WCSB, Wageningen University	EPP-SOP-WU01
	Version 1.0

EPP - Standard Operating Procedure

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

Title: Oxygen gradients for adaptation of bacteria to varying oxygen availability

Oxygen gradients are a fast and easy way to assess the capabilities of a strain in oxic, micro-oxic or anoxic circumstances. It also allows for strain adaptation to lower oxygen levels.

distribution list			
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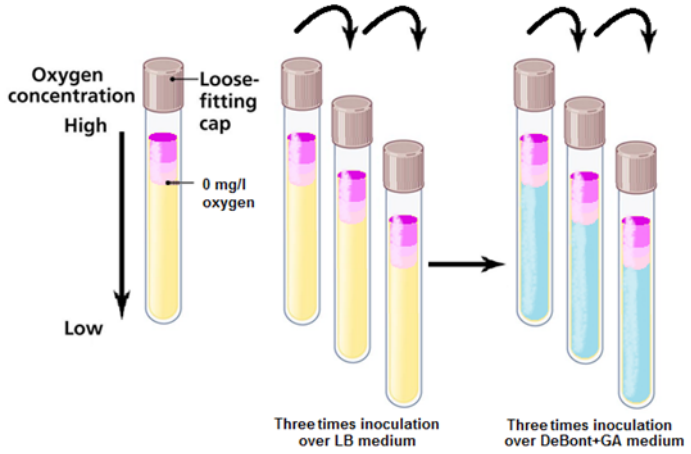
Instruction

Oxygen gradients

1 Introduction / Purpose

Oxygen gradients are a fast and easy way to assess the capabilities of a strain in oxic, micro-oxic or anoxic circumstances. It also allows for strain adaptation to lower oxygen levels.



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2 Equipment and chemical

2.1 Equipment

- ⇨ Glass test tubes, loosely capped or closed off with cotton wool
- ⇨ Time-lapse camera setup
- ⇨ 3 ml syringes (Thermo Fisher Scientific, Waltham, MA USA)
- ⇨ 1.5” needles (BD Microlance)
- ⇨ Heating setup to boil water (preferably with a standard for test tubes inside)
- ⇨ pipettes

2.2 Chemicals

- Medium (LB or minimal medium)
- Agarose
- Resazurin
- Sodium thioglycollate
- L-cysteine
- Antibiotics of choice

2.3 Bacterial strains of choice

Applied to WT and recombinant Pseudomonas putida KT2440.

3 Media and buffers

Different media were tested for this experiment, one of the following can be used:

3.1 LB with agar/sodium thioglycollate/L-cysteine/Resazurin

- 10 g/l NaCl
- 5 g/l yeast extract
- 10 g/l tryptone
- 3 g/l agarose (not agar!)
- 0.5 g/l sodium thioglycollate
- 0.5 g/l L-cysteine
- 0.001 g/l resazurin
- Antibiotics (optional)

3.2 M9 minimal medium with agar/sodium thioglycollate/L-cysteine/Resazurin

- 800 ml autoclaved dH_2O with 3g/l agarose (not agar!)
- 56.4 g/l 5x M9 minimal salts
- 30 g/l carbon source (tested: glucose, fructose or gluconic acid)
- 1M MgSO4
- 1M CaCl2
- 1 g/l thiamin (optional, for CC118 *E. coli* strains
- 100x Trace Elements (pH 7.5) (same as used for DB medium)
- 0.5 g/l sodium thioglycollate
- 0.5 g/l L-cysteine
- 0.001 g/l resazurin
- Antibiotics (optional)

3.3 DB minimal medium with agar/sodium thioglycollate/L-cysteine/Resazurin

- 800 ml autoclaved dH_2O with 4 g/l agarose (not agar!)
- 30 g/l carbon source (tested: glucose, fructose or gluconic acid)
- 100x deBont buffer
- 100x $(NH_4)_2SO_4$
- 100x Trace Elements (pH 7.5) (same as used for DB medium)
- 0.5 g/l sodium thioglycollate
- 0.5 g/l L-cysteine
- 0.001 g/l resazurin
- Antibiotics (optional)

4 Procedures

4.1 Oxygen gradient preparation

Half of the test tube volume of medium is prepared per oxygen gradient.
Prepare the medium as normal (LB autoclaved beforehand completely, otherwise all separate components which are added after autoclaving).
For minimal medium, add all components to the medium while it's still hot, directly after autoclaving, to ensure all oxygen is expelled as much as possible.

Distribute the hot medium over sterile glass test tubes, close them off from contamination using either a not air-tight cap or cotton wool.
Heat the oxygen gradients in 100˚C water (just at a boil) until all resazurin colour is dispelled.
Resazurin is a redox indicator which is coloured blue-black to purple when there is much oxygen, pink in micro-oxic conditions and colourless below 0 g/l dissolved oxygen. Discolouration of the resazurin indicator can take easily 15 – 20 minutes.

From this point on: keep the oxygen gradients as stable as possible! Don't shake, stir, rotate or move the oxygen gradients as much as possible, allow the oxygen gradient to form naturally.

Cool the oxygen gradients so that they are warm to the touch. The resazurin colouring should indicate the dissolving of oxygen, indicating the oxygen gradient is formed. When the resazurin turns pink in 1/5th of the volume (the first centimetre of 5 ml volume), inoculate using 500 µl of an o/n preculture. Inoculate dropwise using a pipette. It's important that the droplets slowly sink through the whole gradient, to allow growth at each level from the start.

Incubate for 4-7 days. Preferably use a time-lapse photography setup in a lighted incubator to follow the strain progress through the oxygen gradient.

4.2 Sampling and continuation of strain

Sampling of the gradients is done using a 3 ml syringe (Thermo Fisher Scientific, Waltham, MA USA) and 1.5" needle (BD Microlance) to extract the bacteria of interest, from the layer of oxygen depletion wanted. The bacterial sample is used to passage over a new oxygen gradient or stored at -80˚C.

4.3 Result analysis with imageJ

Time-lapse photos analysed using FIJI (imageJ 64)
> Open virtual imageJ stack
> Image
  > type
    > 8-bit
> image
  > adjust
    > threshold [40]_[255]
    > black background
> edit
  > selection
    > specify (each vial specified)
      >[20-450-vial specific-260]
      > 1 image/20 min

> 210 pixels = 5 cm
> duplicate
> stk
> plot z-axis profile
data analysed in excel

5 *remarks/troubleshooting:*

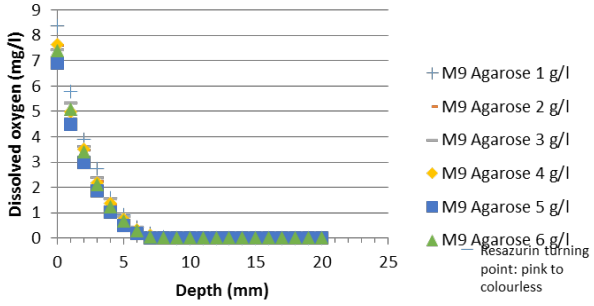
5.1 General remarks

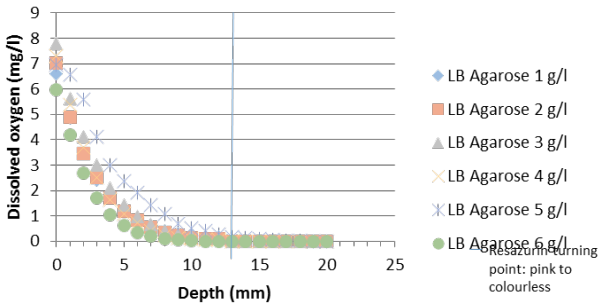
The oxygen gradients were tested with multiple different media, and for each media multiple different types of agar and agarose, different agar and agarose concentrations and different carbon sources were tested. The concentration of dissolved oxygen was determined by micro-electrode. Agarose was determined as most effective and robust.

Instead of 0.5 g/l L-cysteine and 0.5 g/l sodium thioglycollate, 0.75 g/l L-cysteine can be used instead. However, the protocol was not adjusted for this during this setup, so might need additional adaptation.

It is important that the oxygen gradients do not solidify. The small amount of agarose is only meant to stabilize the gradient, so that slight movement won't disturb the gradient, and so that the oxygen doesn't dissolve too fast through the test tube.

For minimal media, a higher agarose concentration is required than for LB medium.





5.2 Troubleshooting

5.2.1 Oxygen gradients turn colour too fast

This is an indication that the gradients are not stable enough. Add more agarose to your medium.

5.2.2 The gradient isn't straight or clear

This indicates a disturbance of the oxygen gradients or no resazurin in the medium.
Disturbance of the oxygen gradients: You can either prepare new oxygen gradients or boil these oxygen gradients again to expel all oxygen. Do not mix, stir or resuspend the oxygen gradients: preferably do not move them at all after heating them. The oxygen gradient should restore relatively quickly after heating, but not too quickly (there should be a gradient). Adding slightly more agarose helps stabilizing the gradient, but the medium should not solidify too much.
No resazurin in the medium : add 0.001 g/l resazurin to the medium. Do not autoclave the resazurin beforehand: this can influence it colouration.

6 Biosafety

No biosafety issues were associated with this protocol when applied to *Pseudomonas putida.* The protocol was developed and performed at an MLI /BSLII laboratory. The boiling of the oxygen gradients was done in the fume hood, but more due to the strong odour of L-cysteine.

7 Acknowledgements

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