



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

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

3

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16 **Abstract**

17 *Objective*

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

21 *Results*

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

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

29 *Conclusions*

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

33

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

35

36 **Introduction**

37 Monoamine oxidases (MAO) belong to the oxidoreductase class and are able to undergo
38 selective oxidative deamination or deracemisation of primary, secondary and tertiary amines.

39 The resulting imines are important precursors or by-products in the pharmaceutical industry
40 and organic chemistry. One of the best characterised MAOs, MAO-N from *Aspergillus niger*

41 (Schilling and Lerch 1995), was the object of extensive engineering. Enzyme variants with

42 improved activity and specificity, including the variant MAO-N-D5, were obtained via directed

43 evolution (Atkin et al. 2008); immobilisation of whole cell biocatalysts or crude enzyme extract

44 using LentiKats[®] technology were evaluated (Zajkoska et al. 2015; Markošová et al. 2016).

45 Recently, a kinetic model for substrate specificity of MAO-N in different biocatalytic reactions

46 was described (Rios-Solis et al. 2015), while other groups analysed the influence of cultivation

47 medium on enzyme activity (Ramesh et al. 2016).

48 MAO-N has been expressed in *Escherichia coli*; however, since the enzyme originates from a

49 filamentous fungus, using a eukaryotic host for MAO production represents an interesting

50 possibility, due to the increased compatibility between the expression host and the protein of

51 interest. Among the possible hosts for recombinant protein production (RPP), one of the most

52 widely utilised is the methylotrophic yeast *Pichia pastoris*, recently reclassified as
53 *Komagataella phaffii* (Kurtzman 2009). *Pichia pastoris* presents many advantages of higher
54 eukaryotic expression systems, specifically good posttranslational modifications, correct
55 protein folding, generally high protein expression levels (gram of protein per litre) and the
56 possibility of relatively efficient protein secretion; moreover, its cultivation is cost-effective,
57 similar to *E. coli* fermentation, and reaches high cell densities (Cereghino and Cregg 2000).
58 This work aims to explore the production of a well-characterised monoamine oxidase variant,
59 MAO-N D5, using a eukaryotic expression system, comparing its activity against the more
60 characterised production in *E. coli*: in particular we report for the first time the use of *P. pastoris*
61 as an expression system for MAO-N D5 enzyme. Enzyme production and activity in selected
62 high-producing clones for MAO-N D5 were evaluated in small scale cultures and bioreactors,
63 scaling production up by applying an optimised fermentation protocol (Markošová et al. 2015).
64 Finally, a crude enzyme extract obtained from optimised fermentation was tested in a model
65 biotransformation of a secondary amine, comparing the activity and volumetric productivity
66 with the prokaryotic expression system, *E. coli* (Zajkoska et al. 2015; Markošová et al. 2016).

67

68 **Materials and methods**

69 Microorganisms and Media

70

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

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

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

79 Fed-batch fermentations were carried out in BSM (Basal Salt Medium) medium and
80 supplemented with 4.35 ml of PTM₁ (trace salts solution) per litre of BSM medium. Methanol
81 added in fed-batch experiments was also supplemented with PTM₁ (1.2 ml pure methanol/l).

82 The composition of each media are fully described in Supplementary material 1.
83

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

85

86 The coding sequence of MAO-N D5, encoding the monoamine oxidase-N with five amino acid
87 mutations (Ile246Met/Asn336Ser/Met348Lys/Thr384Asn (Atkin et al. 2008)), was codon-
88 optimised according to DNA 2.0 optimisation algorithm and cloned in pPpT4-S or pPpT4-S-
89 Alpha vectors (Weis et al. 2004) downstream of the AOX1 promoter (*EcoRI*, *NotI* restriction
90 sites) or in-frame with the alpha mating factor from *S. cerevisiae* (*XhoI*, *NotI* restriction sites).

91 Plasmidic DNA was isolated from *E. coli* TOP10F transformed cells using Thermo Scientific
92 GeneJET Plasmid Miniprep Kit and checked by Sanger sequencing. Plasmids were linearised
93 using *SwaI* (Fast Digest, Thermo Scientific, USA) and approximately 500 ng of linearised
94 plasmid pPpT4-S-MAO-N D5 were used to transform *Pichia pastoris* CBS7435 Mut^S
95 competent cells following the standard condensed protocol (Lin-Cereghino et al. 2005).
96 Transformed cells were plated on YPD plates with zeocin (100 µg/ml) and incubated at 30°C
97 for 48 hours.

98

99 Screening in deep-well plates

100

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

117

118 MAO activity assay – deep well plate cultivation

119

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

126 of ABTS (220 μ g/ μ l) and 10 μ l of cell lysate. The reaction was performed at 30°C and 405 nm
127 for 17 min (shaking 2 seconds at 500 rpm before each cycle). Activity was calculated using
128 36.8 M⁻¹ cm⁻¹ as molar coefficient for ABTS, and was normalised on the total protein amount
129 measured using dual wavelength Bradford method (Zor and Selinger 1996).

130

131 Cultivation

132

133 An initial screening for MAO-N D5 high producer was performed in 500 ml shake flasks. Then,
134 100 ml of BMGY was inoculated with a single colony of *P. pastoris* carrying either pPpT4-S-
135 MAO D5 or pPpT4-S-Alpha-MAO D5 and cultivated at 28°C and 250 rpm for 17 hours. Cell
136 suspension was then centrifuged at 7000 g for 10 min and resuspended in 100 ml of BMMH
137 medium. Finally, 100 μ l of pure methanol was added 3 times per day for 6 days for the induction
138 of MAO-N D5 expression.

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

141

142 Crude enzyme extract preparation

143

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

150 (0.1 M, pH 8). Cell disruption took place at 4°C, in one cycle at 40 kPSI. The resulting lysate
151 was ultracentrifuged at 50,000 g, 4°C for 30 minutes.

152

153 Biotransformation

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

156

157 Analysis

158

159 Glycerol and methanol concentrations were measured by HPLC with an Agilent Technologies
160 1220 Infinity LC apparatus with an Agilent Technologies 1260 Infinity RI detector (Agilent
161 Technologies, Germany), using a WATREX Polymer IEX H form 8 µm, 250×8 mm as the main
162 column and a WATREX Polymer IEX H form 8 µm, 40x8 mm as a guard column, at a flow
163 rate of 0.8 ml/min of 9 mM sulphuric acid at 45°C.

164 Imine concentration was measured using the GC Agilent Technologies 6890N Network GC
165 System (column: CAM 0.25 µm, 30 m x 0.32 mm, 1.6 ml/min of hydrogen as carrier gas with
166 pressure 34.9 kPa, temperature profile of 110°C for 4.2 min, and a gradient of 30°C/min until
167 200°C). The total time was eight minutes and at the end, the temperature was cooled to 100°C.
168 The volume of injection was 1 µl with split 1:50.

169

170 Nucleotide sequence accession number

171

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

174

175 **Results**

176 MAO cloning, screening of clones in DWP plates

177

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

185

186 *-insert Fig.1-*

187

188 Screening of selected clones in 500-ml shake flasks

189

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

195

196 *-insert Table 1-*

197

198 Scale-up in 1.5 l fermenter

199

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

204

205 *-insert Fig. 2-*

206

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

209

210 Five ml of cell suspension from the fermentation broth was used as a whole cell biocatalyst to
211 measure MAO activity. Surprisingly, the specific activities of the enzyme (Fig.2) rapidly
212 decreased over time, even if the induction proceeded and the biomass was obviously growing.
213 This phenomenon was observed using whole cells and might be due to either a transcriptional
214 silencing effect of the recombinant enzyme, leading to less recombinant protein expressed in
215 the unit of time. In the economy of the process aiming to develop an efficient catalyst, a shorter
216 process increases the overall productivity. That's why such decreased specific activity of the
217 catalyst was circumvented by establishing an expression routine with a harvest at 50 hours.

218 Since the expressed MAO-N D5 is produced intracellularly, two disruption methods were tested
219 for effective crude enzyme production: a glass beads disruption protocol versus a high pressure
220 continual cell disrupter, normalising the activity of crude enzyme extracts on dry cell weight.
221 Table 2 shows the activities obtained over time using the two disruption methods, clearly
222 reporting the superiority of the glass-bead disruption protocol over high pressure disrupter or
223 whole cells as a biocatalyst.

224

225 -insert Table 2-

226

227 Comparison of the results with *E. coli*

228

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

236

237 -insert Fig. 3- -insert Table 3-

238 **Discussion**

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

274 Compared with the fermentation preparation of MAO-N D5 using *E. coli* (Zajkoska et al. 2015),
275 the dry cell weight reached in the LB medium was 2.4 g/l, which is about 10.3 times lower. It
276 was previously shown that glycerol has a negative effect on the activity of MAO-N D5; even
277 when the *E. coli* cells were washed with buffer to remove the residual glycerol from the
278 fermentation medium, the conversion never reaches 100% (Ramesh et al. 2016). To overcome
279 this problem, the production of the MAO-N D5 with *P. pastoris* fed batch process where
280 methanol is the carbon source and also inducer for the protein expression is more appropriate
281 (this study). Another interesting phenomenon is that the IPTG (Isopropyl β -D-1-
282 thiogalactopyranoside) induction in *E. coli* did not show increased activity (Markošová et al.
283 2016), so there is no way to obtain higher amounts of the enzyme. The specific productivity
284 achieved in our experiments using *P. pastoris* as an expression host was 83 times higher
285 compared to reported expressions in *E. coli*.

286

287 **Conclusions**

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

294

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306

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357

358 **Table and figure captures**

359

360 **Table 1** The activity of different clones of *P. pastoris* expressing MAO-N D5

361 **Table 2** The activity of MAO-N D5 during the fermentation process using different forms of
362 biocatalyst

363 **Table 3** Comparison of MAO-N D5 production in *E. coli* and *P. pastoris*

364 **Fig. 1** Specific MAO activity in *P. pastoris* crude lysates (deep well plate cultivations). MutS:
365 CBS 7435 Mut^S strain, negative control

366 **Fig. 2** Fermentation of *Pichia pastoris* CBS 7535 Mut^S, clone C4, expressing MAO-N D5 with
367 fed-batch methanol feeding according to actual level of dissolved oxygen. Conditions: 1.5 l
368 BSM, 30°C, pH 5 (ammonia solution), DO= 20% by agitation cascade 50-1,000 rpm, 5%
369 inoculum

370 **Fig. 3** Fermentation of *Pichia pastoris* expressing MAO-N D5 with fed-batch methanol feeding
371 according to actual level of dissolved oxygen. Conditions: 1.5 l BSM, 30°C, pH 5 (ammonia
372 solution), DO= 20% by agitation cascade 50-1,000 rpm, 5% inoculum

373

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)
<i>E.coli</i> BL21 (DE3)	20*	none*	2*	1.99**	39.68**
<i>P.pastoris</i> Mut ^S CBS7435	49 (23+26)	26	1.5	164.43	8057.43

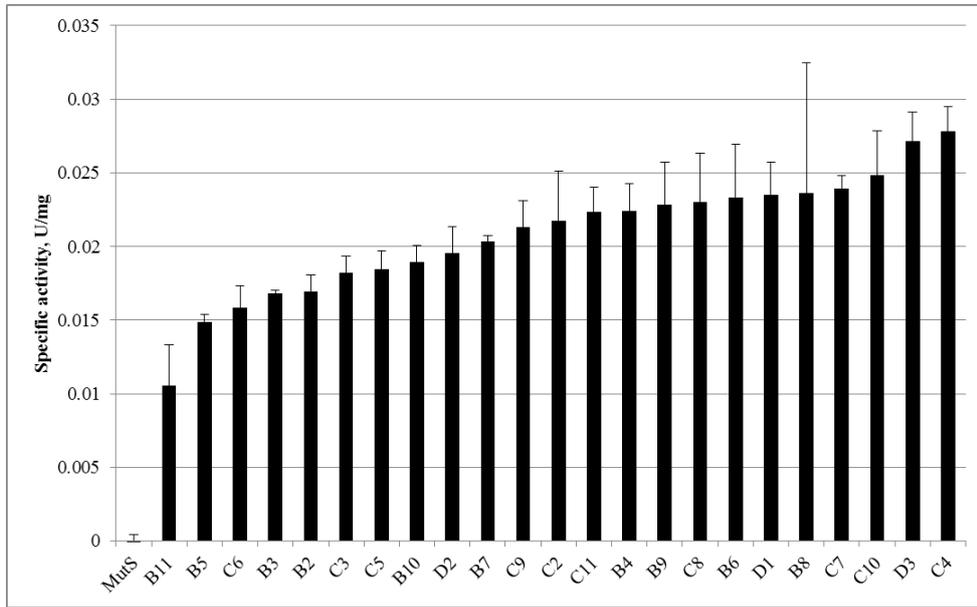
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382

383

384 **Fig. 1**

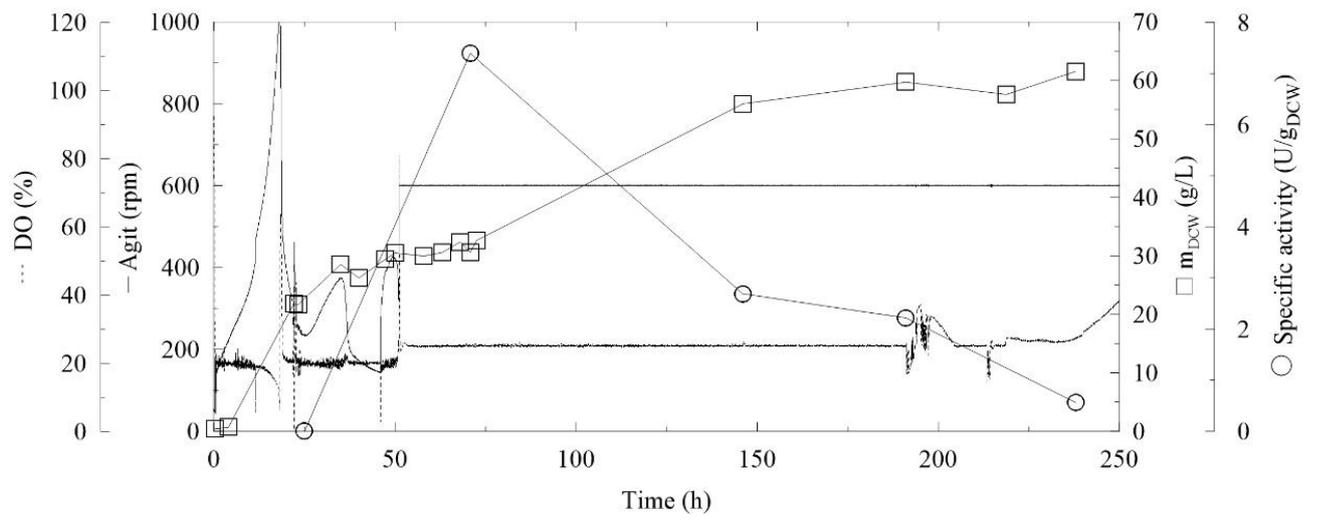
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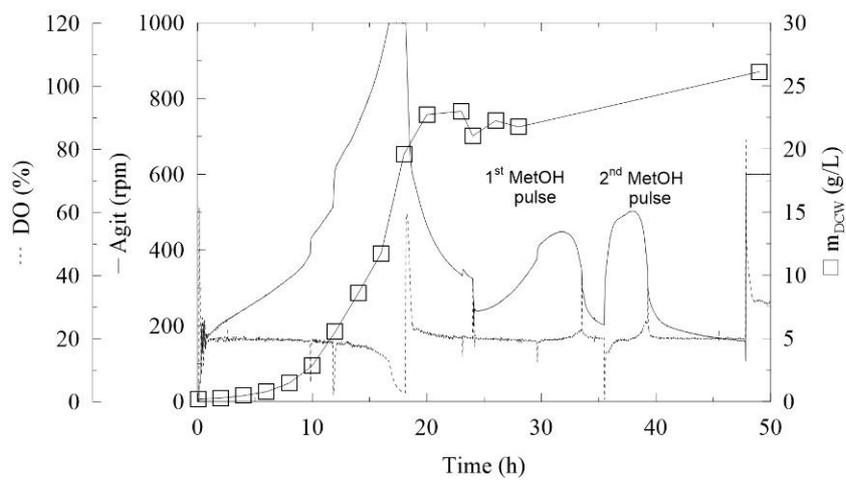
388 **Fig.2**



389

390

391 **Fig. 3**



392

393

394

395 Supplementary material 1:

396

397 **Material and methods:**

398 Media

399 **LB** [Luria-Bertani Medium: 1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract and
400 2% (w/v) agar]

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

404 **BMD** [1% (w/v) glucose, 200mM potassium phosphate buffer, pH=6, 1.34% (w/v) YNB (Yeast
405 Nitrogen Base, Invitrogen, USA) and 4.10⁻⁵% (w/v) biotin]

406 **BMM2** [1% (v/v) methanol, 200mM potassium phosphate buffer, pH=6, 1.34% (w/v) YNB
407 and 4.10⁻⁵% (w/v) biotin]

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

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

412 **BMMH** [Buffered Minimal Methanol Medium: 100 mM potassium phosphate, pH 6.0, 1.34%
413 (w/v) YNB, 4 × 10⁻⁵% (w/v) biotin, 0.5% (v/v) methanol].

414 **BSM** (Basal Salt Medium per l: 26.7 ml 85% H₃PO₄, 0.93 g CaSO₄, 18.2 g K₂SO₄, 14.9 g
415 MgSO₄ · 7 H₂O, 4.13 g KOH, and 40 g glycerol) medium and supplemented with 4.35 ml of
416 PTM₁ (trace salts solution per l: 6 g CuSO₄ · 5 H₂O, 0.08 g NaI, 3 g MnSO₄ · H₂O, 0.2 g
417 Na₂MoO₄ · 2 H₂O, 0.02 g H₃BO₃, 0.5 g CoCl₂, 20 g ZnCl₂, 65 g FeSO₄ · 7H₂O, 0.2 g biotin, 9.2 g
418 H₂SO₄) per litre of BSM medium. Methanol added in fed-batch experiments was also
419 supplemented with PTM₁ (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 PTM₁ solution per l of media was inoculated with
427 5% inoculum (OD₆₀₀ 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 13300g. 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).

455

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