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Cloning and upscale production of monoamine oxidase N (MAO-N D5) by *Pichia pastoris*

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Abstract

Objective

To clone monoamine oxidase- N, enzyme catalysing the selective oxidative deamination or deracemisation of amines into imines, in *Pichia pastoris* and prove the importance of choosing the proper expression system for its recombinant production.

Results

The monoamine oxidase originating from *Aspergillus niger* and subjected to directed evolution (MAO-N D5) has been cloned and successfully expressed in *Pichia pastoris* CBS7435 Mut^S strain for the first time. Various transformants were screened at microscale level. The production of the clone expressing the most active enzyme was scaled-up to a 1.5 l fermenter and preparation of MAO-N D5 as a crude enzyme extract was optimised. The obstacles in the

production of the enzyme in both expression systems, *Escherichia coli* and *Pichia pastoris*, were discussed and demonstrated.

Conclusions

There was an improvement in specific productivity, which was 83 times higher in *P. pastoris*, clearly proving the importance of choosing the right expression host system for the specific enzymes.

Keywords: cloning, monoamine oxidase-N D5, *Pichia pastoris*, upscale

Introduction

Monoamine oxidases (MAO) belong to the oxidoreductase class and are able to undergo selective oxidative deamination or deracemisation of primary, secondary and tertiary amines. The resulting imines are important precursors or by-products in the pharmaceutical industry and organic chemistry. One of the best characterised MAOs, MAO-N from *Aspergillus niger* (Schilling and Lerch 1995), was the object of extensive engineering. Enzyme variants with improved activity and specificity, including the variant MAO-N-D5, were obtained via directed evolution (Atkin et al. 2008); immobilisation of whole cell biocatalysts or crude enzyme extract using LentiKats® technology were evaluated (Zajkoska et al. 2015; Markošová et al. 2016). Recently, a kinetic model for substrate specificity of MAO-N in different biocatalytic reactions was described (Rios-Solis et al. 2015), while other groups analysed the influence of cultivation medium on enzyme activity (Ramesh et al. 2016).

MAO-N has been expressed in *Escherichia coli*; however, since the enzyme originates from a filamentous fungus, using a eukaryotic host for MAO production represents an interesting possibility, due to the increased compatibility between the expression host and the protein of interest. Among the possible hosts for recombinant protein production (RPP), one of the most

widely utilised is the methylotrophic yeast *Pichia pastoris,* recently reclassified as *Komagataella phaffii* (Kurtzman 2009). *Pichia pastoris* presents many advantages of higher eukaryotic expression systems, specifically good posttranslational modifications, correct protein folding, generally high protein expression levels (gram of protein per litre) and the possibility of relatively efficient protein secretion; moreover, its cultivation is cost-effective, similar to *E. coli* fermentation, and reaches high cell densities (Cereghino and Cregg 2000).

This work aims to explore the production of a well-characterised monoamine oxidase variant, MAO-N D5, using a eukaryotic expression system, comparing its activity against the more characterised production in *E. coli*: in particular we report for the first time the use of *P. pastoris* as an expression system for MAO-N D5 enzyme. Enzyme production and activity in selected high-producing clones for MAO-N D5 were evaluated in small scale cultures and bioreactors, scaling production up by applying an optimised fermentation protocol (Markošová at al. 2015). Finally, a crude enzyme extract obtained from optimised fermentation was tested in a model biotransformation of a secondary amine, comparing the activity and volumetric productivity with the prokaryotic expression system, *E. coli* (Zajkoska et al. 2015; Markošová at al. 2016).

Materials and methods

Microorganisms and Media

P. pastoris CBS7435 Mut^S and *E. coli* TOP10F were used in this work.

LB [Luria-Bertani Medium] and YPD [Yeast Extract Peptone Dextrose Medium] in addition to zeocin (50 mg/l for *E. coli* and 100 mg/l for *P. pastoris*) were used to respectively preserve *E. coli* and *P. pastoris* clones throughout the study. Screenings in deep well plates were performed in BMD, BMM2 and BMM10 according to Weis et al., 2004.

Shake flasks and fermenter cultures of *P. pastoris* were performed in BMGY medium [Buffered Glycerol-complex Medium]; the induction media was BMMH [Buffered Minimal Methanol Medium].

Fed-batch fermentations were carried out in BSM (Basal Salt Medium) medium and supplemented with 4.35 ml of PTM_1 (trace salts solution) per litre of BSM medium. Methanol added in fed-batch experiments was also supplemented with PTM_1 (1.2 ml pure methanol/l).

The composition of each media are fully described in Supplementary material 1.

Cloning of DNA encoding monoamine oxidase in *E. coli* and *P. pastoris*

The coding sequence of MAO-N D5, encoding the monoamine oxidase-N with five amino acid mutations (Ile246Met/Asn336Ser/Met348Lys/Thr384Asn (Atkin at al. 2008)), was codon-optimised according to DNA 2.0 optimisation algorithm and cloned in pPpT4-S or pPpT4-S-Alpha vectors (Weis et al. 2004) downstream of the AOX1 promoter (*Eco*RI, *Not*I restriction sites) or in-frame with the alpha mating factor from *S. cerevisiae* (*Xho*I, *Not*I restriction sites). Plasmidic DNA was isolated from *E. coli* TOP10F transformed cells using Thermo Scientific GeneJET Plasmid Miniprep Kit and checked by Sanger sequencing. Plasmids were linearised using *Swa*I (Fast Digest, Thermo Scientific, USA) and approximately 500 ng of linearised plasmid pPpT4-S-MAO-N D5 were used to transform *Pichia pastoris* CBS7435 Mut^S competent cells following the standard condensed protocol (Lin-Cereghino et al. 2005). Transformed cells were plated on YPD plates with zeocin (100 μg/ml) and incubated at 30°C for 48 hours.

Screening in deep-well plates

P. pastoris transformants were screened in 96 deep-well plates. Briefly, single colonies were inoculated into 250 µl of BMD1 media per well and cultivated at 28°C, 320 rpm and 80% relative humidity. After 48 hours, methanol induction started by adding 250 µl of BMM2/ well. After 58, 70 and 80 hours, another 50µl of BMM10/ well was added. After approximately 92 hours, activity assay was performed on cell lysates. Then, 300 µl of cell suspension was centrifuged for 10 minutes, at 25°C and 16000 *g* and the supernatant was removed by pipetting. Cell pellets were re-suspended in 100 µl of Glass Beads Extraction Buffer [50mM potassium phosphate buffer, pH 7.9, 5% (w/v) glycerol, 1 mM EDTA, 2 mM DTT, without PMSF] and transferred into a clean tube containing a 1:1-volume of glass beads. Disruption was performed by 8 cycles of vortexing at maximum speed for 30 seconds, followed by 30 seconds of incubation in ice. Cell lysates were separated from glass beads by piercing the tube bottom with a sterile needle and centrifuging the pierced tube on a clean tube for 1 minute at 3000 *g*. As an alternative lysis method, YPER (Y-PER™ Yeast Protein Extraction Reagent, Thermo Fisher Scientific, USA) was tested for cell lysis, incubating pellets from deep-well plate cultivation in 200 µl of YPER under vigorous shaking, followed by a centrifugation step (3000 *g*) to remove major debris and supernatants collection.

MAO activity assay – deep well plate cultivation

The activity assay of the enzyme extracts was performed using a plate reader (BMG Labtech, FLUO Star Omega) in 96 well microplates (GREINER 96F-Bottom), employing an activity assay with HRP Via and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Bateman and Evans 1995). The composition of one reaction was as follows: 48 µl of potassium phosphate buffer (0.1 M, pH 7.9 or 7.7), 35 µl of 3-Azabicyclo [3,3,0]octane HCl, 98% (20 mM, AK Scientific Inc., USA) as substrate for MAO-N D5, 2 µl of HRP VIa (2 mg/ml), 5 µl

of ABTS (220µg/µl) and 10 µl of cell lysate. The reaction was performed at 30°C and 405 nm for 17 min (shaking 2 seconds at 500 rpm before each cycle). Activity was calculated using 36.8 M^{-1} cm^{-1} as molar coefficient for ABTS, and was normalised on the total protein amount measured using dual wavelength Bradford method (Zor and Selinger 1996).

Cultivation

An initial screening for MAO-N D5 high producer was performed in 500 ml shake flasks. Then, 100 ml of BMGY was inoculated with a single colony of *P. pastoris* carrying either pPpT4-S-MAO D5 or pPpT4-S-Alpha-MAO D5 and cultivated at 28°C and 250 rpm for 17 hours. Cell suspension was then centrifuged at 7000 *g* for 10 min and resuspended in 100 ml of BMMH medium. Finally, 100 µl of pure methanol was added 3 times per day for 6 days for the induction of MAO-N D5 expression.

The cultivation scale-up was performed as described in Markošová et al. 2015, described in detail in Supplementary material 1.

Crude enzyme extract preparation

Crude enzyme extract from the fermentation media was prepared according to two different types of disruption protocols. Glass beads disruption protocol was applied as described above, except for the sediment from 100 µl of media, which was resuspended in 300 µl of Glass Bead Extraction Buffer; as an alternative, high pressure continual cell disrupter (Constant cell disruption systems, Constant Systems LTD, UK) was tested in parallel. Biomass was first centrifuged at 7000 *g* for 10 min at 4°C and then resuspended in potassium phosphate buffer

(0.1 M, pH 8). Cell disruption took place at 4°C, in one cycle at 40 kPSI. The resulting lysate was ultracentrifuged at 50,000 *g*, 4°C for 30 minutes.

Biotransformation

Biotransformation reaction and processing of the samples were performed as in Zajkoska et al. 2015; described in Supplementary material 1.

Analysis

Glycerol and methanol concentrations were measured by HPLC with an Agilent Technologies 1220 Infinity LC apparatus with an Agilent Technologies 1260 Infinity RI detector (Agilent Technologies, Germany), using a WATREX Polymer IEX H form 8 μm, 250×8 mm as the main column and a WATREX Polymer IEX H form 8 μm, 40x8 mm as a guard column, at a flow rate of 0.8 ml/min of 9 mM sulphuric acid at 45°C.

Imine concentration was measured using the GC Agilent Technologies 6890N Network GC System (column: CAM 0.25 μm, 30 m x 0.32 mm, 1.6 ml/min of hydrogen as carrier gas with pressure 34.9 kPa, temperature profile of 110°C for 4.2 min, and a gradient of 30°C/min until 200°C). The total time was eight minutes and at the end, the temperature was cooled to 100°C. The volume of injection was 1 μl with split 1:50.

Nucleotide sequence accession number

The codon-optimized nucleotide sequence of MAO-N D5 gene reported here has been deposited in the GenBank nucleotide sequence database with accession number MF472009.

Results

MAO cloning, screening of clones in DWP plates

P. pastoris CBS7435 MutS clones, transformed with pPpT4-S-MAO-N D5, were obtained and cultivated as described in the Methods section. Upon cultivation and methanol induction, 23 independent colonies were picked for deep-well plate cultivation and activity screening at the microscale level. A higher throughput YPER-mediated cell lysis, previously reported for *P. pastoris* cells (McKinney et al. 2004), was attempted but yielded no detectable MAO activity (data not shown); on the contrary, lysates from glass beads disruption method showed significant MAO activity compared to the control strain (Fig. 1).

-*insert Fig.1*-

Screening of selected clones in 500-ml shake flasks

Five independent clones, spanning the whole range of measured activities from deep-well plate cultivation, were selected for shake flask cultivation. After 6 days of induction, MAO activity in crude lysates for oxidative desymmetrisation of Azabicyclo [3,3,0] octane HCl was tested, and specific activities, normalised on cell dry weight of the respective clones, were calculated (Tab. 1).

-*insert Table 1*-

Scale-up in 1.5 l fermenter

Clone C4, as a representative clone for the selected MAO-N D5 producing panel, was cultivated in BSM media using a 3 l laboratory fermenter (1.5 l working volume). The fermentation was performed according to Markošová et al. (2015) (Fig.2). The methanol feeding was set according to the yeast metabolic activity reflected by the DO level.

-*insert Fig. 2*-

Comparison of biotransformation performance between crude enzyme extract and whole cells bioconversion

Five ml of cell suspension from the fermentation broth was used as a whole cell biocatalyst to measure MAO activity. Surprisingly, the specific activities of the enzyme (Fig.2) rapidly decreased over time, even if the induction proceeded and the biomass was obviously growing. This phenomenon was observed using whole cells and might be due to either a transcriptional silencing effect of the recombinant enzyme, leading to less recombinant protein expressed in the unit of time. In the economy of the process aiming to develop an efficient catalyst, a shorter process increases the overall productivity. That´s why such decreased specific activity of the catalyst was circumvented by establishing an expression routine with a harvest at 50 hours.
Since the expressed MAO-N D5 is produced intracellularly, two disruption methods were tested for effective crude enzyme production: a glass beads disruption protocol versus a high pressure continual cell disrupter, normalising the activity of crude enzyme extracts on dry cell weight. Table 2 shows the activities obtained over time using the two disruption methods, clearly reporting the superiority of the glass-bead disruption protocol over high pressure disrupter or whole cells as a biocatalyst.

-*insert Table 2*-

Comparison of the results with *E. coli*

By applying an optimised fermentation protocol (Markošová et al. 2015) for *P. pastoris* expressing MAO-N D5, a reliable source of monoamine oxidase was obtained (Fig 3). Table 3 summarises a comparison of MAO-N D5 prepared using either *E. coli* (Zajkoska et al. 2015; Markošová et al. 2016) or *P. pastoris* (present work). The productivity of the enzyme prepared in *Pichia* is 83 times higher than the one prepared in *E. coli*. Also, the total produced activity of crude enzyme extract (per 1 l of fermentation media), even though it was after a longer period of time, is 203 times higher.

-*insert Fig. 3*- -*insert Table 3*-

Discussion

Monoamine oxidases (MAOs), enzymes catalysing the asymmetrical oxidation of a variety of amines, represent a relevant tool for biocatalysis. Wildtype or engineered MAOs have been expressed in *Escherichia coli* and characterised in terms of substrate specificity and regioselectivity; MAO from *Aspergillus niger*, in particular has been engineered to considerably extend its substrate specificity (Atkin et al. 2008; Zajkoska et al. 2015; Ramesh et al. 2016; Rios-Solis et al. 2015). Since the gene is originated from a filamentous fungus *A. niger* (Atkin et al. 2008), the use of *E. coli* as a host for protein expression might have limited the overall yield of such catalysts. The gene for MAO-N D5 was codon-optimised and was successfully cloned into *Pichia pastoris* CBS7435 Mut^S strain under the AOX promoter. From the 23 screened colonies, 5 were selected in order of specific MAO activity in their lysates and scaled up to shake flask and bioreactor scale. The production in a 3 l laboratory fermenter was

250 set according to fermentations with a previously reported *P. pastoris* Mut^S protocol (Markošová
251 et al. 2015). MAO activity was monitored throughout a batch fermentation (Fig.2), comparing
252 activities from whole cell biocatalyst with crude enzyme extracts obtained following two
253 different protocols. The activity using the whole cell biocatalyst reached its maximum of 7.4
254 U/g_{DCW} and was significantly decreased over time, even with the ongoing methanol induction.
255 A possible explanation for this contradiction could be the difficulties of substrate/product
256 transport through the yeast membrane (Markošová et al. 2016), since cell wall during
257 cultivation on methanol may become thicker, as observed e.g. for *Saccharomyces* cultivated on
258 ethanol (Aguilar-Uscanga and François 2003). However, the specific activity of extracted
259 enzyme normalized by the biomass did not decrease over time. Alternatively, the toxicity of the
260 produced imine might have a larger negative impact on the whole cell biocatalyst (Ramesh and
261 Woodley 2014). When observing the activity of the crude enzyme extract prepared by continual
262 disrupter there is the same trend of decreasing activity (Table 2), which also confirms possible
263 difficulties due to a more rigid cell wall. Comparing the activity with the enzyme prepared in
264 flask experiments in the BMGY media, after 168 hours (24 h of cultivation and 144 of
265 induction), where the activity was 15.9 U/g_{DCW}, the activity of the enzyme prepared in the
266 fermenter after 144 hours (23 h of cultivation and 121 h of induction), was only 13 U/g_{DCW} and
267 decreasing over time. The best way to obtain an active enzyme extract of MAO-N D5 was the
268 glass beads disruption protocol, with which we were able to obtain the activity of 44.1 U/g_{DCW}.
269 Even with the increasing trend of biomass and ongoing methanol induction, the activity
270 remained at the same value. While total activity per reactor volume can be increased with longer
271 cultivation time there is no increase in activity normalized by biomass. Two methanol pulses
272 of 3 g/l are sufficient for the maximum activity obtained. For whole cell activity the longer
273 cultivation in the presence of methanol shows negative effect.

Compared with the fermentation preparation of MAO-N D5 using *E. coli* (Zajkoska et al. 2015), the dry cell weight reached in the LB medium was 2.4 g/l, which is about 10.3 times lower. It was previously shown that glycerol has a negative effect on the activity of MAO-N D5; even when the *E. coli* cells were washed with buffer to remove the residual glycerol from the fermentation medium, the conversion never reaches 100% (Ramesh et al. 2016). To overcome this problem, the production of the MAO-N D5 with *P. pastoris* fed batch process where methanol is the carbon source and also inducer for the protein expression is more appropriate (this study). Another interesting phenomenon is that the IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction in *E. coli* did not show increased activity (Markošová et al. 2016), so there is no way to obtain higher amounts of the enzyme. The specific productivity achieved in our experiments using *P. pastoris* as an expression host was 83 times higher compared to reported expressions in *E. coli*.

Conclusions

The recombinant monoamine oxidase (MAO-N D5) from *Aspergillus niger* was for the first time cloned and expressed in *P. pastoris* Mut^S expression system. In this paper it was clearly demonstrated that the production of MAO-N D5 is better in eukaryotic expression system of *P. pastoris* in comparison with prokaryotic *E. coli*. The achieved productivity was 83 times higher in *P. pastoris* and also the total produced activity of crude enzyme extract per 1 l of fermentation media was 203 times higher.

Acknowledgements

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357

Table and figure captures

374 **Table 1**

	C2	C3	C4	B7	B11
Activity (U/g_{DCW})	14.2	14.9	15.9	15.2	14.6

375

376

377 **Table 2**

Time (h)	Activity (U/g_{DCW})		
	Whole cell	CE (continual disrupter)	CE (glass beads)
70.75	7.40	-	-
146	2.69	13.03	44.12
191	2.22	11.44	-
238	0.56	-	45.50

378

379

380 **Table 3**

Strain	Fermentation time (h)	Induction (h)	Volume (L)	Productivity (U/L/h)	Total produced activity of CE/ 1L of fermentation media (U/L)
E.coli BL21 (DE3)	20*	none*	2*	1.99**	39.68**
P.pastoris Mut^S CBS7435	49 (23+26)	26	1.5	164.43	8057.43

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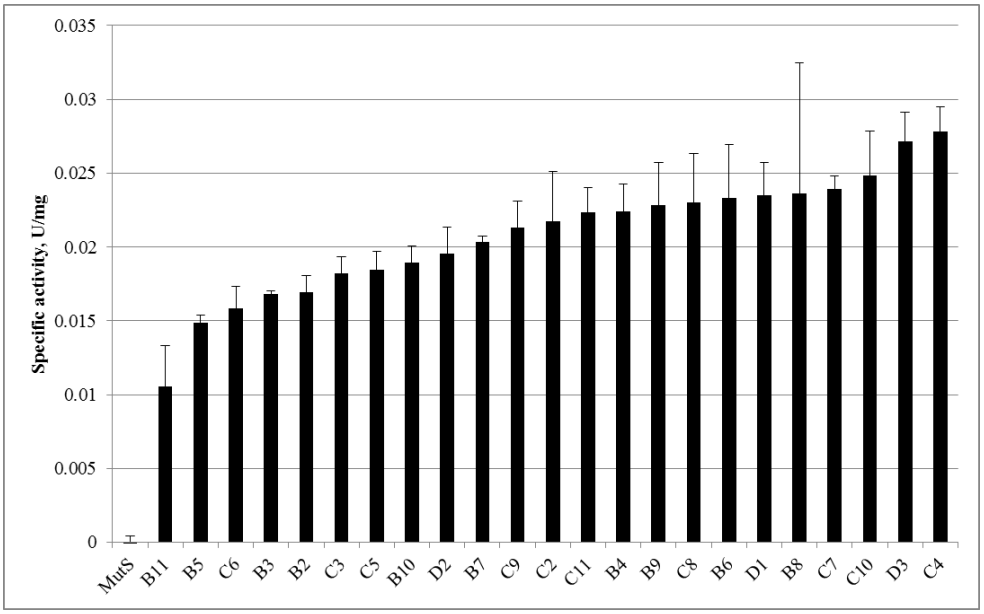
383

384 **Fig. 1**

385



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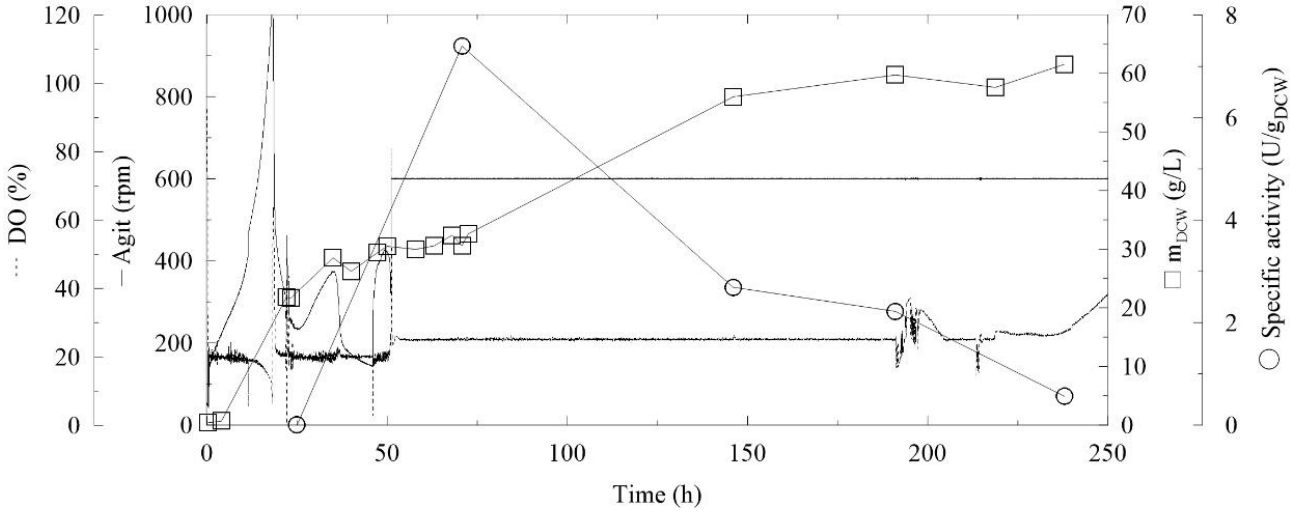
388 **Fig.2**



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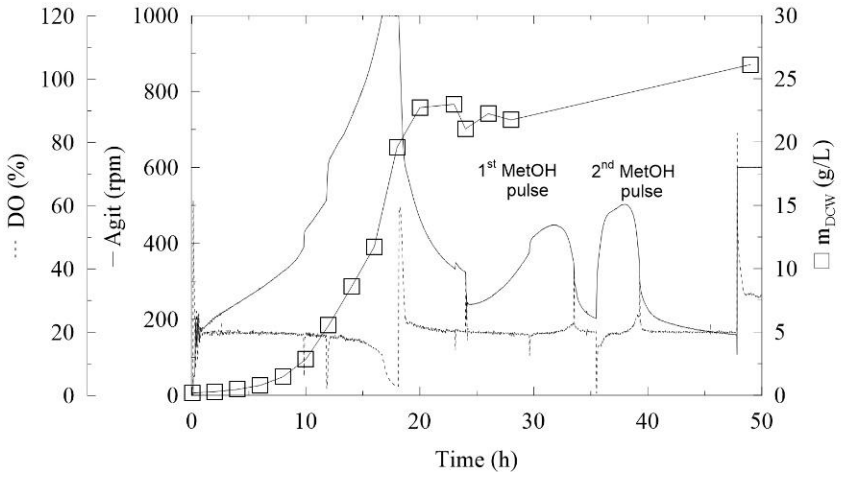
391 **Fig. 3**



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Supplementary material 1:

Material and methods:

Media

LB [Luria-Bertani Medium: 1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract and 2% (w/v) agar]

YPD [Yeast Extract Peptone Dextrose Medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar] in addition to zeocin (50 mg/l for *E. coli* and 100 mg/l for *P. pastoris*).

BMD [1% (w/v) glucose, 200mM potassium phosphate buffer, pH=6, 1.34% (w/v) YNB (Yeast Nitrogen Base, Invitrogen, USA) and 4.10^{-5}% (w/v) biotin]

BMM2 [1% (v/v) methanol, 200mM potassium phosphate buffer, pH=6, 1.34% (w/v) YNB and 4.10^{-5}% (w/v) biotin]

BMM10 [5% (v/v) methanol, 200mM potassium phosphate buffer, pH=6, 1.34% (w/v) YNB and 4.10^{-5}% (w/v) biotin] according to Weis et al., 2008 (Näätsaari et al. 2012).

BMGY [Buffered Glycerol-complex Medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol]

BMMH [Buffered Minimal Methanol Medium: 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 0.5% (v/v) methanol].

BSM (Basal Salt Medium per l: 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4$. 7 H_2O, 4.13 g KOH, and 40 g glycerol) medium and supplemented with 4.35 ml of PTM_1 (trace salts solution per l: 6 g $CuSO_4$ .5 H_2O, 0.08 g NaI, 3 g $MnSO_4.H_2O$, 0.2 g $Na_2MoO_4.2$ H_2O, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4.7H_2O$, 0.2 g biotin, 9.2 g H_2SO_4) per litre of BSM medium. Methanol added in fed-batch experiments was also supplemented with PTM_1 (1.2 ml pure methanol/l).

420

421 Cultivation scale-up

422

423 The inoculum for the fermentation cultivation was prepared in 100ml of YPD medium. The
424 fermentation was performed according to our previous report (Markošová et al. 2015) in 3-l
425 laboratory fermenters (Brunswick BioFlo® 115, Eppendorf, Hamburg, Germany). Then, 1.5l of
426 BSM media supplemented with 4.35ml of PTM_1 solution per l of media was inoculated with
427 5% inoculum (OD_{600} approx. 6-8) and the fermentation conditions were as follows: 30°C, pH
428 5 maintained by ammonia solution (28-30%), DO 20% maintained by agitation cascade from
429 50 to 1000 rpm, aeration 0.66 v/v/m with the addition of 200µl of Struktol J650 as an antifoam.
430 After the complete depletion of glycerol (approx. 22-24 hours), two methanol pulses of 3g/l
431 were added after 23 and 35.5 hours. After depletion of the second pulse, the agitation was fixed
432 at 600 rpm, the agitation cascade was stopped and an additional methanol (3g/l) was added. The
433 methanol feed was connected to the actual level of dissolved oxygen as described (Markošová
434 et al. 2015). Whenever the level of DO rose above 32%, the methanol feeding was turned on
435 by an automated program, and when the DO level rose above 45%, signalling the overflow of
436 methanol and the incapability of the culture to utilise it, the pump was again turned off. In this
437 way, the culture was never inhibited by methanol concentrations higher than 3 g/l, which is the
438 maximum tolerable concentration for Mut^S-type strains (Khatri and Hoffmann 2006).

439

440 Biotransformation

441

442 Enzyme activity was measured by monitoring oxidation of 3-Azabicyclo [3,3,0] octane HCl,
443 98% as a substrate. The reaction mixture consisted of 2.96 g of the substrate/l, 0.1M potassium
444 phosphate buffer (pH 8) and biomass from 5ml of fermentation media (whole cell

445 bioconversion) or crude enzyme extract as a catalyst. The reaction took place in a 50ml Falcon
446 tube, at 37°C and 250 rpm. Reactions were monitored using GC, processed as follows: 8 µl of
447 10 M NaOH and 1 ml of tert-butyl-methyl-ether were added to 200 µl of the sample. Samples
448 were vortexed and centrifuged for 1 min at 13300*g*. The organic phase was collected into a
449 clean tube with 0.2 g sodium sulphate (non-aqueous) and analysed by gas chromatography (GC)
450 for substrate and product detection. One unit of MAO-N D5 represents the amount of enzyme
451 catalysing the formation of one µmol of imine per minute at 37°C and pH 8. The specific
452 activity was related to either cell dry weight of biomass used for the biotransformation (whole
453 cell conversion) or biomass disrupted to prepare crude enzyme extracts. All activities were
454 calculated as previously reported (Zajkoska et al. 2015).
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