



High-level expression of -amino acid oxidase in

Sandra Abad, Jozef Nahalka, Margit Winkler, Gabriele Bergler, Robert Speight,
Anton Glieder, Bernd Nidetzky

► To cite this version:

Sandra Abad, Jozef Nahalka, Margit Winkler, Gabriele Bergler, Robert Speight, et al.. High-level expression of -amino acid oxidase in. *Biotechnology Letters*, 2010, 33 (3), pp.557-563. <10.1007/s10529-010-0456-9>. <hal-00638146>

HAL Id: hal-00638146

<https://hal.science/hal-00638146v1>

Submitted on 4 Nov 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Section: Microbial and Enzyme Technology

High-level expression of *Rhodotorula gracilis* D-amino acid oxidase in *Pichia pastoris*

Sandra Abad¹ · Jozef Nahalka^{1, 2, 4} · Margit Winkler¹ · Gabriele Bergler¹ · Robert Speight⁵ · Anton Glieder^{1,3} · Bernd Nidetzky^{1,2,§}

¹ Austrian Centre of Industrial Biotechnology, Petersgasse 14, 8010 Graz, Austria

² Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria

³ Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010 Graz, Austria

⁴ Current address: Institute of Chemistry, Center of Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, SK-84538 Bratislava, Slovak Republic

⁵ Ingenza Ltd., Wallace Building, Roslin BioCentre, Roslin, EH25 9PP, UK

[§] Corresponding author

Email: bernd.nidetzky@tugraz.at, tel: +43 316 873 8400; fax: +43 316 873 8434

Keywords: D-amino acid oxidase · permeabilization · *Pichia pastoris* · *Rhodotorula gracilis* · stability

Section: Microbial and Enzyme Technology

Abstract By combining gene design and heterologous over-expression of *Rhodotorula gracilis* D-amino acid oxidase (RgDAO) in *Pichia pastoris*, enzyme production was enhanced by one order of magnitude compared to literature benchmarks, giving 350 kUnits/l of fed-batch bioreactor culture with a productivity of 3.1 kUnits/l h. *P. pastoris* cells permeabilized by freeze-drying and incubation in 2-propanol (10% vv/) produce a highly active (1.6 kUnits/g dry matter) and stable oxidase preparation. Critical bottlenecks in the development of an RgDAO catalyst for industrial applications have been eliminated.

Introduction

D-Amino acid oxidases (DAO) are flavoenzymes of significant interest for application in biotechnology (Pollegioni et al. 2008; Tishkov and Khoronenkova 2005). The most widely quoted use of DAO is in the industrial manufacture of 7-amino-cephalosporanic acid, an important intermediate in the production of semi-synthetic antibiotics (Riethorst and Reichert 1999). For this process, DAO from the yeast *Trigonopsis variabilis* (TvDAO) is regarded as the most suitable enzyme, essentially because it displays superior specific activity towards the bulky cephalosporin C substrate (Pollegioni et al. 2004). More recently, TvDAO has also been considered as catalyst for dynamic kinetic resolution of amino acid racemates (Beard and Turner 2002). However, for this application to become economically viable, there is a clear demand for enhancement of total turnover number (TTN) of the TvDAO preparation used under the industrial process conditions (Fig. 1; Fotheringham et al. 2006). We have developed a *Pichia pastoris* whole-cell catalyst of TvDAO in which significant improvement of TTN results from two main effects of strain engineering (Abad et al. 2010b). Firstly, *P. pastoris*, containing recombinant TvDAO localized in their peroxisomes, displayed oxidase activity in markedly stabilized form, as compared to the free enzyme. Secondly, combining TvDAO overexpression with suitable permeabilization of the *Pichia* cells provided a catalyst having useful specific activity (per unit mass of preparation; ~600 Units/g). Further enhancement of TTN would be achievable through molecular stabilization of the enzyme. With TvDAO, however, this goal remains elusive (Nidetzky 2007).

(Figure 1)

We therefore consider DAO from the yeast *Rhodotorula gracilis* (RgDAO) is the most thoroughly characterized microbial member of the DAO group of enzymes (Fitzpatrick 2010; Pollegioni et al. 2002; Umhau et al. 2000). Taking into account that loss of FAD appears to be the preponderant path of inactivation of TvDAO under a wide range of conditions (Betancor et al. 2003; Dib and Nidetzky 2008; Dib et al. 2006; Pollegioni et al. 2004), the ability of RgDAO to bind its cofactor by one order of magnitude more tightly than TvDAO made the enzyme from *R. gracilis* a promising candidate for development of DAO catalysts showing further enhanced robustness. However, exploitation of the extra stability of RgDAO as compared to TvDAO is hampered by relatively inefficient production of the *R. gracilis* enzyme in both native (Molla et al. 2003) and *E. coli* (Wang et al. 2008) host strains. We show here that optimized expression of RgDAO in *P. pastoris* eliminates this bottleneck. By applying cell permeabilization with 2-propanol (Abad et al. 2010b), a

potentially useful oxidase preparation was obtained that is superior to reported whole-cell DAO catalysts with respect to specific activity and stability.

Materials and methods

Chemicals and media

Oligonucleotides were from IDT Integrated DNA Technologies BVBA (Leuven, Belgium). DNA-modifying enzymes were from Fermentas (Burlington, Ontario, Canada). Unless otherwise stated, chemicals were from Carl Roth GmbH (Karlsruhe, Germany), Becton, Dickinson and Company (Franklin Lakes, NJ, USA) or Sigma-Aldrich (St Louis, MO, USA).

Construction of *P. pastoris* expression cassettes

E. coli TOP10F' (Carlsbad, CA, USA) was the host for cloning. The *RgDAO* gene (GenBank: CAA96323) was codon-optimized using methods described in Abad et al. (2010b) for expression in *P. pastoris* (see Supporting Information) and synthesized by GenScript (Piscataway, NJ, USA). To facilitate isolation of recombinant *RgDAO*, the gene was further modified to also encode an *N*-terminal *Strep* II tag. PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), digested with *Eco*RI and *Not*I (Fermentas, Burlington, Canada), and ligated via *Eco*RI/*Not*I restriction sites into in-house *E. coli*/*P. pastoris* shuttle vectors pPpT2 and pPpB1 that provide single and multi-copy chromosomal integration of target genes in *P. pastoris*, respectively (Abad et al. 2010b). Details of these vectors are available upon request.

Transformation of *P. pastoris* and copy number determination

A Mut^S strain derived from *P. pastoris* CBS 7435 (TU Graz strain collection number: BT 3132) was used for transformation applying the protocol of Lin-Cereghino et al. (2005). Electro-competent cells were transformed using pPpT2 or pPpB1 previously linearized by *Bgl*II and purified by QIAquick PCR Purification Kit. After electroporation and regeneration for 2 h at 30°C using agitation at 60 rpm, aliquots (0.2 ml) were plated on solid selection medium containing 100 mg Zeocin/L. The number of copies of *RgDAO* expression cassettes integrated into the *P. pastoris* genome was determined by quantitative real-time PCR as described in Abad et al. (2010a), employing the *AOX1* promoter as target and the endogenous *ARG4* gene as a reference.

P. pastoris cultivation at different scales, and enzyme purification

P. pastoris transformants were cultivated in deep well plates (96-well format) or 2 l shake-flasks using conditions described in Weis et al. (2004). Sample preparation for intracellular activity measurement and the assay for DAO activity were as reported by Abad et al. (2010b). Bioreactor cultivations were performed with a 1.5-l fed batch-Pro Bioreactor system (Dasgip AG, Juelich, Germany) applying the protocol of Hellwig et al. (1999). A methanol feed of 3 ml/h was used (Abad et al. 2010b). Recombinant RgDAO was purified from cells obtained in shake-flask culture, using the procedure described in Dib et al. (2007) expanded by a final purification step on MonoQ HR 5/5 column.

Permeabilization of *P. pastoris* cells and activity measurement for whole-cell enzyme preparations

Cells from bioreactor cultivations were used. Treatment with 2-propanol (10% v/v) in water for 3 h was employed to achieve permeabilization, applying the conditions described in Abad et al. (2010b). Assays for activity and stability of soluble as well as whole-cell oxidase preparations were based on direct measurement of O₂ consumption during oxidation of D-methionine or D-alanine (Abad et al. 2010b). A bubble-aerated magnetically stirred glass mini-reactor with a working volume of 30 ml was employed to conduct these assays (Nahalka et al. 2008).

For the activity measurements, the reactions were started by adding 20 µL of appropriately diluted *P. pastoris* cell extract or 4 µg purified protein. Initial rates of O₂ consumption were recorded for activity determination, therefore no aeration was used. For the stability measurements, the reaction mixture contained 20 or 100 mM substrate dissolved in 10 mM or 100 mM Tris/HCl buffer (pH 7.5), respectively, and air was continuously sparged at 30 l/h. Reactions were started by adding 100 µg purified protein.

Results and discussion

Expression design

A previously successful approach of high-level TvDAO expression in *P. pastoris* combined gene design for optimum codon usage with protein targeting to peroxisomes (Abad et al. 2010b). The codon-optimized sequence of RgDAO is shown in the Supporting Information. RgDAO contains the canonical PTS1 peroxisomal targeting motif (-Ser-Lys-Leu) at its C-terminal end that is known to be well recognized by the

corresponding transport machinery in *P. pastoris* (Monosov et al. 1996). Therefore, unlike *TvDAO*, in which the absence of a suitable PTS1 motif necessitated enhancement of sub-cellular targeting by protein engineering (Abad et al. 2010b), the native primary structure of *RgDAO* was expected to support efficient translocation of recombinant enzyme to host cell peroxisomes. Expression of the *RgDAO* gene from the methanol inducible alcohol oxidase promoter was chosen. The number of integrated expression cassettes necessary for high-level recombinant protein production cannot be foreseen in *P. pastoris*. The codon-optimized *RgDAO* gene was therefore cloned into plasmids enforcing low and multi-copy gene integration into the host cell genome. Screening for transformants displaying high oxidase activity was performed in high through-put format (Weis et al. 2004). Two strains thought to feature single and multi-copy gene integration events were chosen and are henceforth named *RgDAO_sc* and *RgDAO_mc*, respectively. Quantitative real-time PCR analysis (Abad et al. 2010a) confirmed the presence of 1 (*RgDAO_sc*) and 12-13 (*RgDAO_mc*) copies of the expression cassette integrated in the *P. pastoris* genome.

Characterization of the selected *RgDAO* production strain

Controlled fed-batch bioreactor experiments, following a standard protocol for high-cell density cultivation of *P. pastoris* (Abad et al. 2010b), were carried out to evaluate strains *RgDAO_sc* and *RgDAO_mc* with respect to production of biomass and oxidase activity. Results are summarized in Table 1. The intracellular activity of *RgDAO_mc* surpassed that of *RgDAO_sc* by a factor of 6, demonstrating the benefit of multi-copy gene integration for strain construction. However, the relationship between enzyme titer and the number of integrated expression cassettes is not completely linear. Interestingly, in *P. pastoris* expressing *TvDAO* (Abad et al. 2010b), change from a single-copy strain (767 U/g wcw) to a multi-copy strain harboring 16 – 17 copies of the expression cassette resulted in only moderate (1.7-fold) enhancement of specific oxidase activity. The volumetric productivity of *RgDAO_mc* (3.1 kU/L/h) was about 9-fold that of *RgDAO_sc*. *RgDAO_mc* showed about 8.5-fold expression improvement as compared to a previously reported *E. coli* strain (Molla et al. 1998). Its specific oxidase activity was about twice that of the corresponding multi-copy strain of *P. pastoris* producing *TvDAO* (Abad et al. 2010b).

(Table 1)

Table 1 shows that enhancement of specific oxidase activity in *RgDAO_mc* as compared to *RgDAO_sc* went along with a 1.6-fold decrease in biomass yield. This finding is in agreement with

observations from previous studies of DAO expression in *P. pastoris* (Abad et al. 2010b; Zheng et al. 2006) and supports the idea that high levels of DAO activity are not readily accommodated by the growing *P. pastoris* cell. Abad et al. (2010b) showed that the negative effect of DAO over-expression in *P. pastoris* on biomass formation can be alleviated by using a mixed substrate feed of glycerol and methanol. However, optimization of the yield of *RgDAO*_mc biomass was beyond the scope of this study. Notwithstanding, a volumetric yield of 350 kU/l for *RgDAO* activity represents a significant improvement, by one order of magnitude, as compared to literature benchmarks (Wang et al. 2008). Enzyme production should therefore no longer be a critical bottleneck in the development of *RgDAO* preparations for applied biocatalysis.

Quality control for DAO production in *Pichia pastoris*

RgDAO and *TvDAO*, each harboring N-terminal Strep-tag II and containing the SKL peroxisomal targeting motif at their C-terminus, were produced in *P. pastoris* and purified to apparent electrophoretic homogeneity, as demonstrated in Fig. 2. The *TvDAO* derived from *P. pastoris* migrated in SDS PAGE with a relative mobility that was identical to that of a reference preparation obtained from *E. coli*. Specific activities for O₂-dependent conversion of D-Ala and D-Met are shown in Table 2. Preference for reaction with D-Met as compared to D-Ala is consistent with reported biochemical data for each enzyme (Tishkov and Khoronenkova 2005). The measured values of specific activity for each oxidase were in good agreement with literature (Tishkov and Khoronenkova 2005), indicating that the functionality of enzymes produced in *P. pastoris* was comparable to preparations obtained through other expression hosts. This is clearly worth noting considering that *in vivo* assembly of a functional DAO molecule relies on availability of sufficient amounts of FAD cofactor, which can be limiting in a high level expression approach. Furthermore, as shown for *TvDAO* (Nidetzky 2007), the target protein may be sensitive to oxidative modification, which appears not to be an issue for the supported peroxisomal localization. We conclude therefore that high-level recombinant production of *RgDAO* and *TvDAO* in *P. pastoris* peroxisomes occurs with excellent retention of quality in enzyme preparations as isolated.

(Figure 2)

(Table 2)

Data supporting the notion that *RgDAO* is kinetically more stable than *TvDAO* were obtained under temperature stress conditions in the absence of the enzyme catalyzed (Betancor et al. 2003; Pilone and Pollegioni 2002). However, resistance to high-temperature inactivation may not be an adequate measure of stability under process conditions. Using reported methodology (Nahalka et al. 2008), we therefore determined the loss of activity of purified *RgDAO* and *TvDAO* during conversion of D-Met using O₂ supply by bubble aeration (Fig. 3). Because enzyme inactivation occurs at a rate much faster than the rate of depletion of substrate, the observed increase in the concentration of O₂ as a function of the incubation time reflects the activity loss (Nahalka et al. 2008). Time-course data for the increase in O₂ concentration were fitted exponentially, yielding the inactivation rate constants (k_{in}) summarized in Table 2. At 100 mM D-Met, *RgDAO* displayed about five-fold superior stability (lower k_{in}) as compared to *TvDAO*. Because at a lower substrate concentration of 20 mM, both enzymes showed similar resistance to inactivation, the difference in enzyme stability at the high substrate level must result from significant destabilization of *TvDAO* under these conditions.

(Figure 3)

Pichia pastoris* whole-cell catalyst expressing *RgDAO

Abad et al. (2010b) have shown that whole cells of *TvDAO*_mc are poorly active catalysts for the conversion of D-Met because the high level of intracellular oxidase activity was almost completely masked by mass transfer limitations. Lyophilization of cells followed by incubation of dried biomass in the presence of 10% 2-propanol caused dramatic (125-fold) enhancement of utilization of the enzyme activity present (see Table 1). The catalytic effectiveness factor (η) for permeabilized *TvDAO*_mc as compared to total intracellular enzyme activity was 0.49 (= 627/1283). Applying the same sequence of treatment to *RgDAO*_mc, the data shown in Table 1 were obtained. The steps of lyophilization and treatment with organic solvent each contributed about half of the total (58-fold) enhancement of whole-cell oxidase activity achieved in comparison to untreated *P. pastoris* biomass. The η value of 0.59 (= 1624/2762) for permeabilized *RgDAO*_mc even surpassed the corresponding effectiveness factor for *TvDAO*_mc. (Note: Because *RgDAO*_mc surpassed *RgDAO*_sc with respect to production of intracellular oxidase activity (Table 1), permeabilization of the single-copy strain was not further pursued.) Stability tests for permeabilized *RgDAO*_mc were performed as described above for the purified enzyme, taking care that the steady-state

level of dissolved O₂ was always limited by the amount of enzyme activity present. Inactivation rate constants for permeabilized *RgDAO_mc* were at the lower limit of detection of the used assay ($k_{in} \leq 5 \times 10^{-4} \text{ min}^{-1}$), indicating a very useful operational stability of this whole-cell catalyst.

Conclusions

Optimized expression in *P. pastoris* alleviates the likely bottleneck of enzyme production in the development of *RgDAO* catalysts for application in fine chemical synthesis. The best producing *P. pastoris* strain obtained in this study had 12-13 expression cassettes integrated into its genome and delivered an enzyme yield of 350 kU/L of bioreactor culture, exceeding relevant literature values by one order of magnitude. After permeabilization, this strain constituted a stable whole-cell catalyst preparation that could make use of 60% of its total intracellular oxidase activity. Its specific activity surpassed that of comparable whole-cell systems by a factor of 2 or more. *RgDAO* was obtained from *P. pastoris* as a high-quality protein preparation. At high D-Met substrate loading, isolated enzyme showed superior operational stability as compared to *TvDAO*, which is currently used in industry.

Acknowledgements

The authors would like to thank Thorsten Bachler and Margaretha Schiller for their excellent technical support. The Österreichische Forschungsförderungsgesellschaft mbH (FFG), the provinces of Vienna, Styria and Tyrol as well as the Steirische Wirtschaftsförderungsgesellschaft mbH (SFG) and Ingenza Ltd. are acknowledged for financial support. BN and JN acknowledge the Slovak-Austrian travel project SK-AT-0024-08.

References

- Abad S, Kitz K, Hormann A, Schreiner U, Hartner FS, Glieder A (2010a) Real-time PCR-based determination of gene copy numbers in *Pichia pastoris*. *Biotechnol J* 5:413-420
- Abad S, Nahalka J, Bergler G, Arnold SA, Speight R, et al. (2010b) Stepwise engineering of a *Pichia pastoris* D-amino acid oxidase whole cell catalyst. *Microb Cell Fact* 9:24
- Beard TM, Turner NJ (2002) Deracemisation and stereoinversion of α -amino acids using D-amino acid oxidase and hydride reducing agents. *Chem Commun (Camb)* 7:246-247

- Betancor L, Hidalgo A, Fernandez-Lorente G, Mateo C, Rodriguez V, et al. (2003) Use of physicochemical tools to determine the choice of optimal enzyme: stabilization of D-amino acid oxidase. *Biotechnol Prog* 19:784-788
- Dib I, Nidetzky B (2008) The stabilizing effects of immobilization in D-amino acid oxidase from *Trigonopsis variabilis*. *BMC Biotechnol* 8:72
- Dib I, Slavica A, Riethorst W, Nidetzky B (2006) Thermal inactivation of D-amino acid oxidase from *Trigonopsis variabilis* occurs via three parallel paths of irreversible denaturation. *Biotechnol Bioeng* 94:645-654
- Dib I, Stanzer D, Nidetzky B (2007) *Trigonopsis variabilis* D-amino acid oxidase: control of protein quality and opportunities for biocatalysis through production in *Escherichia coli*. *Appl Environ Microbiol* 73:331-333
- Fitzpatrick PF (2010) Oxidation of amines by flavoproteins. *Arch Biochem Biophys* 493:13-25
- Fotheringham I, Archer I, Carr R, Speight R, Turner NJ (2006) Preparative deracemization of unnatural amino acids. *Biochem Soc Trans* 34:287-290
- Hellwig S, Robin F, Drossard J, Raven NP, Vaquero-Martin C, et al. (1999) Production of carcinoembryonic antigen (CEA) N-A3 domain in *Pichia pastoris* by fermentation. *Biotechnol Appl Biochem* 30:267-275
- Lin-Cereghino J, Wong WW, Xiong S, Giang W, Luong LT, et al. (2005) Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. *Biotechniques* 38:44, 46, 48
- Molla G, Motteran L, Piubelli L, Pilone MS, Pollegioni L (2003) Regulation of D-amino acid oxidase expression in the yeast *Rhodotorula gracilis*. *Yeast* 20:1061-1069
- Molla G, Vegezzi C, Pilone MS, Pollegioni L (1998) Overexpression in *Escherichia coli* of a recombinant chimeric *Rhodotorula gracilis* D-amino acid oxidase. *Protein Expr Purif* 14:289-294
- Monosov EZ, Wenzel TJ, Luers GH, Heyman JA, Subramani S (1996) Labeling of peroxisomes with green fluorescent protein in living *P. pastoris* cells. *J Histochem Cytochem* 44:581-589
- Nahalka J, Dib I, Nidetzky B (2008) Encapsulation of *Trigonopsis variabilis* D-amino acid oxidase and fast comparison of the operational stabilities of free and immobilized preparations of the enzyme. *Biotechnol Bioeng* 99:251-260
- Nidetzky B (2007) Stability and stabilization of D-amino acid oxidase from the yeast *Trigonopsis variabilis*. *Biochem Soc Trans* 35:1588-1592

- Pilone MS, Pollegioni L (2002) D-amino acid oxidase as an industrial biocatalyst. *Biocatal Biotransform* 20:145-159
- Pollegioni L, Caldinelli L, Molla G, Sacchi S, Pilone MS (2004) Catalytic properties of D-amino acid oxidase in cephalosporin C bioconversion: a comparison between proteins from different sources. *Biotechnol Prog* 20:467-473
- Pollegioni L, Diederichs K, Molla G, Umhau S, Welte W, et al. (2002) Yeast D-amino acid oxidase: structural basis of its catalytic properties. *J Mol Biol* 324:535-546
- Pollegioni L, Molla G, Sacchi S, Rosini E, Verga R, Pilone MS (2008) Properties and applications of microbial D-amino acid oxidases: current state and perspectives. *Appl Microbiol Biotechnol* 78:1-16
- Riethorst W, Reichert A (1999) An industrial view on enzymes for the cleavage of cephalosporin C. *Chimia* 53:600-607
- Tishkov VI, Khoronenkova SV (2005) D-Amino acid oxidase: Structure, catalytic mechanism, and practical application. *Biochemistry (Moscow)* 70:40-54
- Umhau S, Pollegioni L, Molla G, Diederichs K, Welte W, et al. (2000) The x-ray structure of D-amino acid oxidase at very high resolution identifies the chemical mechanism of flavin-dependent substrate dehydrogenation. *Proc Nat Acad Sci USA* 97:12463-12468
- Wang SJ, Yu CY, Kuan IC (2008) Stabilization of native and double D-amino acid oxidases from *Rhodospiridium toruloides* and *Trigonopsis variabilis* by immobilization on streptavidin-coated magnetic beads. *Biotechnol Lett* 30:1973-1981
- Weis R, Luiten R, Skranc W, Schwab H, Wubbolts M, Glieder A (2004) Reliable high-throughput screening with *Pichia pastoris* by limiting yeast cell death phenomena. *FEMS Yeast Res* 5:179-189
- Zheng HB, Wang XL, Chen J, Zhu K, Zhao YH, et al. (2006) Expression, purification, and immobilization of His-tagged D-amino acid oxidase of *Trigonopsis variabilis* in *Pichia pastoris*. *Appl Microbiol Biotechnol* 70:683-689

Table 1: RgDAO expression in *P. pastoris*. Results are mean values from three independent determinations using D-Met as substrate and have S.D. < 5%.

Strain	CN ^a	Intracellular	Fresh	Lyophilized	Permeabilized	Biomass	Reference
		activity	cells	cells	cells		
		[U/g wcw] ^b				[g wcw/batch] ^b	
<i>RgDAO_sc</i>	1	450	n.d. ^c	n.d. ^c	n.d. ^c	210	this study
<i>RgDAO_mc</i>	12-13	2762	28	769	1624	128	this study
<i>TvDAO_mc</i>	16-17	1283	5	321	627	117	(Abad et al. 2010b)

^a CN: copy number for expression cassette integration

^b wcw, weight cell weight

^c n.d., not determined

Table 2: Characterization of *Rg*DAO and *Tv*DAO, produced in and isolated from *P. pastoris*. Results are from three independent determinations and have S.D. < 10%.

	Specific activity [U/mg]		Inactivation rate constant [min ⁻¹] ^a	
	20 mM D-Met	20 mM D-Ala	20 mM	100 mM
<i>Tv</i> DAO	112	38	0.0195	0.0778
<i>Rg</i> DAO	117	88	0.0155	0.0153

^a See text for further details

Fig. 1 DAO-based dynamic kinetic resolution of amino acids (Fotheringham et al. 2006). The method relies on the absolute enantioselectivity of the oxidase catalyst. The imino acid produced in the enzymatic reaction is immediately reduced in a non-stereoselective manner. Whole cells of *E. coli* expressing TvDAO were considered for this process but low TTN of this biocatalyst still resulted in an increase of ~ €10/kg to the process cost

Fig. 2 Purified preparations of RgDAO and TvDAO, both produced in *P. pastoris*, analyzed by SDS PAGE. Lanes 1 and 3 show RgDAO and TvDAO, respectively. Lane 2 shows a TvDAO preparation obtained by expression in *E. coli* (Dib et al. 2007). Each of the three enzymes contains an *N*-terminal Strep-tag II. Molecular mass standards (14.4, 20, 30, 45, 66 and 97 kDa) are shown (lane St)

Fig. 3 Stability determination of a purified RgDAO preparation. **A** O₂ consumption measurement during a conversion reaction with 100 mM D-Met as substrate. The time of addition substrate is reflected by immediate decrease of the O₂ level. Increase in O₂ result because of enzyme inactivation. **B** Inverted and normalized O₂ consumption curve (•) and fit of data with an exponential decay function, from which the instability factor (k_{in}) is obtained

Fig. 1

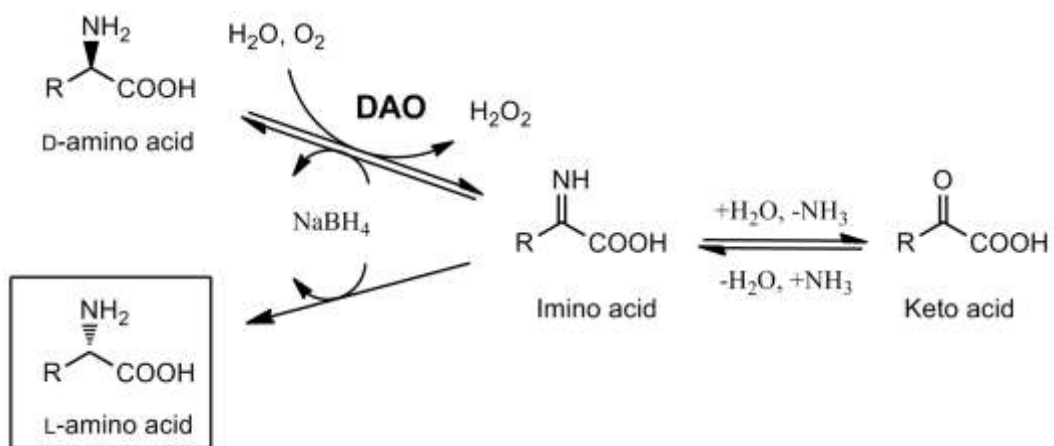


Fig. 2

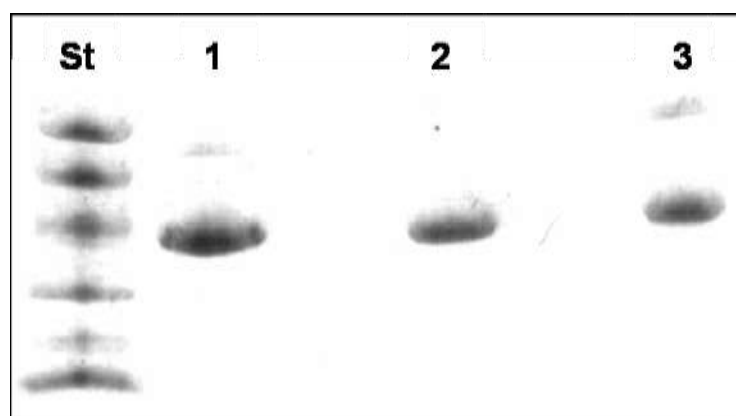
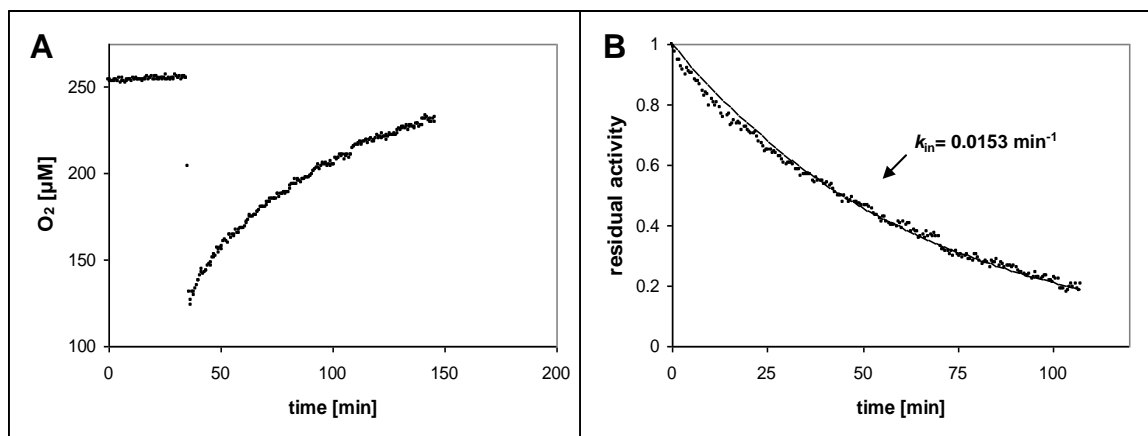


Fig. 3



Supplementary Data

RgDAO sequence (GenBank: CAA96323) re-designed for optimum codon usage in *P. pastoris*:

Atgcactctcagaaacgtgtcgttgcttaggttctgggtgcattgggtctgtcttcagcccttatttggctagaaagggttactcagtcacatcttgccagagac
ttgcctgaagacgtttcttctcaaacctttgcttctccttgggctggagccaactggactccattcatgactttaaccgacgggtccaagacaagctaagtgggagg
aatctactttcaagaaatgggttgaactgtcccaactggtcacgctatgtggttgaagggtactcgtagattcgcctaaacgaggacggtttattgggacattg
gtataaggatatactcctaactacagaccattgccatcttctgaatgccaccagggtgccatcggagtcacttacgatactctgtccgtccatgctccaaagtact
gtcaatacttggctagagaacttcagaaactgggagctaccttcgaaagacgtactgtcacttccctggagcaagcctttgatggagctgacttgggtgtaatgc
aaccggattgggagctaaatctatcgtctggtattgatgaccaagcagctgagcctatcagaggacagactgttcttgaagtccccatgtaagagatgcactat
ggactcctctgacctgcttctcctgcatacatcattccaagacctgggtggagaaagtcatttgggtggaacctacgggtgttgggtgactgggaccttctgttaacc
ctgaaaccgtgcaacgtattcttaagcactgtttgcgttggaccaaccatttctctgacggaaccattgagggatcagggttttgagacacaacgttgggtctt
agacctgctagacgtggtgggtccaagagtggaggcagagagaattgtttgccttagacagaaccaagtccttcattggttagaggttccgctagagc
tgcaaaggagaaggaaagtactttggttcacgcatacgttttctctccgccggttaccagcaatcatgggggtgctgctgaggacgttgcacaactggttgacga
agctttccaacgttatcacgggtgccgctagagagtccaagctgtaa