

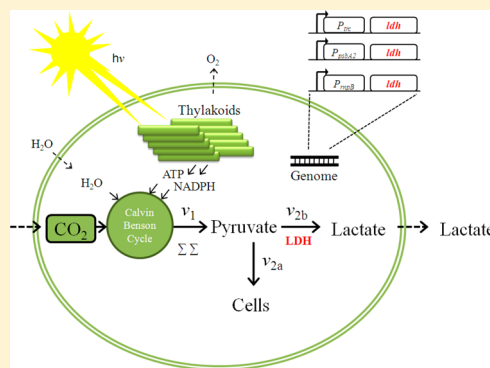
# On the Use of Metabolic Control Analysis in the Optimization of Cyanobacterial Biosolar Cell Factories

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## Supporting Information

**ABSTRACT:** Oxygenic photosynthesis will have a key role in a sustainable future. It is therefore significant that this process can be engineered in organisms such as cyanobacteria to construct cell factories that catalyze the (sun)light-driven conversion of CO<sub>2</sub> and water into products like ethanol, butanol, or other biofuels or lactic acid, a bioplastic precursor, and oxygen as a byproduct. It is of key importance to optimize such cell factories to maximal efficiency. This holds for their light-harvesting capabilities under, for example, circadian illumination in large-scale photobioreactors. However, this also holds for the “dark” reactions of photosynthesis, that is, the conversion of CO<sub>2</sub>, NADPH, and ATP into a product. Here, we present an analysis, based on metabolic control theory, to estimate the optimal capacity for product formation with which such cyanobacterial cell factories have to be equipped. Engineered L-lactic acid producing *Synechocystis* sp. PCC6803 strains are used to identify the relation between production rate and enzymatic capacity. The analysis shows that the engineered cell factories for L-lactic acid are fully limited by the metabolic capacity of the product-forming pathway. We attribute this to the fact that currently available promoter systems in cyanobacteria lack the genetic capacity to provide sufficient expression in single-gene doses.



## INTRODUCTION

For a sustainable future, it is crucial that our society refrains from emitting large amounts of fossil-derived CO<sub>2</sub> into the atmosphere, that is, it is of great importance that the global carbon cycle will be closed. Considering the status of current research into artificial leaves,<sup>1–3</sup> it seems safe to conclude that natural (oxygenic) photosynthesis will have to play a key role to achieve this goal of closing the carbon cycle in a biobased economy. It is therefore significant that during the past few years, several studies have appeared that report that oxygenic photosynthesis in cyanobacteria can be “short-circuited” to construct cell factories that catalyze the (sun)light-driven conversion of CO<sub>2</sub> and water into oxygen and commodity chemicals that can be applied as biofuel, like ethanol, butanol, and so forth, or as a bioplastic precursor like lactic acid.<sup>4–9</sup>

As fossil carbon is primarily used as an energy source and bulk chemicals for energy applications are among the lowest-priced products in the market, it is of primary importance to design cell factories such that they can operate at the maximally achievable efficiency. This latter restriction is relevant for all parts of cellular metabolism, including the light-harvesting machinery of such cells that will have to operate optimally in large-scale photobioreactors<sup>10</sup> under a circadian illumination regime. The same holds for the “dark” reactions of photosynthesis, that is, the conversion of CO<sub>2</sub>, NADPH, and ATP into a specific product. Optimizing the efficiency of the functioning of the antenna complexes in oxygenic photosynthesis is a field of

intense studies in which, for instance, the measurable parameter of nonphotochemical quenching of chlorophyll fluorescence is used to minimize energy losses due to annihilation and/or radiationless recombination.<sup>11,12</sup>

Another aspect of the functioning of a biosolar cell factory (e.g., for ethanol, butanol, or lactic acid), in which the vast majority of the incoming CO<sub>2</sub> is channeled into product rather than into the components of the biomass, is the capacity of the heterologous fermentation pathway that has to be genetically engineered into the cyanobacterium. An exact estimate of this required capacity is important because too little of it will cause energy losses in the upstream metabolism, whereas too high expression levels will function as a biosynthetic burden to the cell, which has associated free-energy costs (compare ref 13 and references therein).

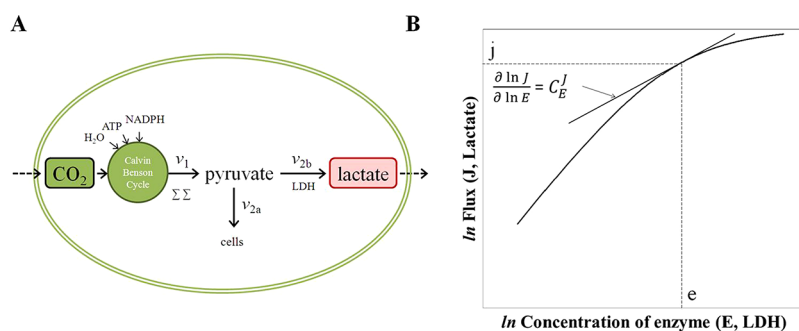
Metabolic control analysis<sup>14,15</sup> provides an elegant and quantitative tool to determine this optimum. Here, we present studies in which this method has been used to analyze the performance of a biosolar cell factory for L-lactic acid. A cyanobacterial cell factory producing lactic acid can be schematically depicted, as shown in Figure 1a. The flow of carbon through such a system can be lumped into two distinct

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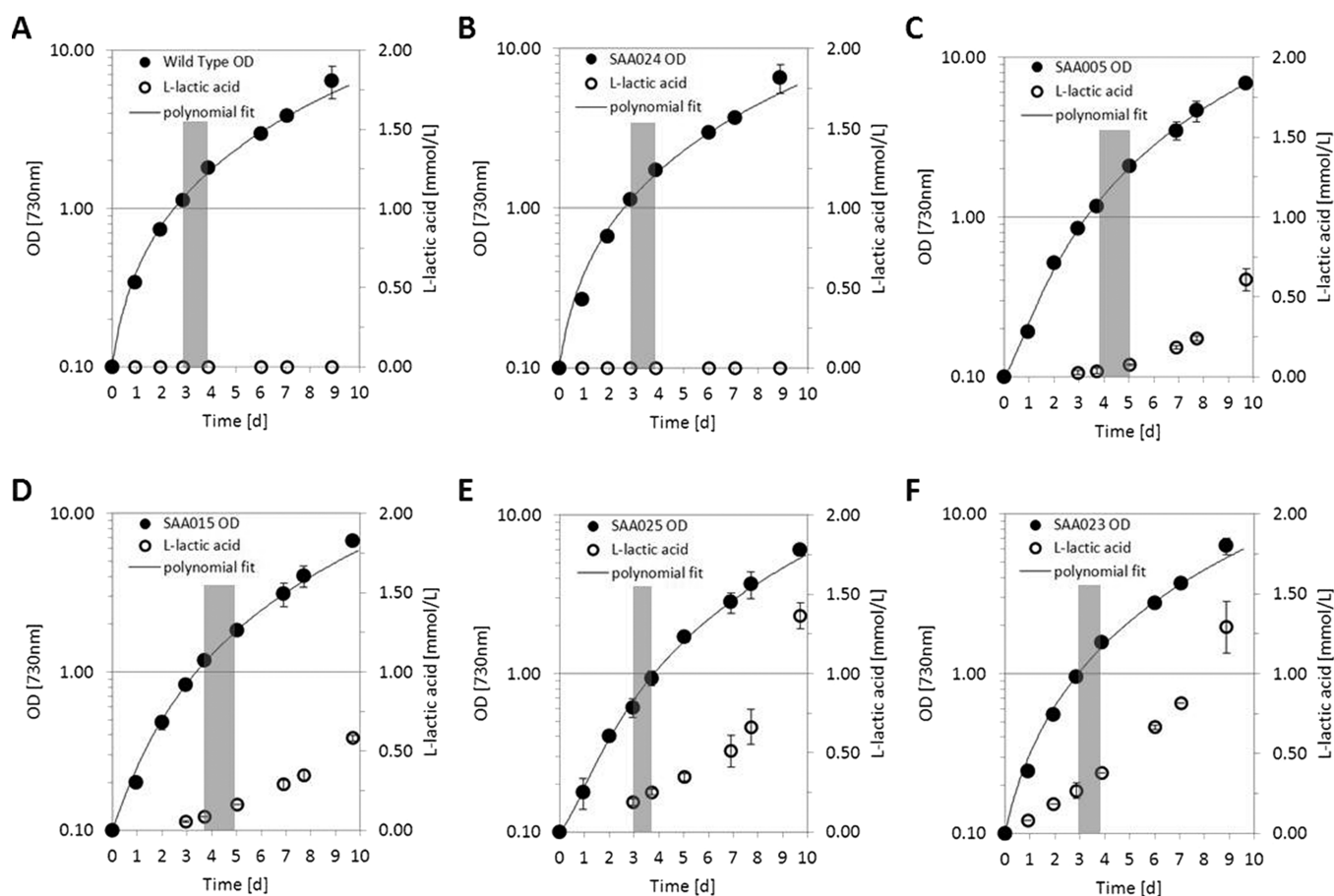
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**Figure 1.** Brief description of the approach of metabolic control analysis. (a) Schematic representation of a lactate-forming cyanobacterial cell factory. Two reactions are taken into account, (i) all reactions necessary for uptake, fixation, and processing of carbon until the metabolite pyruvate (reaction 1) and (ii) the conversion of pyruvate into lactate (for details, see the text), (b) Method for calculation of the flux control coefficients (see also D. Fell (1992)<sup>14</sup>), which indicates quantitatively the contribution of the control of enzyme E (i.e., here LDH) over a flux  $J$  (i.e., lactate formation) through a complex biochemical pathway. To this end, the relative increase in enzyme capacity is plotted against the relative increase in the rate of lactate production. The slope of the tangent to the resulting curve is the flux control coefficient at that level of expression of enzyme E.



**Figure 2.** (a–f) Growth curves and extracellular accumulation of L-lactic acid in batch cultures of wild-type *Synechocystis* and mutants SAA005, SAA015, SAA023, SAA024, and SAA025. The gray bars represent the late exponential growth phase from which the enzymatic activity and production rate are derived. The values are the average of biological replicates; the error bars indicate the standard deviation ( $n = 3$ ); error bars are not visible if they are smaller than the corresponding data point symbol.

steps, all reactions necessary for uptake, fixation, and processing of carbon-containing metabolites, up to the level of pyruvate (reaction 1, Figure 1), and conversion of pyruvate into lactic acid (or lactic acid, catalyzed by a genetically introduced lactate dehydrogenase (LDH); reaction 2b, Figure 1a). In addition, pyruvate (and other metabolites) are converted into new cells (reaction 2a, Figure 1a). This reaction can be ignored in our discussion of the factor(s) that control(s) the rate of lactate production (see below). It should be noted however that the

rate of reaction 2a ( $\nu_{2a}$ ) (Figure 1a) can be read as the growth rate  $\mu$  of the organism. In the following, we will refer to the rate of reaction 2b (Figure 1a) as  $J_l$ .

These two rates (i.e., growth rate  $\mu$  and lactic acid production rate  $J_l$ ) have been determined in a series of mutants expressing different levels of lactate dehydrogenase activity (by varying the strength of the promoter for the *ldh*). Such results allow one to carry out a sensitivity analysis or, in the terminology of metabolic control theory, determine the control of the lactate

**Table 1. Growth Rates and Derived Biomass and Lactate Production Rates from Wild-Type *Synechocystis* PCC6803 and Mutant Strains Derived Thereof, Harvested at the Late-Exponential Growth Phase<sup>a</sup>**

strain	growth rate (±SD)	production rate (±SD)			LDH activity (±SD)		
	$\mu$ (h <sup>-1</sup> )	mmol carbon biomass/ g D W/h	mmol lactate/ g D W/h	relative production rate (%)	nmol/mg of total protein/min	mmol/g D W/h	relative capacity (%)
WT	0.0151 ± 0.0003	0.5993 ± 0.013	0	0	0	0	0
SAA024	0.0153 ± 0.0004	0.6056 ± 0.016	0	0	0	0	0
SAA005	0.0173 ± 0.0007	0.6861 ± 0.028	0.0034 ± 0.0001	19	1.536 ± 0.758	0.046 ± 0.021	9
SAA015	0.0158 ± 0.0008	0.6243 ± 0.030	0.0074 ± 0.0007	42	7.152 ± 0.854	0.215 ± 0.026	41
SAA025	0.0170 ± 0.0015	0.6737 ± 0.061	0.0137 ± 0.0014	78	16.099 ± 2.871	0.483 ± 0.086	91
SAA023	0.0169 ± 0.0008	0.6700 ± 0.030	0.0175 ± 0.0014	100	17.641 ± 3.524	0.529 ± 0.106	100

<sup>a</sup>Enzymatic activities (LDH, in vitro measured under  $V_{\text{MAX}}$  conditions) were determined in cell-free extract of the same cultures. Values for the production rate and enzymatic activity (i.e., LDH capacity) are normalized to those from wild-type. The standard deviation is indicated ( $n = 2$ ).

dehydrogenase enzyme ( $C_E^J$ ) in the overall process of CO<sub>2</sub> driven lactic acid formation. This value is obtained by plotting the relative increase in enzyme (i.e., LDH) capacity ( $E$ ) against the relative increase in rate of lactate production ( $J_l$ ) (compare Figure 1b), or in the formula

$$C_E^J = \frac{\partial \ln J_l}{\partial \ln E} \quad (1)$$

The results obtained, combined with observations from the recent literature, lead to the conclusion that the cyanobacterial cell factories for L-lactic acid formation employing different strength promoters presented here but also those for ethanol<sup>16</sup> and ethylene<sup>17</sup> are (fully) limited by the capacity of the metabolic pathway that leads to product formation. These results then also point to the fact that for efficient use of cyanobacteria as biosolar cell factories, better insight into regulation of gene expression in cyanobacteria is urgently needed.

## ■ RESULTS AND DISCUSSION

We have engineered the *ldh* gene, encoding L-lactate dehydrogenase (LDH) from the lactic acid bacterium *Lactococcus lactis* sp. *cremoris* MG1363 and from the soil bacterium *Bacillus subtilis* sp. 168 into the genome of the cyanobacterium *Synechocystis* sp. PCC6803 (hereafter, *Synechocystis*) under control of selected promoters with different strengths, that is,  $P_{\text{trc}}$ ,  $P_{\text{psbA2}}$ , and  $P_{\text{trc}}$  (for a complete list of strains used, see Table S2 in the Supporting Information). With these strains, we have performed a metabolic control analysis of L-lactic acid formation in *Synechocystis*.

**L-Lactic Acid Production by Mutant *Synechocystis* Strains.** Wild-type *Synechocystis* and also SAA024, the strain expressing the *L. lactis* *ldh* under control of the *rnpB* promoter, do not show lactic acid production. SAA005 and SAA025, the strains expressing the *L. lactis* *ldh* driven by the promoter of *psbA2* and by  $P_{\text{trc}}$ , respectively, build up 0.6 and 1.4 mmol/L of lactic acid within 10 days of culturing. SAA023, the strain carrying the codon-optimized version of the *ldh* gene of *L. lactis*, driven by  $P_{\text{trc}}$ , accumulates 1.35 mmol/L in 10 days, whereas SAA015, the strain expressing the *ldh* from *B. subtilis* under the same promoter, reaches 0.6 mmol/L in the same time period (Figure 2a–f).

As growth does not proceed exponentially during the entire 10 day period of the parallel incubated batch cultures, presumably due to light limitation at the later stages of growth, actual lactic acid production rates of the different mutants were derived from selected time periods, early during batch culturing

(indicated with gray bars in Figure 2). The selected time periods allow a direct comparison between the parallel cultures because all were in the late-exponential growth phase (with 40–45 h of doubling time). Table 1 summarizes these specific growth rates, as well as the derived buildup of (carbon in the) biomass and the lactic acid production rate during this time period.

The maximal production rate is achieved with SAA023, that is, a production rate of 0.0175 mmol lactic acid/g D W/h with LDH under control of the *trc* promoter. With reference to SAA023, the relative production rate of the mutant strain with the non-codon-optimized *ldh* (SAA025) is 78%; the mutant with the *B. subtilis* *ldh* (SAA015) is 42%, and the mutant employing  $P_{\text{psbA2}}$  (SAA005) is 19%. The mutant strain driving the *ldh* expression with  $P_{\text{rnpB}}$  (SAA024) does not show any detectable amount of lactic acid released into the culture medium.

**Lactate Dehydrogenase Capacity of Mutant Cell Extracts.** Detection of the activity of LDH in *Synechocystis* cell extracts, harvested in the late-exponential growth phase (Figure 2), allowed for determination of significant enzyme activity in SAA023 (i.e., 17.6 nmol/min/mg of total protein, relative to a background activity of NADH oxidation of the wild-type cell extract in our experimental setup (which, when expressed in activity is <1.0 nmol NADH oxidized/min/mg of total protein; data not shown)). Following the trend of the lactic acid production rate (see above), the relative capacity in lactate dehydrogenase activity drops from the strongest producer, SAA023, all the way to the wild-type strain (Table 1). In vitro assessment of lactate dehydrogenase activity of the mutant cell extracts for this analysis is best performed under the respective  $V_{\text{MAX}}$  conditions<sup>18,19</sup> and consequently correlates with the amount of enzyme present in the cells. As the LDH enzymes of *L. lactis* and *B. subtilis* display similar affinities for pyruvate (1.7 mM for the former and 0.8 mM for the latter enzyme<sup>20,21</sup>) and cofactor specificity, they qualify for a quantitative comparison, and hence, we included also strain SAA015<sup>6</sup> in this study. Indeed, different maximal specific activities have been determined for these two enzymes, that is 30.6  $\mu\text{mol/min/mg}$  protein for the *L. lactis* LDH<sup>20</sup> and 7.4  $\mu\text{mol/min/mg}$  protein for the *B. subtilis* LDH.<sup>19</sup> Significantly, this would complicate the matter only if we would have based the assay of the enzyme capacity on the amount of enzyme present, as could be determined by a quantitative biochemical assay (e.g., a Western blot).

The relative strength of  $P_{\text{trc}}$ ,  $P_{\text{psbA2}}$ , and  $P_{\text{rnpB}}$  was independently confirmed with reporter enzyme studies and

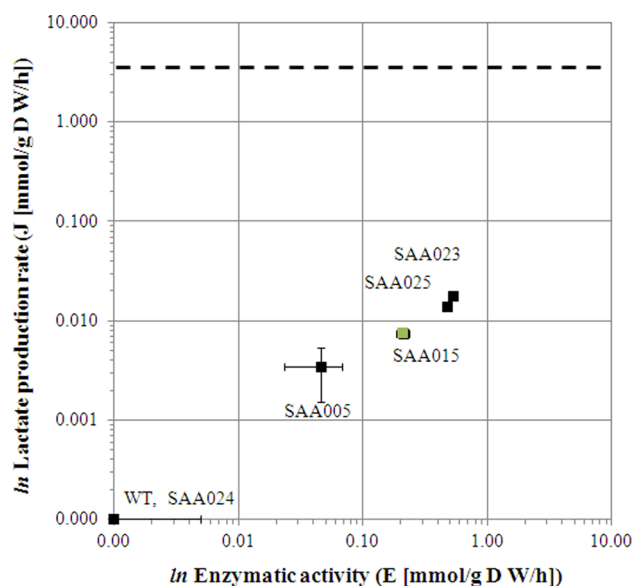


compared to literature data (see Figure S1 in the Supporting Information). SAA024, which did not produce detectable amounts of lactic acid, was confirmed with respect to the correct sequence of the insertion (data not shown; for discussion, see below and compare ref 22, which shows a significantly weaker expression from  $P_{\text{mpB}}$  than that from  $P_{\text{psbA2}}$ ). Codon optimization of the *ldh* gene (compare SAA025 with SAA023) resulted in about 10% higher expression levels of LDH.

**Correlation between the Rate of Lactate Production and LDH Capacity.** Using three different promoters and two different LDH enzymes with very similar kinetics and using a native, nonoptimized, nucleotide sequence and a codon-optimized version<sup>5,23</sup> of the same gene, we have expressed different amounts of LDH enzyme in the *Synechocystis* cells. All three promoters that we used are constitutive. Mutant SAA024, employing  $P_{\text{mpB}}$ , does not show detectable lactic acid production nor LDH activity. Promoter activity data, based on the  $\beta$ -galactosidase assay, shows about 1% activity when compared to  $P_{\text{trc}}$ <sup>24</sup> (see Figure S1 in the Supporting Information); hence, this would predict a lactic acid formation rate of approximately 0.0002 mmol lactate/g D W/h only, which is below the detection limit of our assay. Furthermore, this predicts an enzymatic activity in the cell extract of 0.2 nmol/min/mg of total protein, which is also below the detection limit of the in vitro enzyme assay that we used. It is thus not surprising that mutant SAA024 is indistinguishable from wild-type in our experiments. Nonetheless, the relative expression levels observed in the reporter strains correlate with the LDH activities and the production rates that have been determined.

For the purpose of the analysis of the control of the heterologously expressed LDH on the rate of production of lactic acid by the various mutants, the logarithm of the enzymatic capacity of the mutants is plotted versus the logarithm of the respective production rate (i.e., flux  $J$  toward the product; Figure 3). The experimental data in this figure can be fitted quite well with a straight line with a slope of 1, implying a linear relationship between the intracellular enzyme concentration and the rate of product formation. This linear relationship indicates that the control of the tested enzyme (i.e., LDH) over the entire pathway (from carbon fixation until lactic acid release (Figure 1a)) in the constructed mutant strains is fully embedded in this last step of the pathway (i.e., the LDH enzyme) and hence must approximate 1. It can be anticipated that a further increase of the enzymatic capacity of LDH will lead to higher product formation rates.

Indeed, similar studies on the rate of production of ethanol and ethylene also suggest that even the strongest native and artificial promoters of *Synechocystis* are not strong enough to express sufficient enzyme able to shift a significant part of the control of the carbon metabolism in *Synechocystis* to the upstream part (i.e., reaction 1 in Figure 1a). Gene duplication leads to a corresponding increase of the (rate of) production.<sup>16,17</sup> The need for stronger expression systems, such as gene multiplication, improved ribosome binding sites, multiple promoters,<sup>25</sup> or “amplification-of-expression” strategies such as  $P_{\text{T7}}$ , in combination with T7 polymerase, which has been realized in the heterocyst-forming cyanobacterium *Anabaena*,<sup>26</sup> is evident from these results. Moreover, such an orthogonal system is expected to minimize two-way interference of heterologous and endogenous metabolism.<sup>24</sup>

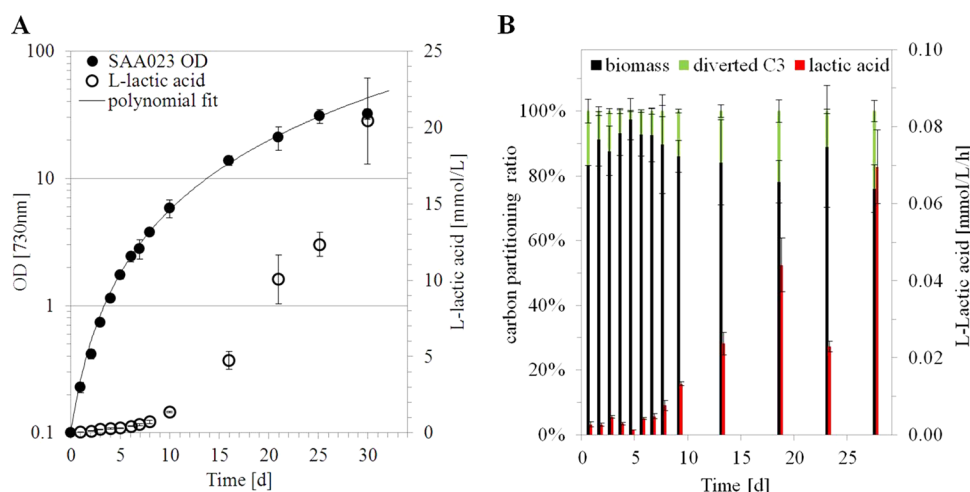


**Figure 3.** Double logarithmic plot of the maximal capacity versus actual production rate of different *Synechocystis* mutant strains that produce lactate. Error bars indicate the standard deviation; error bars are not visible if they are smaller than the corresponding data point symbol.

The stippled line in Figure 4 represents the rate of carbon fixation in wild-type *Synechocystis* when this organism grows at  $\mu_{\text{MAX}}$  (calculation based on its elemental composition<sup>27,28</sup>). In mutant strains with an extra carbon sink, that is, engineered for product formation, this rate can even be higher, as has been observed for the overproduction of sucrose<sup>29</sup> and ethanol (S. A. Angermayr, data not shown). This rate limitation of the rate of carbon fixation is important to know in order to make an estimate as to how much the rate of lactic acid production in these engineered cyanobacterial strains could further be increased by expressing more LDH enzyme. Our conclusion at this point is that presumably a 1 order of magnitude increase should be achievable, provided suitable expression systems can be developed (see above). An exact determination of the contribution of the product-forming enzyme(s) to the overall control over carbon flow in biosolar cell factories is crucial to prevent the situation in which the overexpressed enzyme(s) become(s) an excessive protein burden to the cell.<sup>13</sup> For this reason we anticipate that a control of the LDH enzyme over lactate formation of 0.2–0.3 will be optimal in this system.

**Long-Term Culturing and Carbon Partitioning.** The general interest in  $\text{CO}_2$  based product formation in cyanobacterial cell factories has prompted several groups to do long-term batch culture experiments.<sup>16,30</sup> The reported maximal product titer for any cyanobacterial cell factory has been achieved with ethanol production by *Synechocystis*<sup>16</sup> (i.e., approximately 120 mmol/L after 4 weeks). For lactic acid, the highest reported yield is 3 mmol/L of L-lactic acid in 3 weeks.<sup>6</sup> We therefore decided to test lactic acid formation also over a similar period. Figure 4 shows that with a batch culture of strain SAA023, incubated in relatively low light intensity (see the Material and Methods section), more than 20 mmol/L is produced in 4 weeks.

Careful evaluation (based on a polynomial fit) of the growth and lactate production data allows one to estimate the partitioning of the carbon influx (in the form of  $\text{CO}_2$ ) over biomass (i.e., cells) and product formation (here, extracellular



**Figure 4.** L-Lactic acid production in *Synechocystis* SAA023 upon extended incubation in a batch culture. (a) Growth curve and lactate accumulation in a batch culture of *Synechocystis* SAA023, carrying the codon-optimized *ldh* of *L. lactis*, with the expression driven by  $P_{trc}$ . (b) Partitioning of fixed carbon over the biomass and heterologous product (i.e., lactic acid) and the corresponding volumetric production rate. The values are the average of biological replicates; the error bars indicate the standard deviation ( $n = 3$ ); error bars are not visible if they are smaller than the corresponding data point symbol.

lactic acid; see Figure 4b). During the long-term batch culturing, the carbon partitioning in SAA023 into lactic acid was between 8 and 20% (with slight growth-phase dependence; see Figure 4b). The maximal volumetric production rate achieved in this experiment is 0.07 mmol lactic acid/L/h.

The electrons that are necessary for the conversion of pyruvate into lactic acid in SAA023 are derived from NADH. Besides increasing the rate of lactate production through increases in the LDH capacity of the cells (see above), it can be anticipated, based on results achieved with the formation of products such as ethanol<sup>16</sup> and butanediol (Savakis, Angermayr, Hellingwerf, manuscript submitted, and ref 30), that further increases in the lactate production rate (and presumably also the level) can be achieved via the use of an NADPH-dependent enzyme.

## CONCLUSION

In this study, we have addressed the current limitations present in cyanobacterial cell factories. The *Synechocystis* strains presented here, which were engineered toward L-lactic acid production, display a linear relation between the lactic acid production rate and the LDH enzymatic capacity of the cells (Figure 3). Significantly, even when expressed from the strongest promoter known in *Synechocystis*, that is, the *trc* promoter, the heterologous LDH still exerts full control over lactic acid formation. Increased LDH expression, to remedy this situation, may be achieved, for example, through gene<sup>15</sup> or promoter<sup>25</sup> multiplication, be it that this may lead to unwanted recombination events in *Synechocystis*.<sup>31,32</sup>

Heterologous gene expression in non-natural hosts and denovo gene synthesis has made codon optimization possible to increase expression.<sup>23</sup> Comparisons between the inserted native sequence of the *L. lactis* *ldh* and a codon-optimized version (for details, see Material and Methods in the Supporting Information) has resulted in a mild effect only (Table 1 and Figure 4). Genes from eukaryotic hosts however, for example, the plant isoprene synthase for cyanobacterial isoprene formation, have benefited significantly from codon optimization for the host organism.<sup>5</sup>

Similar analyses as the one presented here will be of great interest not only for other products that can be made using the cyanobacterial cell factory approach (see above) but also for other parts of its carbon and energy metabolism. A case in point is the stoichiometric use of the available photons, which could be significantly lower than unity because of loss of excitons in the antenna system due to exciton annihilation, radiationless decay, and/or charge recombination. Should this be observed, it is a challenge to engineer antenna phycobilisome and antenna expression in a clever way to minimize these losses in large-scale photobioreactors.

## MATERIAL AND METHODS

For a detailed description, see Material and Methods in the Supporting Information.

**Growth Conditions.** For measurement of lactic acid production by mutant *Synechocystis* sp. PCC6803 strains in their late-exponential phase of growth, precultures were rediluted to an OD<sub>730</sub> of 0.1 in a BG-11 medium (Sigma-Aldrich) supplemented with 10 mM TES-KOH buffer (pH 8.0) in 25 mL flasks. Cultures were grown in white fluorescent light at 30–40  $\mu\text{E}/\text{m}^2/\text{s}$  at 30 °C in a shaking incubator. Every 24 h, 2% of the culture volume was removed for analysis.

**L-Lactic Acid Quantification.** Supernatant samples of the cultures were subjected to the D-/L-lactic acid (Rapid) assay (Megazyme), following the manufacturers' instructions. The assay was adapted for use in a 96-well plate and a corresponding 96-well plate reader.

**Curve Fitting of OD<sub>730</sub> Data (i.e., growth) and Calculation of the Rate of L-Lactic Acid Production.** For growth rate calculations, a second-order polynomial curve was fitted to the growth data. Derived data have been used for lactate production rate determination and enzymatic activity determination as well as growth rate determination. An average elemental cell composition of  $\text{C}_4\text{H}_7\text{O}_2\text{N}$  was assumed.

**Cell Extract Preparation and Enzymatic Activity Assay.** Crude cell-free extract from cultures growing in late from the late-exponential growth phase were obtained by disrupting the cells with a bead-beater and pelleting the cell debris by centrifugation. A BCA protein assay kit (Pierce) was

used to determine the total protein content of the soluble fraction in the supernatant. The enzyme activity assays in the crude cell-free extract were performed in duplicate employing an NADH-dependent assay essentially as described in ref 6. The enzymatic activity was derived from the monitoring of the consumption of NADH per second.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Full description of the material and methods; complete list of strains used; promoter activity data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare the following competing financial interest(s): K.J.H. is co-founder of the spin-off company Photanol B.V.

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