IBVT Universität Stuttgart	SOP-Shaking flask experiments
	Version 1.0

EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: Shaking flask cultures of *Pseudomonas putida*

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Instruction

Shaking flask cultures of *Pseudomonas putida*

1 Introduction / Purpose

Standard cultivation protocol for shaking flask experiments with *Pseudomonas putida*. Experiments are performed in DeBont minimal medium with glucose as carbon and energy source. For efficient precultures a working cell bank can be created for the tested strains in minimal medium with 30% glycerol and stored at -80°C. An aliquot of the working cell bank is then thawed at room temperature (RT), centrifuged and transferred to DeBont medium with glucose for preculturing, and finally cultured in 500 ml baffled shaking flasks with DeBont medium.

2 Equipment and chemicals

2.1 Equipment

- LB agar plates
- Screw cap tubes filled with 300 µl sterile glycerol
- Liquid nitrogen
- 500 mL baffled shaking flasks with cellulose plugs.
- 100 mL baffled shaking flasks with cellulose plugs
- 30°C incubator with a shaker, preferably usable at 175 rpm
- spectrophotometer

2.2 Chemicals

- see 2.1 Media recipes for exact media components

2.3 Bacterial strains

- *Pseudomonas putida* KT2440 mt-2 WT
- engineered *P. putida* strains suitable for minimal media

3 Media and buffers

3.1 Overview

- Luria Bertani (LB) medium
- Luria Bertani (LB) medium with 1.5% (w/v) agar
- DeBont medium 100x Buffer
- DeBont medium 100x ammonium sulfate
- DeBont medium 100x trace metal solution
- 50% (w/v) glucose solution
- Sterile demineralized water

3.2 Media recipes

A) Luria Bertani (LB) medium:

Tryptone/peptone	10 *g* L^{-1}
NaCl	10 *g* L^{-1}
Yeast extract	5 *g* L^{-1}

Medium can be autoclaved at 121°C for 20 minutes and stored at RT.

B) Luria Bertani (LB) agar medium:

Tryptone/peptone	10 *g* L^{-1}
NaCl	10 *g* L^{-1}
Yeast extract	5 *g* L^{-1}
Agar	15 *g* L^{-1}

Medium can be autoclaved at 121°C for 20 minutes and stored at RT.

C) 100x DeBont Buffer

K_2HPO_4	*388 g* L^{-1}
NaH_2PO_4	*163 g* L^{-1}

Medium can be autoclaved at 121°C for 20 minutes and stored at RT.

D) 100x ammonium sulfate

$(NH_4)_2SO_4$	*200 g* L^{-1}

Medium can be autoclaved at 121°C for 20 minutes and stored at RT.

E) 100x de Bont trace metals

EDTA	*1 g* L^{-1}
$MgCl_2 \cdot 6H_2O$	*10 g* L^{-1}
$ZnSO_4 \cdot 7H_2O$	*200 mg* L^{-1}
$CaCl_2 \cdot 2H_2O$	*100 mg* L^{-1}
$FeSO_4 \cdot 7H_2O$	*500 mg* L^{-1}
$Na_2MoO_4 \cdot 2H_2O$	*20 mg* L^{-1}
$CuSO_4 \cdot 5H_2O$	*20 mg* L^{-1}
$CoCl_2 \cdot 6H_2O$	*40 mg* L^{-1}
$MnCl_2 \cdot 2H_2O$	*100 mg* L^{-1}

Medium can be autoclaved at 121°C for 20 minutes and stored at RT.

F) Glucose solution:

(50% w/v, per liter)

Dissolving the glucose takes time: use a heating stirrer to speed up the dissolving. Solution is autoclavable at 121°C for 20 minutes. Filter sterilization is also possible.

4 Procedures

4.1 Create working cell bank

used strain should be plated out earlier on an LB-agar supplemented with the appropriate antibiotic.

- Transfer a single colony of the test strain from the LB-agar plate into 10 ml De Bont minimal medium with 5.4 g L^{-1} glucose (and antibiotics, if necessary) in a 100-ml baffled shaking flask
- Incubate overnight at 30°C and 175 rpm
- Inoculate 50 ml De Bont medium with 5.4 g L^{-1} glucose (and antibiotics, if needed) in a 500-ml baffled shaking flask to a start OD_{600} of 0.1 – 0.5
- Incubate at 30°C and 150 rpm and monitor growth via OD_{600}-measurement. Calculate the maximum growth rate μ_{max} in the exponential phase
- At mid-exponential phase (around OD 3.0), transfer 600 µl of culture broth into a sterile screw cap tube with 300 µl glycerol, mix and snap-freeze in liquid nitrogen. Record the cell density at the timepoint of the freezing

4.2 Preculturing with DeBont medium with glucose

- Prepare DeBont minimal medium with 5.4 g L^{-1} (30 mM) glucose. The medium can be stored at room temperature.
- Take one aliquot of the working cell bank from -80°C and thaw at room temperature
- Centrifuge the tube at 5000-10000 x g for 2 min and resuspend the cell pellet in DeBont medium with 5.4 g L^{-1} glucose.
- Transfer a volume of the cell suspension into 10 ml DeBont medium with glucose, for a Start-OD_{600} of ~0.01 (wildtype cells) - 0.1 (producing cells). Add antibiotics, if necessary

- *Note: the goal is to get a preculture in mid-exponential phase at the time of inoculation of the main culture. The final OD_{600} of the preculture can be controlled by the cell suspension volume used for inoculation of the preculture. For a rough estimate, the differential growth rate µ for the first 14 h after thawing is about 70% of μ_{max} determined for the 'Creating working cells banks' step (see above).*

- *Note: as an alternative to the working cell bank, the preculture (5 mL LB reaction tube) can be inoculated directly from the LB-agar plate. These cells can be used to inoculate the bigger preculture at a defined OD_{600}. Adding an additional preculture (using complex or minimal medium) allows the growth rates of the preculture to be closer to μ_{max} due to shorter lag-times*

- Incubate the preculture for about 14 h at 30°C and 150 rpm.

4.3 Batch shaking flask experiments

- If necessary, add antibiotics to the DeBont medium
- Measure OD_{600} of the DeBont precultures. Use appropriate dilutions to measure OD_{600} between 0.1 and 0.3,
- Select the DeBont preculture with an OD_{600} below 4.0 to inoculate the shaking flasks. Ideally, the selected preculture should still be in exponential phase, thereby minimizing the lag-phase of the following shaking flask experiment.
- Determine the amount of preculture that has to be added. The starting OD_{600} of the shaking flask experiment should be 0.1 (but can be higher if necessary). Pelletize the cells in the required amount of volume by centrifugation at room temperature (2 min at 5000-10000 g). Resuspend the pelletized cells in 1 ml of fresh medium.

- Inoculate the shaking flasks with the resuspended cells.
- Check the inoculation OD of the culture by sampling at least 2 mL and measure the OD_{600} in technical duplicates.
- Incubate the shaking flasks at 30°C on the shaker (175 rpm).
- After measuring the OD_{600}, transfer the undiluted sample into a microreaction tube and spin down at max x g for 10 min. Decant the supernatant and store it in a separate tube at -20°C for measuring extracellular concentrations of glucose and organic acids/isobutanol.
- Check the growth of the culture by every hour sampling 1-1.5 mL of broth and measuring the OD_{600} (collect culture supernatants, if needed). Use appropriate dilutions to assure the measured OD_{600} remains below 0.3.
- Keep on taking samples until the OD_{600} reaches a plateau or starts to drop. At this point, the cells have reached the stationary phase and the glucose in the medium is depleted. A few additional measurements should be taken several hours after the stationary phase has been reached to track changes that occur after growth.

5 remarks/troubleshooting:

6 Biosafety

There are no **special** Biosafety rules to follow for this SOP. All work should take place in certified S1 laboratories and all transformed strains that contain plasmids and genomic knockouts should be handled as GMO’s and disposed as such.