Synthetic biology of cyanobacterial cell factories

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Chirality matters: Synthesis and consumption of the D-enantiomer of lactic acid with *Synechocystis* sp. PCC 6803

Both enantiomers of lactic acid, L- and D-lactic acid, can be produced in a sustainable way by a photosynthetic (microbial) cell factory, thus from CO₂, sunlight and water. Several properties of polylactic acid (a polyester of polymerized lactic acid) depend on the controlled blend of the two enantiomers of this chiral weak acid. Genetically modified strains of the cyanobacterium *Synechocystis* sp. PCC 6803 have recently been employed to form either of these (pure) enantiomers. This report elaborates on the D-lactic acid production achieved by the introduction of a D-*ldh* from the lactic acid bacterium *Leuconostoc mesenteroides* in *Synechocystis*. A typical batch culture of this recombinant strain initially shows lactic acid production, followed by a phase with transient lactic acid consumption, until production ‘outcompetes’ consumption at later growth stages, leading to an accumulation of extracellular lactic acid. Interestingly, *Synechocystis* is able to use D-lactic acid, but not L-lactic acid as a carbon source for growth. This could be the consequence of the specificity of an endogenous D-lactic acid dehydrogenase enzyme. Deletion of the putative endogenous D-LDH (encoded by *slr1556*), however, does not eliminate this consumption.

Accordingly, this study highlights the need to match a product of interest of a cyanobacterial cell factory with the host used for its synthesis, and emphasizes the need to understand the physiology of the production host in detail. It therefore implicitly accentuates well-studied model organisms such as *Synechocystis* sp. PCC 6803 as such hosts.

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This chapter has been submitted for publication.

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6.1 Introduction

To date, lactic acid can be produced with almost 100% conversion efficiency by several chemotrophic fermentative bacterial- or yeast cell factories, growing on various sugars (191, 192). Consequently, a large amount of effort is currently undertaken to produce lactic acid with lactic acid bacteria (193), in combination with the use of cheap substrate(s) such as lignocellulosic feedstock, a feedstock that requires an energy-intensive pre-treatment of the biomass (102, 191). Lactic acid is used in food preservation, in the chemical- and pharmaceutical industry, and as building block for polymers, the latter as an alternative to petroleum-derived plastics. It is compelling that the biodegradability and heat stability of this (bio)plastic depends on the blend of the two optical active isoforms of lactic acid (194).

Employing photosynthetic microorganism as the production host has the advantage to enable the direct conversion of CO$_2$ into lactic acid (100, 133, 154, 195). Production, that is dependent on cyanobacterial cell factories, allows compound formation without the need to generate complex (plant)biomass first, only to break it down again later for its utilization by a chemotrophic fermentative microorganism (123).

For living organisms, especially regarding their amino acids, chirality plays an essential role, because exclusively L-enantiomers are incorporated into proteins. Likewise L-lactic acid seems to be the dominant enantiomeric form of this weak acid in nature and thus production hosts for D-lactic acid are more challenging to find and construct (196). Nonetheless, biosynthetic routes for both enantiomers, and thus for the corresponding products, exist in various (micro)organisms, facilitated by enantiomer-specific lactate dehydrogenases (103), whereas chemical synthesis routinely results in a racemic mixture (197). By employing the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*), we have constructed several L-lactic acid producing strains earlier (133, 155). In the framework of these experiments, we have also tested the D-LDH of *E. coli*, but, at that time, were not successful in producing D-lactic acid in *Synechocystis* (133). However, in another cyanobacterium, *Synechococcus elongatus* PCC 7942, synthesis of this enantiomer has been achieved, although the extracellular accumulation required the co-expression of a transporter (100). More recently, D-lactic acid formation in *Synechocystis* has also been accomplished, with the authors arguing that suitable and effective D-enantiomer specific lactate dehydrogenases (LDH) are more difficult to find than the corresponding L-LDH enzymes (154). In this latter study D-lactic acid formation was achieved by the incorporation of a mutated glycerol dehydrogenase, GlyDH*, from *Bacillus coagulans* (198). Here we have chosen to use the D-LDH of *Leuconostoc mesenteroides* (*L. mesenteroides*) (199), because of its superior kinetics compared to other characterized D-LDH enzymes (128). As an alternative, we decided to also overexpress the putative native D-LDH (*slr1536*) of *Synechocystis*, arguing that a native enzyme might be optimized for the respective conditions present in the cyanobacterial cytosol.
Interestingly, various cyanobacteria have been shown to possess a dark and anaerobiosis-induced fermentation capacity, including the capacity to form lactic acid (19, 200, 201). Further, it has been shown that selected cyanobacterial strains can utilize lactic acid to form biomass in the dark. For example, resting cells suspensions of the thermophilic cyanobacterium *Synechococcus* sp. PCC 6716 can (aerobically in the dark) take up lactic acid and use it to form biomass, CO₂, and acetate (202). Moreover, the same study showed that cell extracts of various cyanobacteria, including *Synechocystis*, display lactate dehydrogenase activity. Combined with the fact that *slr1556* is annotated as a putative D-LDH (52), the corresponding enzyme could present a competing pathway, assuming that it functions as a D-lactic acid consumption pathway, utilizing this carbon source for growth as seen for *Synechococcus* sp. PCC 6716.

For an optimized production system employing photosynthetic microorganisms, it is of importance to improve the insights in the physiology of these production hosts. As elegantly summarized by Keasling: For a future industrial application of metabolically engineered microorganisms, the development of the metabolic route, the production host and the envisioned product, have to be matched to achieve optimality (7). Here, we describe the construction of a D-lactic acid producing strain of *Synechocystis*, (over)expressing an NADH-dependent D-enantiomer specific *ldh* of *L. mesenteroides*. However, we also show that production of D-lactic acid is not matching perfectly to this specific host, as *Synechocystis* is capable of D-lactic acid consumption. It appears that a significantly higher capacity of production has to be engineered in order to achieve significant net production, as compared to *Synechocystis* strains that produce L-lactic acid.

### 6.2 Materials and Methods

#### 6.2.1 Growth conditions

For sub-cloning procedures, plasmid propagation and isolation, *Escherichia coli* strains XL-1 blue (Stratagene) and *E. coli* EPI400 (Epicentre) were cultivated at 37°C in Lysogeny Broth (LB) liquid medium or on LB agar. If appropriate, ampicillin was used at a concentration of 100 µg/ml, kanamycin at 50 µg/ml and spectinomycin at 25 µg/ml, either separate or in combination.

The glucose tolerant derivative of *Synechocystis* sp. PCC6803 (wild type, kindly provided by Devaki Bhaya, Stanford, USA) was grown in liquid BG-11 medium (Sigma-Aldrich), supplemented with 10 mM TES-KOH (pH 8). If appropriate, kanamycin was used at a concentration of 20 µg/ml, and spectinomycin at 20 µg/ml, either separate or in combination. Cultures were incubated in a shaking incubator at 120 rpm at 30°C (Innova 43, New Brunswick Scientific) equipped white-light illumination (~35 µE/m²/s). BG-11 solid medium was prepared by adding 1.5% (w/v) agar and 10 mM TES-KOH (pH 8), 5 mM glucose, and 0.3% (w/v) sodium thiosulfate. For the construction and maintenance of the *Synechocystis Δslr1556* mutant strain on plates, the addition of glucose was omitted. Growth of *Synechocystis* wild type and mutant strains was followed by OD₇₃₀ determination (Spectrophotometer Lightwave II, Biochrom) at selected time points. Roughly, every 24 hours approximately 3% of the culture volume was removed for the analysis of growth, and subsequent determination of the lactic acid concentration, when applicable. The water evaporation rate was determined and found to be 1% of the culture volume per day (Supplemental material, Figure S5), which hence increases the lactic acid concentration only marginally. The rate calculations of product formation have been reported earlier (133, 155).
6.2.2 Strain and plasmid construction

*Synechocystis* mutant strains were constructed essentially as described before (133). Briefly, we constructed plasmids to be used for natural transformation with subsequent integration of the gene cassette of interest and a kanamycin resistance marker at the slr0168 locus of the *Synechocystis* genome. Additionally, plasmid pGemT_slr1556_Sp122 targets slr1556 with a streptomycin/spectinomycin resistance marker to create the particular knockout strain. Before selecting one clone for further investigation, routinely three individual colonies originating from the transformation were verified with respect to full segregation and correct sequence alteration and compared with respect to growth and, if applicable lactic acid production. Segregation was evaluated by colony PCR with primers flanking the integration site (Supplemental material, Table S1) employing *Taq* DNA polymerase (Thermo scientific). For verification of correct insertions the sequence was evaluated by capillary electrophoresis sequencing (Macrogen Netherlands) of relevant PCR fragments (for flanking primers see supplemental material, Table S1) amplified employing *Pfu* DNA polymerase (Thermo scientific).

The plasmid used to make the *Synechocystis* strain DLEU is basically a derivative of the previously published integration vector pHKH001 (133). The (protein) sequence of the D-LDH of *Leuconostoc mesenteroides* was taken from UniProt database entry Q2ABS1_LEUME based on (203). The (gene) sequence was codon optimized using a codon usage table generated from a subset of highly transcribed *Synechocystis* genes (for the exact procedure see (129)). Relevant restriction sites, needed for sub-cloning procedures have been avoided. The gene was further flanked on the 5'-end with *Ptra* and an RBS and at the 3'-end with a transcriptional terminator (BBa_B0014, http://parts.igem.org/). The gene cassette was synthesized and directly cloned into pHKH001 at the *SacII/EcoRV* site by Genscript (NJ, USA). The plasmid used to make the *Synechocystis* strain DSYN was constructed from a derivative of pHKDLEU which carries, additionally to NdeI site of the synthesized construct at the 5'-end, a *BamHI* site at the 3'-end for exact ORF replacement. First, *Synechocystis slr1556* was amplified from pIBA43plus_slr1556 with primers introducing the flanking restriction sites NdeI and *BamHI* employing *Pfu* DNA polymerase (Thermo scientific). The gene was then sub-cloned into the pHKDLEU derivative, replacing the respective ORF creating pHKHDYN. pIBA43plus_slr1556 was constructed as follows To generate a Slr1556 over-expressing *E. coli* strain the slr1556 gene was amplified by PCR using genomic DNA from *Synechocystis* sp. PCC 6803, gene specific primers with added appropriate cleavage sites Table S1 (Supplemental material), and proof reading Elongase enzyme (Invitrogen). The resulting fragments were first cloned in pGemT-vector (Promega). After sequence confirmation, the coding sequence of slr1556 was transferred to pIBM43plus using EcoRI/XhoI. The verified recombinant pIBM43plus vector were transformed into *E. coli* strain BL21 DE3. The plasmid inserts where verified by sequencing of the relevant part of the plasmid. We used pGemT_slr1556_Sp122 to make the *Synechocystis* strain ΔDSYN, disrupting Δslr1556. pGemT_slr1556_Sp122 inserts a streptomycin/spectinomycin resistance cassette (for strain construction compare also (204)) replacing a 585 nucleotide-long region [3359843..3360428] on the *Synechocystis* genome and thereby disrupting the ORF of slr1556. The gene slr1556 is the putative *ddh* (encoding the D-isomer specific lactate dehydrogenase) of *Synechocystis*, as annotated in the genome, and essentially inferred by automated homology determination (52). All restriction enzymes and the ligase used were from Thermo scientific.

6.2.3 Protein purification

5 ml overnight culture of *E. coli* BL21 harboring pIBM43plus_slr1556 for the heterologous production of Slr1556 was diluted in 200 ml LB medium. After incubation of 6 hours at 30°C shaking at 170 rpm, expression of the recombinant protein was induced over night with the addition of 200 nm/ml anhydrotetracyclin (Clontech). Cells were harvested by centrifugation, and resuspended in 5 ml homogenization buffer consisting of 20 mM Sodium phosphate buffer (pH 7.4) and 500 mM NaCl and lysed by ultrasonic treatment (Labsonic U, B Braun). Cell debris was removed by centrifugation (30 min at 10,000 rpm at 4°C). Subsequently, the soluble lysates were loaded on a Ni-NTA Sepharose column primed with ProBond slurry
(Invitrogen), washed with sterile water and equilibrated with homogenization buffer. After careful addition of soluble fraction of the cell-free extract the column was washed with the same buffer, but supplemented with 20 mM imidazole, and next with a buffer supplemented with 40 mM imidazole. The protein bound to the column was eluted with elution buffer consisting of 20 mM Sodium phosphate (pH 7.4), 500 mM NaCl and 300 mM imidazol, and collected in 500 μl fractions. The fraction containing the his-tagged protein was collected on a washed PD-10-desalting column (GE Healthcare) and the buffer was exchanged by applying approx. 2 volumes of 20 mM Sodium phosphate buffer (pH 7.4) in a sequential manner. For protein storage 20% (v/v) glycerol were added. The purity of the protein was verified by visual inspection of Coomassie Brilliant blue-stained SDS-PAGE. Srl1556 protein concentration was determined by the method of Bradford (205) with bovine albumin as standard protein.

6.2.4 Determination of lactic acid

The lactic acid concentration in supernatant samples of Synechocystis cultures was determined essentially as described earlier (133). Briefly, aliquots of supernatant were assayed with the D-/L-lactic acid (Rapid) assay (Megazyme), adapted to 96-well plate format. Sporadically the supernatant was subjected to HPLC analysis. Samples were separated on a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex) at 45°C at a flow rate of 0.5 ml/min. Signals were obtained by a refractive index detector (RI-1530, Jasco) and analyzed with the Azur 4.5 software package (Datalys). In both detection methods the sample signal was compared to a set of known standard concentrations of D-/L-lactic acid (Megazyme) for quantification.

6.2.5 Cell extracts and lactate dehydrogenase enzymatic activity assays

Crude cell-free extracts of Synechocystis cells were obtained essentially as described earlier (155). Briefly, 10 ml Synechocystis cultures growing in the late exponential phase of growth (OD 730 of ~1.0) were harvested and pelleted at 4°C. Aliquots of the supernatant were kept for lactic acid determination and the excess supernatant was discarded. The cell pellets were re-suspended in the respective pre-chilled buffer (see below) and disrupted with glass beads of 100 μm diameter (Sigma) in a Precellys®24 bead beater (Bertin technologies). Cell debris was removed by thorough centrifugation at 4°C and supernatant was collected and used either on the same day for the LDH enzymatic activity assay (stored at 4°C) or flash-frozen in liquid nitrogen and stored at -20°C for longer periods. The protein concentration was determined with the standard BCA protein assay (Pierce). Absorption at 570 nm was determined in a 96-well plate reader (Fluostar Optima, BMG labtech) and compared to a set of known standard concentrations of bovine serum albumin for quantification.

LDH enzymatic activity assays were performed essentially as described earlier (155). Briefly, the consumption (or formation) of NADH at 340 nm was followed in a 96-well plate reader (Multiscan FC microplate reader, Thermo scientific) set to 30°C. For cell-free extracts containing the LDH of L. mesenteroides the reaction mixture contained 100 mM Tris-HCl buffer at pH 7.5, 5 mM MgCl2, and 300 μM NAD(P)H. For cell-free extracts of wild type, DSYN and ΔDSYN the reaction mixture contained 100 mM TEA buffer at pH 7.0, 5 mM MgCl2, and 300 μM NAD(P)H. After a background reading, allowing time for equilibration, the reaction was started by the addition of 30 mM sodium pyruvate (Sigma). For the D-lactic acid consumption assay the same reaction mixture was used, but replacing NAD(P)H with NAD(P)+. The reaction was started by the addition of 100 mM lithium D-lactic acid (Roche). For the D-lactic acid consumption assay the same reaction mixture was used, but replacing NAD(P)H with NAD(P)+. The reaction was started by the addition of 100 mM lithium D-lactic acid (Roche). For the reaction destined for HPLC analysis 50 µg/ml cell-free extract were incubated in 100 mM TEA buffer at pH 7.0, 5 mM MgCl2, 1 mM NAD(P)H, and 30 mM sodium pyruvate for 3 hours at gentle agitation at 30°C.

The LDH enzymatic activity assays, and the K_M determination for the purified enzyme, were performed as follows: 50 µl of purified protein were added to 900 ul of 100 mM MES-buffer (pH 6.5) containing (freshly added) DTT at 1 mg/ml, and NADH at 187 µM. After 2 min of equilibration 50 µl of pyruvate (20 mM) were added to start the reaction (206). The decrease of absorption at 340 nm was observed with a Cary-50-spectrophotometer (Varian) at 30°C and the resulting activity was expressed in μmol/min/mg.
considering the equimolar conversion of NADH and pyruvate into NAD and lactic acid. For kinetic parameters concentrations from 0.5 to 50 mM for glyoxylate or 0.1 to 2.5 mM for pyruvate were used, whereby the NADH concentration was kept constant. For D-lactic acid concentrations from 1 to 30 mM were used, and the NAD+ concentration was kept constant. The kinetic parameters were calculated using Sigma Plot 11.0.

6.2.6 Sample preparation for SDS-PAGE analysis

Aliquots (18 ul) of identical protein concentration (2 ug/ul) of the crude cell-free extract samples were supplemented with 2 ul protein solubilisation buffer, consisting of 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 50 mM EDTA, 2% (w/v) sodium dodecylsulphate, and 10% (v/v) glycerol. Samples were boiled at 95°C and mixed thoroughly before application on a 12% (w/v) SDS-polyacrylamide (PA) gel. Samples were electrophoresed on these SDS-PAGE gels, fixed with an isopropanol/acetic acid solution and were subsequently stained with CBB G-250 (PageBlue Staining solution, Thermo scientific) according to the manufacturer’s protocol.

6.3 Results

6.3.1 Mutant construction for D-lactic acid producing Synechocystis strains

Motivated by previous work (133), we anticipated to construct a Synechocystis strain that is capable of D-lactic acid production, in order to have an enantiomeric complement to our L-lactic acid producing strains. Based on data available in BRENDA (128), we chose the D-isomer specific lactate dehydrogenase (LDH) of L. mesenteroides because of favourable kinetic properties, such as a low K_M value for pyruvate, and a high molecular turnover number (199). To avoid potential competition between related pathways we additionally deleted the putative endogenous LDH, coding from ddh (slr1556) (52) of Synechocystis. Additionally, we constructed a slr1556 overexpression strain. The resulting Synechocystis mutant strains are listed in Table 1.

Table 1: Strains and plasmids used and constructed in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant feature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli XL-1 blue</td>
<td>Cloning host</td>
<td>Stratagene</td>
</tr>
<tr>
<td>E. coli EPI400</td>
<td>Cloning host</td>
<td>Epicentre</td>
</tr>
<tr>
<td>Synechocystis sp. PCC6803</td>
<td>Wild type, glucose tolerant derivative</td>
<td>D. Bhaya (Stanford)</td>
</tr>
<tr>
<td>pHKH001</td>
<td>Δslr0168::Kan^R, in E. coli additionally Amp^R</td>
<td>(133)</td>
</tr>
<tr>
<td>pIBA43plus_slr1556</td>
<td>slr1556, Amp^R</td>
<td>This study</td>
</tr>
<tr>
<td>pGemT_slr1556_Sp122</td>
<td>Δslr1556::Sp^c, in E. coli additionally Amp^R</td>
<td>This study</td>
</tr>
<tr>
<td>pHKHDLEU</td>
<td>Δslr0168::Ptrc::Lm ldh_co::tt::Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pHKHDSYN</td>
<td>Δslr0168::Ptrc::Sp slr1556_na::tt::Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>Synechocystis DLEU</td>
<td>Prrc::Lm ldh_na::tt::Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>Synechocystis DSYN</td>
<td>Prrc::Sp slr1556_na::tt::Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>Synechocystis ΔDSYN</td>
<td>Δslr1556::Sp^c</td>
<td>This study</td>
</tr>
<tr>
<td>Synechocystis DLEU/ΔDSYN</td>
<td>Prrc::Lm ldh_na::tt::Kan^R, Δslr1556::Sp^R</td>
<td>This study</td>
</tr>
</tbody>
</table>

For these overexpression strains slr0168 served as the neutral integration site on the Synechocystis chromosome and slr1556 was disrupted by the deletion of a part of its open reading frame (ORF) and the integration of a kanamycin resistance cartridge (Figure 1A). Colony PCR with flanking primers confirmed full segregation of the respective gene cassettes (Figure 1B).

**Figure 1:** (A) Schematic representation of the genetic engineering approach at the neutral site (slr0168) and the disruption of the putative ddh (slr1556) in the genome of Synechocystis sp. PCC 6803. Primers used in (B) are indicated. (B) Colony PCR result of relevant strains showing complete segregation at both sites, confirming the gene insertion in DSYN, DLEU and DLEU/ΔDSYN and the deletion of slr1556 in ΔDSYN and DLEU/ΔDSYN, respectively.

### 6.3.2 D-lactic acid production with a genetically engineered Synechocystis strain

The Synechocystis strain DLEU, expressing the D-LDH of L. mesenteroides, shows D-lactic acid production (Figure 2), accumulating an extracellular concentration of 0.60 ± 0.19 mmol/L within 1 week and 3.93 ± 0.27 mmol/L D-lactic acid within 2 weeks. Strikingly, however, upon exit of the initial phase of exponential growth, D-lactic acid is actually transiently consumed, before the production resumes after the ‘transition phase’ in the later growth phase (Figure 2).

The Synechocystis genome harbours the gene slr1556, annotated as D-LDH, which could be held responsible for the observed D-lactic acid consumption. Therefore, we constructed a strain with a disruption of slr1556, resulting in DLEU/ΔDSYN. Significantly, here we show that this double mutant, DLEU/ΔDSYN, displays the same D-lactic acid production characteristics as the single mutant, DLEU (Figure 2). Hence, the putative D-LDH activity coding from slr1556 does not generate the (only) pathway for D-lactate consumption under these conditions.
Figure 2: (A) Time course of D-lactic acid production by a Synechocystis strain overexpressing solely D-LDH of L. mesenteroides (DLEU) and the latter enzyme in combination with an slr1556 disruption (DLEU/ΔDSYN). In the exponential growth phase D-lactic acid accumulates, whereas in the subsequent transition phase lactic acid is consumed. At later growth stages lactic acid production dominates, which leads to steady lactic acid accumulation. (B) Close-up of (A), allowing a detailed view of the changes in D-lactic acid concentration during the early growth phase. Error bars indicate the SD (n = 3); if error bars are not visible they are smaller than the data point symbol.

Consistent with previous observations (133) we could not detect any D-lactic acid formation in wild-type (and ΔDSYN) culture(s) (Figure 3A and 3B). The overexpression of slr1556 also did not result in a lactic acid producing strain (Figure 3C). The overproduction of the respective enzymes in DSYN, DLEU, and DLEU/ΔDSYN was confirmed by an SDS-PAGE analysis (Supplemental material, Figure S1) and LDH activity assays (see below). To further investigate the potential lactic acid consumption by Synechocystis and correlate the decrease in its concentration with the production that is observed here, we analysed the mixotrophic growth characteristics of the organism (see further below).

6.3.3 D-lactic acid allows mixotrophic growth of Synechocystis

When growing mixotrophically in the light, Synechocystis is able to assimilate glucose to form biomass, or it can utilize glucose heterotrophically in the dark, if provided with short periods of blue light illumination (207). Also acetate can be considered as a preferred organic carbon source for mixotrophic growth for selected cyanobacteria (19). If lactic acid is added to Synechocystis wild-type cultures growing under a continuous light regime, the cultures consume (and grow on) D-lactic acid, but not L-lactic acid (Figure 3A). Final yields of biomass after 1 week are significantly higher for cultures supplemented with D-lactic acid than those without addition of an organic carbon source or with the addition of L-lactic acid (Figure 3A). Interestingly, the slr1556 deletion mutant, ΔDSYN, shows the same lactic acid consumption pattern as the wild type (Figure 3B), suggesting that, in the mutant strain, no elements of the ability to consume (and grow on) D-lactic acid are encoded by slr1556. Furthermore, the slr1556 overexpression strain, DSYN, showed an identical lactic acid consumption pattern.
Chirality matters: D-lactic acid synthesis

Figure 3: Photoautotrophic (AT) growth and mixotrophic growth by addition of D- (+D-LA) and L-lactic acid (+L-LA). (A) Synechocystis wild type, (B) ΔDSYN and (C) DSYN strains show D- but not L-lactic acid consumption. No D- or L-lactic acid formation was observed for any of the strains. Exp.: exponential growth phase; trans.: transition phase; late: later growth phase. Error bars indicate the SD (n = 3), except for DSYN where n = 2; if error bars are not visible they are smaller than the data point symbol.

As evident from the growth and lactic acid data shown in Figures 2 and 3, the production and consumption rates for lactic acid are not constant over the course of a typical batch culture experiment (see materials and methods for detailed culturing conditions). Table 2 summarizes the respective rates, indicating production, consumption and production at the different subsequent stages of growth. The D-lactic acid consumption rate per cell (i.e. per gDW, compare Table 2) varies over the course of a typical batch culture experiment, which is suggesting a correlation between growth (phase) and lactic acid consumption.

Table 2: Rate of consumption of D-lactic acid of the wild-type, the ΔDSYN, and the DSYN strains and rate of production and consumption of the D-lactic acid producing strains DLEU and DLEU/ΔDSYN.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild type</th>
<th>ΔDSYN</th>
<th>DSYN</th>
<th>DLEU</th>
<th>DLEU/ΔDSYN</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-lactic acid</td>
<td>External</td>
<td>External</td>
<td>External</td>
<td>Endogenous</td>
<td>Endogenous</td>
</tr>
<tr>
<td>Exp. growth phase</td>
<td>- 0.0079 ± 0.0010 **</td>
<td>- 0.0068 ± 0.0010 *</td>
<td>- 0.0039 ± 0.0062</td>
<td>+ 0.0635 ± 0.0228 ***</td>
<td>+ 0.0470 ± 0.0047 **</td>
</tr>
<tr>
<td>Trans. growth phase</td>
<td>- 0.0119</td>
<td>- 0.0106</td>
<td>- 0.0083</td>
<td>- 0.0022</td>
<td>- 0.0019 ± 0.0025</td>
</tr>
<tr>
<td>Late growth phase</td>
<td>- 0.0051</td>
<td>- 0.0036</td>
<td>- 0.0011</td>
<td>+ 0.0045</td>
<td>+ 0.0037 ± 0.0016 **</td>
</tr>
</tbody>
</table>

Production rates are annotated with a (+), consumption rates with a (-). Rates are expressed as mmol lactic acid/gDW/h. Exp.: exponential growth phase; Trans.: transition phase; Late: later growth phase. The standard deviation (SD) of the values is also indicated (n = 3); * indicates a p<0.4, ** indicates a p<0.04, and *** indicates a p<0.002 (students t-test) for the difference of the consumption rate during the transition phase opposed to the preceding exponential growth phase or the subsequent later growth phase, respectively.

Substantiating the dynamics of growth on D-lactic acid further, we show that the growth rate for wild type with D-lactic acid differs over the course of the batch culture
experiment from the wild-type cultures not supplemented with D-lactic acid (see supplemental material Figure S2).

This transient increase in growth rate for the culture supplemented with D-lactic acid results also in the before-mentioned increased final yields of biomass seen in Figure 3A.

The capacity of D-lactic acid consumption in the transition phase is 0.0119 ± 0.0006 mmol lactic acid/gDW/h for the wild-type strain. A slightly lower consumption rate is found for the preceding (short) exponential growth phase. However, this rate decreases significantly after exiting the transition phase and the entrance into the late phase of growth (Table 2). If the production capacity of lactic acid in the DLEU strain would not be affected in the transition phase, the production rate should only be reduced by roughly that value. However, we observe a decrease from a production rate of 0.0635 ± 0.0228 to a consumption of 0.0022 ± 0.0035 mmol lactic acid/gDW/h.

This finding indicates that the rate of production must have dropped as well, here by an approximated 85%, as determined from comparing the rate data, relative to the wild-type consumption rate. After the growth phase transition the D-lactic acid production rate increase again to 0.0045 ± 0.0018 mmol lactic acid/gDW/h for the DLEU strain indicating an increases in D-lactic acid accumulation again (see also Figure 2).

It should be noted, however, that a forthright comparison of production and consumption rates is precarious, because the production achieved by the heterologous enzyme directly provides intracellular D-lactic acid, whereas the supplemented D-lactic acid (the lithium salt, see materials and methods for detailed culture conditions) first needs to enter the cells, in order to be utilized, which could possibly be facilitated by a (yet uncharacterized) transporter.

6.3.4 Lactate dehydrogenase activity of purified Slr1556 and Synechocystis cell extracts of wild type and D-LDH carrying strains

It has been found earlier that cell extracts of Synechocystis sp. PCC 6803, at that point also named Aphanocapsa PCC6803 have specific activity towards D-lactic acid but not L-lactic acid (202).

Here we show that cell-free extract of wild type is able to convert pyruvate into lactic acid in an NADH-dependent manner (Figure 4). Significantly, the crude cell-free extract of the ΔDSYN mutant strain lacks this ability. The slr1556 overexpression strain, DSYN, shows an activity more than 650-fold higher than wild-type cell-free extract (i.e. 1.13 ± 0.09 µmol/mg/min). Notably the determined lactic acid dehydrogenase capacities for DLEU and DLEU/ΔDSYN are another 10-fold higher. The activity of DLEU is 14.22 ± 3.29 µmol/mg/min. The activity of DLEU/ΔDSYN is 13.09 ± 2.59 µmol/mg/min (Figure 4A).
Further investigation of the soluble fraction of the crude cell-free extracts shows that pyruvate-dependent LDH activity resulted in the build-up of D-lactic acid (Figure 4B), in case of wild-type cell-free extract, but not with the ΔDSYN mutant strain. Distinct from activity with NADH, the utilization of NADPH as the co-factor resulted in a minor build-up of lactic acid in case of wild-type extract. A D-lactic acid utilization assay of cell-free extracts confirms this NAD(P)⁺ dependent activity (Supplemental material, Figure S3). Additionally, an NADPH-dependent LDH assay shows activity in cell-free extracts of DSYN, suggesting that Slr1556 can utilise both NADH and NADPH. The NADPH dependent activity of wild-type cell extract was not detected; however, observation of this activity is blurred by the high detection threshold of this assay, which is due to (unspecific) background oxidation of NAD(P)H (Supplemental material, Figure S4).

Purified, recombinant Slr1556 can utilize pyruvate but also glyoxylate and hydroxypyruvate as a substrate. Most efficiently, the enzyme uses pyruvate with the by far highest affinity (lowest $K_M$ value), using NADH as co-factor and essentially making this enzyme rather a pyruvate dehydrogenase than a lactate dehydrogenase (Table 3). Compatibly, the activity detected with D-lactic acid was very low, and the high $K_M$ that was determined value makes *in vivo* lactate utilization activity unlikely. Collectively our biochemical data suggest that its preferred activity is not the conversion of D-lactic acid into pyruvate to fuel intermediary metabolism, but rather the reverse reaction.

**Table 3:** Characterisation of the kinetic properties of purified, recombinant Slr1556. Presented are mean values of $n = 3$.

<table>
<thead>
<tr>
<th></th>
<th>Pyruvate</th>
<th>Hydroxypyruvate</th>
<th>Glyoxylate</th>
<th>D-lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (mM)</td>
<td>0.69</td>
<td>7.73</td>
<td>5.10</td>
<td>30</td>
</tr>
<tr>
<td>$V_{MAX}$ (µmol/mg/min)</td>
<td>397</td>
<td>88</td>
<td>333</td>
<td>27</td>
</tr>
</tbody>
</table>
6.4 Discussion

\( \text{D-lactic acid is consumed by all } \textit{Synechocystis} \text{ strains studied in this investigation, and cultured under mixotrophic conditions. The } \textnormal{D-lactic acid dependent NAD(P)H generation in vitro, hence the LDH activity, is intact in the soluble fraction of the wild type cell extract but abolished in the soluble fraction of } \textit{ΔDSYN} \text{ cell extract. This leads to the speculation that } \text{Slr1556 could be a (cytosolic) D-lactic acid specific LDH. However, the } \textnormal{D-lactic acid consuming reaction during mixotrophic growth is not encoded by } \text{slr1556}, \text{ because the } \textit{ΔDSYN} \text{ deletion strain still consumes D-lactic acid. This } \textit{in vivo} \text{ finding is supported by our } \textit{in vitro} \text{ data which suggests that Slr1556 rather constitutes a pyruvate reductase, thus favouring the catabolic direction towards D-lactic acid. We could not verify significant activity with D-lactic acid with the purified enzyme. Strikingly, the activity of cell-free extract of the DLEU mutant strain (14.22 ±3.29 μmol/mg/min) is several orders of magnitudes higher than LDH activities observed in related studies (be it for L-lactic acid production; (133, 155). Interestingly, recently a LDH activity in the same range has been reported (i.e. 51.92 ± 2.31 μmol/mg/min) in a strain expressing the } \textit{DldhE} \text{ of } \textit{E. coli} \text{ K12, but lactic acid production data have not been incorporated in that manuscript (175). Here it can be concluded that the D-LDH of } \textit{L. mesenteroides} \text{ possibly does not function at } \text{V}_{\text{MAX}} \text{ conditions in the cyanobacterial cytoplasm, and/or that competing pathways in the cell interfere with D-lactic acid production. Nonetheless, considering the high activity of cell-free extract of the DLEU mutant strain, it is of interest to note that this is more than 6,000-fold higher than the actual maximal production rate achieved (compare Table 2) and more than 35-fold higher than the theoretical maximal carbon fixation rate (assuming a minimal doubling time of about 8 hours, thus considering a maximum CO}_2 \text{ fixation rate of 3.70 mmol/gDW/h (85) plus the fact that lactic acid is a C3 compound). Significantly, we have shown in the past that the LDH activity in a } \textit{Synechocystis} \text{ strain expressing the D-LDH of } \textit{E. coli} \text{ (Synechocystis strain SAA013) is about 14-fold higher than the activity of wild-type cell-free extract, but no D-lactic acid formation could be observed. The protein level of the D-LDH of } \textit{E. coli} \text{ is similar to the heterologous LDH amount of the mutant strains presented here (Figure S1). Interestingly, in the same study the LDH activity in the strain expressing the L-LDH of } \textit{B. subtilis} \text{ is 23-fold higher than wild type and is achieving L-lactic acid formation (133). Moreover, the LDH activities determined for } \textit{Synechocystis} \text{ strains expressing the L-LDH of } \textit{L. lactis} \text{ are in the very same range, and the } \text{log} \text{ of this activity correlates linearly with the } \text{log} \text{ of the production rate (155). For D-lactic acid formation, however, a huge overcapacity seems to be necessary, as evident from the activities observed here (Figure 4A).}} \)

The transient decrease in product formation, here resulting even in transient net product consumption, during the transition phase of a batch culture, remains an area for further investigation. Possible reasons for the transiently low production rate can be (i) the
shortage of the precursor (pyruvate) because of carbon limitation, (ii) light limitation due to self-shading of cells, (iii) changes in the co-factor availability and ratio, and (iv) a combination of all the limitations appearing at this stage of growth. Carbon limitation can lead to a rather sharp decrease in growth rate (208) and the re-direction of the carbon flux towards 2-oxoglutarate (209). The fixation of carbon can resume after the onset and full exploitation of the carbon concentration mechanism (210). Interestingly, a lowering or even a stagnation of production rate has been observed before for other products in batch cultures exiting exponential growth phase. Examples are the NAD(P)H-dependent formation of products like L-lactic acid (155), ethanol and meso-butanediol (129) in our lab. Also other groups report non-constant production rates in batch cultures; e.g. for ethanol (140) or 2,3-butanediol (148) production.

In a recent computational metabolic network analysis (31) based on a genome scale model of *Synechocystis*, the only annotated route involving lactic acid (from DHAP via methylglyoxal, (R)-S-lactoylglutathione, and lactic acid to pyruvate) does not show any flux in the conditions tested (continuous light and day/night cycle), and thus appears non-essential for (optimal) growth. Accordingly, in this scheme the metabolite lactic acid resides between pyruvate, linked by the (reversible) putative lactate dehydrogenase Slr1556, and (unidirectional towards lactic acid) (R)-S-Lactoylglutathione hydrolase a metabolic route that, in many organisms, is functioning as a glutathione dependent detoxifying mechanism for methylglyoxal. In *Synechocystis* the glyoxylases I (*slr0381*) and II (*sll1019*) are annotated in the genome (52) and have recently been studied showing their respective activity (211). Thus, this pathway could be considered a stress response pathway, only employed under specific conditions, and possibly not a pathway that carries significant amounts of carbon flux under physiological conditions. Significantly, based on the annotated unidirectional reaction towards lactic acid, Slr1556 has been hypothesized before to participate in a competing pathway for the production of alka(e)nes. However, the deletion of *slr1556* failed to show a clear benefit. Although an increase of alka(e)ne production was observed, this, however, was essentially achieved by gene duplication of the production pathway employed (151).

We can safely assume that a part of the produced D-lactic acid (by the heterologously expressed D-LDH in the DLEU and the DLEU/ΔDSYN strains) is utilized by the cells’ metabolism and will support growth, possibly through a similar route as extracellular D-lactic acid (be it that first import is necessary in the latter case). This may resemble a system that is more optimal for the continuous production of cultures growing in a diurnal regime. Typically, cyanobacteria grow during the light phase and biomass does not increase significantly in the dark phase. The D-lactic acid producing mutant might be able to sustain some growth during the night, leading to higher biomass build-up, which in turn may lead to higher volumetric production of D-lactic acid in the long run. The potential of cyanobacteria to utilize different sugars for growth has recently been investigated by genetically engineered *Synechococcus elongatus* PCC 7942 (212) in order to achieve growth in the dark period.
Here, D-lactic acid supports growth in *Synechocystis*, by a yet to be resolved pathway. Inferring from the investigations of the soluble fractions of the cell extract it can be concluded that the involved enzyme is not a soluble LDH, but might be a membrane bound LDH or a different enzyme. A possibility is that D-lactic acid is incorporated as a building block of the peptidoglycan layer (213). In *L. plantarum*, for example, the deletion of two lactate dehydrogenases has been shown to have a negative impact on peptidoglycan formation (214) indicating that at least in this lactic acid bacterium D-lactic acid is essential for efficient peptidoglycan biosynthesis.

### 6.5 Acknowledgements

We are thankful to Chris Olberts for his help with initial enzymatic activity assays. Further we are grateful to the members of the Molecular Microbial Physiology group for inspiring and helpful discussions. We want to thank Johan H. van Heerden, Lucas Stal and Hans Matthijs for helpful comments. We further want to thank Sérgio M. R. Domingos for the discussion on the topic of chirality. This project was carried out partially supported by the research program of BioSolar Cells, co-financed by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

### 6.6 Supplemental material

Supplemental material for this chapter may be requested per Email for personal use: angermayr@gmail.com.