When Do Two-Stage Processes Outperform One-Stage Processes?

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Apart from product yield and titer, volumetric productivity is a key performance indicator for many biotechnological processes. Due to the inherent trade-off between the production of biomass as catalyst and of the actual target product, yield and volumetric productivity cannot be optimized simultaneously. Therefore, in combination with genetic techniques for dynamic regulation of metabolic fluxes, two-stage fermentations (TSFs) with separated growth and production phase have recently gained much interest because of their potential to improve the productivity of bioprocesses while still allowing high product yields. However, despite some successful case studies, so far it has not been discussed and analyzed systematically whether or under which conditions a TSF guarantees superior productivity compared to one-stage fermentation (OSF). In this study, we use mathematical models to demonstrate that the volumetric productivity of a TSF is not automatically better than of a corresponding OSF. Our analysis reveals that the sharp decrease of the specific substrate uptake rate usually observed in (non-growth) production phases severely impacts the volumetric productivity and thus raises a big challenge for designing competitive TSF processes. We discuss possible approaches such as enforced ATP wasting to improve substrate utilization rates in the production phase by which TSF processes can become superior to OSF. We also analyze additional factors influencing the relative performance of OSF and TSF and show that OSF processes can be more appropriate if a high product yield is an economic constraint. In conclusion, a careful assessment of the trade-offs between substrate uptake rates, yields, and productivity is necessary when deciding for OSF vs. TSF processes.

1. Introduction

The development of sustainable bio-based production processes that can compete with classical petrochemical synthesis remains a global challenge of the 21st century. Among other key

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performance indicators, the successful implementation of new bioprocesses requires the maximization of product yield, titer, and productivity. This challenge is usually tackled by a combination of methods for strain design (metabolic engineering) and for optimizing bioprocess conditions and strategies (e.g., temperature, pH, substrate concentrations, volume, feeding strategies, etc.). Strain design strategies often focus on the construction of suitable microorganisms that achieve high product yields while maintaining some minimal growth.[1–3] Due to the inherent trade-off between the production of biomass (needed as catalyst to synthesize the product) and the actual target product, yield, and volumetric productivity cannot be maximized simultaneously.[3–6] In this regard, two-stage fermentations, i.e., the separation of growth and production within a biotechnological process, in combination with dynamic metabolic engineering have recently gained much interest due to their high potential for improving the productivity of biotechnological production processes.[5,7,8] A typical example of a two-stage process is an aerobic cell growth phase followed by an anaerobic production phase. Processes of this kind have been employed to produce different target compounds like succinate, lactate, or amino acids.[9–11] The recent advent of new experimental techniques for the dynamic regulation of metabolic fluxes has greatly broadened the spectrum of dynamic process strategies[12–23] and includes, for example, genetic toggle switches[17] and tunable cell density sensors for controlling metabolic fluxes.[18,23] Apart from applications for increasing volumetric productivity, two-stage fermentations (TSFs) are also useful or even essential if a knockout of a gene for redirecting flux from biomass to product involves essential genes.[23] A TSF is then the only way to enter the production phase with sufficient biocatalyst. In this contribution, we mainly focus on TSF as a tool to maximize productivity.

The great potential of TSFs for improving the performance of industrial fermentations has been emphasized recently by many groups, however, it has not been discussed whether or under which conditions a TSF guarantees superior productivity. Indeed, relevant but significantly reduced substrate uptake

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rates are often observed in the production phase where cells are in a non-growing metabolic state. For example, typical values for *Escherichia coli* have been reported to be in the range of 0.5–4.3 mmol glucose/gDW/h in the production phase[5] compared to 10–15 mmol/gDW/h usually observed in the growth phase. For example, in a recent study implementing a TSF process for production of itaconate with *E. coli*, a drastically reduced glucose uptake rate (below 1 mmol/gDW/h) was observed when switching from growth to production.[24] Stoichiometric calculations indicate that substrate uptake in non-growing cells mainly covers the demand of ATP required for non-growth associated maintenance processes.[25,26] Clearly, a sharp decrease of the substrate uptake rate in the production phase impacts the volumetric productivity and may severely hamper the success of a TSF. Therefore, a careful assessment of the trade-offs between substrate uptake rates, yields, and productivity is necessary to evaluate at which conditions TSFs really outperform one-stage fermentations (OSFs).

Several theoretical papers studied the potential of TSFs[4,27–29] and optimized, for example, the number of different phases,[29] or determined optimal time points for switching between the phases.[28] However, these studies usually assume constant maximal substrate uptake rates for growth and production phase, which is not supported by the data mentioned above. Herein, we will employ a model-based approach to analyze and compare the performance of OSFs vs. TSFs with the substrate uptake rate as the crucial parameter. Further we analyze which additional factors influence the relative performance between OSF and TSF. In particular, we will examine the role of enforced ATP wasting as a means to increase the specific and volumetric productivities in OSFs and TSFs.

2. Mathematical Models

In the following, we describe generic mathematical models to simulate one-stage (with coupled growth and product synthesis) and two-stage (first phase: growth; second phase: production) batch fermentation processes. These process models are kept as simple as possible to illustrate the influence of certain parameters on the (average) volumetric productivity (defined as ratio of final product titer and total process time) in both process designs. In order to make the OSF and the TSF model directly comparable, their model structure is very similar and we demand that the target total product yield is the same in OSF and TSF. This ensures that the performance comparison of TSF and OSF really focuses on volumetric productivity without the need to evaluate also different yields. For concrete simulations, we will investigate OSF and TSF processes for the production of lactate from glucose by *E. coli*.

2.1. Model for One-Stage Fermentation

For the one-stage process we assume that a production strain has been designed which couples growth with product synthesis in a fixed (yield) ratio. A number of such growth-coupled strain designs have been constructed in the past, often with the help of computational strain design methods.[1–3] Accordingly, $Y_{X/S}^{true}$ describes the (true) biomass (X) yield per substrate (S) used, and $Y_{P/X}^{true}$ the true product (P) yield per biomass produced (for parameters and their units see **Table 1**). Importantly, both $Y_{X/S}^{true}$ and $Y_{P/X}^{true}$ are *true* (maximum theoretical) yields thus not accounting for other uses of substrate including the synthesis of ATP for non-growth associated maintenance (NGAM). This demand is quantified by a specific ATP consumption rate, m_{ATP}, and this amount of ATP must be generated by the cell from the substrate. The true ATP yield per substrate is quantified by $Y_{ATP/S}^{true}$. In many (e.g., anaerobic) fermentations, ATP synthesis is also coupled with product synthesis (as in the concrete lactate production scenario investigated below) and we therefore quantify the product yield per mole ATP produced by the parameter $Y_{P/ATP}^{true}$. In some simulations, we will also analyze the effect of an increased ATP demand for NGAM induced, for example, by enforced *ATP wasting* as proposed in Refs [30,31].

With the explanations given above, it follows that the true product yield *per substrate* via the *growth-dependent* route (accounting for growth-associated (but not NGAM) ATP demand) is given by $Y_{P/X}^{true} \cdot Y_{X/S}^{true}$ and the true product yield *per substrate* along *ATP-producing* pathways (used by the cell to satisfy the ATP demand for NGAM) reads $Y_{P/ATP}^{true} \cdot Y_{ATP/S}^{true}$. The observed (total) product yield is then a weighted measure of both these yields, depending on the growth rate and the NGAM ATP value m_{ATP} (see eq. [3] below).

The process model comprises three state variables: the concentrations of biomass (X), substrate (S), and product (P). The differential equations for the state variables read

Table 1. Parameters and variables of the models.

	Unit	Explanation
Parameters		
$Y_{X/S}^{true}$	(gDW/mmol)	(True) biomass yield from substrate
$Y_{P/X}^{true}$	(mmol/gDW)	(True) product yield per biomass produced (under growth-coupling)
$Y_{P/ATP}^{true}$	(mmol/mmol)	(True) product yield per mole ATP produced
$Y_{ATP/S}^{true}$	(mmol/mmol)	(True) ATP yield per substrate
$r_{S,max}$	(mmol/gDW/h)	Maximal substrate uptake rate
m_{ATP}	(mmol/gDW/h)	Demand of ATP for non-growth associated maintenance (NGAM)
State variables		
X	(gDW/L)	Biomass concentration in bioreactor
S	(mmol/L)	Substrate concentration in bioreactor
P	(mmol/L)	Product concentration in bioreactor
Other variables		
μ	(h^{-1})	Specific growth rate
r_S	(mmol/gDW/h)	Specific substrate uptake rate
r_P	(mmol/gDW/h)	Specific product synthesis rate
q_S	(mmol/L/h)	Volumetric substrate uptake rate
q_P	(mmol/L/h)	Volumetric product synthesis rate

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$$dX/dt = \mu \cdot X$$
$$dS/dt = -q_S \qquad\qquad (1)$$
$$dP/dt = q_P$$

where μ is the growth rate and q_S and q_P are the volumetric substrate uptake and product excretion rates, respectively. μ, q_S, and q_P as well as the specific substrate uptake (r_S) and specific product excretion (r_P) rate are variables that can be calculated from the state variables and parameters via algebraic equations. It is assumed that the substrate S is taken up with (known) maximal uptake rate ($r_{S,max}$) and then metabolized to produce ATP for NGAM (m_{ATP}) whereas the rest of the substrate is used for growth.

For the sake of simplicity, we assume that the metabolism and growth is stopped when the substrate concentration in the medium is zero and neglect that the uptake rate will usually decrease for low substrate concentrations, however, this does not affect the key results of this study. We thus obtain the following equations for the five dependent variables:

if $S > 0$
$$\mu = Y_{X/S}^{true}\left(r_{S,max} - m_{ATP}/Y_{ATP/S}^{true}\right)$$
$$r_S = r_{S,max} = \mu/Y_{X/S}^{true} + m_{ATP}/Y_{ATP/S}^{true}$$
$$r_P = \mu \cdot Y_{P/X}^{true} + m_{ATP} \cdot Y_{P/ATP}^{true}$$
else
$$\mu = r_S = r_P = 0 \qquad\qquad (2)$$
end
$$q_S = r_S \cdot X$$
$$q_P = r_P \cdot X$$

2.2. Model for Two-Stage Fermentation

The TSF model has the same variables and parameters as the OSF model. However, for some parameters and equations we have to differentiate between the first (growth) and the second (production) stage. For typical TSF processes, there will be uncoupled growth and ATP synthesis in the first stage, i.e., no or only low amounts of product are formed as byproduct of biomass or ATP synthesis (hence, $Y_{P/X}^{true}$ and $Y_{P/ATP}^{true}$ will normally be [close to] 0). Furthermore, the biomass yield $Y_{X/S}^{true}$ and the ATP yield $Y_{ATP/S}^{true}$ are usually much larger in the growth phase than in the production phase of TSF or under OSF (see also **Table 2** for the parameter values used in the simulations in section 3). Similar as for OSF, for the TSF growth phase we assume that the substrate is taken up with maximal uptake rate ($r_{S,max}$) and then metabolized to satisfy the ATP demand for NGAM and the rest is used for growth.

We assume that the growth phase switches instantaneously to the production phase once the substrate level reaches a certain threshold value S^* (see below). In the production phase, the growth rate is set to zero and thus the biomass yield parameter $Y_{X/S}^{true}$ as well as the biomass-coupled product yield $Y_{P/X}^{true}$ are of no

relevance. Since growth does not take place, the cell consumes substrate exclusively to satisfy the ATP demand for NGAM and the maximal substrate uptake will usually not be reached. We here initially assume that the dynamic metabolic switch from the TSF growth to TSF production phase induces coupling of ATP and product synthesis, hence, the ATP demand for NGAM drives product synthesis. Coupling of product and ATP synthesis often occurs, for example, for classical fermentation products including lactate considered in the simulations below. The case of ATP-independent product synthesis during the production phase will be discussed later in the section 3.

The ODEs (for both stages) of the TSF are identical to the ODEs of the OSF model (eq. [1]). Differences occur only in the algebraic functions of the dependent variables to properly reflect growth and production stage of TSF (indices "1" and "2" at the parameters indicate potentially different values for first (growth) and second (production) phase):

if $S > S^*$ //**growth phase**
$$\mu = Y_{X/S,1}^{true}\left(r_{S,max,1} - m_{ATP,1}/Y_{ATP/S,1}^{true}\right)$$
$$r_S = r_{S,max,1} = \mu/Y_{X/S,1}^{true} + m_{ATP,1}/Y_{ATP/S,1}^{true}$$
$$r_P = \mu \cdot Y_{P/X,1}^{true} + m_{ATP,1} \cdot Y_{P/ATP,1}^{true}$$
else if $S > 0$ //**production phase**
$$\mu = 0$$
$$r_S = m_{ATP,2}/Y_{ATP/S,2}^{true} \qquad\qquad (3)$$
$$r_P = m_{ATP,2} \cdot Y_{P/ATP,2}^{true}$$
else //**substrate exhausted** $(S = 0)$
$$\mu = r_S = r_P = 0$$
end
$$q_S = r_S \cdot X$$
$$q_P = r_P \cdot X$$

We still need to specify the threshold for the substrate level where the switch from growth to production takes place. Importantly, to make the simulations for OSF and TSF comparable, we not only demand that they start with the same amount of substrate and biomass but also that the target amount of synthesized product (and thus the overall product yield) of the TSF is identical to that reached by the OSF. The *observed* final product yield of OSF ($Y_{P/S}^{obs(OSF)}$) can ad hoc be calculated from OSF variables and parameters:

$$Y_{P/S}^{obs(OSF)} = \left(\mu \cdot Y_{P/X}^{true} + m_{ATP} \cdot Y_{P/ATP}^{true}\right)/r_{S,max} \qquad (4)$$

With the initial substrate concentration we can then calculate the final product concentration

$$P_{target,OSF} = S_0 \cdot Y_{P/S}^{obs(OSF)} = P_{target,TSF} \qquad\qquad (5)$$

Based on that, we can now specify the substrate concentration S^* at which the TSF must switch to production stage, namely when the remaining substrate is just sufficient to reach the target product concentration (through product synthesis in the second stage)

Table 2. Parameter values, initial concentrations, and selected results of the simulation scenarios (substrate S: glucose; product P: lactate).

	Unit	Scenario 1 (standard NGAM ATP demand)			Scenario 2 (enhanced NGAM ATP demand)		
		OSF (anaerobic)	TSF–stage1 (aerobic)	TSF–stage2 (anaerobic)	OSF (anaerobic)	TSF–stage1 (aerobic)	TSF–stage2 (anaerobic)
Parameter value							
$Y_{X/S}^{true}$	gDW/mmol	0.022 (m)	0.098 (c)	–	0.022 (m)	0.098 (c)	–
$Y_{P/X}^{true}$	mmol/gDW	69.3 (m)	0 (k)	–	69.3 (m)	0 (k)	–
$Y_{P/ATP}^{true}$	mmol/mmol	1 (k)	0 (k)	1 (k)	1 (k)	0 (k)	1 (k)
$Y_{ATP/S}^{true}$	mmol/mmol	2 (k)	23.5 (c)	2 (k)	2 (k)	23.5 (c)	2 (k)
$r_{S,max}$	mmol/gDW/h	13.3 (m)	10 (k)	–	15.2 (m)	10 (k)	–
m_{ATP}	mmol/gDW/h	7.7 (m+c)	7.7 (m+c)	7.7 (m+c)	16.5 (m+c)	7.7 (m+c)	16.5 (m+c)
Initial values							
S_0	mmol/L	10	10		10	10	
X_0	gDW/L	0.01	0.01		0.01	0.01	
P_0	mmol/L	0	0		0	0	
Results of simulations							
Final product conc.	mmol/L	16.62	16.62		17.82	17.82	
Final product yield	mmol/mmol	1.662	1.662		1.782	1.782	
Process duration	h	13.51	15.67		15.71	12.11	
Average productivity	mmol/L/h	1.23	1.06		1.13	1.47	

For the parameter values, it is indicated whether they have been measured (m) in Ref.,[32] are known (k), or have been calculated (c) from a stoichiometric model of *E. coli*'s central carbon metabolism. In some cases (m+c), measured fluxes were used together with the stoichiometric model to calculate unknown fluxes (e.g., NGAM ATP demand).

$$S^* = \left(P_{target,TSF} - P(t)\right) / \left(Y_{ATP/S,2}^{true} \cdot Y_{P/ATP,2}^{true}\right) \tag{6}$$

3. Results

3.1. Scenario 1: Lactate Synthesis from Glucose

We use the generic OSF and TSF model introduced in section 2 to simulate lactate production from glucose with the *E. coli* lactate producer strain KBM10111 constructed and analyzed in Ref [30]. This strain has knockouts in the genes *ackA-pta* and *adhE*. Under anaerobic conditions, it produces large amounts of lactate in a growth-coupled manner (lactate is a mandatory by-product of biomass formation) whereas production of other fermentation by-products (ethanol, acetate, formate, succinate) is almost completely blocked. Hence, if grown under anaerobic conditions, the strain exhibits coupled growth and lactate synthesis representing a typical OSF process. The parameters for the OSF model are shown in Table 2. Some of them (e.g., $r_{S,max}$) have been reported in the reference,[30] others such as $Y_{ATP/S}^{true}$ or $Y_{X/S}^{true}$ are either well-known from biochemistry or have been calculated from the *EColiCore2* model.[32] For the TSF model we assume that the growth phase of KBM10111 takes place under aerobic conditions without any lactate production. Again, the TSF parameters (Table 2) are either well-known (including the

maximum substrate uptake rate of glucose, $r_{S,max}$, which is usually set to 10 mmol/gDW/h under aerobic conditions) or have been calculated from the *EColiCore2* model. For the production phase, a switch to anaerobic conditions with growth-arrested cells is assumed (e.g., due to micronutrient limitation). Since ATP synthesis in the KBM10111 strain is also stoichiometrically coupled to lactate synthesis, the demand of ATP for NGAM becomes the driving force for substrate uptake and product (lactate) synthesis.

We choose identical initial substrate (10 mmol/L) and biomass (0.01 gDW/L) concentrations for OSF and TSF. The results of the simulations for scenario 1 are shown in **Figure 1**. As intended, both process strategies have the same final product concentration (and thus the same final product yield; see Table 2); this enables a direct comparison of both strategies with respect to volumetric productivity without any bias due to different product yields. With the chosen parameters, the resulting biomass concentrations/yields are also very similar in both processes. As the most important result, we observe that the OSF reaches its final product titer before TSF and has thus a larger average volumetric productivity (Figure 1 and Table 2). Initially, TSF has a higher volumetric substrate consumption rate (q_S) and (directly after the switch from growth to production phase) also a higher volumetric productivity (q_P) because the final concentration of biomass has already been reached. In fact, there is a time window, where the intermediate product concentration and thus the average volumetric productivity is
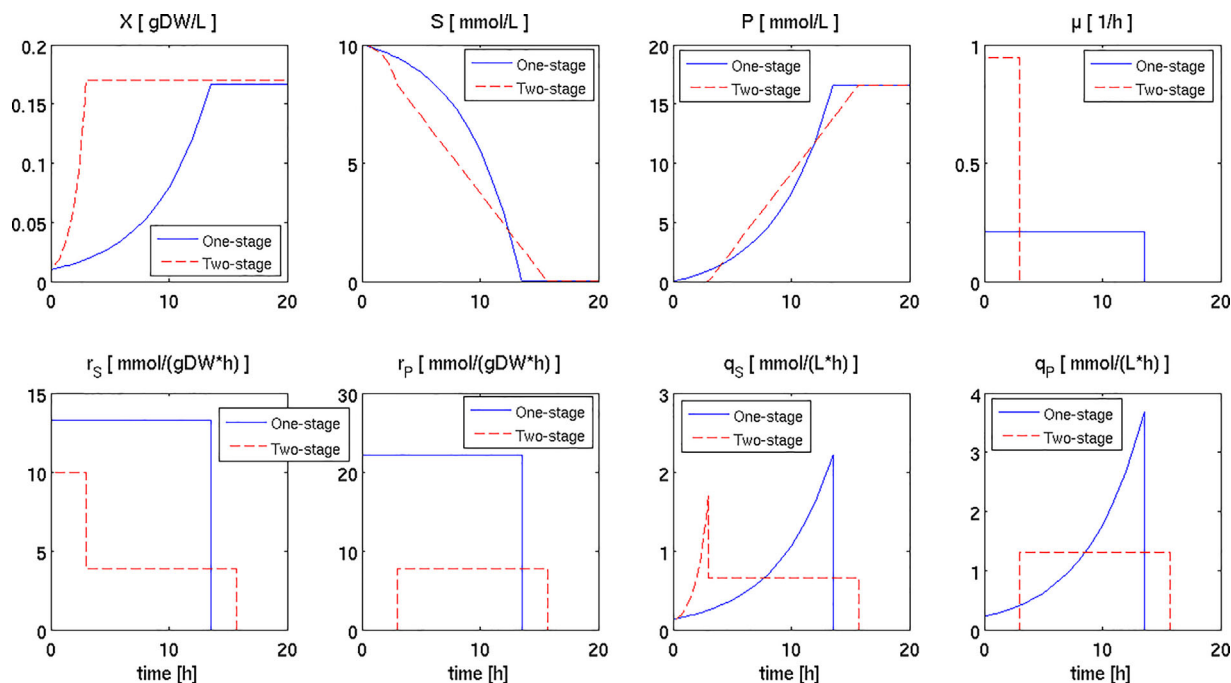
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Figure 1. Simulation of scenario 1: production of lactate (P) from glucose (S) in OSF and TSF.

higher for TSF. However, due to the low specific substrate uptake rate of the cells in the production phase (the substrate is just used to produce ATP for NGAM which is coupled with formation of the target product), the volumetric production rate in OSF increases with the growing biomass and eventually overtakes that of TSF. At a later time point, the same holds true for the product concentration (reflecting the average volumetric productivity). Also, the specific substrate uptake (r_S) and product synthesis (r_P) rates are always higher in OSF. It should be noted that the simulated specific glucose uptake rate r_S of the non-growing cells in the production phase in TSF (≈ 4 mmol/gDW/h) is at the upper bound of what has been reported from experiments (0.5-4.5 mmol/gDW/h).[5] Thus, for many realistic processes, the effect might be even more pronounced.

What is apparently limiting the performance of the TSF is the low specific substrate uptake and specific productivity in the production phase.

3.2. Scenario 2: Lactate Synthesis from Glucose with Enforced ATP Wasting

It has been observed that, in cells where biomass *and* ATP synthesis are coupled with product synthesis, enforced ATP wasting due to futile cycles or other mechanisms dissipating ATP may (1) boost the specific substrate uptake and product synthesis rate and (2) increase the product yield.[30,36] These results were obtained for OSF processes but they may have a favorable effect also for TSF processes, especially due to the potential to boost substrate consumption in the production phase. In a second scenario we therefore considered the same production process as in scenario 1 but here with an activated ATP wasting mechanism. In Ref.,[30] an ATP futile cycle

consisting of the pyruvate kinase and phosphoenolpyruvate synthase was established in the lactate producer strain KBM10111 (carrying the gene knockouts already mentioned in scenario 1). Since ATP synthesis in the considered strain KBM10111 is stoichiometrically coupled to lactate synthesis under anaerobic conditions, ATP futile cycling indeed improved specific substrate uptake and product synthesis rates. According to the results presented in Ref.,[30] two parameters needed to be adapted compared to scenario 1. First, it was reported that the ATP futile cycle increased the non-growth associated ATP demand by a value of 8.8 mmol/gDW/h, which was therefore added to the m_{ATP} value (Table 2). In addition, the experimentally observed increase in the substrate uptake rate was also accounted for in the $r_{S,max}$ parameter. All other OSF parameters were kept as in the previous scenario 1. For the TSF scenario, we assumed that the ATP futile cycle was only induced during the production phase, hence, the m_{ATP} value was only increased for the second stage.

Figure 2 shows the simulation results for the OSF and TSF model with activated ATP wasting mechanism. First of all, both processes are again directly comparable as they have the same overall product (lactate) yield and again similar biomass yield. However, compared to scenario 1, due to the induced ATP wasting, the product yield increases at the expense of biomass yield, because more substrate must be invested for ATP synthesis whose coupling with product synthesis ($Y_{P/ATP}^{true} \cdot Y_{ATP/S}^{true} = 2$) is higher than for biomass production ($Y_{P/X}^{true} \cdot Y_{X/S}^{true} = 1.52$). Furthermore, this time TSF finishes clearly before and thus outperforms OSF in terms of volumetric productivity (see also Table 2). Remarkably, TSF in scenario 2 is even faster than TSF in scenario 1, despite the reduced biomass and increased product yield. The reason is that the higher ATP
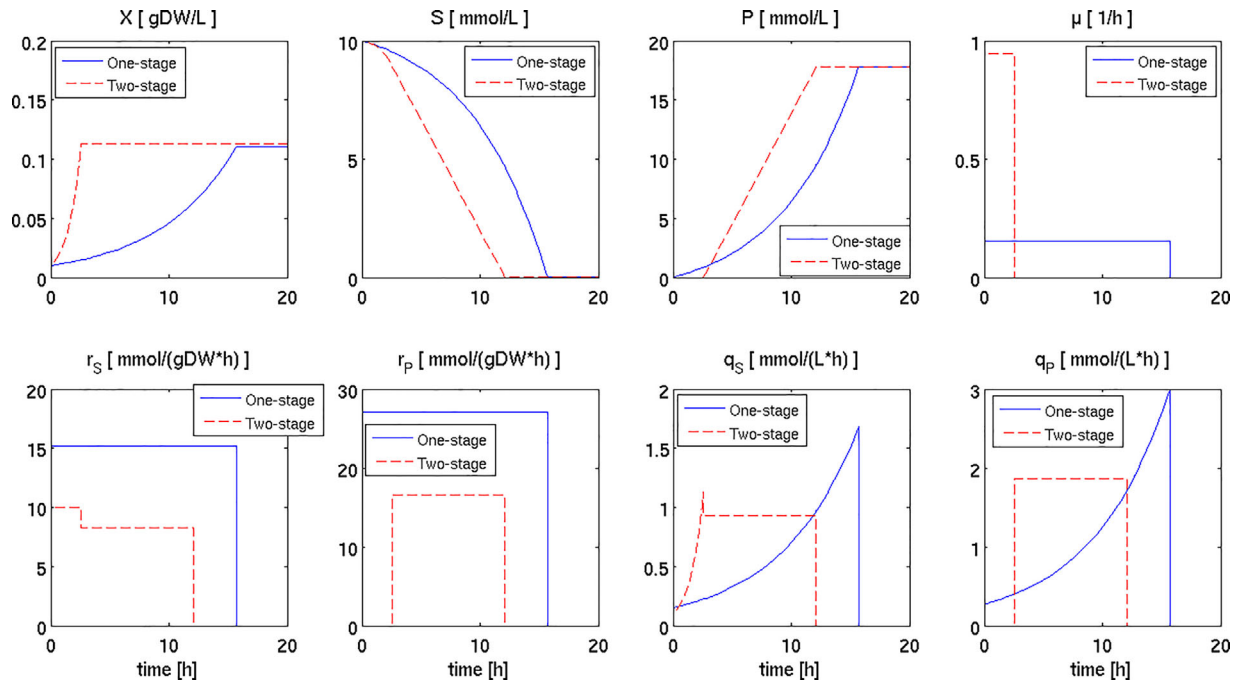
Figure 2. Simulation of scenario 2: production of lactate (P) from glucose (S) in OSF and TSF under enforced ATP wasting.

consumption increases the specific substrate uptake rate r_S in the production phase of TSF and thus, due to coupling of ATP and product synthesis, also the specific lactate production rate. The superior performance holds true despite the increased specific substrate uptake rate in OSF. In fact, the OSF finishes even later (has a lower overall productivity) than in scenario 1: ATP wasting is only partially counterbalanced by the increased substrate uptake rate. Therefore, the growth rate is reduced and biomass accumulates slower thus decreasing the overall volumetric productivity. We also analyzed the hypothetical case where the cell could even further increase the maximal substrate uptake $r_{S,max}$ in OSF to fully counterbalance the drain of ATP (corresponding to $r_{S,max}$=17.7 mmol/gDW/h). This would indeed improve the volumetric productivity of OSF but TSF still had superior performance in this scenario (data not shown).

3.3. Influence of Other Parameters

There are other parameters influencing the relative performance of OSF vs TSF. In particular, this pertains to the total length of the batch process which is mainly governed by the total amount of substrate (i.e., the initial substrate concentration) converted in the process. It is well-known from other simulation studies,[4] that the larger the total amount of substrate converted and, thus, the higher the final biomass concentration, the better the relative performance of TSF against OSF because TSF reaches the target amount of biomass much faster. Hence, a significant increase of the initial substrate concentration will be beneficial for the relative performance of TSF against OSF.

So far we have studied the case where product synthesis is coupled with growth (OSF) and coupled with ATP synthesis

(OSF and second stage of TSF). In another simulation (scenario 3), we therefore analyzed the case that there is (additionally) also some uncoupled constant flux from substrate to product in the OSF and second stage of the TSF. Obviously, this leads to a higher product yield in both fermentations. But only for TSF, the overall productivity increases compared to scenario 1 and the process finishes earlier because the specific substrate consumption in the TSF production phase is now higher (Figure S1, Supporting Information). In contrast, the overall productivity for OSF reduces because partial redirection of flux from substrate to product reduces the growth yield and thus leads to a delayed accumulation of biomass. Generally, whether TSF becomes eventually superior to OSF in this scenario depends on the magnitude of the uncoupled product synthesis flux.

We finally analyzed for scenario 1 how the average volumetric productivity changes for varying target product yields. For TSF, the average productivity is a function of the product yield and can directly be calculated and plotted (red curve in **Figure 3**). For OSF, this gets more complicated. First, we have to take into account that the observed product yield is a combination of growth- and ATP-coupled product synthesis (eq. [4]), and, moreover, that, under growth-coupled product synthesis, the biomass yield $Y_{X/S}^{true}$ and the growth-coupled product yield $Y_{P/X}^{true}$ cannot be varied independently of each other. We therefore used a stoichiometric model of the central metabolism of *E. coli*[32] to calculate the yield space diagram for lactate and biomass synthesis (Figure S2, Supporting Information). This diagram can be used to determine the maximal (true) lactate yield feasible under a given biomass yield. For the sake of simplicity, we assumed that, by appropriate strain designs, this maximum lactate yield can be achieved for a given
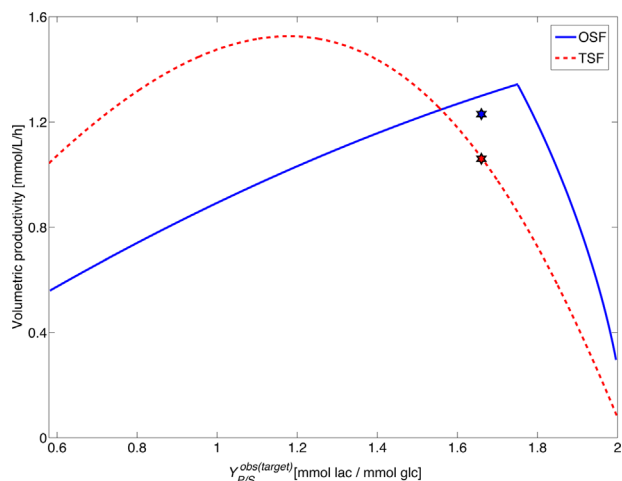
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Figure 3. Dependency of the maximal average volumetric productivity of OSF and TSF on the target product (lactate) yield for scenario 1. The blue/red star indicates the obtained values for OSF/TSF in the simulations of scenario 1 (cf. **Table 2**).
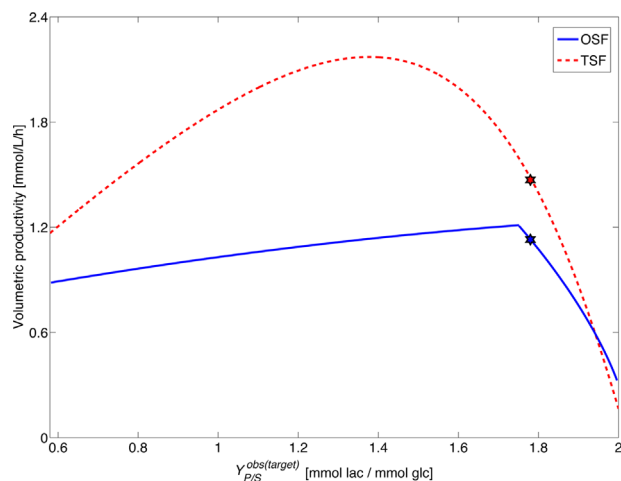

Figure 4. Dependency of the maximal average volumetric productivity of OSF and TSF on the target product (lactate) yield for scenario 2. The blue/red star indicates the obtained values for OSF/TSF in the simulations of scenario 2 (cf. **Table 2**).

biomass yield and that the true maximum lactate yield per produced ATP is again also reached ($Y_{P/ATP}^{true} = 1$; cf. Table 2). With these assumptions, we get the blue curve in Figure 3 showing that the maximum average productivity for OSF (1.34 mmol/L/h) is reached at a relatively high product yield (1.75 mmol/mmol glucose; this point corresponds to the vertex of the Pareto front in the yield diagram; see Figure S2, Supporting Information). The productivity of the OSF obtained in the simulations for scenario 1 (blue star in Figure 3) lies close to the calculated idealized curve and it can be seen that an even higher productivity would be possible when choosing a slightly higher product yield. A unique point of maximum productivity can also be seen for TSF (1.53 mmol/L/h) which, compared to OSF, is reached for smaller product yields at around 1.2 mmol lactate/(mmol glucose) and exceeds the maximum productivity of OSF. On the other hand, Figure 3 also indicates that there is a critical product yield (at around 1.56 mmol lactate/[mmol glucose]) beyond which OSF becomes superior to TSF. Furthermore, for both OSF and TSF, given that their respective maximum productivity is taken at an intermediate product yield, there is range of product yields where an increase of product yield enhances simultaneously the productivity of the respective process. However, further increasing the product yield beyond the point of maximum productivity implies a loss of productivity reflecting the trade-off between high product yield and high productivity.

The yield-productivity curves for scenario 2 (ATP wasting activated) are shown in **Figure 4**. Compared to scenario 1 (Figure 3), we see significantly increased productivities for TSF for fixed product yields (the maximum productivity is approximately 40% higher than the maximum for TSF in scenario 1) while the OSF productivities remain roughly constant (with slightly reduced maximum). Therefore, with enforced ATP wasting, TSF outperforms OSF for a broader range of target product yields and only for product yields beyond 95% of the maximum yield OSF reaches higher productivities than TSF.

4. Discussion and Conclusion

Volumetric productivity is, apart from product yield and titer, a key performance indicator for many biotechnological processes. For this reason, combined with a multitude of fascinating new genetic tools for implementing dynamic metabolic control modules, two-stage fermentations with separated growth and production phase have become of high interest because they hold great potential to significantly improve the productivity of bioprocesses. The intention of the present study was two-fold: first, we wanted to demonstrate that the volumetric productivity of a TSF process is not automatically better than in the corresponding OSF process; second, we wanted to evaluate the impact of substrate utilization rate (e.g., modulated by higher ATP consumption) in the production stage on the TSF productivity. The first point was illustrated by a realistic application example (scenario 1) where the OSF batch process finishes substrate conversion before the TSF process (with identical overall product yield). The advantage of an OSF process with respect to productivity becomes especially significant if one aims for higher product yields (Figure 3). Given that we assumed an ideal (i.e., instantaneous and cost-neutral) ON-OFF switch for the TSF process, which is not realistic in practice, the relative performance advantage of the OSF in the considered scenario can be expected to be even larger. The low *specific* substrate conversion rate of non-growing cells during the production phase is the key reason for this unfavorable performance. Stoichiometric calculations with the *EColiCore2* model[32] indicate that the minimum glucose uptake rate of non-growing *E. coli* cells with an ATP demand for NGAM of up to 8.39 mmol/gDW/h (used in Ref.[33]) is less than 0.5 (2.8) mmol glucose/gDW/h under aerobic (anaerobic) conditions. These values are close to experimentally measured glucose uptake rates of *E. coli* wild type and *E. coli* production strains under stationary conditions[5,25,26] indicating that resting cells consume only relatively small amounts of substrate to satisfy the ATP demand for non-growth associated maintenance processes. The

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considerably reduced metabolic activity raises a big challenge for designing competitive TSF processes.

Several recent works therefore addressed the construction of chassis hosts exhibiting larger substrate uptake rates also under non-growth conditions. For example,[34] showed that the glucose uptake rate of *E. coli* in a nitrogen-limited stationary phase could be increased to 2.5 mmol/gDW/h (and thus by a factor of five compared to the wild type) by overexpressing *ptsI*, a component of the glucose uptake system. Likewise Michalowski et al.[35] constructed an *E. coli* HGT (high glucose throughput) strain by modulating the stringent response regulation program and decreasing the activity of pyruvate dehydrogenase. This strain exhibits up to three-fold higher rates of cell-specific glucose uptake under nitrogen limitation and appears promising for the construction of strains for products downstream of pyruvate. However, for each application, it needs to be shown that those strains maintain their higher substrate uptake rates also under production of the target compound (which usually involves further genetic engineering).

Herein, we have demonstrated that enforced ATP wasting could be another promising strategy to establish a "driving force" that pushes the cell into a state with high substrate uptake (and then product synthesis) rates also in the production phase (scenario 2). This approach requires (a) coupling of ATP synthesis with product synthesis in the production phase, and (b) a suitable mechanism for dissipation of ATP (or, more generally, any mechanism that increases the ATP demand of the cell). This mechanism must be robust enough to ensure that the amount of ATP wasted is not too high as it would drive the cell into an unstable state. Regarding OSF processes, enforced ATP wasting strategies have a beneficial effect on product yield as well as on specific substrate uptake and production rates (see simulations in Figure 2 and experimental proofs in Refs.[30,36]; in addition, increased substrate uptake rates as consequence of enforced ATP futile cycling (but without production of a target compound) was earlier reported by[37–39]). However, lower overall volumetric productivities can be an undesired side effect[30] because biomass accumulates more slowly unless the cell is able to increase the specific substrate uptake rate to a value where the loss of ATP is (almost) fully compensated by the additional substrate taken up for ATP synthesis. Liu et al.[36] showed that this might indeed be feasible. While the potential of ATP wasting strategies has already been demonstrated for OSF, applications for TSF processes have not been reported yet although our simulations suggest that the performance gain could be even much more significant. Hence, these results motivate experimental verification of the role of ATP wasting strategies in improving TSF productivities.

Another suitable driving force for higher substrate consumption rates could be to consider a growth-coupled production phase (instead of a pure production phase) for the second stage of the TSF. As for OSF, one may expect that the cells will maximize their growth rate which implies maximal substrate uptake and, therefore, also maximal product synthesis rates. However, to reach the product yield of a corresponding OSF process, a higher growth-coupled product yield and thus a lower biomass yield compared to the OSF would then be required in the production phase of the TSF process to counterbalance the low (or even zero) product yield during the TSF growth phase.

Recently, an approach to design such TSF processes using the principle of orthogonality between the biomass and the production pathways was outlined.[40] The use of such orthogonal pathway design might be valuable to minimize potential interactions with the native biomass synthesis pathways and allow for more efficient implementation of TSF processes especially for cases where the OSF is sub-optimal. Further development of computational methods for the systematic design of TSF processes might also elucidate the relative merits of TSF and OSF for different biochemical products.

In our study, we assumed that product synthesis is coupled with growth (OSF) and with ATP synthesis (OSF and second stage of TSF), a scenario relevant and feasible for many production processes.[41] However, coupling might not always be possible or desirable. Product synthesis with high specific productivity in the TSF production phase requires then activation of a deregulated product pathway along which the substrate is converted with high rates. However, it remains to be shown that strains with high fluxes along uncoupled pathways (without any benefit for the cell, i.e., where neither ATP nor biomass precursors are produced) can be engineered in a robust manner, especially if these pathways involve many steps. Moreover, those strains cannot be further optimized via adaptive evolution.[42,43]

Generally, when designing new or/and comparing possible variants of bioprocesses, target ranges for productivity, biomass yield, product yield, titer, and process duration (which are obviously not independent) and ideally also an objective function based on these performance indicators must be specified. The specified constraints serve as inputs for (computational) strain and process design. We suggest that, in addition to the usual specification of bioprocess metrics, one should consider the type of process (OSF vs. TSF) in the analysis since it is not obvious which process reaches the highest performance under the given constraints. In particular, the role of mechanisms to improve substrate utilization rate (such as enforced ATP wasting) and their impact on the overall productivity should be assessed. Enhancing the substrate uptake rate in the production stage is a key requirement to achieving high productivities in the TSF and can tilt the balance toward a TSF process. On the other hand, if the substrate uptake rate is low or/and if a high product yield is an essential economic requirement, as is the case for products such as biofuels, then an OSF process might be more appropriate. The generic models used herein may serve as a simple yet valuable tool to investigate the relative performance of OSF vs. TSF and thus to support the rational design of bioprocesses.

Abbreviations

NGAM, non-growth associated maintenance; OSF, one-stage fermentation; TSF, two-stage fermentation.

Supporting Information

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