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Broadening the Scope of Enforced ATP Wasting as a Tool for Metabolic Engineering in *Escherichia coli*

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The targeted increase of cellular adenosine triphosphate (ATP) turnover (enforced ATP wasting) has recently been recognized as a promising tool for metabolic engineering when product synthesis is coupled with net ATP formation. The goal of the present study is to further examine and to further develop the concept of enforced ATP wasting and to broaden its scope for potential applications. In particular, considering the fermentation products synthesized by *Escherichia coli* under anaerobic conditions as a proxy for target chemical(s), i) a new genetic module for dynamic and gradual induction of the F_1-part of the ATPase is developed and it is found that ii) induction of the ATPase leads to higher metabolic activity and increased product formation in *E. coli* under anaerobic conditions, and that iii) ATP wasting significantly increases substrate uptake and productivity of growth-arrested cells, which is vital for its use in two-stage processes. To the best of the authors' knowledge, the glucose uptake rate of 6.49 mmol $gCDW^{-1}$ h^{-1} achieved with enforced ATP wasting is the highest value reported for nongrowing *E. coli* cells. In summary, this study shows that enforced ATP wasting can be used to improve yield and titer (in growth-coupled processes) as well as volumetric productivity (in two-stage processes) depending on which of the performance measures is more crucial for the process and product of interest.

1. Introduction

The development of bio-based production processes for fuels, commodity chemicals, and high-value products plays a pivotal role in making the chemical industry more sustainable and environmentally friendly.[1–3] To be economically competitive with conventional fossil-based manufacturing and to justify industrial applications, these processes need to be optimized in terms of the three key performance measures: yield, titer, and (volumetric) productivity.[1,4] Metabolic engineering of the microbial production organisms is one of the main approaches to improve these parameters.[5]

Manipulating the adenosine triphosphate (ATP) pool has been a major target for enhancing the performance of production organisms.[6–12] For pathways with ATP limitations, increasing the pool of available ATP may improve the production of desired compounds, e.g., of succinic acid[7,13] or of recombinant proteins.[14] A contrary strategy for metabolic engineering has been proposed more recently based on enforced ATP consumption (or enforced ATP wasting).[8,10,15,16] The main idea behind this approach is as follows: if product synthesis is coupled to net ATP synthesis, an increased ATP drain should—not only for thermodynamic reasons, but also due to evolutionary pressure—lead to an increased flux along the product pathway. Enforced ATP hydrolysis has been implemented either by the introduction of short futile cycles[8,15,17] or by a more "direct" approach via expressing ATP-hydrolyzing enzymes such as the (uncoupled) cytosolic F_1-subunit of the ATPase from *Escherichia coli*.[9,10,18] Early studies[9,18,19] focused mainly on the physiological response of the cells upon exposure to elevated ATP drain and did not aim to increase the synthesis of certain products (**Table 1**). In these works, the authors consistently found that the substrate uptake rates in *E. coli* increased with elevated ATP consumption, indicating the potential of ATP wasting to improve the properties of microbial cell factories. Koebmann et al.[9] already envisioned the application of ATP wasting for metabolic engineering purposes; however, only very recently, first concrete application examples have been published targeting lactate synthesis in *E. coli*,[8] acetoin synthesis in *Lactococcus lactis*,[10] and ethanol production in *Saccharomyces cerevisiae*[15] (Table 1). In general, using enforced ATP wasting as a tool for metabolic engineering requires that synthesis of the desired product is coupled with net ATP synthesis, ideally in an obligatory manner where the pathway from substrate to product is the only

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Table 1. Aspects investigated in this study and other published papers on enforced ATP wasting.

	Chao et al.[19]	Koebmann et al.[9]	Holm et al.[18]	Hädicke et al.[8]	Liu et al.[10]	Semkiv et al.[15]	This study
Direct ATP wasting via F_1-ATPase	No (futile cycle)	Yes	Yes	No (futile cycle)	Yes	No (futile cycle)	Yes
Inducible ATPase	No	No	No	No	No	No	Yes
Organism	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*L. lactis*	*S. cerevisiae*	*E. coli*
Anaerobic conditions	No	No	No	Yes	No	Yes	Yes
Use of ATP wasting for growth-coupled product synthesis	No	No	No	Yes (lactate)	Yes (acetoin)	Yes (ethanol)	Yes (fermentation products)
Use of ATP wasting for growth-decoupled product synthesis	No	No	No	No	No	No	Yes (fermentation products)

pathway that leads to net ATP production. In the case of anaerobic ethanol production with yeast,[15] this is naturally the case. For lactate production with *E. coli* and acetoin production with *L. lactis*, alternative (fermentation) pathways for ATP synthesis have to be blocked by knocking out certain metabolic genes. Note that, to obtain coupling, ATP synthesis need not necessarily be achieved along the pathway from a precursor to the product. For example, the pathway form pyruvate to lactate does not lead to the production of ATP; however, ATP synthesis from glucose via glycolysis under anaerobic conditions essentially requires the lactate pathway for balancing redox and replenishing NAD if all alternative pathways have been blocked.

In the above-cited works,[8,10,15] it could be shown that in strains where ATP synthesis is coupled with product synthesis, a higher ATP demand imposed by a futile cycle or an uncoupled ATPase forces the cells to generate more ATP, leading to higher (specific) productivities, substrate uptake rates, and product yields accompanied with decreased growth rate and biomass yield. Based on the results of these proofs of principle, we here aim to further develop and broaden the scope of ATP wasting as a tool for metabolic engineering. One major aspect studied herein concerns the fact that due to the reduced formation of biomass (the catalyst of the bioconversion), the volumetric productivity (amount of product per time and volume) will likely decrease compared to the strain without ATP wasting.[6] One approach to overcome such inherent trade-offs between high product yield and high volumetric productivity is to use two-stage fermentation (TSF)[20–22] with decoupled growth and production phase in contrast to one-stage fermentation (OSF) with growth-coupled product synthesis as used in the aforementioned studies. In a recent theoretical study where the productivities of OSF and TSF processes were systematically compared,[22] we found that enforced ATP wasting in the second (production) phase can significantly increase the productivity and thus the competitiveness of TSFs as it keeps a high driving force for substrate uptake also in the production phase where cell growth (usually consuming a large fraction of the substrate taken up) is missing.[23,24] Accordingly, we here want to give experimental evidence that ATP wasting can serve as a tool to elevate substrate uptake and product synthesis rates in growth-arrested cells. A second important goal of this study is to test the use of an inducible ATPase as an ATP wasting mechanism under anaerobic conditions, the preferred operation mode for industrial applications. So far there is only one study examining ATP wasting under anaerobic conditions in *E. coli*[8] (see also Table 1). This study used the pyruvate kinase/phosphoenolpyruvate synthase futile cycle, which is an indirect way to waste ATP and could have undesired side effects. In this study, we therefore intended to use the ATP-hydrolyzing F_1-subunit of the *E. coli* ATPase as a direct mechanism for enforced ATP wasting. This method was described earlier for use in *E. coli* and *L. lactis*,[9,10] however, only under aerobic cultivation conditions. In addition, in these studies, the ATPase was set under the control of a constitutive promoter. If a TSF or multistage fermentation process is meant to be applied, one needs dynamic control over the relevant reactions for the synthesis of the product.[21,25] Therefore, we put the ATPase encoding genes *atpAGD* under the control of a promoter inducible by *m*-toluate.[26]

As an application example to analyze the effect of ATP wasting with an inducible ATPase under anaerobic conditions in both growth-coupled (OSF) as well as growth-decoupled (for TSF) mode, we chose the *E. coli* wild-type strain MG1655 and considered the standard fermentation byproducts (ethanol, formate, acetate, lactate, and succinate) as proxies for product synthesis since formation of these metabolites is naturally coupled to ATP synthesis under anaerobic conditions.[27] ATP wasting was induced in growth-coupled fermentation conditions (OSF) and in conditions where growth was arrested by the lack of a nitrogen source in the medium. We found that in growth-coupled conditions, yield, titer, as well as specific productivity can be improved by ATP wasting, while volumetric productivity decreased due to a lower growth rate. In the growth-decoupled production phase, however, even volumetric productivity could be more than doubled by ATP wasting as high glucose uptake rates could be maintained during stationary phase. Our results demonstrate that ATP wasting is a promising general approach for metabolic engineering, as it can be used to adjust both factors of the trade-off of optimizing yield/titer or productivity, depending on which of the parameters is more crucial for the process and product of interest.

2. Experimental Section

2.1. Strain and Plasmid Construction

The strains, plasmids, and primers used in this study are listed in **Table 2**. The standard molecular cloning techniques followed the protocols described earlier.[30] Polymerase chain reactions (PCRs) were performed using the Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's protocol. For construction of pSB38.2, the kanamycin resistance cassette was cut out from pSB-M1g-1–17 with restriction enzyme *Pst*I (New England Biolabs) and substituted with the *Pst*I-digested ampicillin resistance cassette created by PCR-amplification from pKD3 with the primer pair Amp_PstI_fw/Amp_PstI_rv (Table 2). For the construction of the ATPase expression plasmid pSB44.1, genes encoding the ATPase F_1-subunit were amplified by PCR from pCP41::*atpAGD* with primer pair atpAGD_mono_fw/atpAGD_mono_rv (Table 2). *gfpmut3* was cut out from plasmid pSB38.2 with restriction enzymes *Nde*I (New England Biolabs) and *Bam*HI (New England Biolabs) and substituted with the *Nde*I/*Bam*HI-digested *atpAGD* PCR product. For construction of the control vector pSB43.1, the 5′-overhangs of the *Nde*I/*Bam*HI-digested plasmid pSB38.2 were filled-in using the Klenow Fragment (Thermo Scientific) and the blunt-ended DNA fragment was self-ligated. pSB43.1 and pSB44.1 were transformed into the *E. coli* wild-type strain MG1655, generating the control strain and the ATPase strain, respectively (Table 2).

2.2. Media and Cultivation Conditions

When needed, 100 μg mL^{-1} of ampicillin was added to cultures. For growth assays, 3 mL of LB_0 medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 5 g L^{-1} NaCl) was inoculated with the corresponding strain at 37 °C and 150 rpm for 5 h. 100 μL of the LB_0 culture was used to inoculate 50 mL of minimal medium (MM) adapted from Tanaka et al.[31] with the pH adjusted to 7.0 and 0.4% of glucose added as the sole carbon source. The expression of ATPase was induced with 0.1 mM (for growth-coupled production) or 0.5 mM (for growth-decoupled production) of *m*-toluate, and the medium was incubated without shaking at 37 °C overnight. For growth-coupled production, cells from the overnight culture were washed and used to inoculate fresh MM (with 0.1 mM of *m*-toluate) to an optical density at 420 nm (OD_{420}) of 0.2. The medium was filled into 5 mL screw-cap glass vials (completely filled to the top), and the vials were incubated at 37 °C without shaking. For every time point, new vials were opened to guarantee anaerobic conditions. For growth-decoupled production, cultivation conditions were the same as described above, but MM without $(NH_4)_2SO_4$ as a nitrogen source was used. The medium was inoculated with an OD_{420} of 2, and 0.5 mM of *m*-toluate was added for ATPase expression.

Cell growth was monitored measuring the OD_{420} and using a factor of 0.22 to convert one OD_{420} unit to gram cell dry weight (gCDW) L^{-1}. All cultivations were performed in biological triplicates.

2.3. Analytical Methods

Extracellular glucose and ethanol concentrations in the medium were measured using the corresponding kits from Megazyme. Extracellular lactate, acetate, formate, and succinate were quantified by the high-performance liquid chromatography (HPLC) method described by Harder et al.[32] and using a mix of the organic acids as an external standard. Pyruvate, orotate, and fumarate were measured by the same method but were not secreted in significant amounts by the strains.

Table 2. Strains, plasmids, and primers used in this study.

Strain or plasmid	Relevant characteristics	Source
E. coli NEB 5-alpha	Competent cells for heat shock transformation	New England Biolabs
E. coli MG1655	*E. coli* wild type	[28]
Control strain	*E. coli* MG1655 transformed with pSB43.1	This study
ATPase strain	*E. coli* MG1655 transformed with pSB44.1	This study
pCP41::*atpAGD*	*atpAGD* under control of CP41-*lacLM* promoter, Erm^r	[9]
pSB-M1g-1–17	*m*-Toluate inducible *xylS*/*Pm* promoter (variant ML1–17), *gfpmut3*, Kan^r	[26]
pKD3	Donor for Amp^r-casette, Amp^r	[29]
pSB38.2	pSB-M1g-1–17 derivative, kanamycin resistance cassette switched to ampicillin resistance cassette, Amp^r	This study
pSB43.1	pSB38.2 without *gfpmut3* reporter gene (empty control plasmid), Amp^r	This study
pSB44.1	pSB43.1 with *atpAGD* gene (ATPase plasmid), Amp^r	This study

Primer	Sequence (5′ → 3′)
Amp_PstI_fw	CGTACTGCAGAATGTGCGCGGAACCCCTATTTG
Amp_PstI_rv	CGTACTGCAGCGTACTATCAACAGGTTGAAC
atpAGD_mono_fw	CATGAACATATGCAACTGAATTCCACCGAAATC
atpAGD_mono_rv	CTAGAGGATCCTTAAAGTTTTTTGGCTTTTTCC

The ATPase activity of the cell lysate was measured using the ATPase Activity Assay Kit (Colorimetric) from BioVision (#K417). Cells from 15 mL of medium (OD_{420} 1.7–2.3) were harvested by centrifugation, and the cell pellet was resuspended in the supplied buffer (175 µL buffer/OD_{420}). The cells were disrupted by sonication, and the ATPase activity in the lysate was measured according to the manufacturer's protocol. ATPase activity was normalized to the overall protein content of the lysate, which was measured by the method described by Bradford.[33]

For determination of intracellular adenosine monophosphate (AMP), adenosine diphosphate (ADP), and ATP concentrations, cells (≈0.5 mg of biomass) were applied to filter disks (polyvinylidene difluoride, 0.45 µm, 25 mm; Merck-Millipore), while a N_2 flow was used to keep the environment oxygen-free. The medium was removed by suction filtration, and the filter disks were immediately transferred to 1 mL of a −20 °C cold acetonitrile/methanol/water (40:40:20) quenching solution. After incubation at −20 °C for 30 min, the samples were shaken vigorously, and 500 µL of the mixture was centrifuged at 13 000 rpm and −9 °C for 15 min. Next, 400 µL of the supernatant was kept at −80 °C until metabolite quantification by liquid chromatography-tandem mass spectrometry, which was performed as previously described[34] using an Agilent 6495 triple quadrupole mass spectrometer (Agilent Technologies). Absolute ATP, ADP, and AMP concentrations were determined with 13-C internal standard and authentic standards.[34] The intracellular adenosine energy charge was calculated with the formula ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]).

For growth-coupled production, the growth, productivity, and glucose uptake rates were calculated for the exponential growth phase, while the yield and titer were determined at the end of the cultivation. For growth-decoupled production, biomass concentration was assumed to be constant and the average of the measured biomass concentrations during the cultivation was used for calculating productivity and glucose uptake rate.

3. Results

3.1. Construction of an Inducible ATP-Wasting Mechanism for *E. coli*

In order to have a dynamic and gradual control over the expression of the ATP wasting machinery, the genes encoding the F_1-part (consisting of the α-, γ-, and β-subunits) of the ATPase from *E. coli* (*atpAGD*) were cloned into the XylS/*Pm* expression system, inducible by the addition of *m*-toluate.[26] Herein, we used higher inducer concentrations for the growth-decoupled than for the growth-coupled batch process to obtain the highest ATPase expression under starvation.

3.2. Effect of ATP Wasting on Growth-Coupled Product Formation

The effect of ATPase overexpression was first tested during growth-coupled production of fermentation products (OSF) under anaerobic conditions. Plasmids pSB43.1 (empty vector) and pSB44.1 (ATPase expression vector) were transformed into *E. coli* MG1655; in the following, these two strains are referred to as control strain and ATPase strain, respectively. Both strains were cultivated anaerobically, and as the formation of the main fermentation products—ethanol, formate, acetate, lactate, and succinate—is naturally coupled to ATP synthesis, they were used as proxies to evaluate the influence of ATP wasting on product synthesis.

Overexpression of the ATPase increased the specific glucose uptake rate by 18.5% and the specific productivity by 17.2% compared to the control strain (**Table 3** and **Figure 1**C). Note that for a better comparability, the sum of all carbon atoms incorporated in the five main fermentation products was considered to be the overall product. At the same time, the yield and titer also increased by 6.8% and 8.7%, respectively (Figure 1E,F). With a yield of 88% product C-atoms/glucose C-atoms, the control strain is already close to the theoretical maximum yield. With the help of ATP wasting, more than half of the remaining 12% could be captured in the form of fermentative products.

In contrast, the expression of the ATPase decreased the growth rate, which dropped from 0.40 h^{-1} (control strain) to 0.28 h^{-1} (ATPase strain) (Figure 1A and Table 3). Similarly, although the specific productivity increased significantly, the volumetric productivity of the ATPase strain decreased by 25.3% compared to the control strain during exponential growth (Figure 1D). This is due to the lower growth rate and the associated decrease in biomass, which acts as the biocatalyst. At the beginning of the cultivation (the first ≈3.5 h), the higher specific productivity can compensate for the reduced biomass formation since product concentrations are slightly higher in the ATPase strain than in the control strain even though less biomass is present (Figures 1B and 2B). In addition to the volumetric productivity, the volumetric glucose

Table 3. Summary of performance parameters with and without ATP wasting under growth-coupled and growth-decoupled production conditions.

	Growth-coupled production		Growth-decoupled production	
	Control strain	ATPase strain	Control strain	ATPase strain
μ [h^{-1}]	0.40 ± 0.01	0.28 ± 0.01	≈0	≈0
$r_{Glucose}$ [mmol $gCDW^{-1}$ h^{-1}]	11.68 ± 0.35	13.84 ± 0.51	2.82 ± 0.02	6.49 ± 0.19
$q_{Glucose}$ [mmol L^{-1} h^{-1}]	1.97 ± 0.11	1.46 ± 0.04	1.29 ± 0.01	2.75 ± 0.08
$r_{\Sigma\ C\text{-atoms in products}}$ [mmol $gCDW^{-1}$ h^{-1}]	66.31 ± 2.09	77.75 ± 2.52	15.37 ± 0.71	37.30 ± 2.49
$q_{\Sigma\ C\text{-atoms in products}}$ [mmol L^{-1} h^{-1}]	11.19 ± 0.60	8.36 ± 0.33	7.02 ± 0.39	15.79 ± 0.99
$Y_{\Sigma\ C\text{-atoms in products/glucose C-atoms}}$ [mol mol^{-1}]	0.88 ± 0.03	0.94 ± 0.02	0.92 ± 0.03	1.01 ± 0.08

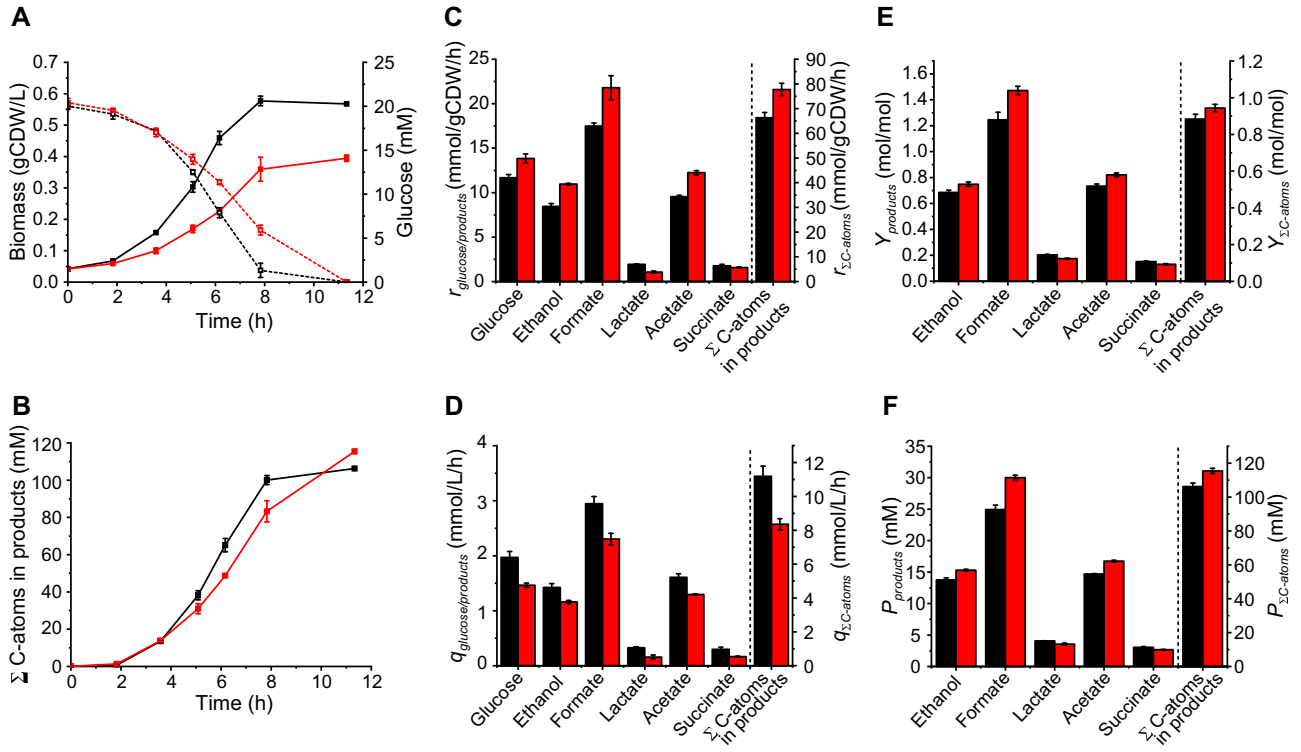

Figure 1. Concentration profiles, productivities, yield, and titer of the control strain (black) and the ATPase strain (red) under growth-coupled production conditions. A) Biomass concentration (solid line) and glucose concentration (dashed line) during cultivation, B) product (sum of carbon atoms incorporated in main fermentation products) concentration during cultivation, C) specific glucose uptake rate and specific productivity, D) volumetric glucose uptake rate and volumetric productivity, E) yield, and F) titer.

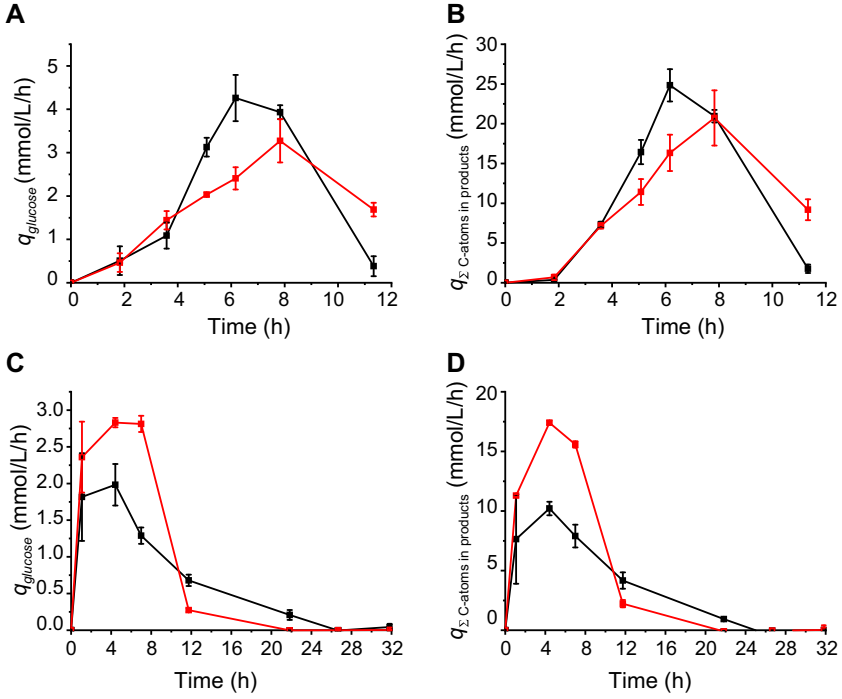

Figure 2. A,C) Volumetric glucose uptake rate and B,D) volumetric productivity (A,B) during growth-coupled and (C,D) growth-decoupled production of the control strain (black) and the ATPase strain (red).

uptake rate of the ATPase strain is also higher at the beginning of the cultivation (**Figure 2**A). However, when the difference in biomass surpasses a certain value, the higher specific productivity cannot make up for the reduced amount of biomass and only at the end of the cultivation, the product concentration of the ATPase strain surpasses the control strain, leading to a higher yield and titer.

3.3. Determination of ATPase Activity and Intracellular ATP Concentration

To examine whether the observed effects on growth, productivity, and yield are due to an increased ATPase activity in the cytosol and not a result of the burden to overexpress three proteins (sum of molecular weights of the three ATPase subunits: 137.12 kDa), the ATPase activity in the lysate of both strains was measured. With 21.5 mU mg^{-1} protein, the ATPase activity was almost twice as high in the ATPase strain as in the control, showing that the overexpression leads indeed to an increased ATPase activity in the cytosol (**Figure 3**B).

Furthermore, the influence of the expressed ATPase on the intracellular concentrations of AMP, ADP, and ATP was examined. Samples for intracellular metabolite quantification were taken at the end of the exponential growth phase (after 7.3 h). The measurements revealed that the concentration of intracellular ATP in the ATPase strain was 34.7% lower than in the control strain (Figure 3A). Likewise, the [ATP]/[ADP] ratios also dropped in the ATPase strain (2.99 ± 0.28) compared to the control strain (3.74 ± 1.09), while the adenosine energy charge decreased only slightly from 0.78 ± 0.04 (control strain) to 0.76 ± 0.02 (ATPase strain). The only mild reduction of the energy charge is a consequence of the fact that the total adenosine pool [ATP] + [ADP] + [AMP] in the ATPase strain was significantly smaller.

3.4. Effect of ATP Wasting on Growth-Decoupled Product Formation

The issue of decelerated cell growth of the ATPase strain and the associated lower volumetric productivity in an OSF batch process can be circumvented if growth and production phases are decoupled in a two-stage fermentation (TSF). We therefore examined the behavior and performance of the ATPase strain under growth-arrested conditions (mimicking the second [production] phase of a TSF) by using production medium without the addition of a nitrogen source. The medium was inoculated with a tenfold higher cell density compared to the growth-coupled production conditions. Except for a small biomass increase of the control strain at the beginning of the cultivation, the biomass concentration stayed constant or decreased slightly throughout the 32-h cultivation period (**Figure 4**A). ATP wasting under these conditions led to a peak glucose uptake rate of 6.78 ± 0.28 mmol $gCDW^{-1}\,h^{-1}$, which stayed in that range until all of the glucose was consumed (Figures 2C and 4C). The control strain reached a peak value of only 4.22 ± 0.60 mmol $gCDW^{-1}\,h^{-1}$ at the beginning of the cultivation, which then continuously decreased and went down to close to zero after 32 h, although one-fourth of the added glucose was still present in the medium (Figures 2C and 4 A). Considering the mean values within the first 11.75 h of cultivation, the specific glucose uptake rate with ATP wasting was more than twice as high (6.49 ± 0.19 mmol $gCDW^{-1}\,h^{-1}$), than without wasting (2.82 ± 0.02 mmol $gCDW^{-1}\,h^{-1}$; Figure 4C). Consequently, the specific productivity of the ATPase strain was more than 142% higher than of the control strain. Interestingly, the productivity of lactate was especially upregulated and more than twelve times higher (Figure 4C). We hypothesize that under nitrogen starvation conditions, the lactate pathway (with a yield of 2 mol ATP per mol glucose) may either have a higher saturation or/and it is preferred for redox balancing, as it requires only one enzymatic step from pyruvate opposed to several enzyme steps needed for the ATP yield-optimal pathway (2.5 mol ATP mol^{-1} glucose) with formation of formate, ethanol, and acetate. The slightly lower ATP yield may pay off due to the reduction in enzyme synthesis costs, which are especially crucial under nitrogen limitation. In contrast to the growth-coupled production conditions, where a higher biomass concentration of the control strain led to a higher volumetric productivity, biomass concentrations in growth-decoupled production conditions were the same for both strains and stayed constant. Thus, the differences in specific glucose uptake rate and specific productivity between the control and the ATPase strain could be directly transferred to the volumetric uptake rate and volumetric productivity (Figures 2D, 4D). The yield of the ATPase strain reached the maximum with 1.01 ± 0.08 product C-atoms/glucose C-atoms. In theory, the control strain should also reach the maximal possible yield[22] but stayed with 0.92 ± 0.03 product C-atoms/

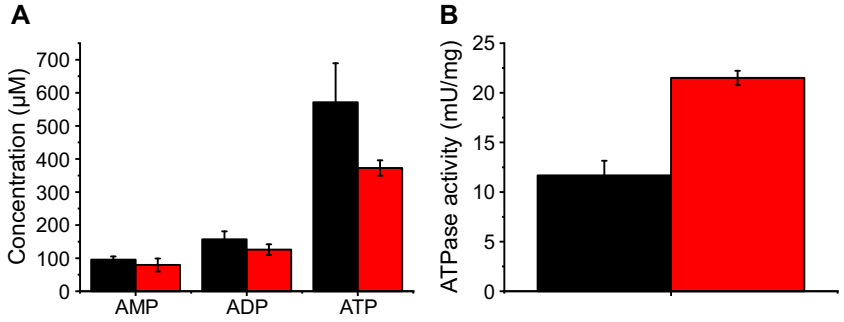

Figure 3. A) Intracellular concentrations of AMP, ADP, and ATP in control strain (black) and ATPase strain (red). B) ATPase activity of cell lysate of control strain (black) and ATPase strain (red).

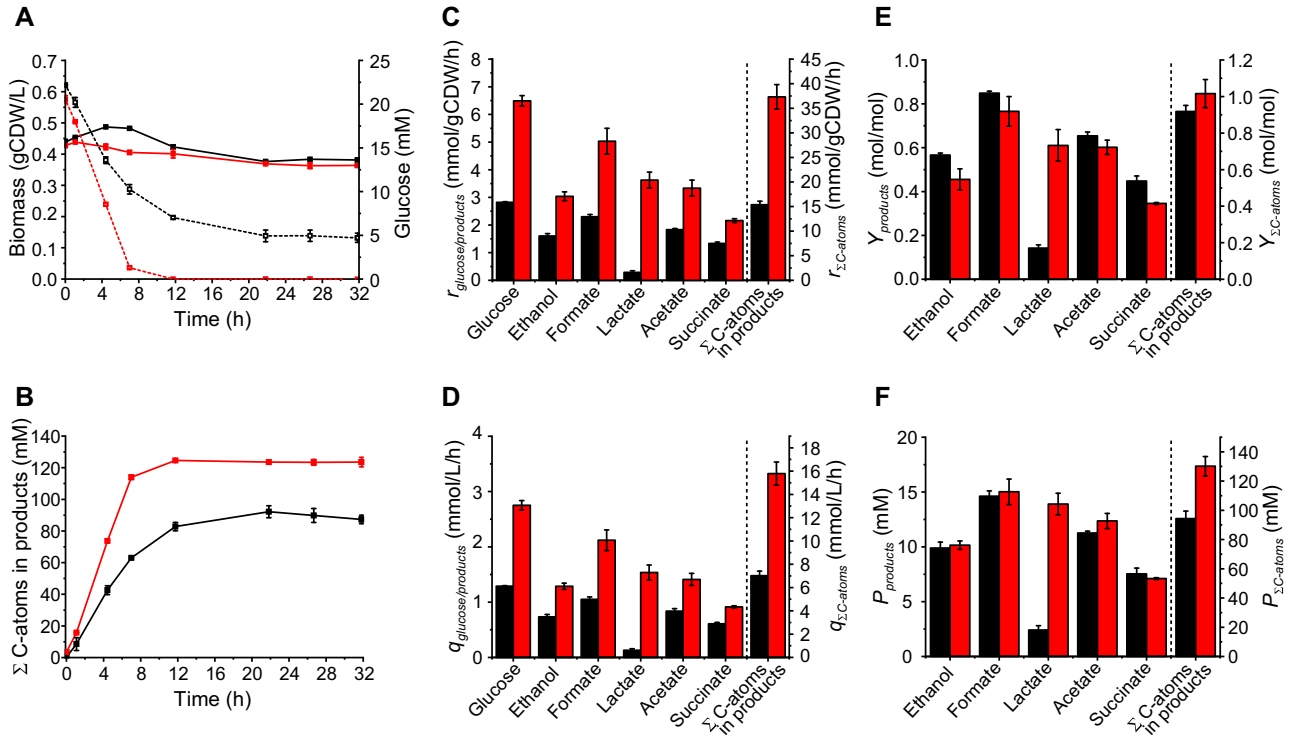

Figure 4. Concentration profiles, productivities, yield and titer of the control strain (black) and the ATPase strain (red) under growth-decoupled production conditions. A) Biomass concentration (solid line) and glucose concentration (dashed line) during cultivation, B) product (sum of carbon atoms incorporated in main fermentation products) concentration during cultivation, C) specific glucose uptake rate and specific productivity, D) volumetric glucose uptake rate and volumetric productivity, E) yield, and F) titer.

glucose C-atoms somewhat below the maximum, which could be due to the increase of biomass (and thus a flux of carbon to the biomass) at the beginning of the cultivation as mentioned above. Because not all of the added glucose was consumed by the control strain, the product titer was 27.57% lower than in the ATPase strain (Figure 4B,F).

Using the experimentally determined exchange rates and a stoichiometric model of the central metabolism of *E. coli*[35] and the software *CellNetAnalyzer*,[36,37] we estimated the nongrowth associated ATP maintenance demand in both strains (in the ATPase strain, this value includes the amount of ATP hydrolyzed by the overexpressed ATPase) and obtained a value of 5.34 mmol ATP $gCDW^{-1}\,h^{-1}$ in the control strain and 12.62 mmol ATP $gCDW^{-1}\,h^{-1}$ in the ATPase strain (data not shown). Hence, the expression of the ATPase more than doubles the ATP demand (and metabolic activity) in the nongrowing cells. The factor for the ATP demand is thus well in the range of the measured doubled ATPase activity (Figure 3B) in the ATPase strain.

4. Discussion

The goal of the present study was to further examine and further develop the concept of enforced ATP wasting and to broaden its scope for potential applications in metabolic engineering. We studied aspects that have not been investigated before or/and not in the combination as used herein (see Table 1). In particular, i) we developed a new genetic module for dynamic induction of an ATPase; ii) we showed that the F_1-part of the ATPase leads to higher metabolic activity and increased product formation in *E. coli* also under anaerobic conditions; and iii) we tested and proved suitability of ATP wasting also for growth-arrested production which is vital for its use in TSF processes. To keep things simple, in our example process, we considered the total amount of fermentation products as a proxy for the target chemical(s); however, in realistic applications, one may focus on a particular metabolite by deleting pathways to other fermentation products as was done, e.g., for lactate.[6]

Our results with the ATPase strain for growth-coupled product synthesis confirmed earlier results of increased ATP demand in *E. coli* or *L. lactis*,[8–10] namely an increase in substrate uptake, specific productivity, product yield, and titer accompanied with a reduced growth rate and biomass yield, causing reduced volumetric productivities. Hence, generalizing results found for aerobic conditions,[9] the flux through glycolysis in *E. coli* wild-type cells is governed by ATP-consuming processes even under anaerobic conditions. However, as the necessary redirection of carbon flux from biomass to ATP formation (coupled with product synthesis) reduced the growth rate of the ATPase strain, less biomass as biocatalyst was present and the volumetric productivity dropped by 25.3% compared to the control strain. Lowered volumetric productivities were also observed in Hädicke et al.[8] and Liu et al.,[10] however, Liu et al.[10] also reported that fine-tuning of the ATPase activity by selecting a (constitutive) promoter with moderate strength led to a *L. lactis* strain that exhibited even a

slightly higher volumetric productivity for acetoin than the control strain. It is important to note that the amount of substrate used, the reference time point, growth inhibition by the accumulated product and other factors determine the relative volumetric productivity of an OSF with ATP wasting against an OSF without ATP wasting. As was already mentioned in Section 3, when we stop the fermentation within the first 3 hours, the volumetric productivity of the ATPase strain would be higher than of the control strain. However, in general, it is very likely that the volumetric productivity of a strain with enforced ATP wasting will be lower than a strain without increased ATP demand, reflecting the trade-off between high product yield and high volumetric productivity. Here, adjusting the strength of the ATPase activity enables one to adjust product yield and productivity at a desired point with an optimal trade-off. Compared to the approach followed by Liu et al.,[10] where different strains were constructed each having a static constitutive promoter of a specific strength, our developed system with an inducible ATPase promoter offers a more practical solution for fine tuning the ATPase expression and for studying the influence of different expression levels on cell physiology. Moreover, the inducible promoter now allows applications with dynamic control of the ATPase activity, which will be essential for TSF processes where enforced ATP wasting is desired only in the production phase. For industrial applications, however, external inducers are often too costly, which may favor the use of autonomous switches, e.g., based on quorum sensing.[38]

Generally, TSF processes separating growth and production may help to overcome the drawback of reduced volumetric productivity during OSF.[20,21,39,40] In Klamt et al.[22] we hypothesized that ATP wasting in the production phase could further boost the performance of TSFs. We therefore analyzed the effect of overexpressed ATPase under growth-arrested conditions (mimicking the production phase of a TSF) caused by nitrogen starvation, where the biomass of the ATPase strain and the control strain remain constant. As was shown earlier, the metabolism usually shuts down in nongrowing cells just to cover cellular maintenance, leading to low substrate uptake rates and productivities and thus severely limiting TSF processes.[23,24] This can also be observed in the present study: the specific glucose uptake rate of the control strain slows down under nitrogen starvation conditions until it comes almost to a complete rest even though the substrate was not completely consumed. In recent years, there have been several attempts to maintain high metabolic rates in the stationary phase, e.g. by overexpression of PtsI in *E. coli*, which is involved in the regulation of the glucose uptake machinery,[41,42] by modulating the stringent response program leading to a "high glucose throughput (HGT)" strain,[43] and by directed evolution.[44] To the best of our knowledge, the glucose uptake rate of 6.49 mmol $gCDW\,h^{-1}$ we achieved with enforced ATP wasting is the highest reported for nongrowing *E. coli* cells. For example, although not directly comparable (anaerobic conditions in our study and aerobic conditions in the studies mentioned above), the specific glucose uptake rate in the PtsI overexpression strain was $\approx$2.5 mmol $gCDW^{-1}\,h^{-1}$,[41] 3.27 mmol $gCDW^{-1}\,h^{-1}$ in the HGT strain,[43] and 1.55 mmol $gCDW^{-1}\,h^{-1}$ in the evolved strain.[44] Moreover, the ATPase strain keeps these high rates—in contrast to the control strain—until all of the glucose is completely consumed. The resulting high volumetric productivity during stationary phase could help make TSF processes more efficient as envisioned in the theoretical study of our group.[22] In contrast, if yield and titer are more important than productivity (e.g., for high-value products), ATP wasting can be applied in a growth-coupled mode in an OSF as described above.

Our measurements of the adenosine pools in the ATPase and the control strain in the growth-coupled process showed, as expected, reduced ATP levels and a lowered ATP/ADP ratio and thus followed the same trend as in Koebmann et al.[9] Interestingly, and also consistent with the study of Koebmann et al.,[9] we found that the concentrations of AMP, ADP, and ATP were all lower in the ATPase strain compared to the control strain (Figure 3A). As a consequence, energy charges of the ATPase strain (0.76), as well as the control strain (0.78), differed only to a minor extent, meaning that *E. coli* is still able to counterbalance the increased ATP demand caused by the expressed ATPase, at least in terms of energy charge. In future studies, the tipping point between maximizing the glucose uptake rate and overburdening the cells with too high ATP wasting rates should be determined by titrating the expression level of the ATPase. Furthermore, we plan to test the approach for other (heterologous) products and substrates in *E. coli* and other organisms and to fine-tune the ATP wasting level for the respective product.

In summary, we showed that yield and titer, as well as volumetric productivity, can be improved by enforced ATP wasting depending on which cultivation approach (OSF or TSF) is being applied. We believe that this concept can become a general approach in bioprocess and metabolic engineering to construct microbial cell factories with superior performance.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

ATPase, ATP wasting, *Escherichia coli*, metabolic engineering, nitrogen starvation, one-stage fermentation, two-stage fermentation

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