Supporting Information

Linking Increased Isotope Fractionation at Low Concentrations to Enzyme Activity Regulation: 4-Cl Phenol Degradation by *Arthrobacter chlorophenolicus* A6

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SUMMARY

12 pages, 7 figures

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SI Table S6 – Biomass and yield measured at different dilution rates after achieving steady states in chemostat.

Supporting References

SI Materials and methods:

HPLC method for 4-CP concentration measurement

The eluents were acetonitrile, as well as water acidified with 1% of acetic acid. The initial gradient contained 10% acetonitrile (1 min) and was increased to 80% acetonitrile (2-6 min), after which the level was maintained for 3 min. The gradient was returned to 10% acetonitrile (8-13 min) with a post-time run (13-16 min). The injected sample volume was 20 µL. The flow rate was 0.7 mL $min^{-1,}$ and oven temperature was set to 45 °C. The compounds were detected by UV absorbance at 222 nm, and the peaks quantified using LabSolutions V 5.71 SP2 (Shimadzu Corp., Japan).

Estimation of growth kinetic parameters

Growth kinetics of *A. chlorophenolicus* A6 was described by Haldane kinetics[1]

$$\mu = \frac{\mu_{max} \cdot S}{(S + K_S) \cdot (1 + \frac{S}{K_I})} \quad (1)$$

where μ is the specific growth rate, S is the substrate (i.e., 4-CP) concentration, μ_{max} is the maximum growth rate, K_S is the substrate affinity and K_I is an inhibition constant. The three parameter K_s, K_I and μ_{max} were estimated from fitting the experimentally measured µ and S (i.e., 4-CP concentration) in batch experiments to the model (eq.1). The specific growth rate was calculated as

$$\mu = \frac{dC_x/dt}{C_x} \quad (2)$$

where C_x is the cell concentration. The model implementation, fitting parameter estimations and model analysis was performed using Python and employing the built-in functions in scientific libraries NumPy and SciPy[2]. The three parameters parameter K_s, K_I and μ_{max} in the Haldane kinetics model (eq.1) were estimated from the experimentally measured μ and S (4-CP concentration) data by minimizing the Root mean squared error (RMSE) as objective function.

$$\mathrm{RMSE} = \sqrt{\frac{\sum(\vartheta_{exp} - \vartheta_{sim})^2}{N}} \qquad (3)$$

The "brute force" optimization method was used to find the global minimum of the objective function to compute the objective function's value at each point of a multidimensional grid of points, to obtain the global minimum of the function. This multidimensional grid contained ranges of μ_{max} (0.002 to 0.9), K_i (1 to 100) and K_s (0.5 to 100) with linear grid space of 0.005 and 1, respectively. Thereafter, the result of "brute force" minimization was fed as initial guess to obtain a more precise (local) minimum using the downhill simplex algorithm[3].

EA-IRMS measurement for determination of reference values

4-CP (Sigma-Aldrich, Germany) used in the cultivation was characterized with elemental analyzer coupled with isotope ratio mass spectrometry (EA-IRMS), which was used as a standard for isotope analysis during ongoing degradation. A EuroEA (Euro Vector, Milano, Italy) was coupled with Finnigan MAT 253 IRMS via a FinniganTM ConFlow III interface (Thermo Fisher Scientific, Bremen, Germany). Calibration of standards was performed against the organic referencing materials USG 40 (L-glutamic acid), USG 41 (L-glutamic acid) and IAEA 600 (caffeine) provided by the International Atomic Energy Agency (IAEA, Vienna). $\delta^{13}C$ of 4-CP in per mil (‰) relative to PeeDee Belemnite (V-PDB) by using the following equation-

$$\delta^{13}C = \frac{\left(\frac{c^{13}}{c^{12}}\right)_x}{\left(\frac{c^{13}}{c^{12}}\right)_{ref}} - 1 \qquad (2)$$

Determination of $\delta^{13}C$ values was relative to the laboratory CO_2 monitoring gas, which was introduced at the beginning and the end of each analysis run. The laboratory CO_2 was calibrated to VPDB by reference CO_2 standard (RM8563) supplied by the IAEA. 4-CP samples were measured four times, and the instrumental isotope values of 4-CP $\delta^{13}C$ = -28.12‰± 0.03‰.

Method for analysis of the carbon isotope in 4-CP samples

The IRMS was set to a vacuum of 1.9×10^{-6} mbar, an accelerating potential of 9 kV and an emission energy of 2 mA. Helium grade 5.0 was used as a carrier gas with a flow rate of 1 mL min^{-1}. Liquid samples (volume in between 1 to 2 µL) were injected with a GC pal autosampler (CTC analytics). The injector was held at 200°C with a split ratio of 1:10. The GC program was as follows: start 70°C (hold 1 min), ramp 1 15°C/min to 130 °C (hold for 1 min), ramp 2 40°C/min to 220°C (hold for 3.5 min). GC-program: start 80°C (hold 4 min) → ramp 1 4°C/min to 150 °C (hold for 5 min)→ ramp 2 2°C/min to 200°C (hold for 1 min). A commercial ceramic tube/reactor with CuO/NiO/Pt-wire (Thermo Fisher Scientific, Bremen, Germany) operated at 940°C was used for isotope analysis. Prior to the isotope analysis, the reactor was oxidized at 940°C for 6 hours. Determination of $\delta^{13}C$ values for all the samples was relative to our laboratory CO_2 monitoring gas, as described earlier (eq.2).

Lipid extraction and membrane fatty acid analysis

A sample volume of 2 ml was centrifuged at 4°C for 10 min. The fatty acids were separated from the rest of the lipid by saponification with sodium hydroxide. To this end, the bacterial pellet was

treated with 1 mL of a solution consisting of 45 g of NaOH in 150 mL of methanol and 150 mL of MilliQ water. The mixture was vortexed briefly, heated in a water bath (100 °C, 5 min), vigorously vortexed for 5-10 seconds and heated again for another 25 min in the water bath (100 °C). To methylate the saponified free membrane fatty acids and generate FAMEs, 2 mL of a solution consisting of 6.0 N HCl and methanol in a volumetric ratio of 1.2:0.8 was added and after brief vortexing, the sample was heated at 80 °C (10 min) in the water bath again. After rapidly cooling the sample in running water, the generated FAMEs were extracted from the bacterial cell membrane by adding 1.25 mL of a mixture of hexane and diethyl ether (1:1, v/v) and gentle tumbling in a rotator (10 min). The aqueous lower phase (containing other cell components) was discarded. Subsequently, the organic phase was base-washed with at least 3.0 mL of a solution of 10.8 g NaOH in 900 mL H_2O. The sample was mixed again in a rotator for 5 min. Two third of the upper, organic phase containing FAME was transferred into a 1 mL GC Vial containing a 200 µL inlay and was analyzed using a GC-MS equipped with a split/ splitless injector (FinniganTrace Ultra and Trace DSQ, Thermo Electron Coorperation, Waltham, MA, USA). To separate the FAMEs, a CP-Sil 88 capillary column was used (from Agilent Technologies, Netherlands; length, 50 m; inner diameter, 0,25 mm; 0,20 µm film). The GC conditions were as follows: the detector temperature was held at 200 °C, and the injector temperature was held at 240 °C. The injection was splitless for 1 min, and the carrier gas was helium at a flow of 0.3 mL min^{-1}. The temperature program was as follows: 40 °C, 3 min isothermal; 6 °C min^{-1} to 220 °C and finally 4 min at 220 °C isothermal. The pressure program was as follows: 186 kPa, 2 min isobaric; 4 kPa min^{-1} to 310 kPa and finally 3 min at 310 kPa isobaric. To determine the relative amounts of FAMEs, the peak areas of the fatty acids in total were used.

Proteomics analysis

LC-MS/MS analysis was performed on a QExactive HF mass spectrometer (Thermo Fisher Scientific) online coupled to an Ultimate 3000 nano-RSLC (Thermo Scientific). The acquired spectra were loaded to the Progenesis QI software (version 4.0, Nonlinear Dynamics, part of Waters) for label-free quantification and analyzed as described previously [4]. All MS/MS spectra were exported as Mascot generic files and used for peptide identification with Mascot (version 2.6.2) in the UniProt *A. chlorophenolicus* A6 protein database (1479563 residues, 4608 sequences). Search parameters used were: 10 ppm peptide mass tolerance and 0.02 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation was set as fixed modification, methionine oxidation and asparagine or glutamine deamidation were allowed as variable modifications. A Mascot-integrated decoy database search calculated an average false discovery of 0.59 % when searches were performed with the mascot percolator algorithm and $p<0.05$. Peptide assignments were re-imported into the Progenesis QI software. Raw abundance data of all unique peptides allocated to each protein were normalized and summed up.

SI Figures:

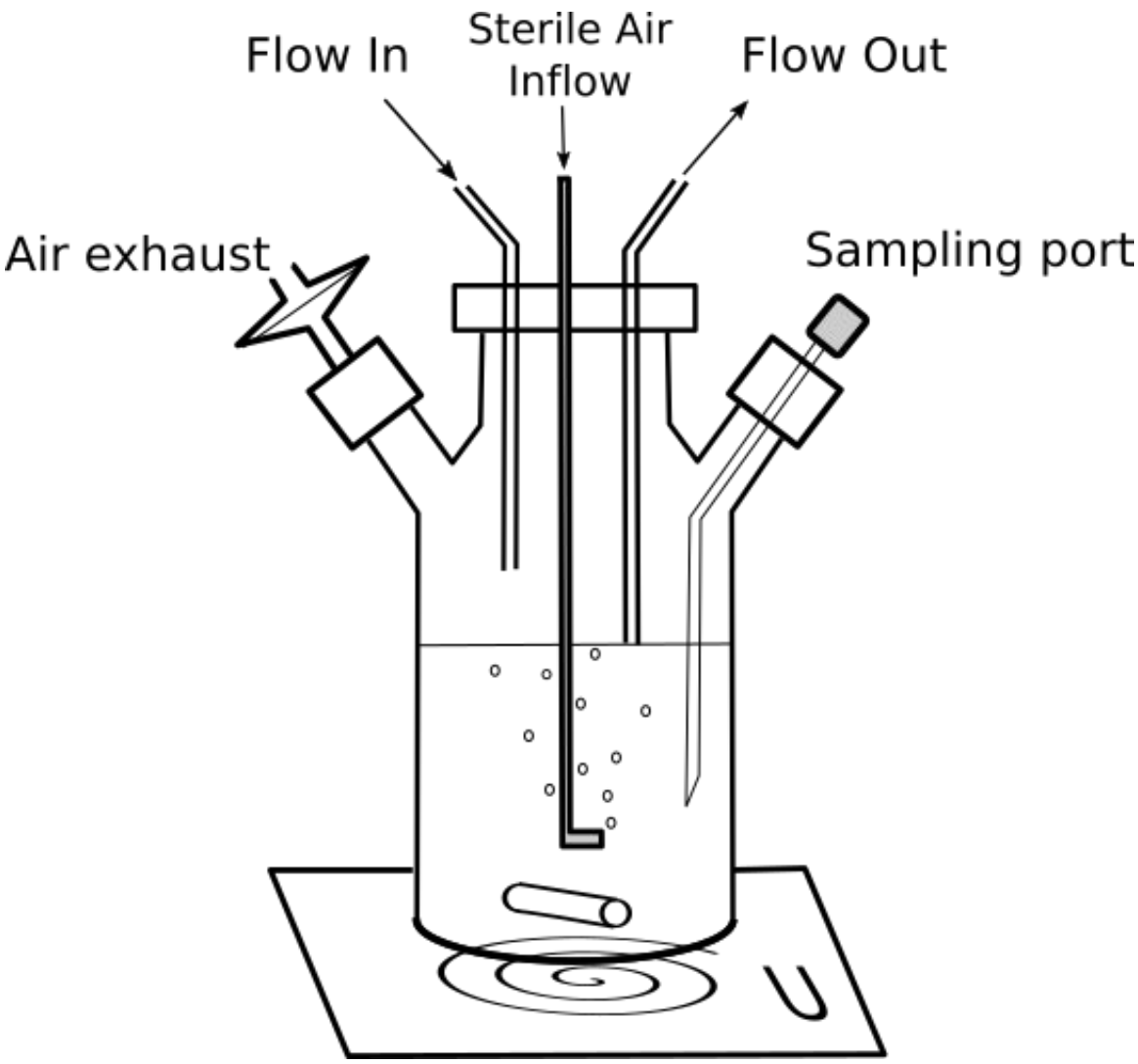

Figure S1: Schematic diagram of custom-made bioreactor used in this study.

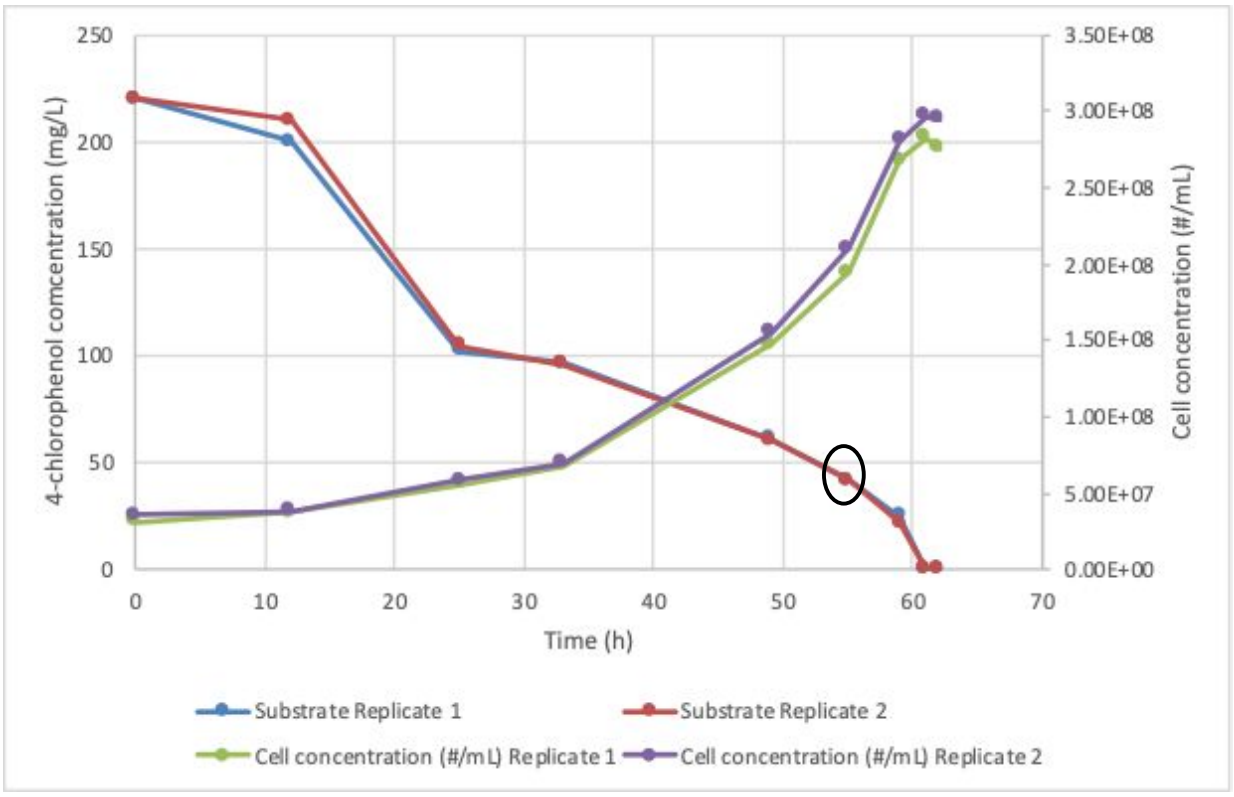

Figure S2: Degradation of 4 chlorophenol and cell concentration in batch cultivation experiment. After a lag phase of 12 hours, a slow increase in cell concentrations was observed at ~100 mg L^{-1} 4-CP (transition lag phase), whereas growth became rapid (exponential phase) at lower 4-CP concentrations of ~ 40 mg L^{-1}.The circle denotes the sampling point for fatty acid and proteome analysis.

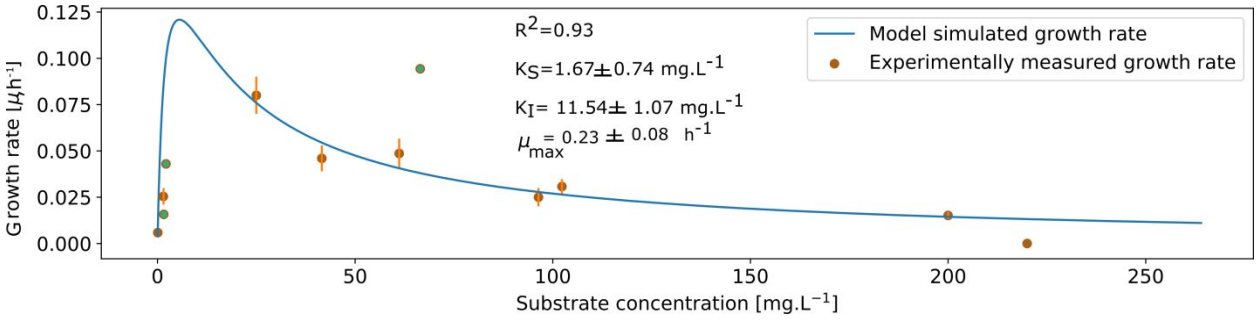

Figure S3: The observed specific growth rate at different residual 4-CP concentrations in batch was modelled using Haldane inhibition[1] kinetics. The symbols indicate observed growth rate (μ, calculated according to eq.2) based on cell concentrations and residual substrate (4-chlorophenol) concentration in batch cultivation. The line indicates μ according to Haldane inhibition kinetics model (eq. 1) using fitted growth parameters of (μ_{max}), Monod affinity constant (K_s) and

158 inhibition constant (K_I) in batch cultivation. Data points represent the mean ± standard deviation
159 of samples. The fitted kinetic parameters imply that 4-CP concentrations above K_I ~ 12.0 mg L^{-1}
160 (0.09 mM) reduce growth of *A. cholorophenolicus* A6. Symbols in green indicate growth rates
161 observed in chemostat cultivations (Fig.1, see the main manuscript).

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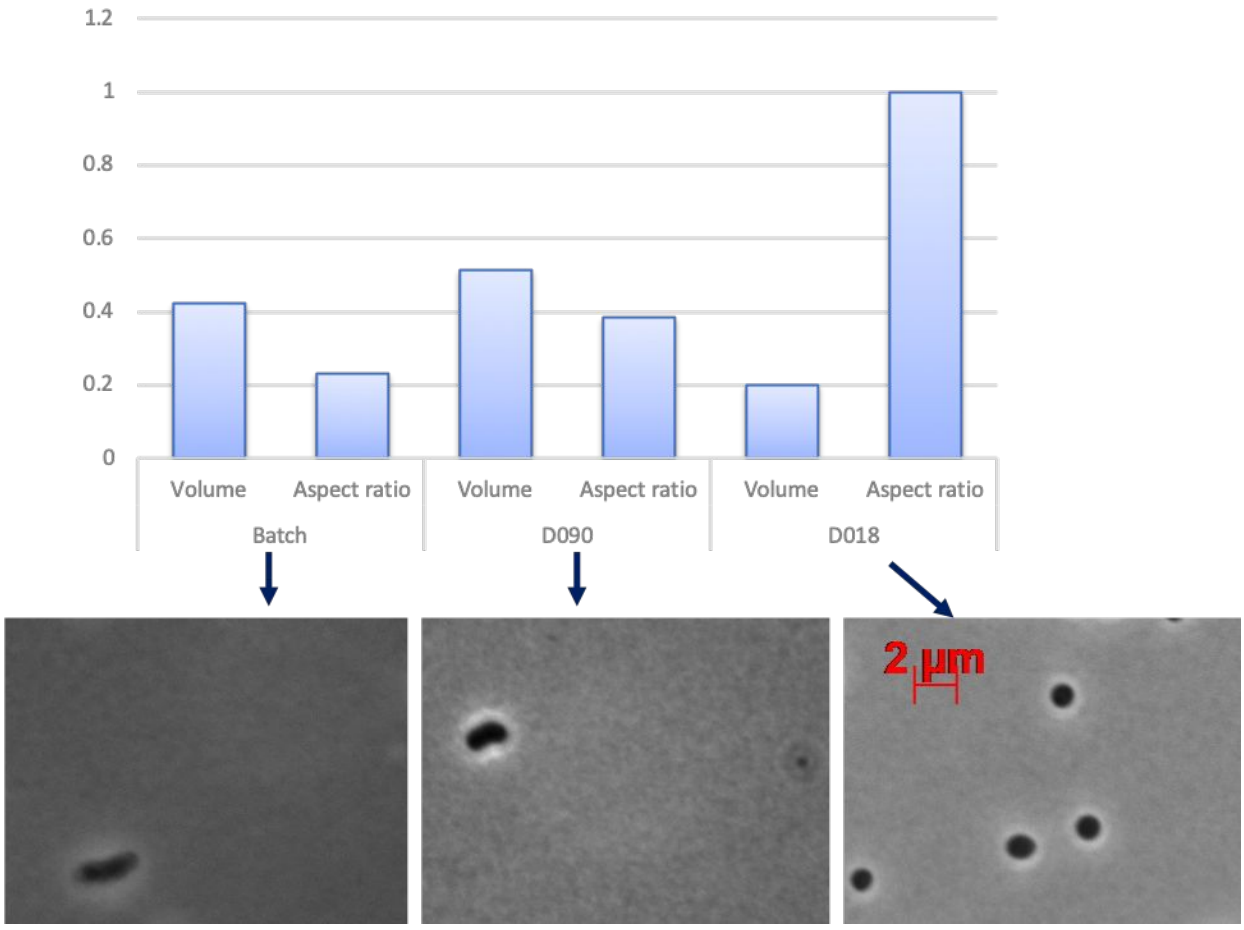

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165 **Figure S4:** .Change in morphology at different dilution points in chemostats and batch. D090 and
166 D018 denote dilution rate of 0.090 and 0.018 h^{-1}, respectively.

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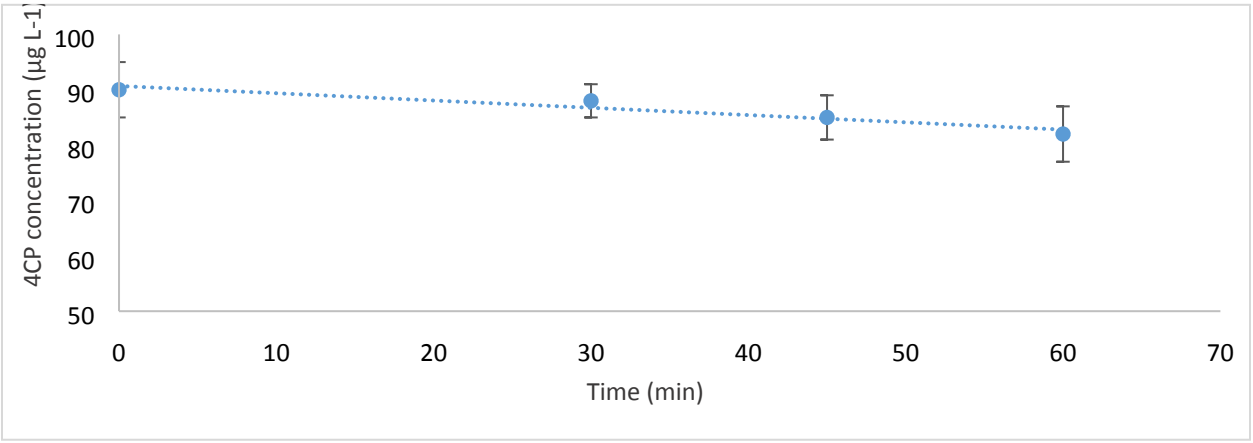

Figure S5: Degradation profile of 4-chlorophenol with an effluent concentration of 95 µg L^{-1} in the chemostat under no addition of feed shows slow enzymatic turn-over.

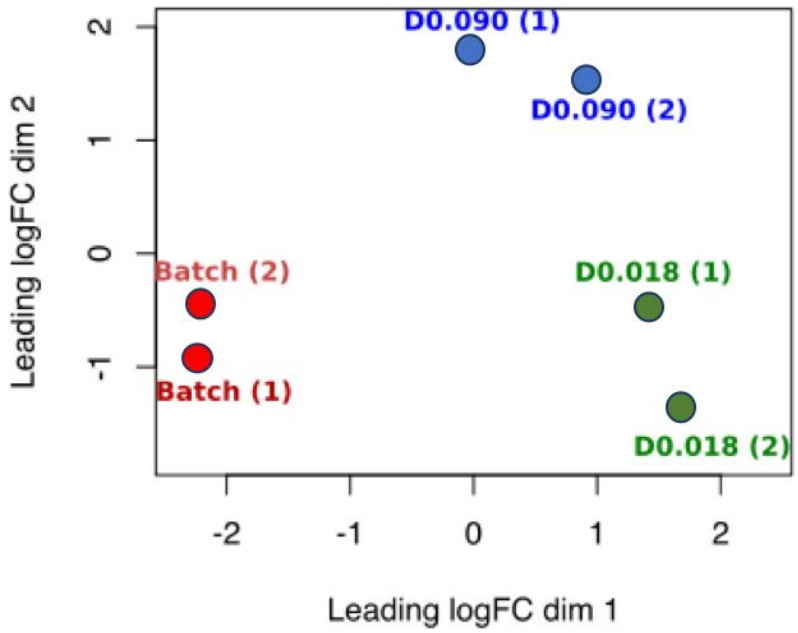

Figure S6: Non-parametric clustering (NMDS) of all conditions used for proteomics analysis. The biological replicates are placed closer on the plot, which shows similarity, hence, can be used for further analysis. The similar clustering between biological replicates are shown in Fig.S6 based on

Euclidean distance. The correlation coefficient was similarly high in biological replicates (0.9 for Batch, D0.018 and 0.7 for D0.090)

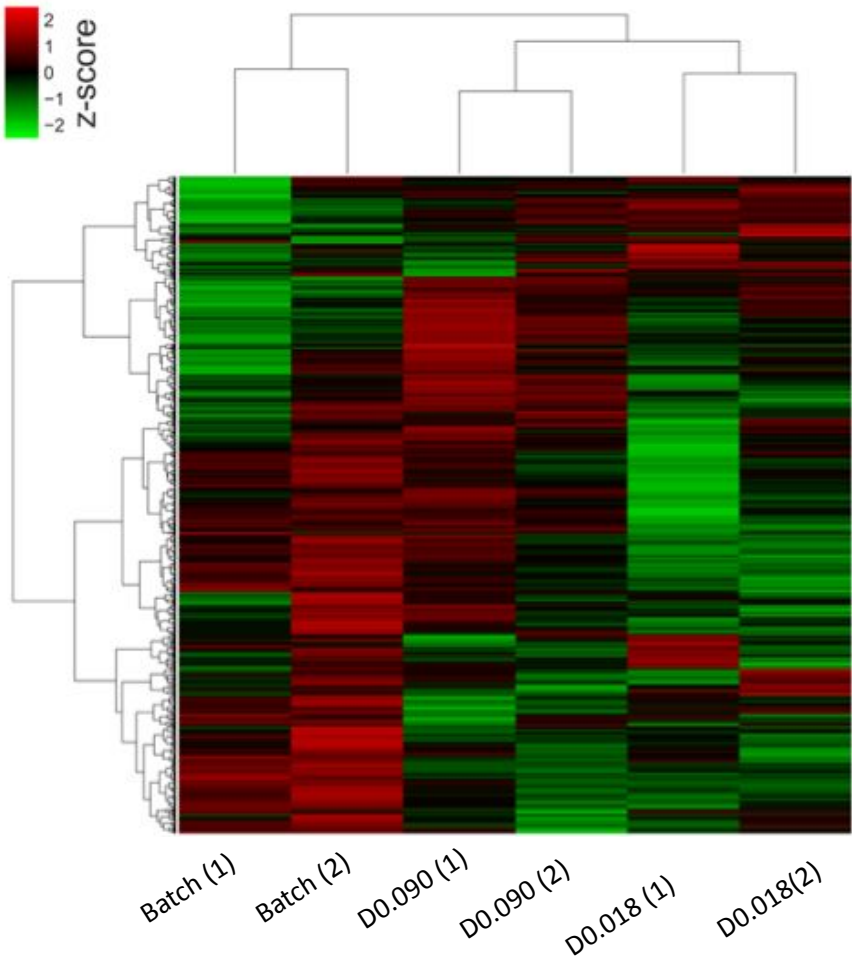

Fig. S7: Heat map representing the clustering of quantified proteins (in total 1201) in all samples - Batch, D0.018 and D0.09. Protein abundance is displayed in the heat map as z-scores (i.e., calculated based on how many SD units a protein's abundance is away from the mean abundance derived from all conditions) in the range between 2 (of significantly higher abundance, red) and -2 (of significantly lower abundance, green). Each batch and chemostat cultivation was performed in replicates as indicated by dilution rates in the brackets below the heat map.

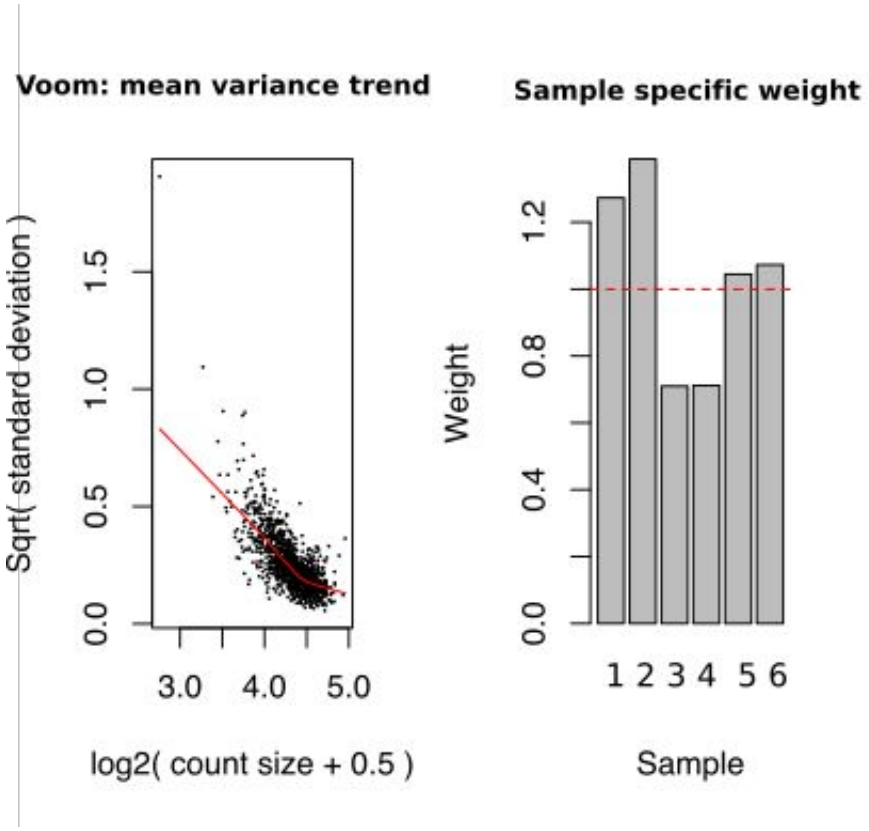

Figure S8: Voom transformation of the proteomics data. Mean variance trend in the data is shown (left panel) and down weight low-intensity observation by implying sample-specific weight (right panel). 1,2: Batch replicates, 3,4 : two replicates for chemostat at D 0.018 h^{-1} and 5,6: two replicates for chemostat at D0.090 h^{-1}.

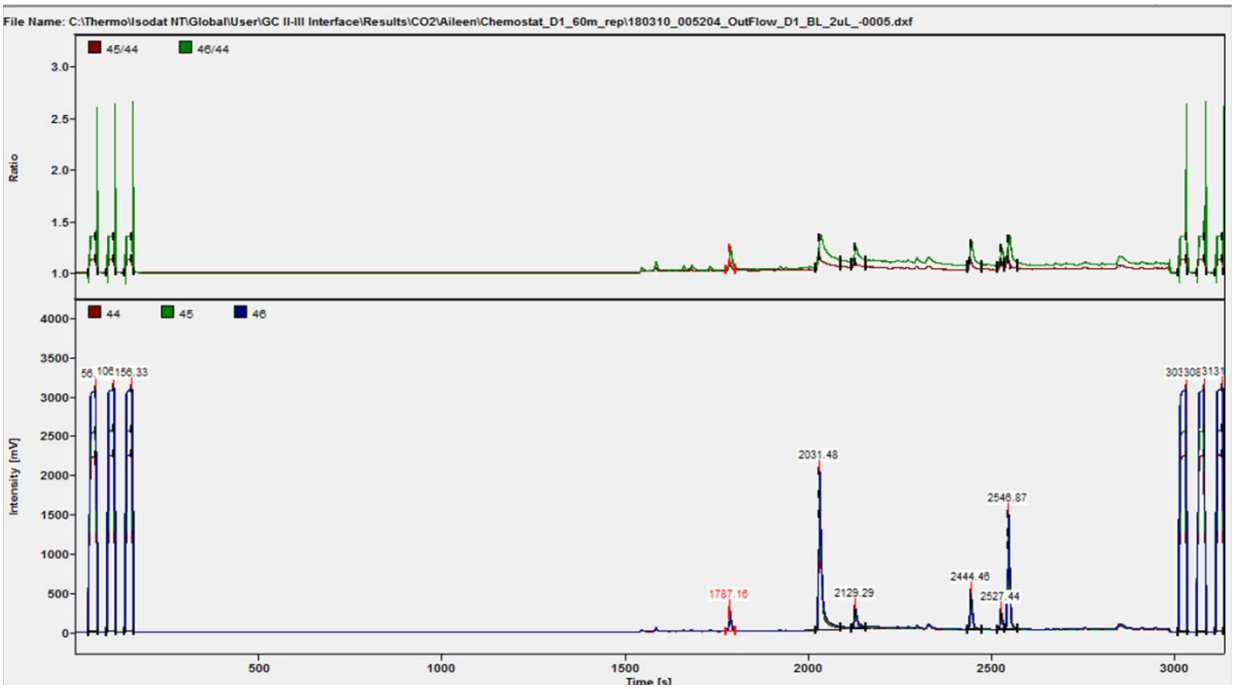

Figure S9: IRMS chromatograms from measurements of 4-Cl phenol extracts of 90 µg/L from chemostat samples. The 4-Cl peak appears at a retention time of 2031 seconds.

SI Tables

Table S6 – Biomass and yield measured at different dilution rates after achieving steady states in chemostat.

Dilution rate (h^{-1})	Biomass (mg L^{-1})	Yield (mg-Biomass^{-1} mg-substrate)
0.018	6.012	0.0273
0.038	5.377	0.0245
0.090	1.933	0.0146

REFERENCES

(1) Haldane, J. B. S. Enzymes. 1930. *Repr. by MIT, Cambridge* **1965**.

(2) Oliphant, T. E. Python for Scientific Computing. *Comput. Sci. Eng.* **2007**, *9* (3), 10–20. https://doi.org/10.1109/MCSE.2007.58.

(3) Nelder, J. A.; Mead, R. A Simplex Method for Function Minimization. *Comput. J.* **1965**, *7* (4), 308–313. https://doi.org/10.1093/comjnl/7.4.308.

(4) Merl, J.; Ueffing, M.; Hauck, S. M.; von Toerne, C. Direct Comparison of MS-Based Label-Free and SILAC Quantitative Proteome Profiling Strategies in Primary Retinal Müller Cells. *Proteomics* **2012**, *12* (12), 1902–1911.