*) in time variant environments (*dynamic models*) and even considering spacial variations (*spatio-temporal models*) [38].

The simulation of co-cultures using GEMs is still an emerging field. Every approach has its strengths and weaknesses and must be chosen dependent on the application. In *Steady-state models* like compartmentalized community-level metabolic modeling a common objective function for all involved species must be formulated. This approach has a relatively low computational effort but shows drawbacks in competitive co-cultures where a common objective can not easily be formulated. The spacial dimension of *Spatio-temporal models* results in very high computational effort and it has to be evaluated if this enhances the accuracy of the estimated behavior in that case.

The class of *dynamic models* described by Zomorrodi et al. uses a system of ordinary differential equations (ODE) to extend the static simulation approach of FBA with external system dynamics like the exchange of metabolites and bacteria growth and is called dynamic flux balance analysis (DFBA) [38].

Mehadevan et al. describes two basic categories of DFBA approaches: *dynamic optimization approach* (DOA) and *static optimization approach* (SOA) [25].

In DOA the linear programming (LP) problem to predict the behavior of the bacteria and the differential equations are reformulized to a non-linear programming problem (NLP). This approach has a very high computational effort [18] compared to SOA and has only been used for relatively small GEMs with up to 13 modeled fluxes and 8 metabolites [24] [23].

In SOA the simulation time is discretised by a defined time interval and a FBA is executed for each time point. The results are used to iteratively solve the discretised differential equations to update the environmental conditions at each time point. The described integration method in SOA is similar to Euler-Cauchy. In this approach it is assumed that the cell internal dynamics

are much faster than the external dynamics.

Höffner et al. basically generalizes SOA to arbitrary integration methods for solving the system of ODEs by using a third-party solver and names it *direct approach* (DA) [18].

Henson et al. introduces a further method which shows similarities to SOA and DA but enhances the efficiency of the algorithm [18] by reformulating the LP and ODE to a differential-algebraic equation system [16].

The DA described by Höffner et al. has been successfully applied to simulate alcohol fermentation in a wine approximating setup [32] [31] and to predict growth of Escherichia coli and Saccharomyces cerevisiae bacteria in a co-culture [12]. Zhuang et al. implemented the method in the *Dynamic Multispecies Metabolic Modeling* (DMMM) framework which is publicly accessible and written for Matlab [36]. It uses the Matlab toolbox *COBRA* [14] to implement FBA. The method developed by Henson et al. compared to DMMM has the potential to increase the accuracy by fixed calculation runtime but since the implementation of DMMM is publicly accessible and relatively simple to implement, this method is used in the simulator in this work.

The next chapter will first introduce basic technologies as GEMs and FBA needed as a basis in further descriptions. These sections are followed by an overview of the implemented algorithms and techniques in the implemented simulator and differences to DMMM. The chapter is concluded by an description of the simulation including Saccharomyces cerevisiae and Lactobacillus plantarum as conterminant in a co-culture which shall be serve as a use-case. As a first step Sacharomyces cerevisiae is simulated with different densities of lactic acid to tune the inhibition of the yeast model by the acid. In a second step S. cerevisiae and L. plantarum are simulated in a co-culture. The results are compared to the outcomes of *in vivo* experiments and discussed in chapter III. Chapter IV gives an outlook on further improvements of the implementation.

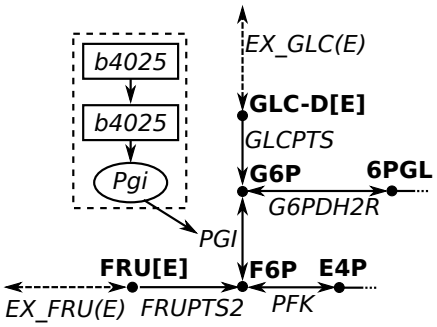
II. Methods

A. Genome-Scale Models

A genome-scale model (GEM) is an analytical reconstruction of ideally all cellular process of a cell. All available biochemical, genetic and genomic (BiGG) information of the processes are analyzed to identify gene-to-protein-to-reaction (GPR) associations and involved compounds. Based on this data a network composed of compounds (nodes) and reactions (links) is created and added to the reconstruction [30]. Figure 1a shows an example for a metabolic network. The GPR association is shown for the enzymatic process *PGI*.

Tools like *Pathway Tools* [21], *SEED* [15] or *AUTOGRAPH* [28] are used to generate a reconstruction based on a (annotated) genome. The produced models contain many wrong assignments, gaps and inconsistencies due to many organism-specific choices and must be refined by hand. A biomass function to model growth and input/output reactions are added to the network [33].

The reconstruction is the basis to create geno- and phenotypes of a cell and to transform it into mathematical models for further analysis. An important mathematical representation is



(a) Example of a metabolic network with genome association of the enzymatic process *PGI*

	EX_GLC(E)	GLCPTS	PGI	FRUPTS2	EX_FRU(E)	PFK	bio
$GLC-D[E]$	1	0	0	0	0	0	0
$G6P$	0	−1	−1	0	0	0	0
$F6P$	0	0	1	1	0	−1	1
$FRU[E]$	0	0	0	−1	1	0	0
$6PGL$	0	1	0	0	0	0	1
$E4P$	0	0	0	0	0	1	0

(b) Possible stoichiometric matrix of the metabolic network in 1a

Figure 1: Example of a genome-scale model

the stoichiometric matrix. It uniquely represents the metabolic network of the cell, based on the stoichiometric constants of its reactions [30]. Figure 1b shows a possible stoichiometric matrix for the exemplary metabolic network in figure 1a.

B. Flux Balance Analysis

Flux Balance Analysis (FBA) is a mathematical tool which analysis possible flux distributions through the metabolic network based on the stochiometric matrix of a GEM.

The system of linear equations built by the stoichiometric matrix S of a GEM is usually underdetermined. To choose one flux distribution from the solution space spanned by S, it is assumed the organism adapted its metabolic fluxes to a certain environment and is now in a steady state where the fluxes are optimal distributed recording to some goal. In FBA this flux distribution is chosen by solving a optimization problem as in equation 1 using linear programming (LP) [29].

$$\begin{aligned} \max_{\boldsymbol{v}} \quad & a = \boldsymbol{w}^T \boldsymbol{v} \\ \text{s.t.} \quad & S\boldsymbol{v} = \boldsymbol{0}, \\ & \boldsymbol{v_{min}} \leq \boldsymbol{v} \leq \boldsymbol{v_{max}} \end{aligned} \tag{1}$$

The fluxes in the metabolic network $\boldsymbol{v}$ are optimized so that a is maximized where $\boldsymbol{w}$ is a weighting vector which defines the adaption goal of the cell. A often used goal is to maximize the growth of the organism. The optimization is done using mostly two constraints, (1) the stoichiometric constants

S for each reaction which is defined by the used GEM and (2) lower and upper bounds for each flux, $\boldsymbol{v_{min}}$ and $\boldsymbol{v_{max}}$, in the metabolic network but further constraints can be added as well.

The lower and upper bounds can be used to simulate gene know-outs by setting the upper bound of reactions dependent on this gene to zero or to define environmental conditions like the availability of certain metabolites.

The chosen solution by the LP solver represents a phenotype of the given organism due to adaption to its environment but FBA has its limitations. Usually GEMs does not model regulatory structures as activation of enzymes or regulation of gene expressions. As the GEM does not contain kinetic parameters of the involved compounds, their densities can not be considered and must be modeled externally [29].

C. Simulation Algorithm

Table I: Overview of implemented features compared to DMMM

Feature	DMMM	This project
Model		
arbitrary many GEMs	yes	yes
arbitrary many metabolites in environment	yes	yes
mortablility of bacteria	yes (in output flux)	yes
input/output flux of bacteria and metabolites	yes	no
parameterized initial state of environment composition	yes	yes
Michaelis-Menten kinetics	yes	yes
Toxicity	no	yes
Algorithm		
ODE solver	yes	yes
different ODE solvers	yes	no
analytical solver	yes	no

As described by Zhuang et al. in [37] the algorithm uses an ODE solver with embedded FBA. An FBA is solved for each GEM in the model and for each time step in the discretised simulation time interval considering the changed metabolite and bacteria densities in the shared environment. The results of the FBAs are used by the ODE solver to solve the differential equations

$$\frac{\mathrm{d}X_M}{\mathrm{d}t} = (\mu_M - \mathrm{Mort}(\mu_M))X_M \tag{2}$$

$$\frac{\mathrm{d}S_m}{\mathrm{d}t} = \sum_{M \in models} v_{M,m} X_M \tag{3}$$

which models the dynamics of the bacteria's environment [36]. Equation 2 updates the bacteria masses X_M in the shared medium where M are the bacteria models and μ_M the growth rate of bacteria M. Mortality is considered using a function $\mathrm{Mort}()$ dependent on the growth rate μ_M for each organism M in this implementation while Zhuang et al. modeled this using the output flux of bacteria out of the system. Yeast cells have a mean lifetime of 26 cell divisions [20], making the mortality dependent on the biomass production μ_M. We have a doubling rate of $t_d = \frac{\log(2)}{b}$, i.e. every cell doubles every t_d hours. Hence, cells have a lifetime of $t_l = 26 \cdot t_d$ hours. Consider a population of N cells, which have been born equidistantly. On average, $\frac{N}{t}$ cells die every hour. The populations mortality is therefore $\frac{N}{t} \cdot \frac{1}{N} = \frac{1}{t_l} = \frac{b}{26 \cdot \log(2)}$. Equation 3 updates the amount of molecules S_m of metabolites in the shared medium where $v_{M,m}$ are the output fluxes of metabolite m by bacteria model M.

In each time step each bacteria's metabolite intake must be changed dependent on the densities of the metabolites in the shared environment. To model saturation of metabolite intake for high metabolite densities Zhuang et al. implemented Michaelis-Menten kinetics [19]

$$b_{M,m} = v_{max,M,m} \frac{s_m}{k_{M,m} + s_m} \prod_{m'} \frac{1}{1 + \frac{s_{m'}}{I_{M,m,m'}}} \tag{4}$$

Formula 4 describes the the dependency of the upper bound of the input flux $b_{M,m}$ for metabolite m of model M on the metabolite density s_m in the shared medium. The formula is the product of two expressions. The left expression describes the actual dependency of the intake flux on the metabolite density and is modeled using Michaelis-Menten kinetics where $v_{max,M,m}$ is the maximal possible input flux and $k_{M,m}$ is the Michaelis-Menten constant. This constant defines the metabolite density where the input flux is exactly at the half of its maximal possible value. The right expression of formula 4 models the inhibition of the input flux of metabolite m of bacteria M by another metabolite m' in the shared medium. This expression can be used to add toxic effects of certain compounds on the metabolism of a bacteria. This therm is defined by the inhibition constant $I_{M,m,m'}$ which, similar to the Michaelis-Menten constant, defines the metabolite density $s_{m'}$ where the value of this therm is exactly 0.5.

Algorithm 1 shows a basic implementation of the differential equations solved by an ODE solver during the simulation similar to DMMM [37].

In a first step, the absolute metabolite counts are converted to densities, and all smaller than zero entries are set to zero. This step is necessary since negative densities are unphysical and forbidden, hence the function is transformed as proposed in [34].

Next, the lower bounds of the intake fluxes are updated for each bacteria M and exchange metabolite m according to the Michaelis-Menten formula 4.

In a further step, the GEMs are optimized for growth using FBA, the results are used as biomass production μ_M and actual input and output fluxes $\boldsymbol{v_M}$ of bacteria M in this time step. The mortality is considered by subtracting the constants $\mathrm{Mort}(\mu_M)$ from the biomass production rates $\boldsymbol{v_M}$. The slopes $\dot{X}_M$ and $\dot{\boldsymbol{S}}$ are calculated according to 2 and 3.

D. Simulation Setup

The goal of the simulation is to validate the basic functionality of the simulator using a simplified setup of a realistic future simulation scenario. As defined in the project goals,

Algorithm 1: Differential equation with embedded FBA

Input: metabolites in shared medium m, bacteria populations X_M, metabolite counts S_m
Output: slope of bacteria masses and amounts metabolite molecules $\dot{X}_M, \dot{S}_m$
Data: bacteria models model_M, Michaelis-Menten constants of model M $\boldsymbol{k}_M$, inhibition matrices of model M $\boldsymbol{I}_M$, Total volume V

$\boldsymbol{s} \leftarrow \frac{\boldsymbol{S}}{V}$
foreach *model* M *in models* **do**
  foreach *metabolite* m *in exchanges of* M **do**
    $b_{M,m} = \text{upper\_bound}(\boldsymbol{s}, M, m)$
  end
end
foreach *Model* M *in models* **do**
  $\mu_M, \boldsymbol{v_M} \leftarrow \text{FBA}(M, \boldsymbol{b_M})$
  $\dot{X}_M = (\mu_M - \text{Mort}(\mu_M)) \cdot X_M$
  $\dot{\boldsymbol{S}} = \dot{\boldsymbol{S}} + \boldsymbol{v_M} \cdot X_M$
end
return $[\dot{\boldsymbol{X}}, \dot{\boldsymbol{S}}]$

this simulation scenario is the dynamic flux balance analysis (DFBA) of a co-culture of Saccharomyces cerevisiae and Lactobacillus plantarum.

The genome-scale models used are iBT904 Lactobacillus plantarum published by Teusink et al. [35], and for the yeast the iFF708 model by Förster et al. [10]. Both models were modified to have a consistent naming of the (relevant) exchange reactions.

The simulation will consider two main input metabolites: oxygen and glucose. Table II and table III contain all values needed to define the initial metabolite conditions and kinetics. Other metabolites, e.g. nitrogen and sulfate sources or vitamins will be provided in abundance.

Table II: Model constants used in the simulation setup

Constant	S. cerev.	L. plant.	Unit	Reference
$v_{max,M,glc}$	18.5	18.5	$\text{mmol}\,\text{g}^{-1}\,\text{h}^{-1}$	[13, Table 1]
$v_{max,M,o}$	2.5	2.5	$\text{mmol}\,\text{g}^{-1}\,\text{h}^{-1}$	[13, Table 1]
$k_{M,glc}$	0.5	0.5	$\text{g}\,\text{L}^{-1}$	[13, Table 1]
$k_{M,o}$	0.005	0.005	$\text{mmol}\,\text{L}^{-1}$	[13, Table 1]
$I_{M,glc,eth}$	10	-	$\text{g}\,\text{L}^{-1}$	[17, Table 1]
$I_{M,glc,lac}$	30	-	$\text{mmol}\,\text{L}^{-1}$	Chapter III-A

Table III: Initial values for simulation

Parameter	Value	Unit	Reference
s_{glc}	272.9 ... 1230.755	$\text{mmol}\,\text{L}^{-1}$	eq. 7, tab. IV
s_o	0.5039	$\text{mmol}\,\text{L}^{-1}$	eq. 8, ta. IV
x_Y	0.5	$\text{g}\,\text{L}^{-1}$	[8, p. 171]
x_L	0.05,0.5,5,50	$\text{g}\,\text{L}^{-1}$	[26]

To verify the results, we compare the simulated data with known experimental outcomes in similar media.

E. Software Stack

This DFBA simulator framework uses Python version 3 as programming language and builds on the CobraPy Framework [9]. As solver for the linear programs during the FBA the GNU Linear Programming Kit (GLPK) is used. To integrate the ODEs, the Python package scipy.integrate.ode is used, employing the dopri5 solver, which is a classical Runge-Kutta explicite ODE solver for non-stiff problems [11].

III. Results

At a first stage, yeast and lactic bacteria monocultures were simulated to verify whether they work independantly of each other. At the second stage, the coculture was simulated to show.

The mediums used contain $50\,\text{l}$ of water mixed with an inital glucose concentration of $1000\,\text{mmol}\,\text{l}^{-1}$.

A. Yeast Monoculture

The yeast monocolture is well-behaved, until the very edges of metabolic activity, where the GEM is blocked. But its growth is almost exponential, as can be seen in Figure 2. The steep curve at the beginning marks the region where oxygen can be metabolised. The slight curvature of the following section can be explained by the self-inhibition of the organism, caused by the toxicity of ethanol, and by the sinking glucose concentration. Concentrations of the relevant metabolites that can be seen in Figure 3.

At time $t \approx 25$ the GEM is blocked since the glucose uptake can not satisfy the ATP maintenance reactions anymore; the population starts to die.

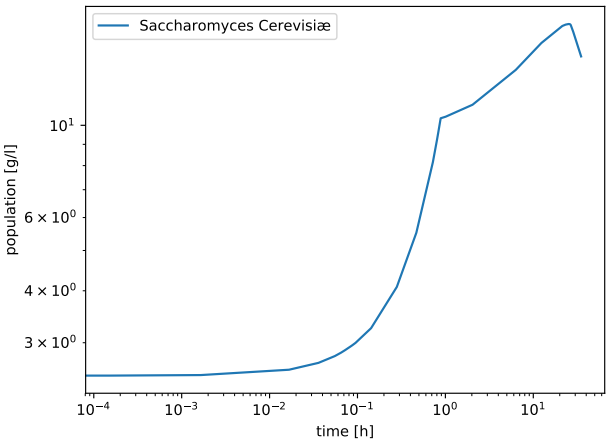


Figure 2: The yeast growth in the medium in a logarithmic scale

In further simulations the concentration of lactate was increased, and the toxicity of the lactate was tuned to best fit the curves given in Figure 4. This was achieved with a number of $I_{Y,\text{glc,lac}} = 30\,\text{mmol}\,\text{l}^{-1}$. Figure 5 shows the tuned curves.

The fit is not perfect on multiple accounts: Firstly, the organism takes longer to metabolise the glucose, which can be traced back to a too high ethanol inhibition constant $I_{Y,\text{glc,etoh}}$

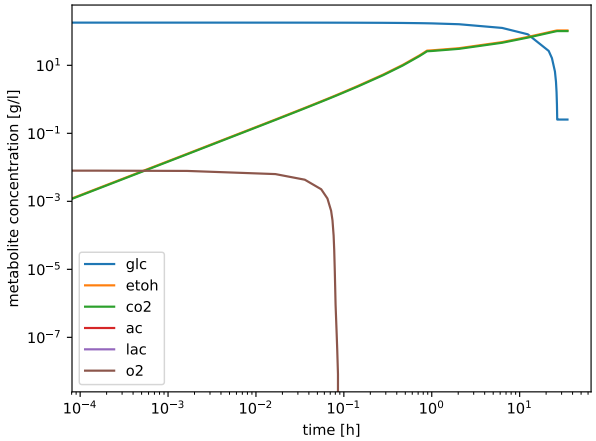


Figure 3: The concentrations of interesting metabolites in the simulated medium for yeast monocultures

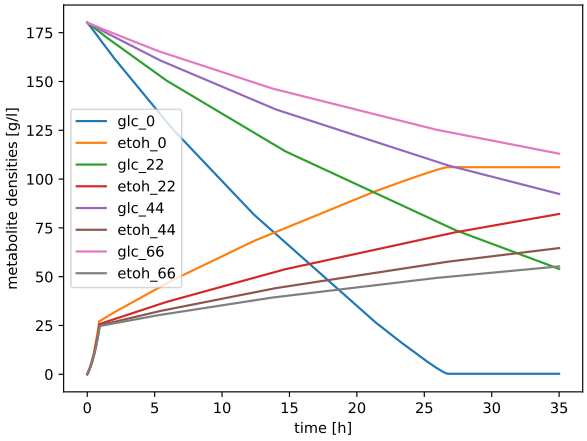


Figure 5: The ethanol and glucose concentration at different concentrations of lactate

Secondly, there are sharp, seemingly non-smooth corners in the plot. These can be explained by wrong Michaelis-Menten constants $k_{Y,m}$

Adapting these constants to $k_{Y,\mathrm{glc}} = 15\,\mathrm{mmol\,l^{-1}}$ and the inhibition constant to $I_{Y,\mathrm{glc,etoh}} = 350\,\mathrm{g\,L^{-1}}$ yields a better fitting result shown in Figure 6

For the remainder of the simulations, the constants were reset to the original values.

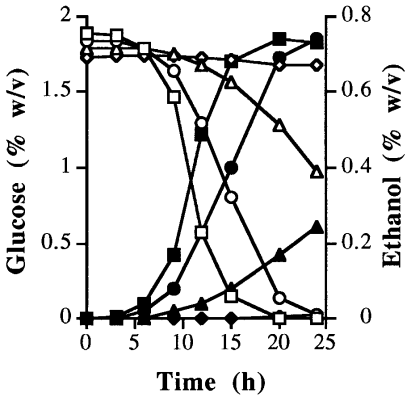


Figure 4: Glucose and ethanol concentrations measured in a real-life scenario with varying lactate concentrations [27].

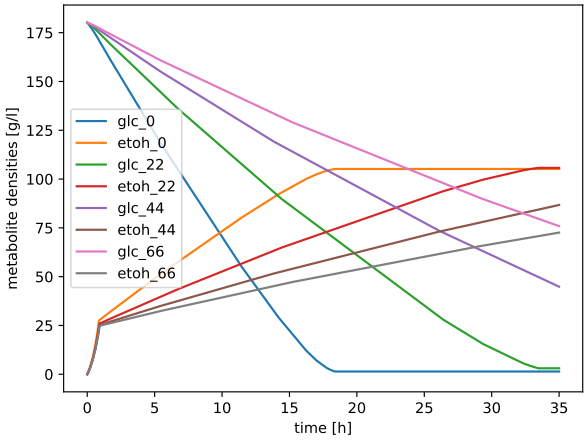


Figure 6: The ethanol and glucose concentration at different concentrations of lactate with the improved constants

B. Lactobacillus Plantarum

If we add Lactobacillus Plantarum to the same medium without the yeast, we get the population curves in Figure 7. These, too, are what can be expected (i.e. exponential growth that gets dampened as the glucose runs out and ad the environment becomes more toxic).

An interesting side-effect of the Lactobacilli is that they not only produce lactate and acetate, but also a rather large amount of ethanol.

C. Cocultures

The simulation of the cocultures shows many effects known by experiment. The yeast shows much higher activity relative to the lactic bacteria when the medium contains oxygen, as can be seen in both Figure 10 and 12. This is congruent with the common brewing practice of aerating the wort before actually fermenting it [8, P. 168]. More oxygen gives the yeast an additional head start in population.

Additionally, whether the Lactobacilli overtake the yeast is dependant on the initial concentration of Lactobacillus Plantarum. This can be seen in Figure 13.

IV. Conclusions

The DFBA framework performs properly, and simulates the Lactobacillus Plantarum and Saccharomyces Cerevisiae monocultures and cocultures approximately as experimental results. Since the framework is generic and flexible, it can be

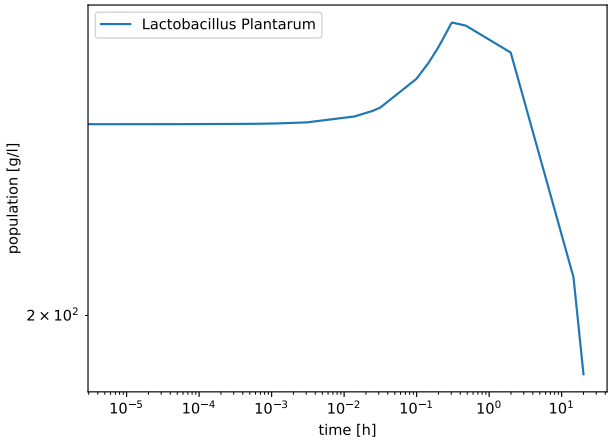


Figure 7: The growth of Lactobacillus Plantarum in the medium in a logarithmic scale

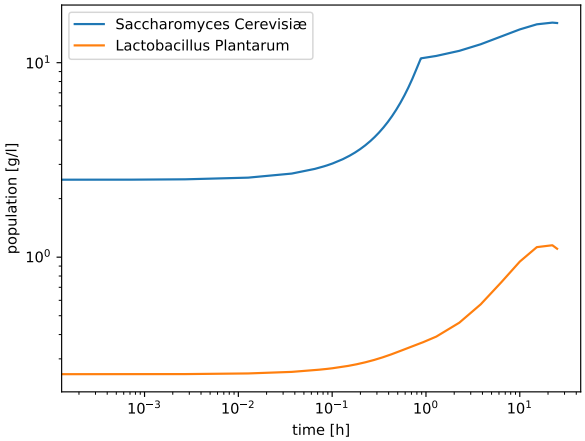


Figure 9: The population of the two organisms with $0.05\,\mathrm{g\,l^{-1}}$ initial lactobacillus population

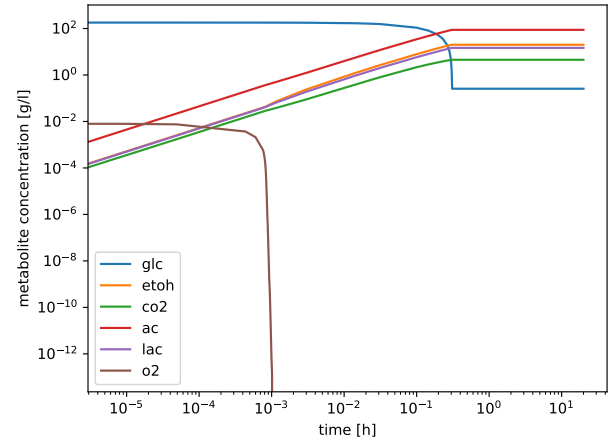


Figure 8: The concentrations of interesting metabolites in the simulated medium for Lactobacillus Plantarum monocultures

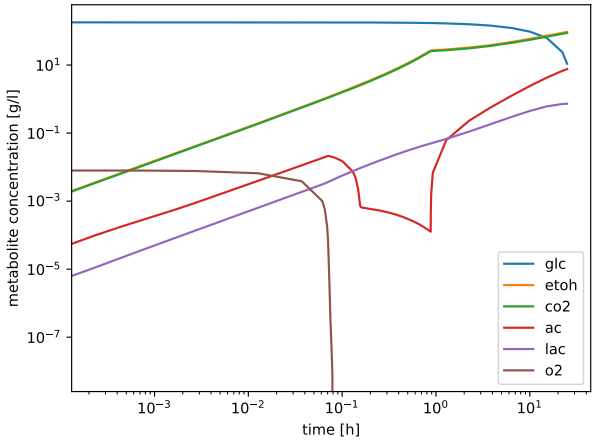


Figure 10: The metabolites in the medium with $0.05\,\mathrm{g\,l^{-1}}$ initial lactobacillus population

easily used to simulate other cocultures beyond yeast and lactic bacteria.

A. *Further Work*

Further efforts can be made by replacing scipy.integrate.ode with a different solver framework (for example odespy [22]), to make implicite solvers with adaptive step-sizes available. Since the problem seems to be somewhat stiff (e.g. the fast changing oxygen depletion combined with the slower glucose usage), the switch will most likely result in a speedup in simulation time.

Furthermore, the system does not allow metabolite exchanges from the medium to the surrounding environment via concentration gradients. Allowing those would open up the simulator to a wider range of applications, e.g. for simulating continuous production of a metabolite with continuous feeding.

Lastly, a method to recycle dead cells could be added, countering the perpetual loss of metabolites in the medium.

APPENDIX A

The following approximation is used to convert °P ("degree plato") to a density measure ($\mathrm{g\,L^{-1}}$) [7].

$$d_{total} = 4.13\frac{g}{l\ {}^{\circ}P}p + 997\frac{g}{l} \tag{5}$$

As the simulation framework expects metabolite densities relative to the total volume of the solution (mmol of metabolite per liter solution, mmol/l) the total density d_{total} must to converted to a density s_{glc}. It is assumed that $V_{total} = V_{glc} + V_W$. Furthermore m is used in the following equations as a measure of mass.

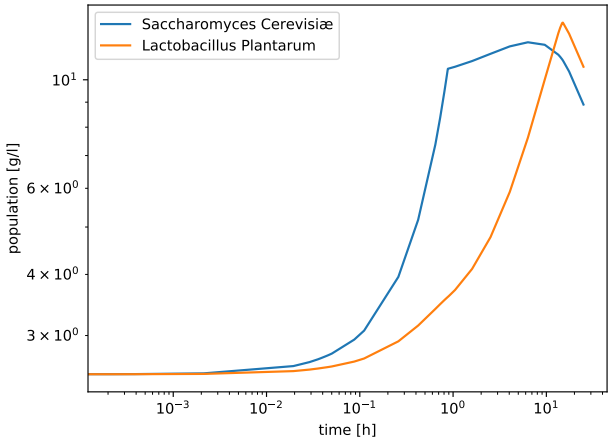


Figure 11: The population of the two organisms with $0.5\,\mathrm{g\,l^{-1}}$ initial lactobacillus population

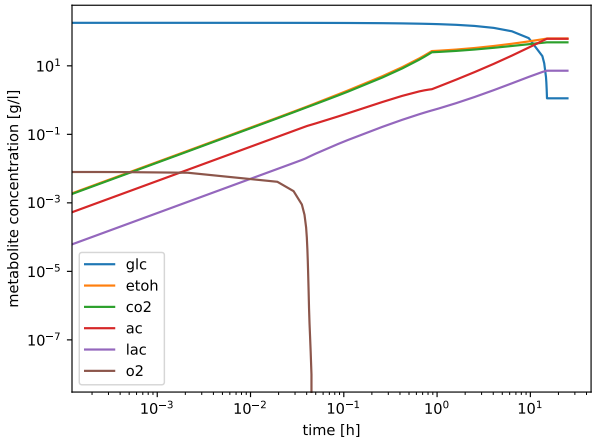


Figure 12: The metabolites in the medium with $0.5\,\mathrm{g\,l^{-1}}$ initial lactobacillus population

$$
\begin{aligned}
d_{total} &= \frac{m_{total}}{V_{total}} \\
&= \frac{m_{glc} + m_w}{V_{total}} \\
&= \frac{m_{glc} + d_w V_w}{V_{total}} \\
&= \frac{m_{glc} + d_w \left(V_{total} - V_{glc}\right)}{V_{total}} \\
&= \frac{m_{glc} + d_w \left(V_{total} - \frac{m_{glc}}{d_{glc}}\right)}{V_{total}} \\
\Leftrightarrow \quad s_{glc} &= \frac{m_{glc}}{V_{total}} = \frac{d_{total} - d_w}{1 - \frac{d_w}{d_{glc}}}
\end{aligned} \tag{6}
$$

Combining equation 5 and 6, including all constants and converting it to mmol/l leads to:

$$
s_{glc} = \left(63.857 \frac{1}{^\circ P} p - 46.385\right) \frac{mmol}{l} \tag{7}
$$

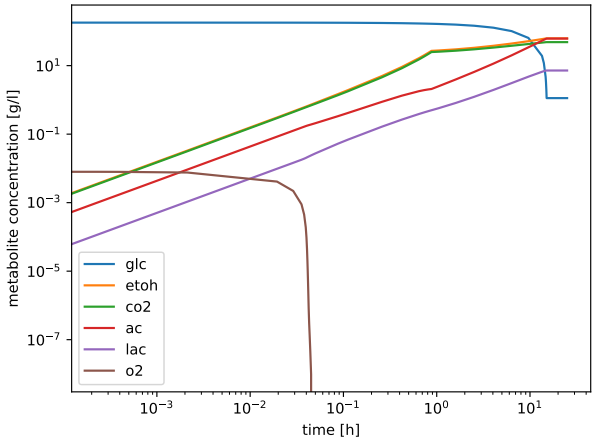


Figure 13: The populations over multiple initial Lactobacillus concentrations (0.05, 0.5, 5, 50)

Table IV: Constants used in this document

Constant	value	reference
Oxygen saturation of water at 20°C ($\mathrm{mg\,L^{-1}}$)	9.077	[1]
Molar mass of water ($\mathrm{g\,mol^{-1}}$)	18.015	[5]
Molar mass of glucose ($\mathrm{g\,mol^{-1}}$)	180.156	[5]
Density of water ($\mathrm{g\,L^{-1}}$)	1.00	[5]
Density of glucose ($\mathrm{g\,L^{-1}}$)	1.56	[5]
Considered wort densities (°P)	5...20	-

To calculate the initial oxygen density in the solution it is assumed that the solution is at 20 °C and fully saturated with oxygen:

$$
\begin{aligned}
s_o &= 9.077 \frac{mg}{l} \\
&= 9.077\ 10^{-3} \frac{g}{l} \\
&= \frac{9.077\ 10^{-3} \frac{g}{l}}{18.015\ 10^{-3} \frac{g}{mmol}} \\
&= 0,5039 \frac{mmol}{l}
\end{aligned} \tag{8}
$$

Appendix B

Acknowledgment

This work was supported by the Technology Development Fund Spring 2018, Háskóli Íslands.

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