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Optimization of lipid production by oleaginous yeast in continuous culture ⋆

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Abstract: Oleaginous yeasts are microbial factories capable of converting carbohydrates and fat substrates into lipids. The transesterification of these lipids results in the production of biodiesel, a renewable energy source that has emerged as a promising and sustainable alternative to overcome the eminent depletion of fossil fuels and to reduce the environmental impacts of petroleum exploitation. To bring biodiesel production as a realistic technology to respond to the worldwide energy demand, process optimization is to be pursued. In this paper, we performed a model-based optimization of lipid productivity in continuous mode operation. It is considered that two input flow rates at different carbon/nitrogen ratio are available as manipulated variables. The optimal control problem was solved numerically by using the control vector parameterization approach. A simple parameterization with piecewise linear functions was found to be suitable for providing optimal performances. We showed that by driving the carbon/nitrogen ratio along the process adequately, optimal performances are attained. The control strategy here developed excels substantially an operation with a single input flow rate with fixed C/N ratio. This work sets up useful guidelines towards optimal bioprocesses for microbial lipid production.

Keywords: biodiesel, chemostat, energy, modelling, optimal control

1. INTRODUCTION

Biodiesel production based on oleaginous microorganisms such as yeast and microalgae offers a high potential and sustainable alternative for energy supply in view of the decrease of petroleum reserves (Chisti, 2007; Meng et al., 2009).

Heterotrophic yeasts are able to produce single cells oils (SCOs) when growing on inexpensive agro-industrial by-products. Some yeasts can accumulate SCO up to 70% of their biomass weight (Ratledge and Wynn, 2002). SCOs are formed by triacylglycerols such as palmitic, stearic and oleic acids, which are feedstocks for biodiesel production. These metabolic capabilities of oleaginous yeast are indeed promising. However, to outcompete with the plant oil production, microbial lipid productivities need to be optimized (Kosa and Ragauskas, 2011).

Mathematical modelling can be of great help in the attempt of optimizing lipid productivities. Different macroscopic models have been proposed to describe microbial growth and lipid production by yeast (*e.g.*, Ykema et al. (1986); Papanikolaou and Aggelis (2003); Economou et al. (2011); Meeuwse et al. (2012); Shen et al. (2013)). These models have shown to represent acceptably the time evolution of both fat-free and lipid biomass. However, there is still a need for improving such models to better understand the process of lipid formation and thus provide robust models that can be used for process optimization.

Among the modelling approaches cited above, the work of Economou et al. (2011) provides an interesting model structure in which biomass synthesis is function of both carbon and nitrogen substrates. In this model, the lipid production is associated to the uptake of carbon substrate. Additionally, a nitrogen regulation term is included to represent the experimental observation that lipids accumulation is favored by nitrogen limitation. The model proposed by Economou et al. (2011) was developed to describe batch cultures of the fungus *Mortierella isabellina*. It is reasonable that this model structure also holds for yeasts. However, for describing the dynamics of yeasts, model parameters may be different reflecting the specificities of the metabolic activities of the microorganisms. We use as a basis this model to perform a numerical optimization study to favor lipid production in continuous mode.

The article is organized as follows. In Section 2, we describe the reactor configuration of our case study and derive the respective model equations. Section 3 details the statement of the optimal control problem and the numerical approaches used for its solution. Finally, in

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Section 4, we discuss about the relevance of our results for the optimization of microbial lipid production.

2. REACTOR SYSTEM AND MATHEMATICAL MODELLING

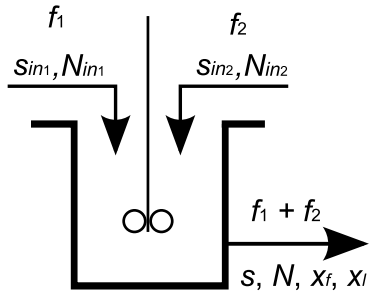

Fig. 1. Reactor scheme for continuous culture of yeast. Two separate inputs flow rates allow to regulate the C/N ratio during the process.

Oleaginous yeasts can be cultured in batch, fed-batch and continuous mode. The drawback of the batch mode is the unfeasability of regulating the C/N ratio in the culture, whereas a fed-batch strategy is efficient for regulating the C/N ratio.

The standard operation in continuous mode involves one input flow rate at fixed C/N ratio, not allowing the regulation of the nutrient flow rate in the process. However, if two inputs flow rates are considered, the C/N regulation is possible. In this work, we study such a reactor scheme, which is depicted in Fig. 1. This chemostat has two input flow rates f_1, f_2 with different concentration of carbon substrate and nitrogen ($s_{\mathrm{in}i}, N_{\mathrm{in}i}$).

By extending the model of Economou et al. (2011) to the reactor scheme in Fig. 1, the model equations read as follows

$$\dot{s} = \frac{f_1 s_{\mathrm{in}1} + f_2 s_{\mathrm{in}2} - (f_1+f_2)s}{V} - \left(\frac{1}{Y_{X/S}}\mu_{\mathrm{S}}(\cdot) + \frac{1}{Y_{L/S}}q_{\mathrm{L}}(\cdot)\right)x_f, \quad (1)$$

$$\dot{N} = \frac{f_1 N_{\mathrm{in}1} + f_2 N_{\mathrm{in}2} - (f_1+f_2)N}{V} - \frac{1}{Y_{X/N}}\mu_{\mathrm{S}}(\cdot)x_f, \quad (2)$$

$$\dot{x_f} = -\frac{(f_1+f_2)x_f}{V} + (\mu_{\mathrm{S}}(\cdot) + \mu_{\mathrm{L}}(\cdot))\,x_f, \quad (3)$$

$$\dot{x_L} = -\frac{(f_1+f_2)x_L}{V} + \left(q_{\mathrm{L}}(\cdot) - \frac{\mu_{\mathrm{L}}(\cdot)}{Y_{X/L}}\right)x_f, \quad (4)$$

where s is the concentration of the carbon substrate (sugars in this case), N is the concentration of nitrogen and V is the volume of the reactor .

The biomass is constituted by two pools, namely fat-free biomass (x_f) and lipid biomass (x_L). The fat-free biomass is the catalytic biomass responsible of the metabolic conversions.

The specific growth rate of fat-free biomass $\mu_{\mathrm{S}}(\cdot)$ on s and N is defined by a double substrate kinetics with inhibition on the carbon substrate

$$\mu_{\mathrm{S}}(\cdot) = \mu_{\mathrm{S\,max}}\frac{s}{Ks_1 + s + s^2/K_{I1}}\frac{N}{K_N + N}. \quad (5)$$

Table 1. Model notation and parameters.

	Definition	Value
State variable		
N	Nitrogen concentration (g/L)	
S	Carbon substrate concentration (g/L)	
x_f	Concentration of fat-free biomass (g/L)	
x_L	Concentration of lipid biomass (g/L)	
Inputs		
D_i	Dilution rate (h^{-1})	
f_i	Input flow rates (L h^{-1})	
$N_{\mathrm{in}i}$	Concentration of nitrogen in the flow rate i	
$s_{\mathrm{in}i}$	Concentration of carbon in the flow rate i	
Parameter		
$\mu_{\mathrm{S\,max}}$	Maximal growth rate on carbon and nitrogen	0.566 h^{-1}
$\mu_{\mathrm{L\,max}}$	Maximal growth rate on lipids	NA
$q_{\mathrm{L\,max}}$	Maximal specific rate of lipid production	0.785 g $\mathrm{L}^{-1}\mathrm{h}^{-1}$
k_1	Constant of carbon regulation for lipid utilization	NA
k_2	Constant of nitrogen regulation for lipid production	835.2 g L^{-1}
Ks_1	Carbon saturation constant for growth on carbon and nitrogen	1.256 g L^{-1}
Ks_2	Carbon saturation constant for lipid production	69.269 g L^{-1}
K_N	Nitrogen saturation constant for growth on carbon and nitrogen	0.085 g L^{-1}
K_L	Lipid saturation constant for growth on lipids	NA
K_{I1}	Inhibition constant for growth on carbon and nitrogen	20.981 g L^{-1}
K_{I2}	Inhibition constant for lipid production	0.399 g L^{-1}
V	Volume of the reactor	1.0 L
$Y_{L/S}$	Yield of lipid biomass wrt carbon substrate	0.242 g g^{-1}
$Y_{X/L}$	Yield of fat-free biomass wrt lipids	NA
$Y_{X/N}$	Yield of fat-free biomass wrt nitrogen	18.209 g g^{-1}
$Y_{X/S}$	Yield of fat-free biomass wrt carbon substrate	0.345 g g^{-1}

Lipids are assumed to be formed during nitrogen limitation. The rate of lipid production $q_{\mathrm{L}}(\cdot)$ is described as

$$q_{\mathrm{L}}(\cdot) = q_{\mathrm{L}\max}\frac{s}{Ks_2 + s + s^2/K_{I2}}\frac{k_2}{k_2 + N}, \tag{6}$$

where the coefficient k_2 represents the effect of down regulation due to the nitrogen concentration.

It is assumed that the accumulated lipids are used for synthesis of fat-free biomass, a process known as turnover.

The kinetic rate $\mu_{\mathrm{L}}(\cdot)$ of lipid turnover is given by

$$\mu_{\mathrm{L}}(\cdot) = \mu_{\mathrm{L}\max}\frac{x_L}{K_L + x_L}\frac{k_1}{k_1 + s}. \tag{7}$$

From the experiments developed by Economou et al. (2011), however it was noticed that the lipids were not consumed for the synthesis of fat-free biomass. Therefore, $\mu_{\mathrm{L}}(\cdot) = 0$.

The notation of the model and values of the parameters are listed in Table 1. They were obtained from batch cultures of *Mortierella isabellina* on sweet sorghum as carbon substrate (Economou et al., 2011). For now, this set of parameters is considered as a prior for the parameters associated to yeast. A further calibration will be needed to represent yeast metabolism.

For the operation on continuous mode here analyzed, we set for both input flow rates a common nitrogen concentration of $N_{\mathrm{in1}} = N_{\mathrm{in2}} = 0.4$ g/L. The sugar concentrations were set to $s_{\mathrm{in1}} = 50$ g/L and $s_{\mathrm{in2}} = 5$ g/L. Thus, the feeding rates f_1, f_2 have a C/N ratio of 50 g/g and 5 g/g respectively. This computation is made by converting the sugar concentration in carbon units.

3. DEFINITION OF THE OPTIMAL CONTROL PROBLEM

Optimizing the process of lipid production of yeast is a two-fold objective. Firstly, we want to maximize the productivity of lipid, who dynamics follows

$$\dot{p}_{\mathrm{L}} = (D_1 + D_2)x_L, \tag{8}$$

with $D_i = f_i/V$ the dilution rate.

During a time of operation t_f, the first objective, that is the volumic productivity, is $P_{\mathrm{L}} = p_{\mathrm{L}}(t_f)/t_f$,

Secondly, we are interested in producing a biomass with high-lipid content. The mass fraction of lipid content of biomass is

$$w_{\mathrm{L}} = \frac{x_{\mathrm{L}}}{x_f + x_{\mathrm{L}}}. \tag{9}$$

By defining a target of lipid content ($w_{\mathrm{L}t}$), we define

$$\dot{p}_{\mathrm{w}} = \frac{w_{\mathrm{L}}(t)}{w_{\mathrm{L}t}}. \tag{10}$$

The second objective is then $P_{\mathrm{W}} = p_{\mathrm{w}}(t_f)$.

To achieve the multi-objective performance in the process the inputs dilution rates ($D_i = f_i/V$) need to be adjusted accordingly. The optimal control problem is then defined as

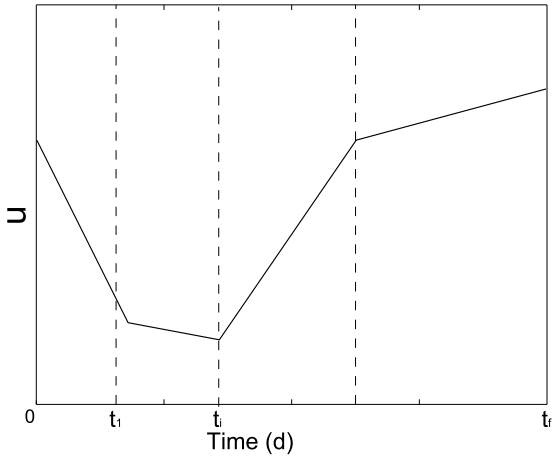

Fig. 2. Approximation of the control inputs by piecewise linear functions.

$$\begin{aligned} &\max_{D_1(t),D_2(t)} \quad P_{\mathrm{L}} + bP_{\mathrm{W}} \\ &\text{s.t.} \\ &\qquad D_{\mathrm{L}} = 0 \le D_i(t) \le D_{\mathrm{U}} = 2.0\mathrm{h}^{-1}, \end{aligned} \tag{11}$$

with D_{L} and D_{U} the lower and upper bounds of the dilution rates. The parameter b is a penalization coefficient. It can be defined as an index of the energetic cost related to the concentration and further separation of the microbial lipids. The target lipid content was set to $w_{\mathrm{L}t} = 40\%$ and the penalization coefficento to $b = 4.0 \cdot 10^{-4}$.

The solution of (11) implies to finding an optimal dilution rate together with an optimal C/N ratio.

The optimal problem (11) was solved numerically by the control vector parameterization (CVP) approach. Piecewise linear functions were used to parameterize the control inputs (Fig 2).

The optimization was performed with the Matlab toolbox SSmGo: (`http://www.iim.csic.es/ gingproc/ssmGO.html`). SSmGo performs global optimization by using a scatter search method (Rodríguez-Fernández et al., 2006; Egea et al., 2007). When the solution of the scatter search method was found, the Nelder-Mead method implemented in the *fminsearch* function (Lagarias et al., 1998) was used to look for improvement of the solution.

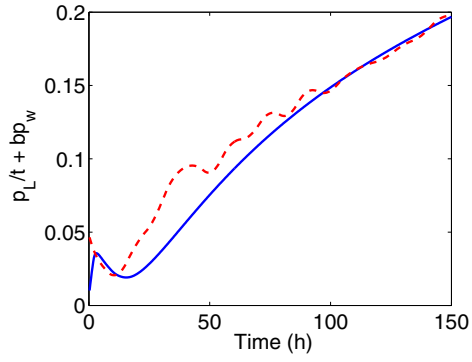

Fig. 3. Objective function for a parameterization with 20 piecewise linear functions (dashed red line) and with three linear piecewise functions (blue solid line).

4. RESULTS AND DISCUSSION

Figure 3 shows the objective function resulting from the piecewise linear approximation of the control inputs using three and 20 time intervals. Until $t = 100$ h, the input with 20 time intervals provides better objective function than that obtained with the input of three time intervals. However, at the final time, both parameterizations provided similar objective functions. Therefore, approximating the control variables $D_1(t), D_2(t)$ to only three piecewise linear functions is a suitable parameterization for attaining an optimal performance. The infinite dimension problem was thus reduced to a problem of dimension 2×6.

Figure 4 shows the response of the state variables of the model when applying the resulting optimal control. At the first hours of operation, the nitrogen and carbon concentrations are increased to favor the exponential growth. At 22 h, the fat-free biomass reaches the 90% of its value at steady state. For the lipid biomass; the 90% of the concentration at steady state is reached at 80 h.

We can observe that the main regulation is performed on D_2 during the first nine hours, while D_1 was almost constant during the operation time. The first nine hours of operation allow a progressive increase of the C/N ratio. This gradual increase favors, at the first hours of the operation, the formation of catalytic biomass until reaching a maximal C/N ratio that contributes to the lipid formation. This result is in line with the metabolic states described for lipid formation in yeasts (Beopoulos et al., 2009).

To have a reference for comparison, an optimal dilution rate was calculated for a configuration with one input flow rate (f_1) set to be constant along the process. The optimal constant dilution rate was found to be 0.027 h^{-1}. Figure 5 shows the dynamics of the variables that determine the objective of lipid productivity (p_{L}) and high lipid content (p_{w}) for both configurations, namely two input flow rates and a constant input flow rate.

The volumic productivity with the two input flow rates is 40% higher than the one obtained with only one input flow rate. By applying the optimal control with two input flow rates, a maximal volumic productivity of $P_{\mathrm{L}} = 0.14$ g(L h)$^{-1}$ is obtained. This value is in the range of volumic productivities for yeasts (Ykema et al., 1988; Papanikolaou and Aggelis, 2002).

The cost function related to the high-lipid content is of the same order for both controllers. The controller with one flow rate provides a higher lipid content at t_f. However, the controller with two flow rates excels between 28-98 h. In summary, the configuration with two input flow rates provides a suitable control strategy for optimizing lipid productivity and a biomass with high lipid content.

As mentioned before, the model parameters correspond to a batch culture with *Mortierella isabellina*, where the yeast did not exhibit lipid turnover (Economou et al., 2011). This was probably due to the exhaustion of an essential component for growth. Furthermore, the coefficient describing the regulation of nitrogen to the lipid production (k_2) has a high value, that is $k_2 >> N$ which implies that under the experimental conditions of the culture, the production of lipids is weakly regulated by the nitrogen. This mechanism, however, is not representative of other oleaginous yeasts. For instance, *Yarrowia lipolytica* when growing on industrial fats was able to utilize its accumulated lipids for the synthesis of fat-free biomass (Papanikolaou and Aggelis, 2003).

It is worth noting that, even if the model parameters from *Mortierella isabellina* implies a low level of regulation of nitrogen for lipid production, the control strategy with the two flow rates resulted in an significant improvement on the lipid production compared to an continuous operation with a single input flow rate with fixed C/N ratio. For strains such as *Y. lipolytica*, which has been identified as a model for biofuel production due to its metabolic capabilities (Beopoulos et al., 2009; Makri et al., 2010), the C/N ratio appears as a crucial operational parameter for driving lipid production. The metabolism of *Y. lipolytica* takes place in three phases, namely the biomass production phase, the lipogenic phase and the citric acid phase. The transitions between these phases, together with the rate of lipid turnover need to be controlled for optimizing the productivity of lipids. The C/N ratio needs to be lower bounded for allowing the building of carbon blocks and upper bounded to prevent the production of citric acid that affects lipid production. The dynamic control here developed provides a solution for such a regulation.

As occurred in microalgae growth processes, optimal inputs are strongly determined by the rates of maintenance and respiration (Muñoz-Tamayo et al., 2013). For yeast growth as described by the model (1-4), respiration and maintenance are aggregated into the kinetics of lipid turnover $\mu_{\mathrm{L}}(\cdot)$. It is therefore crucial to quantify this rate.

For the sake of illustration, in a second scenario, we included the kinetics of lipid utilization. A simple first-order kinetic ($mu_{\mathrm{L}}(\cdot) = k_l x_{\mathrm{L}}$)was used for the numerical study with a constant rate of $k_l = 0.005$ h^{-1}. The yield factor for fat-free biosynthesis was set to $Y_{X/L} = 0.60$ g/g. When applying the previously calculated D_1, D_2, we obtained a substantial decreased in the lipid productivity and in the lipid content of the biomass as illustrated in Fig 6. This results indicates that a characterization of lipid turnover is needed for obtaining feasible solutions.

Finally, the development of genomic based approaches as the one developed by Morin et al. (2011) are intended to improve our understanding of the pathways involved in the *de novo* and *ex novo* lipid synthesis by yeast. The integration of this knowledge will allow us to construct detailed metabolic models (Pan and Hua, 2012) that can be further utilized for prediction and for boosting biodiesel production.

The next step of the work presented consists of the real implementation of the developed control strategy. For that, a mathematical model of the metabolism *Y. lipolytica* will be developed and validated with experimental data. Since the optimal control as presented here is in open loop scheme, adaptation strategies (Chachuat et al., 2009; Jäschke and Skogestad, 2011) will be further investigated for the real implementation. This will allow us to exploit the available information from on-line measurements and to account for model uncertainty.

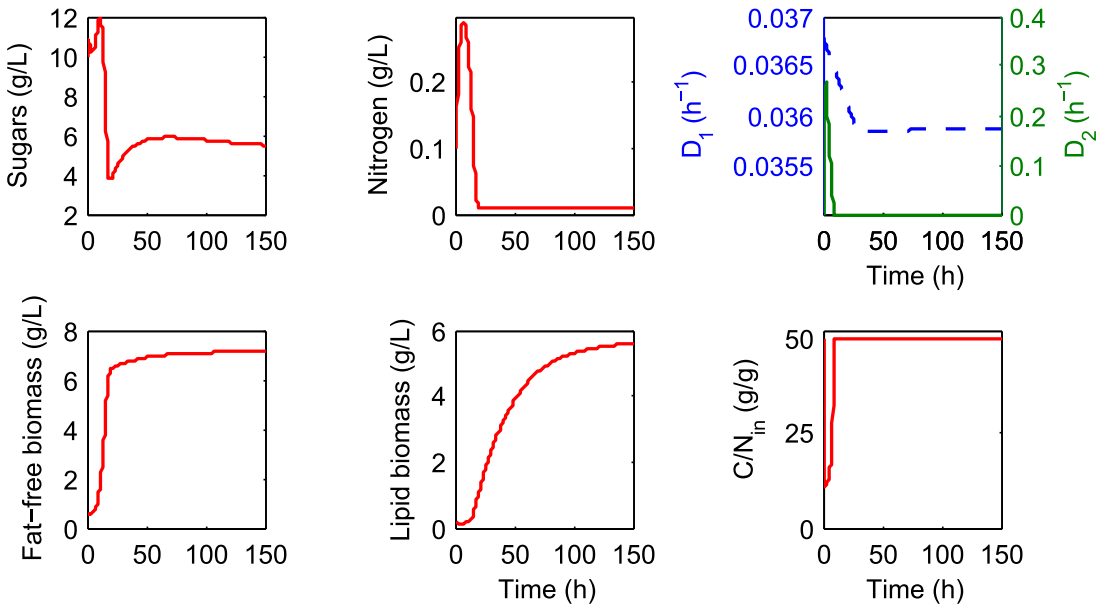

Fig. 4. Model outputs and resulting optimal control. The shape of the controls is described by two piecewise linear functions.

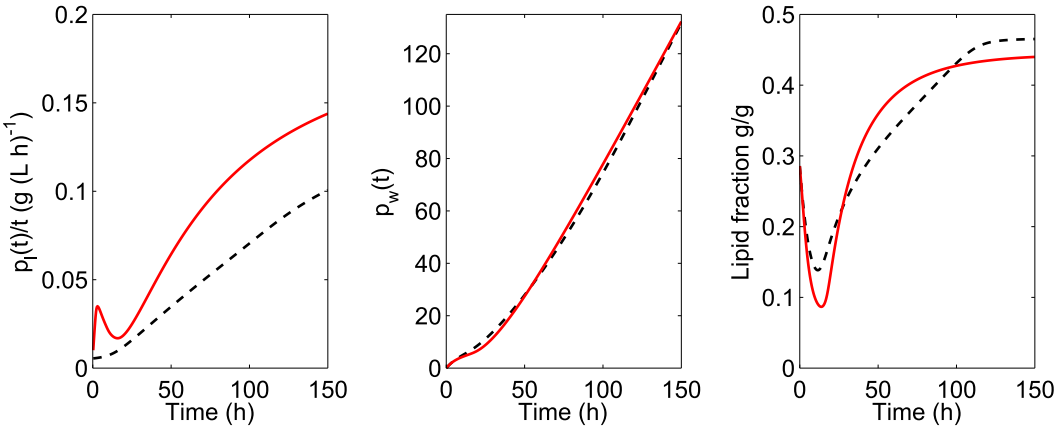

Fig. 5. Objective functions and biomass lipid fraction for the optimal controller with two input flow rates (continuous red line) and for an optimal controller with one input flow rate (black dashed line).

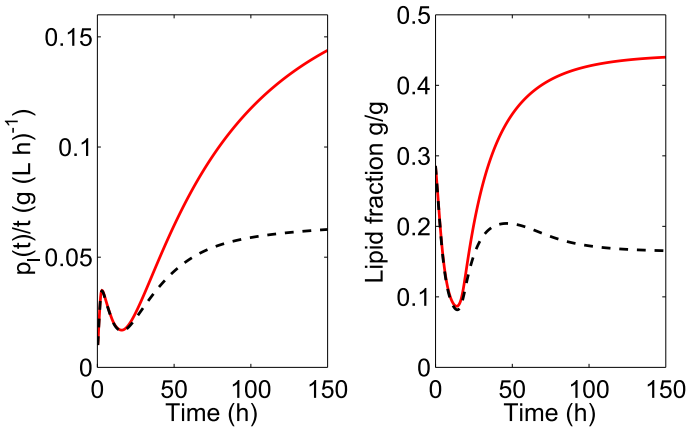

Fig. 6. Optimal performance is very sensitive to lipid turnover. The indices of performance are shown for the model with no lipid turnover (solid red line) and with lipid turnover (dashed black line).

5. CONCLUSIONS

In this work, we tackled the problem of optimizing the production of lipid by oleaginous yeast in continuous culture. Model-based optimization was performed using the control vector parameterization approach. We showed the benefits of having a reactor configuration with two input flow rates with different C/N ratios. This configuration allows to regulate efficiently the C/N ratio along the process, providing a lipid productivity that exceeds in a 40% the productivity obtained with a single flow rate. This approach is a step toward optimal bioprocesses for microbial lipid production.

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