Supplementary Material

Conversion of cyclohexane to 6-hydroxyhexanoic acid using recombinant *Pseudomonas taiwanensis* in a stirred-tank bioreactor

1 Supplementary Data

1.1 Molecular biology methods

The preparation of electrocompetent *Pseudomonas* cells was performed according to Choi *et al.* (Choi and Schweizer, 2006), and the vectors were introduced by electroporation (2500 V, Eppendorf Eporator®, Hamburg, Germany). DNA manipulation methods and agarose gel electrophoresis were performed as described by Sambrook and Russel (Sambrook and Russell, 2001). Enzymes (Phusion High-Fidelity Polymerase, T5 exonuclease, *Taq* ligase, restriction enzymes, Fast Alkaline Phosphatase) and buffers were purchased from Thermo Scientific Molecular Biology (St. Leon-Rot, Germany) or New England Biolabs (Frankfurt/Main, Germany) and oligonucleotides from Eurofins Genomics (Ebersberg, Germany). Plasmids were isolated using the peqGOLD Plasmid Miniprep Kit I from peqLab (Erlangen, Germany) and purified via NucleoSpin Gel and PCR Clean-up from Macherey–Nagel (Düren, Germany) according to supplier protocols. The Gibson Master Mix was prepared according to Gibson *et al.* (Gibson et al., 2009).

1.2 Plasmid construction

Acidovorax sp. CHX100 was cultivated for 4 days in nutrient broth (NB) medium (Sambrook and Russell, 2001) for DNA isolation. The lactonase gene was amplified with the primers PLS017 and PLS018 (Table S2), Gibson Assembly of the resulting fragment and HindIII-digested pSEVA244_T (Schäfer et al., 2020b) gave rise to pSEVA_Lact.

1.3 Semi-dry Western blot analysis

Cell disruption was performed by 3 passes through a French press (Thermo Electron Corporation, Waltham, MA/USA). The protein concentration was determined via Bradford assay (Bradford, 1976), and 20 µg total protein amount were loaded on a SDS-PAGE gel including a 3 µL Strep-Tag protein ladder (iba Lifesciences, Göttingen, Germany). One nitrocellulose membrane (NitroBind, 0.45 µm, 200x200 mm, GVS Filter Technology, Bologna, Italy), six Whatman papers, and the SDS-PAGE gel were pre-incubated in transfer buffer for 5 min at 4°C. The transfer sandwich was assembled as follows: 3 Whatman papers, membrane, SDS page, 3 Whatman papers. The transfer was performed for 30 min at 44 mA and 5 V. Afterward, the membrane was blocked with 20 mL 5 % BSA in T-TBS buffer for 1 h at 4°C. Biotin blocking was performed by adding 20 µL biotin to the blocking solution and incubation for 10 min at 4 °C. One µl Strep-Tactin antibody solution (iba Lifesciences) was added followed by overnight incubation at 4 °C. Five washing steps were performed with T-TBS for 5 min

each. The membrane was imaged via the Fluor Chem FC3 imaging system (ProteinSimple, San Jose, CA/USA) after 5 min incubation in WesternBright ECL (Advansta, San Jose, CA/USA).

2 Supplementary Figures and Tables

2.1 Supplementary Tables

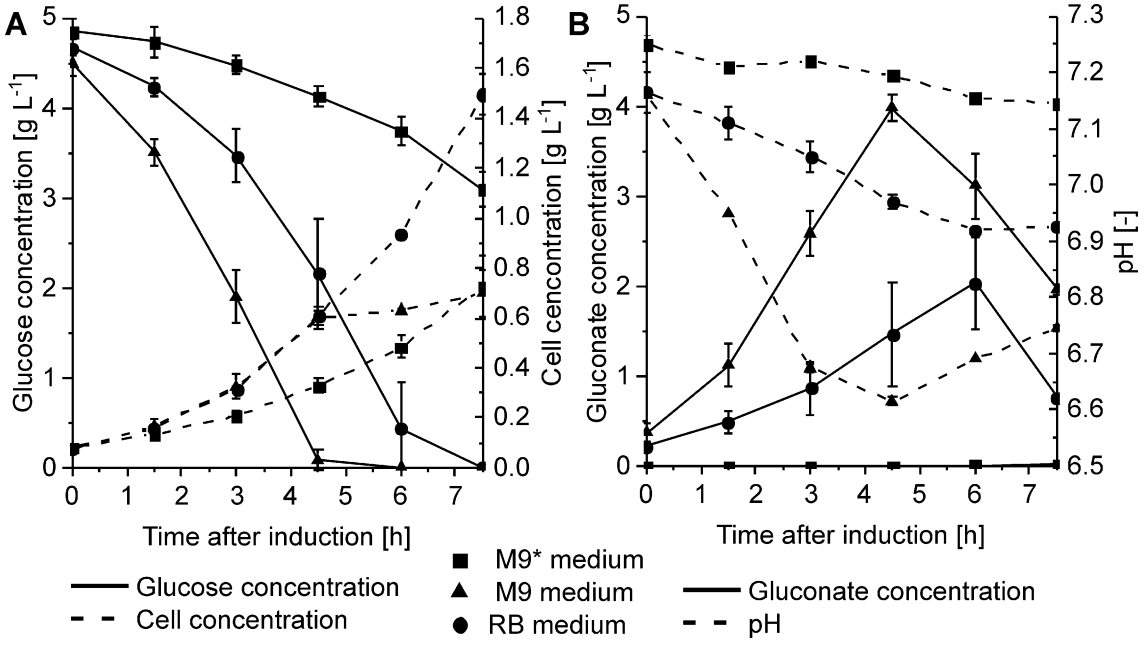
Table S1: Strains and plasmids used in this study.

	Characteristics	**Reference**
Strains		
E. coli DH5α	*sup*E44Δ*lacU*169(Φ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	(Hanahan, 1983)
P. taiwanensis VLB120	solvent tolerant, styrene degrading bacterium, isolated from forest soil	(Köhler et al., 2013)
Plasmids		
pSEVA_Cyp	pRO1600 and ColE1 ori, *lac*-regulatory system (*lacI*^{q}, P_{trc}), BBa_B0015 terminator, RBS*, Cyp genes from *Acidovorax* sp.	(Schäfer et al., 2020b)
pSEVA_CDH	pRO1600 and ColE1 ori, lac-regulatory system (lacI^q, Ptrc), BBa_B0015 terminator, RBS*, CDH gene from *Acidovorax sp.*	(Schäfer et al., 2020a)
pSEVA_CHMO	pRO1600 and ColE1 ori, *lac*-regulatory system (*lacI*^{q}, P_{trc}), BBa_B0015 terminator, RBS*, CHMO gene from *Acidovorax* sp.	(Schäfer et al., 2020a)
pSEVA_Lact	pRO1600 and ColE1 ori, *lac*-regulatory system (*lacI*^{q}, P_{trc}), BBa_B0015 terminator, RBS*, Lact gene from *Acidovorax* sp.	This study
pSEVA_6HA_2	pRO1600 and ColE1 ori, *lac*-regulatory system (*lacI*^{q}, P_{trc}), BBa_B0015 terminator, RBS*, Cyp genes, P_{trc}, *cdh*, *chmo*, *lact* (all with RBS*)	(Schäfer et al., 2020a)

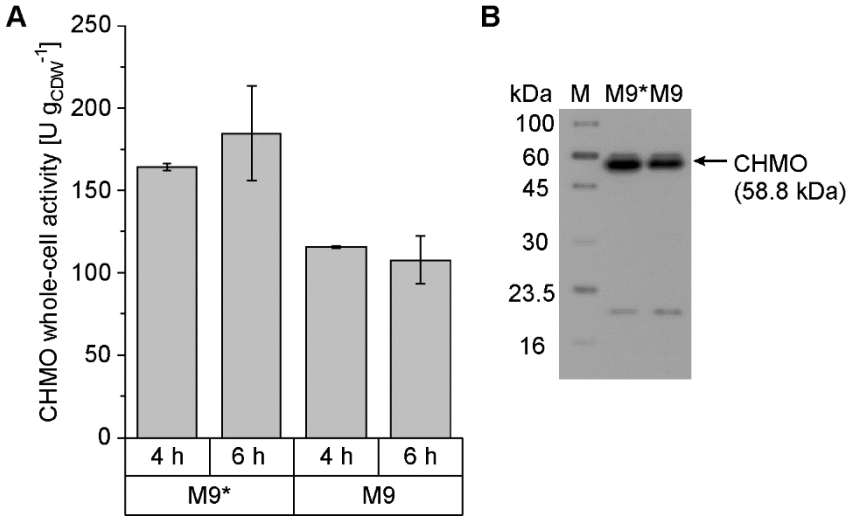
Table S2: Primers used for cloning with indicated **fragment binding region,** overlap to vector, and RBS

Primer#	Function	Sequence
PLS017	Lact fwd	TCTAGAGTCGACCTGCAGGCATGCATAGTGGAGGTTACTAG**ATGGGCACCTCACCCCAATC**
PLS018	Lact rev	TTTTCCCAGTCACGACGCGGCCGCA**TCAGGCGCGCTTGAACCAC**

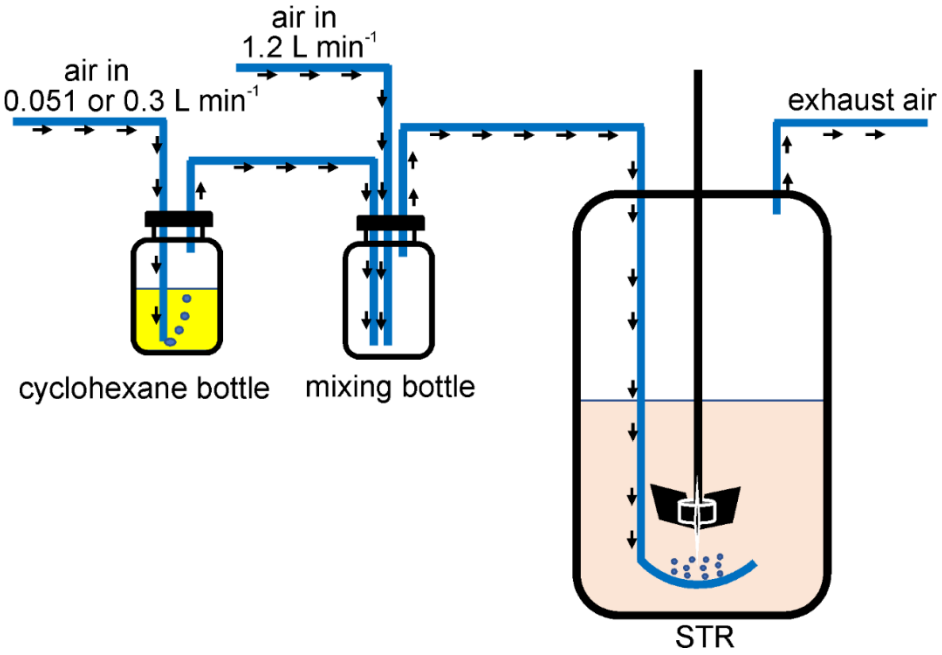
2.2 Supplementary Figures



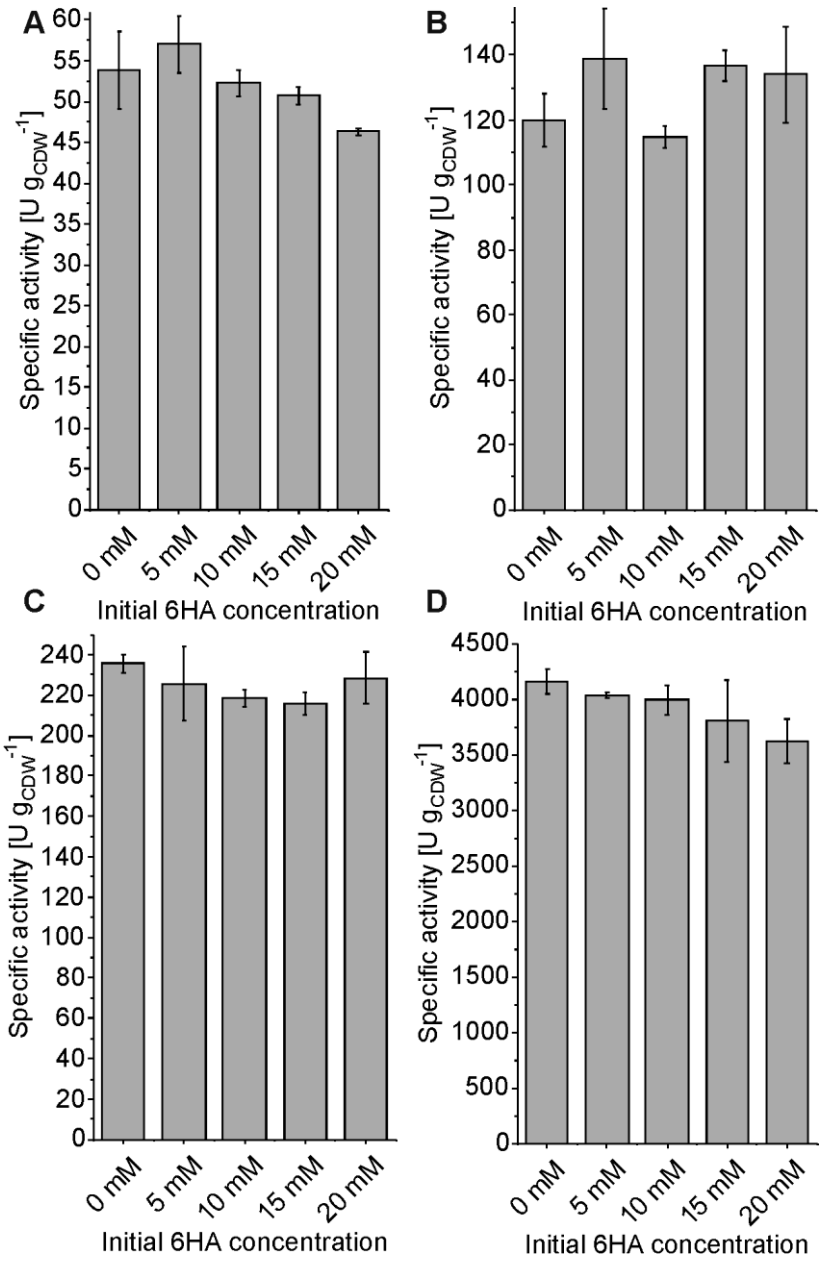
Supplementary Figure S1: Physiological characterization of *P. taiwanensis*_6HA. Cells were cultivated in M9* (square), M9 (triangle), or RB medium (circle) containing 0.5% (w/v) glucose and were induced by IPTG at OD_{450} ~0.5. (A) Time courses of glucose and cell concentration after induction. (B) Time courses of gluconate concentration and pH after induction.



Supplementary Figure S2: Comparison of CHMO activity (A) and protein amount (B) in M9- and M9* grown *P. taiwanensis* VLB120 (pSEVA_CHMO). (A) Cells were cultivated in M9* or M9 medium containing 0.5% (w/v) glucose and were harvested after 4 or 6 h of induction by IPTG. After resuspension in Kpi-buffer containing 1% (w/v) glucose to a biomass concentration of 0.25 g_{CDW} L^{-1}, 1 mL liquid volume was transferred to a Pyrex tube and equilibrated for 10 min at 30°C. Reactions were started by adding 12 mM cyclohexanone and stopped after 10 min. Graph represents average values and standard deviations of two independent biological replicates. The average experimental error over all activity measurements is 7.6%. (B) Cell samples for Western blot analysis were taken after 6 h of induction and disrupted via French press. 20 µg total protein were loaded. CHMO was detected via its C-terminal Strep-tag.



Supplementary Figure S3: Stirred-tank bioreactor (STR) setup for continuous cyclohexane feed via the gas phase. Pressurized air with a defined flow rate (0.051 or 0.3 L min^{-1}) is bubbled through a cyclohexane-filled bottle to obtain a cyclohexane-saturated air stream, which is subsequently mixed in the mixing bottle with the main air stream (1.2 L min^{-1}). By varying the flow rate of the cyclohexane-saturated air stream, defined gas phase cyclohexane concentrations can be directed into the STR.



Supplementary Figure S4: Effect of 6HA on specific Cytochrome P450 monooxygenase (A), cyclohexanol dehydrogenase (B), cyclohexanone monooxygenase (C), and lactonase (D) activity. Cells were cultivated in M9* medium containing 0.5% (w/v) glucose and harvested after 6 h of induction by IPTG. Cells were resuspended to a biomass concentration of 0.25 g_{CDW} L^{-1} in Kpi-buffer containing 1% (w/v) glucose and defined concentrations of 6HA, of which 10 mL were transferred into 100 mL screw-capped Erlenmeyer flasks (A, D) or 1 mL into 2 mL reaction tubes (B, C). After equilibration for 10 min at 30°C, reactions were started by adding pure cyclohexane to a concentration of 5 mM with respect to the aqueous phase volume (A), 5 mM cyclohexanol (B), 5 mM cyclohexanone (C), or 10 mM ε-caprolactone (D) and stopped after 10 min (A) or 5 min (B, C, D). Graphs represent average values and standard deviations of two independent biological replicates. The average experimental errors over all activity measurements is 4.9%.

3 References

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