DOI: 10.1002/adsc.202000558
Advanced Synthesis & Catalysis

Amino Benzamidoxime (ABAO)-Based Assay to Identify Efficient Aldehyde-Producing *Pichia pastoris* Clones

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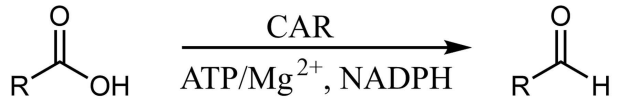
Manuscript received: May 10, 2020; Revised manuscript received: August 13, 2020;
Version of record online: September 24, 2020

Supporting information for this article is available on the WWW under https://doi.org/10.1002/adsc.202000558

Abstract: The chemoselective synthesis of aldehydes is a challenging task. Nature provides carboxylic acid reductases (CARs) as elegant tools for the direct reduction of carboxylic acids to their respective aldehydes. The discovery of new CARs and strains that efficiently produce these enzymes necessitates a robust high-throughput assay with selectivity for aldehydes. We recently reported a simple assay that allows the substrate independent and chemoselective quantification of aldehydes (irrespective of their chemical structure). The assay utilized amino benzamidoxime (ABAO), which forms UV-active and fluorescent dihydroquinazolines. In this study, we adapted the ABAO-assay for the identification and comparison of *Pichia pastoris* clones with the ability to produce aldehydes from carboxylic acids. Specifically, CAR and PPTase from *Mycobacterium marinum* (*Mm*CAR and *Mm*PPTase) were co-expressed using different bidirectional promoters (BDPs). A library of 598 clones was screened for piperonal production with the ABAO assay and the results were validated by HPLC quantification. 1 OD unit of the best *Pichia pastoris* clone 2.A7, regulating *Mm*CAR and *Mm*PPTase expression by two strong constitutive promoters, fully converted 5 mM of piperonylic acid within 2 h.

Keywords: high-throughput screening; carboxylic acid reductase (CAR); aldehydes; enzyme catalysis; gene expression in *Pichia pastoris*

Aldehydes as chemical targets are widely produced by the flavor and fragrance industry[1,2] and used by the pharmaceutical[3] and biofuel industry[4] as reactive intermediates. Industrial scale aldehyde production often resort to ecologically unfriendly, energy-draining and unselective methods.[5] In recent years, the enzyme class of carboxylic acid reductases (CARs, E.C.1.2.1.30) has been explored as milder and more sustainable alternative to the harsh chemical approaches used by industry.[6] CAR enzymes enable the reduction of a broad range of carboxylic acids (**a**) to the respective aldehydes (**b**) in nicotinamide adenine dinucleotide phosphate (NADPH)- and adenosine triphosphate (ATP)-dependent catalytic steps (Scheme 1).[7] The three-domain enzyme class acts as peptidyl carrier protein and requires binding of a 4'-phosphopantetheine moiety from a 4'-phosphopantetheinyl transferase (PPTase) to be activated.[8] Even though CAR reactions might be the selective answer for sustainable production of **b**, whole-cell biocatalysts also need to overcome the host-defence mechanism,



carboxylic acid (**a**) aldehyde (**b**)

Scheme 1. CAR mediated reduction of carboxylic acid to aldehyde

the overreduction of **b** to the less cytotoxic alcohol (**c**, *Scheme S1*).[9]

In CAR research, the main workhorse today is *Escherichia coli (E. coli)*.[10–13] However, the methylotrophic yeast *Pichia pastoris*[14] (syn. *Komagataella phaffii)* as a host organism offers certain advantages over the bacterial host. The eukaryotic organism has been described as the second most commonly used servant for recombinant protein expression[15] due to the following reasons: First, genomic integration of expression cassettes furnishes stable strains, which is vital for industrial use.[16] Second, the combination of high cell-density fermentations of *P. pastoris* utilizing the exceptionally strong *AOX1* promoter yields particularly high protein concentrations of singular heterologous proteins.[17]

A new tool for multi-gene expression in *P. pastoris*, described by Vogl *et al.*,[18] made the yeast species especially attractive for fine-tuning co-expression of proteins in a systematic approach. Within their study, traditional monodirectional promoters were compared to sets of bidirectional ones. The limited capabilities of monodirectional promoters for multi-gene expression were amended. A library of synthetic bidirectional promoters (BDPs) made a rapid screening for diverse expression profiles of dual gene expression as well as whole metabolic pathways feasible.[18]

In *P. pastoris*, specific genomic integrations of the designed expression cassettes are rather rare (less than 30%) compared to the model yeast organism *Saccharomyces cerevisiae* (*S. cerevisiae*).[19,20] Hence, screening efforts for identifying the best *P. pastoris* clones can be time consuming. Recently, we described a high-throughput assay, which takes advantage of the reactive nature of the CAR-produced aldehyde to form UV-detectable and fluorescent dihydroquinazolines with amino benzamidoxime (ABAO).[21] Its first application was subject to a mutagenesis study of the CAR from *Nocardia iowensis* (*Ni*CAR), expressed in *E. coli* K-12 MG1655 RARE (DE3),[22] to enhance activity for the poor substrate 2-methoxybenzoic acid.[23]

CAR proteins are rather large (approx. 130 kDa) and structurally flexible enzymes.[24] Especially fungal CARs tend to low expression levels in the bacterial expression host *E. coli*,[25] and their production could potentially be improved in a eukaryotic host.

Hansen *et al.* observed that different yeast strains have different endogenous abilities to activate CARs. *S. cerevisiae*, e. g., was not able to activate the bacterial *Ni*CAR by endogenous enzymes,[2] leading to the assumption that *P. pastoris* will also require the co-expression of a capable PPTase to activate CAR enzymes. No information is available in literature, which correlates efficient biocatalysts with the co-expression levels of PPTase and CAR enzymes.

In this study, we investigated a set of BDPs for the simultaneous recombinant expression of PPTase (*Mm*PPTase,[26] Accession Nr: WP_094357779) and CAR (*Mm*CAR,[10] Accession Nr: WP_012393886) from *Mycobacterium marinum* in *P. pastoris*.

To identify the best suited promoter pair for dual gene expression and the best *P. pastoris* clones with carboxylic acid reductase activity, an ABAO-based high-throughput assay[21] was applied. Overall 598 clones of 10 different BDP variations (Table 1) were screened in a 96-well format, selected constructs were rescreened, up-scaled to flask cultivations and validated by HPLC-UV analytics (Scheme 2).

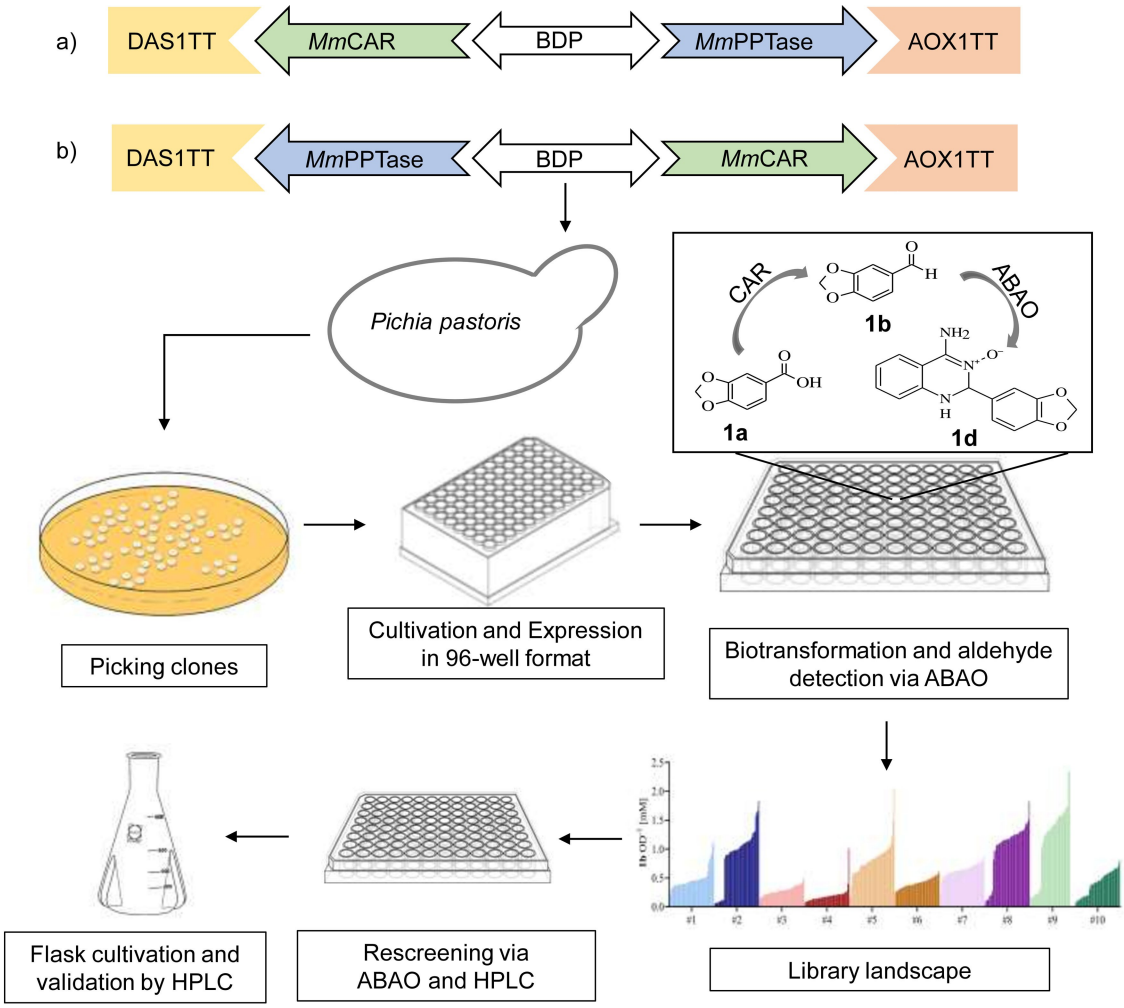
Expression analysis of one colony of each construct was performed to visualize *Mm*CAR and *Mm*PPTase in cell free extracts. For all constructs, *Mm*CAR expression at approx. 130 kDa was evident (*Figure S1A*). *Mm*PPTase (approx. 25 kDa) was not clearly detectable.

To get first insight which promoter combinations furnish high levels of functional *Mm*CAR, 460 carboxylate-reducing *P. pastoris* clones, 46 clones per construct, were screened in a 96-well format for first aldehyde-formation estimates by the ABAO-based screening assay (*Figure S2*). First, the yeast cells were grown in deep well plates (DWPs, a representative plate-layout is found in *Table S1*) and harvested. Next, in whole-cell biotransformation mode, piperonylic acid (**1 a**) was reduced to piperonal **1 b** by *Mm*CAR. In the detection step, **1 b** reacts with ABAO to piperonyl dihydroquinazoline (**1 d**). The level of *Mm*CAR expression *(Figure S1A*) correlated with the mean activity levels detected by ABAO and whole-cell conversions as determined by HPLC *(Figure S1B*). A difference in **1 d** formation by 38-fold from the least to the most efficient carboxylic acid-reducing clone was observed (*Figure S2.*). Constructs #2, #5, #8 and #9 showed the most promising clones, whereas constructs #3, #4 and #6 were dismissed from further experiments due to low **1 d** formation (Figure *S1B*) and poor expression levels detected on an SDS-PAGE (*Figure S1A*).

Table 1. Numeric scheme of BDP combinations for dual gene expression of *Mm*CAR and *Mm*PPTase.

BDP combination	*Mm*CAR:BDP: *Mm*PPTase[a]	*Mm*PPTase:BDP: *Mm*CAR[b]
$P_{CAT1\text{-}AOX1}$	#1	#2
$P_{GAP\text{-}CAT1}$	#3	#4
$P_{CAT1\text{-}PEX5}$	#5	#6
$P_{DAS1\text{-}DAS2}$	#7	#8
$P_{GAP\text{-}TEF}$	#9	#10

[a] *Mm*PPTase forward orientation, *Mm*CAR reverse orientation.
[b] *Mm*PPTase reverse orientation, *Mm*CAR forward orientation. Detailed description and illustrations of the constructs are shown in *Figure S8.*



Scheme 2. Flow scheme for identifying carboxylic acid-reducing *Pichia pastoris* clones using the ABAO-screening assay.[21]

All constructs with high **1 d** concentrations expressed *Mm*CAR via an equally strong or stronger promoter (*P*) compared to *Mm*PPTase. Construct #2 is based on the strongest methanol-inducible promoter P_{AOX1} (*alcohol oxidase 1*)[27] for *Mm*CAR expression and P_{CAT1} (*catalase*)[28] for *Mm*PPTase expression (Table 1). Construct #5 expressed *Mm*CAR via P_{CAT1}, also known as P_{DC}, and is a promoter of medium strength,[18] whereas *Mm*PPTase was expressed by the weak methanol-inducible promoter P_{PEX5}.[29] BDP construct #8 included upstream the P_{DAS2} (*dihydroxyacetone synthase 2*)[18] for *Mm*CAR and downstream the P_{DAS1} (*dihydroxyacetone synthase 1*)[18,27] for *Mm*PPTase expression, which are both strong methanol-induced promoters. Constructs #9 and #10 are regulating dual gene expression of CAR and PPTase by two strong constitutive promoters: P_{GAP} (*glyceraldehyde-3-phoshphate-dehydrogenase*)[30] and P_{TEF} (*translation elongation factor*).[31] It was expected that expression as well as activity levels from both constructs should be rather similar, but this does not seem to be the case. Expression of *Mm*CAR regulated by P_{GAP} and *Mm*PPTase regulated by P_{TEF} with 1% of glycerol feed seemed to generate more clones with higher expression levels leading to higher **1 b** formation. *P. pastoris* clones with *Mm*PPTase expression regulated by P_{GAP} and *Mm*CAR by P_{TEF} produced on average 2.8-fold less of **1 b**. Nevertheless, previously reported expression levels of proteins regulated by P_{GAP} or P_{TEF} were similar, P_{TEF} demonstrated a tighter growth-associated expression mode, possibly limiting *Mm*CAR expression as observed in *Figure S1A*. Recently Vogl *et al.* extensively investigated the influence of integration parameters on protein expression. They came to the conclusion that increased copy number appears to be the dominant positive influence rather than the integration locus, genomic rearrangements, deletions, or single-nucleotide polymorphisms.[32] Therefore, transforming increased DNA concentrations into *P. pastoris* could generate carboxylate-reducing clones with even higher product titers.

To summarize, expression of *Mm*CAR under the control of a strong methanol-induced or constitutive (glycerol) promoter was beneficial for generating aldehyde-forming *P. pastoris* clones. A low expression level of *Mm*PPTase was sufficient for the activation of *Mm*CAR.

For a direct and fair comparison of expression and activity levels of the best clones of selected constructs (#1, #2, #5, #7, #8, #9 and #10, see DWP layout *Table S2*), cultivations, expression and biotransformations were performed on a single plate, to rule out effects that cause cultivation batch variability. After 2 hour biotransformations at 28 °C, aldehyde levels were quantified both via HPLC-UV and the ABAO-assay. The best 7 to 11 clones of the selected constructs were investigated. As shown in *Figure S4*, the clones from library #1, #2 (methanol-induced) and #9 (constitutive regulation on glycerol) were most promising in this direct comparison. Biological duplicates were determined in technical triplicates each. The ABAO-screening method overestimated the concentration of **1 b** compared to HPLC analysis but gave a very well correlating fingerprint: the best clones determined by HPLC were throughout the best clones in the ABAO-assay. Overall, more than 80% of clones showed a perfect correlation of activity landscapes determined by the ABAO-based assay and HPLC analysis. The ABAO-based high-throughput assay proved to be a suitable tool as screening means for aldehyde-forming *P. pastoris* clones.

For rescreening, three single colonies of the 8–9 best clones from library #1, #2 and #9 were cultivated, expressed and used for whole-cell biotransformations of **1 a** in 96-well plates. Results are shown in Figure 1. *Pichia* clones B12 and F12 from construct #1 formed more than 2.5 mM **1 b** and **1 c**. Within the yeast whole-cell, endogenous enzymes reduce **1 b** further to piperonyl alcohol (**1 c**) (*Scheme S1*). Previously, over-reduction of vanillin to vanillyl alcohol was also observed and could only be limited to a minimum by utilizing yeast knock-out strains.[2] In agreement with these observations, up to 1.65 mM **1 c** was detected in methanol-inducible libraries #1 and #2. Carboxylic acid-reducing *P. pastoris* clones D7 and G9 were the most promising of library #2. Construct #9 generated clones with the highest aldehyde-formation potential. Hence, we took a step back and screened more *P. pastoris* clones harboring construct #9. Overall, 184 clones of construct #9 on multiple DWPs were screened with the ABAO-based high-throughput assay (*Figure S5*). A 113-fold difference in aldehyde-formation was detected between the least and highest carboxylate-reducing *P. pastoris* clone. *P. pastoris* clones 2.A7 (clone A7 of DWP No.2) and 2.B4 (clone B4 of DWP No.2) were newly identified, whereas clones 1.D6 and 1.C7 were reconfirmed.

Finally, for confirmation of expression (*Figure S6*) and activity levels (*Figure S7*) of the four best clones from constructs #1, #2 and #9 in 50 mL scale, clones were cultivated in 300 mL baffled shake flasks. As observed in DWP-cultivation, *P. pastoris* clone B12 and F12 (construct #1) showed the highest expression levels, which correlated with the highest product formations among clones from construct #1. Biotransformations with flask-cultivated clones of construct #1 could not replicate **1 b** and **1 c** titers determined in DWP-cultivated clones. Both clones of construct #1 converted approx. 2.0 mM of **1 a**. *Pichia* clones D7 and G9 of construct #2 converted approx. 2.2 mM of **1 a**, which was slightly increased compared to the same clone cultivated in DWP. Again, the best clones of construct #2 screened in DWPs showed also the highest activity levels in flask cultivations. Remarkably, constitutive expression of *Mm*CAR in shake flaks led to approximately twice the activity as compared to methanol induced biocatalysts. Expression levels of *Mm*CAR regulated by P_{GAP}, however, did not surpass expression levels of clones regulated by the strong methanol-inducible promoters, P_{AOX1} or P_{CAT1}. This fact indicates a limitation in activation of *Mm*CAR. The

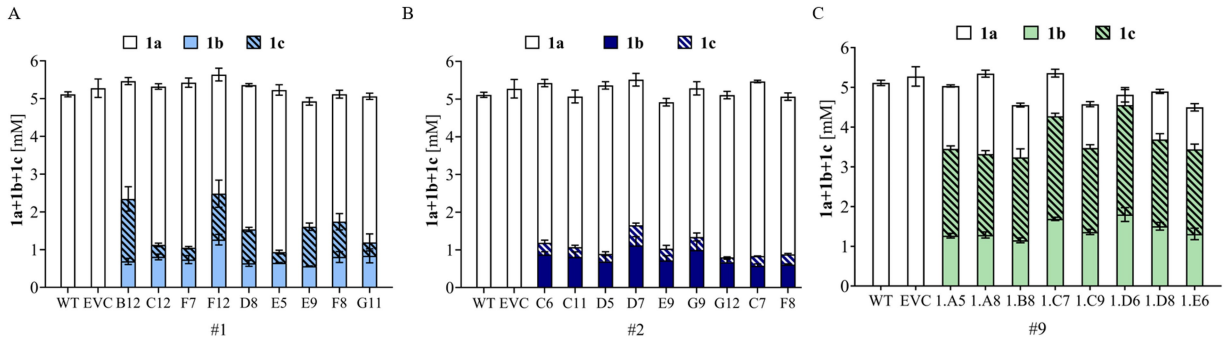


Figure 1. Rescreening of the 8–9 best carboxylic acid-reducing *P. pastoris* clones expressing *Mm*CAR and *Mm*PPTase via construct #1 (A), #2 (B) and #9 (C). **1 a**, **1 b** & **1 c** were detected via HPLC-UV at 254 nm. Error bars are shown for biological triplicates. 1 OD unit of cells was used for whole-cell bioconversion of 5 mM of **1 a**.

best *P. pastoris* clone of library #9 (#9 2.A7) showed full conversion of **1 a** and clone #9 1.D6 converted 4.3 mM of **1 a**. Hence, strong constitutive regulation of *Mm*CAR and *Mm*PPTase expression was beneficial for high product titers. Also, upscaling cultivations from 0.5 mL to 50 mL scale was successful and showed that *P. pastoris* is a suitable host for biocatalytic carboxylate reduction.

Further characterization of the clones is ongoing. In particular, the question whether *Mm*CAR generated through the different constructs show equal or varying specific activities due to differences in PPTase activation remains to be answered. Based on experience from *E. coli*,[26] we may expect differences and the respective experiments are ongoing research. Tailoring cultivation conditions towards each side of the BDP as well as fermenter-controlled cultivations may further aid in optimizing yields.[33,34]

To summarize, approx. 600 carboxylic acid-reducing *P. pastoris* clones of 10 different BDP constructs expressing CAR and PPTase from *Mycobacterium marinum* were screened (Table 2) with the ABAO-based assay and results were confirmed by HPLC-UV analysis. The ABAO-based high-throughput assay was used as screening means for carboxylate-reducing *P. pastoris* clones. Even though aldehyde is also reduced to the respective alcohol in yeast cells, the aldehyde level reflects the total efficiency of the clones very well and best performers could be identified. Expression of *Mm*CAR under the control of a strong methanol-inducible or constitutive (glycerol) promoter was beneficial for generating efficient carboxylate-reducing *P. pastoris* clones. For dual gene expression, BDP *P. pastoris* clone libraries generated a broad expression profile and determined activity differences from the least to the highest carboxylic acid-reducing clone by 113-fold. Clones F12 and B12 of construct #1 and clones D7 and G9 of construct #2 showed promising product titers under methanol-inducing conditions. Under glycerol-feed, construct #9 showed the highest product formation. After screening of 184 clones of construct #9, clones 2.A7 and 1.D6 showed the highest **1 b** and **1 c** concentrations. 1 OD unit of *P. pastoris* #9 2.A7 cells fully converted 5 mM **1 a** within 2 h.

Table 2. Overview of *P. pastoris* aldehyde producing clones screened with ABAO-high-throughput assay in 96-well format.

construct	No. of clones	**1 d** min [mM]	**1 d** max [mM]	fold difference
#1	46	0.213	1.134	5.3
#2	46	0.062	1.832	29.7
#3	46	0.125	0.513	4.1
#4	46	0.084	1.011	12.0
#5	46	0.174	2.037	11.7
#6	46	0.247	0.617	2.5
#7	46	0.229	0.885	3.9
#8	46	0.092	1.827	19.9
#9	184	0.024	2.758	113.0
#10	46	0.076	0.806	10.6
total	598	0.024	2.758	113.0

Experimental Section

A detailed Materials and Methods section can be found in the Supporting Information. Piperonylic acid (**1 a**), piperonal (**1 b**) and piperonyl alcohol (**1 c**) were obtained with the highest purity from Sigma Aldrich (Vienna, Austria). Unsubstituted amino benzamidoxime (ABAO) was synthesized as described.[21] The BDP library was purchased from bisy GmbH (Hofstätten, Austria).

Cultivation and Expression of *Pichia pastoris* Clones

P. pastoris cultivations were performed in small-scale 96-deep well plates (DWP, 0.5 mL cultivation) or in 300 mL baffled flasks (50 ml cultivation). Single colonies were picked for precultures. They were grown in YPD media at 28 °C and 320 rpm (DWP cultivation) or 150 rpm (flask cultivation) for 60 h. For each construct, approximately 46 clones were picked and screened, except for construct #9 (184 clones, Table 2). Each DWP containing *P. pastoris* clones also contained 24 single clones of the empty vector control (EVC) for on-plate calibration as described by Schwendenwein *et al.*[23] Main cultures were cultivated and induced with either BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 10 mM $MgSO_4$ and 1% glycerol) or BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 10 mM $MgSO_4$ and 1% methanol) at 28 °C for 48 h. 1% Methanol or glycerol was added every 12 h. OD_{600} measurements in microtiter plates were performed in a Synergy Mx Plate reader (BioTek, Winooski, USA). *P. pastoris* cells were harvested by centrifugation at 4,000 rpm (3,220 x g) for 10 min in an Eppendorf tabletop centrifuge 5810R for expression analysis, biotransformations or ABAO-screening.

The best 8 clones of constructs #1, #2, #5, #7, #8, #9 and #10 were cultivated on a single DWP in biological duplicates for direct comparison. The best 8 clones of constructs #1, #2 and #9 were streaked on YPD-Zeo^{100} plates. Single colonies were cultivated in biological triplicates in DWPs and used for bioconversions. Flask cultivations were performed for the best four clones of constructs #1, #2 and #9.

ABAO-Based High-Throughput Assay to Identify Efficient Aldehyde Producing *Pichia pastoris* Clone

One OD unit of *P. pastoris* cells was harvested for 10 min at 3,220 x g. Supernatant was discarded. Cell pellets were resuspended in 400 µL of 100 mM MES buffer, pH 6.0, containing 1% glucose, 5 mM of **1 a** and 10 mM $MgSO_4$ (conversion buffer). Bioconversions were incubated at 28 °C and 320 rpm for 2 h. 10 min before bioconversions were finished, **1 b** in concentrations ranging from 0.313 mM to 10 mM were added

to EVC cell suspensions for on-plate calibration. Bioconversions were stopped by adding ABAO-solution (400 µL of sodium acetate, 300 mM, pH 4.5, containing 10 mM ABAO and 5% DMSO). DWP plates were centrifuged for 30 min at 3,220 x g. 150 µL of the supernatant was transferred into a microtiter plate and measured at 405 and 380 nm, respectively (end-point measurements). Due to a higher extinction coefficient of **1 d** at 380 nm ($\varepsilon = 1649\ M^{-1}cm^{-1}$, *Figure S10*) as compared to 405 nm ($\varepsilon = 379\ M^{-1}cm^{-1}$) the 380 nm reads were used for calculations. On-plate calibration was used for determination of **1 d** formation per clone. Selected DWP layouts are shown in *Table S1* and *S2*. A calibration curve representative for on-plate calibrations can be found in *Figure S3*. Product values were OD normalized.

Whole-Cell Biotransformation for HPLC-UV Validation

One OD unit of harvested *P. pastoris* clones were resuspended in 400 µL of conversion buffer and incubated in DWPs at 28 °C and 320 rpm for 2 h. To stop reactions, they were transferred into Eppendorf tubes, 1.2 mL of MeOH was added and the mixtures vortexed. Reaction mixtures were centrifuged at 16,100 rcf for 40 min and 4 °C. 150 µL of the supernatant was transferred into a polypropylene microtiter plate and measured via HPLC-UV. Equipment and method details of HPLC-UV analysis are described in Horvat *et al.*[25]

Acknowledgements

The authors thank Sebastian Hecko for synthesis of ***1d*** *and determination of extinction coefficients. We are thankful to Claudia Rinnofner, Daniel Schwendenwein, Fredi Brühlmann and Andreas Taglieber for valuable discussions and recommendations. The Austrian Research Agency FFG (P860262) and Firmenich SA are kindly acknowledged for financial support. T. S.L. received funding from the Austrian Research Promotion Agency FFG (FEM-Tech stipend, grant no. 877955). SH and FR are grateful for the funding received from TU Wien.*

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