SOPs for the operation of the capillary biofilm reactor available for the PROMICON consortium
Deliverable 4.3

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PROMICON

Harnessing the power of nature through PROductive MIcrobial CONsortia in biotechnology - measure, model, master

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1. Introduction

In industrial biotechnology utilizing whole-cell biocatalysts, most processes rely on mono-septic suspended cultures of the respective production strains. This leads to challenges regarding contaminations and also ongoing evolution or the formation of subpopulations, which negatively impact space-time-yields. Mutually dependent co-cultures can stabilize each other and lead to overall better production. Also, in contrast to suspended cultures it is possible to grow the organisms in a surface attached format, termed biofilm, which represent a very common microbial life style.[1] In biofilms organisms grow in tightly packed aggregates that are often attached to surfaces. Upon attachment these microbes start to excrete extracellular polymeric substances (EPS), mainly composed of carbohydrates, proteins and extracellular DNA, which serve as protection shield and concrete, giving biofilms a three dimensional structure.[2] Hence, biofilms are naturally immobilized high cell density biocatalysts that can be operated in continuous processes in which the substrate is supplied constantly to the biocatalyst.[3]

Here, we strive to establish an artificial microbial consortium, which grows as biofilm and is capable of producing hydrogen in a continuous mode. The most important strain in this consortium is *Rhodopseudomonas palustris* (*R. palustris*), which under redox limiting conditions (anaerobic cultivation) produces hydrogen, while growing on organic acids like acetate or lactate and thus serves as labor strain. *R. palustris* belongs to the Gram-negative purple non-sulfur bacteria and is well known for its exceptional metabolic versatility and robustness, and its ability to flexibly switch between these modes of metabolism depending on the environmental conditions.[4]

R. palustris is cultivated photoheterotrophically under anaerobic and nitrogen limiting conditions. Hence, the strain utilizes light for energy, and obtains carbon and electrons from the organic acids, while fixing nitrogen in a nitrogenase catalyzed reaction. As a side product, hydrogen is produced in reasonable amounts, depending on the specific reaction conditions.

This organism was teamed up with *Pseudomonas taiwanensis* VLB120 as a balancer strain (see also Del 3.7). *P. taiwanensis* is a chemoheterotrophic organism, which is growing aerobically and is well known for its excellent biofilm forming abilities. We hypothesized, that *P. taiwanensis* on the one hand respires the excess oxygen produced by the cyanobacterial farmer, while triggering and improving biofilm formation of laborer and farmer organism. Similar findings have already been reported for a different reaction system.[5]

For the cultivation of the proposed consortium a reactor system had to be developed that meets the consortiums and the reaction needs. Most important is the gas tightness, as our product H_2 is very volatile and escapes easily through many materials and connectors and is only produced under anaerobic conditions. On the other hand, a transfer to outdoor conditions of the reactor system is important for the

[1] Flemming, H.-C. & Wuertz, S. Bacteria and archaea on Earth and their abundance in biofilms. Nat Rev Microbiol (2019)17: 247
[2] The biofilm matrix: multitasking in a shared space. Nat Rev Microbiol (2023) 21:70
[3] Philipp, L.-A., Bühler, K., Ulber, R. & Gescher, J. Beneficial applications of biofilms. Nat Rev Microbiol *accepted*
[4] Brown, B., Wilkins, M., Saha, R. *Rhodopseudomonas palustris:* A biotechnology chassis. Biotechnol Adv (2022) 60: 108001
[5] Hoschek, A., et al. Mixed-species biofilms for high-cell-density application of *Synechocystis* sp. PCC 6803 in capillary reactors for continuous cyclohexane oxidation to cyclohexanol. Bioresour Technol (2019) 282:171

use of sunlight as energy source for a cost-efficient production system. In the following the capillary biofilm reactor (CBR) is described and a standard operation procedure is reported.

2. Methodology

2.1 Strains used in this study

Species	Strain	References
Rhodopseudomonas palustris	DSM127	Malik et al. (1987)[6]
Pseudomonas taiwanensis	VLB120_eGFP	Heuschkel, Hoschek et al. (2019)[7]

2.2 Growth media used

The following media were used for batch cultivation of *R. palustris* (R8AH-H2), batch cultivation of *P. taiwanensis* (M9-Medium) and biofilm cultivation (M27-H2). All media are prepared under sterile conditions.

Table 2: R8AH-H_2 Medium for cultivation of *R. palustris*

Component	**Formula**	**M [g/mol]**	**Conc. [g/L]**	**Volume [mL/L]**
Sodium acetate	$C_2H_3NaO_2$	82.03	1	
Yeast extract	$C_{19}H_{14}O_2$	319.19	1	
Magnesium sulfate	$MgSO_4$ x 7 H_2O	246.48	0.2	
Calcium chloride	$CaCl_2$ x 2 H_2O	147.01	0.07	
Di-sodium EDTA dihydrate	$C_{10}H_{14}N_2Na_2O_8$ x $2H_2O$	372.21	0.023	
Potassium Dihydrogen Phosphate	KH_2PO_4	136.09	0.6	
Di-Potassium hydrogen phosphate	K_2HPO_4	174.18	0.9	
Fe(III) citrate solution	$C_6H_5FeO_7$	244.94	0.01	
R8AH Trace element solution				1.0
R8AH Viatmin solution				7.5

R8AH Trace element solution				
Iron(III)citrate	$C_6H_5FeO_7$	244.94	3	
Manganese sulfate monohydrate	$MnSO_4$ x H_2O	169.02	0.02	
Boric acid	H_3BO_3	61.83	0.01	
Copper sulfate pentahydrate	$CuSO_4$ x 5 H_2O	249.68	0.01	

[6] Malik, K.A., et al. A survey of hydrogen production, nitrogen fixation and hydrogen metabolism in Rhodospirillaceae. In: Neijssel,O.M. (eds). Proc. 4th European Congress on Biotechnology. (1987) 3: 558; Elsevier, Amsterdam

[7] Heuschkel, I. et al. Data on mixed trophies biofilm for continuous cyclohexane oxidation to cyclohexanol using *Synechocystis* sp. PCC 6803. Data Brief (2019) 25: art. 104059

Ammonium molybdate tetrahydrate	$(NH_4)_6Mo_7O_{24}$ x 4 H2O	1235.86	0.02
Zinc sulfate heptahydrate	$ZnSO_4$ x 7 H_2O	287.60	0.018
EDTA	$C_{10}H_{16}N_2O_8$	292.24	0.5
Calcium chloride	$CaCl_2$ x 2 H_2O	147.01	0.2

R8AH Vitamin solution			
Nicotinic acid (B3)	$C_6H_5NO_2$	123.11	0.2
Nicotinamide	$C_6H_6N_2O$	122.12	0.2
Thiamin-HCl dihydrate (B1)	$C_{12}H_{22}Cl_2N_4O_3S$	373.30	0.4
Biotin (B7)	$C_{10}H_{16}N_2O_3S$	244.31	0.008

Table 3: M27-H_2 Medium for the cultivation of dual species biofilms.

Component	**Formula**	**M [g/mol]**	**Conc. [g/L]**	**Volume [mL/L]**
Yeast extract	$C_{19}H_{14}O_2$	319.193	0.3	
Sodium acetate	$C_2H_3NaO_2$	82.0343	1	
Potassium dihydrogen phosphate	KH_2PO_4	136.086	0.5	
magnesium sulfate	$MgSO_4$ x 7 H_2O	246.48	0.4	
Sodium choride	NaCl	58.443	0.4	
Calcium chloride	$CaCl_2$ x 2 H_2O	147.01	0.05	
Fe(III) citrate solution	$C_6H_5FeO_7$	244.944	1	
Viatmin B12-solution	$C_{63}H_{88}CoN_{14}O_{14}P$	1355.38	0.1	
L-Cysteiniumchloride	$C_3H_{10}ClNO_3S$	175.64	300	
Trace element solution SL-6				1

Trace element solution SL-6				
Zinc sulfate heptahydrate	$ZnSO_4$x 7 H_2O	287.60	0.1	
Mangan(II)chloride tetrahydrate	$MnCl_2$x 4 H_2O	197.91	0.03	
Borate	H_3BO_3	61.83	0.3	
Cobalt chloride hexahydrate	$CoCl_2$x 6 H_2O	237.93	0.2	
Copper chloride dihydrate	$CuCl_2$x 2 H_2O	170.48	0.01	
Nickel chloride hexahydrate	$NiCl_2$x 6 H_2O	237.69	0.02	
Sodium molybdate	Na_2MoO_4x 2 H_2O	241.95	0.03	

For the cultivation in the optimized CBR system, the medium is split into carbon source and salt medium. Both are two times concentrated.

Table 4: M9-Medium for the cultivation of *P. taiwanensis*.

Component	**Formula**	**M [g/mol]**	**Conc. [g/L]**	**Volume [mL/L]**

10x M9 salt solution				100
Magnesium sulfate (1M)	$MgSO_4$	120.37		2
US* trace elements solution				1
Thiamine (1 % w/v)	$C_{12}H_{17}N_4OS^+$	265.36		1
Glucose (50% w/v)	$C_6H_{12}O_6$	180.16		4
Water	H_2O	18.02		982

10x M9 salt solution

Disodium hydrogen phosphate heptahydrate	Na_2HPO_4 x 7 H_2O	268.07	128.00	
Potassium dihydrogen phosphate	KH_2PO_4	136.09	30.00	
Sodium chloride	NaCl	58.443	5.00	
Ammonium chloride	NH_4Cl	53.49	10.00	

US* trace elements solution

Hydrochloric acid (37 % w/v)	HCl	12.02		83.20
Iron (II) sulfate dihydrate	$FeSO_4$ x 2 H_2O	187.94	4.87	
Calcium chloride dihydrate	$CaCl_2$ x 2 H_2O	147.01	4.10	
Mangan chloride tetrahydrate	$MnCl_2$ x 4 H_2O	197.91	1.50	
Zinc sulfate	$ZnSO_4$	161.47	1.05	
Borate	H_3BO_3	201.22	0.30	
Disodium molybdate dihydrate	Na_2MoO_4 x 2 H_2O	241.95	0.25	
Copper chloride dihydrate	$CuCl_2$ x 2 H_2O	134.45	0.15	
Disodium EDTA dihydrate	Na_2EDTA x 2 H_2O	372.24	0.84	

3. Standard operation procedure CBR:

SOP for the CBR will be described in 3 consecutive steps, the preparation of the inoculum, the reactor assembly and the reactor run.

1. **Preparation of the inoculum:**
    a. Cultivation of *R. palustris* preculture:
        1. For preculture 1 mL of *R. palustris* cryo culture is inoculated into 50 mL R8AH-H2 Medium in 120 mL serum flask filled with N_2 gas.
        2. Cultivation was performed at 30 °C, 50 µE m^{-2} s^{-1} for 6 days.
        3. From these precultures inoculation cultures in 10 mL M27-H2 medium with an OD_{650}=2 were prepared 12 h before inoculation and cultivated like the preculture.
    b. Cultivation of *P. taiwanensis* preculture:
        1. For the first preculture 5 mL LB medium are inoculated with an inoculation loop of a *P. taiwanensis* preculture and cultivated at 30 °C, 200 rpm (2.5 cm amplitude) over night.

2. 10 mL M9 medium are inoculated with 200 µL of the first preculture and cultivated at 30 °C, 200 rpm (2.5 cm amplitude) for 24 h.
3. 10 mL M9 medium are again inoculated with 200 µL of step 1.b.2 and cultivated for 6-8 h at 30 °C, 200 rpm (2.5 cm amplitude)
4. 10 mL M27-H2 medium are inoculated with an OD_{450}=2 12 h before inoculation and cultivated like described for the precultures.

2. Reactor assembly:

For running two CBR's the use of two 1L split medium bottles and one 2L waste bottle is recommended. Schematic overview figure 1.

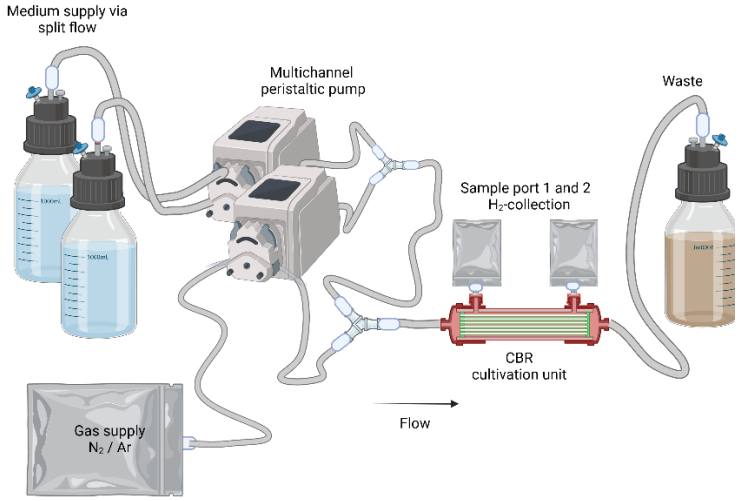

Figure 1: Simplified schematic overview of the CBR system for H_2 production using *R. palustris* and *P. taiwanensis*. The CBR cultivation unit is located in a gas tight, transparent glas casket which allows anaerobic cultivation as it is constantly flushed with either N_2 or Ar depending on the cultivation conditions. The gaseous product is collected in the gas bags connected to either sample port 1 or 2.

1. The tubings for the reactors are assembled according to Heuschkel et al. 2019[8] with the addititon of tubing for the additional medium bottle (2x medium lines, Tygon, 20cm length, Inner diameter: 2mm) and a second t-connector in front of the t-connector that connects the gas peristaltic pump tube (PVC, inner diameter 2.01 mm, Watson Marlow) with the inlet of the CBR that connects the two split medium flows before the reactor inlet.
2. The assembly of the reactor unit is performed before autoclaving. The reactor consists of an outer glass tube with two GL45 connections on the ends.
3. A GL45 3 port cap is attached to the outlet end of the reactor unit with two GL14 hose connectors with PTFE fitting both equipped with a PTFE tube (PTFE, 5 cm length, Inner diameter: 2mm). One connects to the silicon capillary that is located within the outer glass tube, the other is used as outlet for the glass mantle stream of gas. One GL14 screw cap with a hole is supplied with a silicon membrane coated with PTFE for gas sampling of the mantle space.
4. A GL45 2 port cap is attached to the inlet of the glass tube supplied with two GL45 hose connectors with PFTE fitting both equipped with a PTFE tube (PTFE, 5 cm length, Inner diameter: 2mm) as used on the outlet. One is used to connect to the silicon capillary (silicon, length: 22 cm, inner

[8] Heuschkel, I. et al. Data on mixed trophies biofilm for continuous cyclohexane oxidation to cyclohexanol using *Synechocystis* sp. PCC 6803. Data Brief (2019) 25: art. 104059

diameter: 3mm) that is located within the outer glass tube, the other is used as inlet for the glass mantle stream of gas. This PTFE tube is on the outside attached to a peristaltic pump tube (PVC, inner diameter 2.01 mm, Watson Marlow) as used for the medium pumps and a sterile filter. This line can be used to pump any gas or simply air through the mantle of the reactor.

5. After autoclaving, the reactor is assembled under sterile conditions.

3. Preparation for operation:

1. The medium bottles are attached to the CBR setup. The pumps are turned on and the entire system is filled with gas and medium. The gas tubes except for mantle flow (constantly on at 250 µL min^{-1}) are tightly closed using metal clamps and only medium is flushed through the capillary for 1-2 h at a flow rate of 52 µL min^{-1} prior to inocculation.
2. The two inoculation cultures are combined in a luer lock syringe (2 mL of each preculture). A cannula is attached to the luer lock syringe. The safety cap is kept on until inoculation.
3. For inoculation the medium flow is stopped and the 3-way valve in front of the inoculation port is closed. Inoculation takes place next to a flame to ensure sterile conditions. The inoculation port is sterilized with 70 % ethanol solution. The cannulas safety cap is removed and 2 mL of inoculum is injected into each capillary.
4. The CBR's are covered with aluminum foil or any other foil that prohibits light transmission to the capillary and left settling for 24 h.
5. After 24 h the foil is removed and medium flow is started with a final combined flow of 52 µL min^{-1}. If run under indoor conditions the overhead LED's are turned on with 50 µmol m^{-2} s^{-1} The gas tubes are kept closed and the reactor is run in continuous flow mode for 72 h.
6. After these 72 h in constant flow, the metal clamps are removed from the gas tubes and the gas flow is started with a flow of 52 µL min^{-1}.

Sampling options:

1. The gas phase of the reactor mantle and the gas phase of the capillary can be sampled through the membrane in the reactor mantle and the bubble trap at the capillary outlet and either quantified via gas chromatography of membrane inlet mass spectrometry (MIMS). MIMS can additionally be installed in the liquid phase of the reactor to quantify dissolved gasses.
2. The outflow of the reactor can be sampled by the introduction of an additional 3-way valve after the capillary. The cell count and populations can be directly analyzed via flow cytometry. After centrifugation, the cell-free supernatant can be used for quantification of extracellular metabolites, components and salts.
3. During the run, the biofilm thickness can be monitored via optical coherence tomography.
4. After the reactor run, endpoint measurements including omics and EPS analysis of the biofilm can be performed.