ITB, Stuttgart	EPP-SOP-ITB05
	Version 1.0

EPP - Standard Operating Procedure

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

Title: Identification of target alcohol dehydrogenases enzymes (ADHs) involved in small alcohols and terpene alcohols catabolism.

distribution list			
changes to prior version:			
	name	signature	date
experimenter 1	Wendy Escobedo		1st April 2019

Instruction

Identification of target alcohol dehydrogenases enzymes (ADHs) involved in small alcohols and terpene alcohols degradation in *P. putida*

1 Introduction / Purpose

According to computational predictions, performed by Wageningen partner (WUR), (table 1), nineteen different open reading frames, encoding for alcohol dehydrogenase type proteins, were identified in *P. putida* KT2440 type strain. Only a few ADHs have been previously described and characterized, but for most of them such putative function remains unknown. It is well known that ADHs serve to break down alcohols that otherwise are toxic for the cell. This fact should be considered when alcohols are employed as substrates in whole cell biocatalyzed processes. Thus, in order to guarantee viable production conditions, it is crucial to determine how large the substrate degradation in the selected cell factory is. The recent development of molecular genetic tools based in the dCas9 system, has enabled the possibility of tuning specific genes by blocking transcription, making possible a robust and noiseless knockdown of targeted genes. In order to assess the potential degradation of selected small alcohols and terpene alcohols in *P. putida*, we generated two customized ADHs-dCas9 libraries, by transforming a dCas9 plasmid collection, obtained from WUR partner (see EPP-SOP-WU03), into two different *P. putida* genetic backgrounds; EP1 and EM383. Substrate selection was based on potential industrial interest to produce small alcohols or terpene alcohol derivatives. Therefore, we selected two small alcohols (isobutanol and crotyl-alcohol) and five different terpene alcohols (Nerol, *R*-citronellol, *S*-citronellol, Geraniol, Linalool).

Table 1. Alcohol dehydrogenases dCas9 library

	Plasmid	Targeted ADH spacer	Resistance marker
1	pSEVA231	adh1	Kanamycin
2	pSEVA231	adh2	Kanamycin
3	pSEVA231	adh3	Kanamycin
4	pSEVA231	adh4	Kanamycin
5	pSEVA231	adh 4.1	Kanamycin
6	pSEVA231	adh5	Kanamycin
7	pSEVA231	adh6	Kanamycin
8	pSEVA231	adh7	Kanamycin
9	pSEVA231	adh8	Kanamycin
10	pSEVA231	adh9	Kanamycin
11	pSEVA231	adh10	Kanamycin
12	pSEVA231	adh11	Kanamycin
13	pSEVA231	adh12	Kanamycin
14	pSEVA231	adh13	Kanamycin
15	pSEVA231	adh14	Kanamycin
16	pSEVA231	adh15	Kanamycin
17	pSEVA231	adh16	Kanamycin
18	pSEVA231	adh17	Kanamycin
19	pSEVA231	adh18	Kanamycin
20	pSEVA231	adh19 const	Kanamycin
21	pSEVA231	CRISPR (empty, control)	Kanamycin
22	GC-dCas9	META65	Gentamycin

2 Equipment and chemicals

2.1 Equipment

Clean bench Walner electronics
Spectrophotometer ultrospec 3100 pro
Gene pulser electroporation system BioRad
Fumehood Walner electronics
Centrifuge

2.2 Chemicals and antibiotics

Glycerol (Sigma 99.0%, Cat. No. G5516)
DMSO (Dimethyl sulfoxide) (Sigma, Cat. No. 472301)
Isobutanol (2-methyl-1-propanol) (Fluka Chemika 99.5% GC, Cat. No. 58450)
Crotyl-alcohol (2-Buten-1-ol) (Alfa Aesar 96%, Cat. No. A10681)
Linalool (Sigma 97.0%, Cat. No. L2602)
S-citronellol (Sigma 99.0%, Cat. No. 303488)
R-citronellol (Sigma 98.0%, Cat. No. 303461)
Geraniol (Sigma 98.0%, Cat. No. 163333)
Nerol (Sigma Cat. No. 50949)
Kanamycin sulfate (Roth 750 U mg^{-1}, Cat. No. T832.3)
Gentamicin sulfate from Micromonas purpurea (Fluka Chemika 700 U mg^{-1}, Cat. No.48760)

2.3 Bacterial strains

P. putida EP1
P. putida EM383

3 Media and buffers

3.1 High-salt LB agar

10 g L^{-1} tryptone

5 g L^{-1} yeast extract

10 g L^{-1} sodium chloride

15 g L^{-1} agar agar

3.2 High-salt LB medium

10 g L^{-1} tryptone

5 g L^{-1} yeast extract

10 g L^{-1} sodium chloride

3.3 Electroporation solution (sterile)

Glycerol 10% in double distilled water

3.4 Buffer solution for resting cells (pH 7)

5.23 g L^{-1} KH_2PO_4

10.71 g L^{-1} K_2HPO_4

9 g L^{-1}Glucose

3.5 Substrate stocks (prepared in buffer solution)

Isobutanol (200 mM)
1-butanol (200 mM)
Crotyl alcohol (200 mM)

3.6 Substrate stocks (prepared in DMSO)

Linalool (200 mM)
S-Citronellol (200 mM)
R-Citronellol (200 mM)
Geraniol (200 mM)
Nerol (200 mM)

3.7 Antibiotic stocks (prepared in double distilled water and filter sterilized)

Kanamycin sulfate (50 mg mL^{-1})
Gentamicin sulfate (30 mg mL^{-1})

4 Procedures

This protocol is useful to identify target alcohol dehydrogenases enzymes (ADHs) involved in small alcohols and terpene alcohols degradation in *P. putida*

4.1 Library construction

Precultures: *P. putida* strains EP1 and EM383, were inoculated from single isolated colonies in 5 mL high-salt LB media. Precultures were incubated o/n at 30°C and 180 rpm.

Cultures: cultures were inoculated with 1% (v/v) preculture in 25 mL high-salt LB media until stationary phase was reached. Optical density was measured over time, since both strains have different duplication times. Cultures were incubated o/n at 30°C and 180 rpm.

Preparation of competent cells for electroporation: For each strain, EP1 and EM383, cultures were centrifuged (4,000 rpm, 4°C, 20 min). Cell pellets, were sequentially washed and centrifuged three times with cold electroporation solution. Afterwards, cell pellets were suspended in 2,5 mL of cold electroporation solution.

Cell transformation: EP1 and EM383 cells ready for electroporation (100 µL) were deposited in 2 mm gap width electroporation cuvette along with 50 ng of each of the 19 plasmids (1-19 in table 1), or empty spacer plasmid (21 in table 1), or empty dCas9 plasmid (22 in table 1), by separate. Employed electroporation settings were: 25 µF; 200 Ω; 2.5 kV. After applying a pulse, 1 mL of room temperature LB medium was added. Cells were transfered to 2 mL Eppendorf tubes and incubated 2h at 37°C and 180 rpm. Afterwards, cells were harvested (4,000 rpm, 4 min, room temperature), 900 µL of the media was discarded and pellets were suspended in the remaining media. The whole volume was plated on LB plates containing the correspondent antibiotic at final concentrations of 30 and 50 µg mL^{-1} for gentamycin and kanamycin, respectively. Plates were incubated 24 h at 37°C. Controls included cells pulsed without added DNA.

4.2 Resting cells production

Precultures: all the 22 different strains of, both EP1 and EM383, *P. putida* libraries were inoculated by triplicate from single isolated colonies in high-salt LB media tubes (5 mL). Precultures were incubated o/n at 30°C and 180 rpm.

Cultures: biological triplicate cultures were inoculated with 1% (v/v) preculture in 4 mL high-salt LB media, until stationary phase was reached. Optical density was measured over time, since the two strains have different duplication times. Cultures were incubated o/n at 30°C and 180 rpm.

Resting cells production: For each strain, cultures were centrifuged (4,000 rpm, 4°C, 20 min). Cell pellets, were sequentially washed and centrifuged three times with cold buffer solution. Afterwards, cell pellets were suspended in sufficient cold buffer solution to reach an optical density of 0.5.

4.3 Assessment of stability/consumption of small alcohols in the different genetic backgrounds

Substrate degradation screening was performed as follows: EP1-ADHs-dCas9 library was designed for assessing crotyl alcohol degradation, while EM383-ADHs-dCas9 library was generated for isobutanol and all five different terpene alcohols.

For each strain, deep well plates containing 2 mL of resting cell solution (biological triplicates) were supplemented with 20 µL of each one of the different substrates to give a final concentration of 20 mM. Plates were tightly sealed with silicon mats and three layers of aluminum seals on top to avoid substrate decrease due to evaporation. Samples were incubated at 30°C and 180 rpm.

4.4 Analysis

In order to follow potential consumption of small alcohols in the different genetic backgrounds, samples were taken at different time points; 2, 4, 6 h. In the case of terpene alcohols samples were taken only after 2 h. Further sample treatment regarding extraction and GC-MS analysis is described in the complimentary downstream protocol EPP-SOP-ITB07.

5 remarks/troubleshooting:

It is highly recommended to use always freshly prepared substrate stocks, since small alcohols and terpene alcohols are very volatile.

6 Biosafety:

No biosafety issues were associated with this protocol. The protocol was developed and performed at an S1 laboratory. The pipetting of small alcohols was performed in the fume hood due to the harmfulness of vapors (H226-315-318-335-336). For protection against the employed substrates, nitrile gloves are to be worn at all time when handling the samples.

7 Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635536.