Immobilisation and kinetics of monoamine oxidase (MAO-N-D5) enzyme in polyvinyl alcohol gels

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Abstract

This study focused on the production and immobilisation of the crude enzyme extract of recombinant monoamine oxidase (EC 1.4.3.4), originated from *Aspergillus niger* (MAO-N-D5) and expressed in *Escherichia coli*, in PVA gel using the LentiKats® technique. MAO-N are important enzymes in the chemical industry for their stereoselectivity and they are often used for the deracemisation of non-optically pure mixtures of amines. Biomass production, enzyme preparation, immobilisation of the enzyme, process parameters for the immobilised enzyme and characterisation of the enzyme are described in detail. The biomass was prepared in laboratory bioreactors, and a comparison of two different disruption techniques was made. The activity of the enzyme was determined by biotransformation with secondary amine 3-

azabicyclo [3,3,0] octane as a substrate. Crude enzyme extract had 61.5% of the whole cell activity and it was proven that the immobilised enzyme has a wider pH and temperature optimum than the free enzyme. The initial specific activity of immobilised monoamine oxidase crude enzyme extract remained 80% after 12 repeated biotransformations. For the first time the full kinetic parameters of an immobilized MAO-N D5 were obtained based on a ping-pong bi-bi reaction mechanism. The k_{cat} was 0.42 mM/h/U and the K_m was 7.31 mM, which represented an improvement in kinetic efficiency in comparison to cell MAO-N. Characterisation of immobilized MAO-N showed particular benefits in terms of activity and stability in comparison with free and whole cell MAO-N, therefore the immobilization is very suitable for industrial applications.

Key words: monoamine oxidase, polyvinyl alcohol, immobilisation, kinetic parameters

Highlights

– monoamine oxidase enzyme extract successfully immobilised in PVA hydrogel
– immobilised enzyme showed improved pH and temperature stability
– immobilised enzyme retained activity over 12 consecutive batches

1. Introduction

Monoamine oxidases (MAO, EC 1.4.3.4) are enzymes that catalyse the oxidation of primary, secondary and tertiary amines. They belong to the flavin-containing amine reductases family of enzymes since they contain a flavin adenine dinucleotide (FAD) as a cofactor [1]. They are three types of monoamine oxidase, MAO-A, MAO-B and MAO-N. The A and B are human monoamine oxidases, where they play an important role in the central nervous system. For example, abnormal activity of MAO-B is involved in neurologic diseases, such as Parkinson's disease or Alzheimer's disease [2]. MAO-N is a monoamine oxidase originally isolated from the filamentous fungus *Aspergillus niger*. MAO-N was

extensively studied in evolution experiments, leading to the creation of mutants with improved activities and wide substrate specificity [3]. Created mutants that are expressed in *E.coli* are suitable for deracemisation of primary, secondary and tertiary amines within the chemo-enzymatic oxide-reduction cycle (Fig.1) [4].

– Insert Figure 1 –

MAO-N are stereoselective, and therefore, they are often used for the deracemisation of non-optically pure mixtures of amines. Enantioselective MAO only oxidise S-enantiomer to imine, which is subsequently reduced *in situ* back to racemic amine. Optically pure amines are widely used as resolving agents and chiral auxiliary substances, and they can also be valuable intermediates for drug synthesis and agrochemicals [5]. Recombinant MAO are sensitive to oxygen, therefore, they may be influenced by culture aeration [5]. One of the methods that may improve its stability and allow for the repetitive use of this group of enzymes is immobilisation.

Immobilisation of the enzymes allows for improving almost all properties of enzymes, such as their stability, activity, specificity, and selectivity, in addition to inhibition reduction etc., although there can be negative effects due to substrate mass transfer limitations into the immobilisation matrix [6]**.** The main advantages of immobilisation are: increase of enzyme activity in organic solvents, increase of thermal stability, remarkable operational stability, increase of enantioselectivity, easy enzyme separation from the reaction by filtration or centrifugation etc. [7]. From an economic point of view, the reuse of the enzyme is a problem that immobilisation can easily resolve [6]. The biggest advantage in using an immobilized enzyme instead of immobilized biomass is certainly in the low risk of contamination or loss of

the cell viability during storage and repetitive use and with none possibility of the environment contamination with the recombinant bacteria.

LentiKats® technology is a novel method of immobilisation developed in recent years. It is based on the entrapment of the biocatalyst, both enzymes and whole cells, into polyvinyl alcohol hydrogel (PVA), which has excellent physical and mechanical properties, as well as being non-toxic and difficult to biodegrade. Lens shaped particles (diameter 3–4 mm, thickness 200–400 μ m) are also ideal for manipulation and there are low diffusion limitations in the transfer of the substrate and product [8].

The aim of this study was to prepare the recombinant monoamine oxidase (MAO) crude enzyme extract and its immobilisation into lens shaped particles LentiKats®. pH, temperature profiles, enzyme kinetics, substrate toxicity, stability and repetitive use of immobilised enzyme were also investigated with a view to large scale, industrial application of the MAO-N-D5 enzyme.

2. Material and methods

2.1. Chemicals and media

All chemicals used were analytical grade materials. The Luria-Bertani media (LB) (per liter) consisted of 1% (w/v) NaCl, 1% (w/v) tryptone, and 0.5% (w/v) yeast extract, and 2% (w/v) agar was added to the agar plates. After autoclaving to all LB mediums, 100 μ g/mL of filter sterilised ampicillin (Sigma Aldrich, USA) was added.

2.2. Microorganism, culture and inoculum preparation

Escherichia coli BL21(DE3) with pET16b+ expressing MAO-N D5 was provided by Prof. Nicholas Turner, from the University of Manchester (UK). D5 indicates five amino acid mutations in the gene encoding monoamine oxidase N: Ile246Met/Asn336Ser/Met348Lys/Thr384Asn [9]. The microorganism was cryopreserved at -80°C in 50% (v/v) glycerol and was cultivated on LB plates. Inoculum and fermentation biomass grew in LB media supplemented with ampicillin (100 ng/μL). The pre-inoculum culture was prepared as follows: 3 mL of LB in a glass tube was inoculated with a single colony from LB plates and cultivated overnight (approx. 12 hours) in rotary shaker (225 rpm, 37°C). Inoculum preparation: 100 mL of LB medium in a 500 mL flask was inoculated with 1% (v/v) of pre-inoculum culture and cultivated until the OD_{600} reached 0.5 in a rotary shaker (225 rpm, 37°C).

2.3. Biomass production

Batch fermentations were carried out in a 3 L Brunswick Flo® 115 fermentor with 2 L of LB media. The conditions were as follows: 1% (v/v) inoculum, oxygen saturation 5% with cascade agitation 100–1000 rpm, 30°C, 20 hours, 1 vvm aeration and without pH stating.

2.4. Crude enzyme extracts preparation

Prepared biomass was harvested by centrifugation (30 min, 2750 g, 4°C) and the sediment was resuspended in 20 mL of potassium phosphate buffer (0.1 M, pH 8) to a final concentration 0.064 g_{DCW}/mL (0.064 g of dry cell weight per 1 mL of buffer). The cell suspension was disrupted using a high-pressure disruptor French press (Fischer Scientific, USA) in three disruption cycles at 120 kPSI or in a continual cell disruptor (Constant cell disruption systems, Constant Systems LTD, UK) in one disruption cycle at 20 kPSI at 4°C. After disruption, 1 mL of protease inhibitor (Complete EDTA-free, Roche Diagnostics, Germany) per 20 mL of crude enzyme extract was added. The crude enzyme extract was then

ultracentrifuged (Avanti® centrifuge 1-30I, Beckman Coulter, USA) (30 min, 20000 g, 4°C) and the supernatant was stored at -20°C for further experiments. MAO crude enzyme extract was concentrated by ultrafiltration membrane Amicon® Ultra (15 mL, 50 kDa Cut off membrane, Millipore, USA). Then, 14 mL of crude enzyme extract was centrifuged for 1.5 hours at 4000 g and 4°C until it reached a final volume of 7 mL.

2.5. Immobilisation

Polyvinyl alcohol gel (PVA, 4 g) and polyethylene glycol (PEG, 2.4 g) were mixed together with 31.5 mL of water and heated at 90°C for 30 minutes. The mixture was cooled to 40°C and 7 mL of concentrated MAO crude enzyme extract was added. Particles were prepared using laboratory immobilization equipment LentiPriner (www.lentikats.eu). The drops were then dried for 45 minutes at 40°C and hardened in 0.1 M of sodium sulphate. The prepared particles were washed by a sterile potassium phosphate buffer (0.1 M, pH=8) and stored at 4°C in a potassium phosphate buffer (0.1 M, pH=8) with ampicillin (100 ng/ μ L).

2.6. Biotransformations

As a substrate for biotransformations, a secondary amine 3-azabicyclo [3,3,0] octane HCl (AK Scientific, Inc.) was used. The reaction mixture with ultracentrifuged crude enzyme extract contained 2 mL of enzyme extract, 3 mL of potassium phosphate buffer (0.1 M, pH=8) and 2.96 g/L of the substrate. The reaction mixture with concentrated enzyme extract contained 0.5 mL of enzyme, 2 mL of the buffer and 2.96 g/L of substrate. Reactions with the immobilised biocatalyst contained 1 g of immobilised enzyme 5 mL of the buffer and 2.96 g/L of substrate. After each biotransformation, the particles were washed in the potassium phosphate buffer (0.1 M, pH=8) and stored in the same buffer at 4°C overnight. The same particles were used for the next cycle after overnight storage. The biotransformation conditions were as follows: 50 mL plastic Falcon tube, 37°C and 250 rpm in an orbital shaker.

pH profile biotransformations were performed with 0.5 mL of enzyme (prepared as 2.4, non-concentrated), 4.5 mL potassium phosphate buffer (0.1 M) with different pH values (4.6, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9.05) and 2.96 g/L of the substrate. The temperature profile was investigated at pH 8 and at the following temperatures (°C): 25, 30, 37, 41, 45, 50, and 55. The pH profile and temperature profile of the immobilised enzyme was performed with 0.5 g of the particles in 5mL of buffer, with the same conditions as above.

2.7. Kinetic parameter measurements

All kinetic measurements were performed in polypropylene 24 deep well Oxodish (Presens, Regensburg, Germany), which included both pH and oxygen SDR SensorDish. The total volume for all standard bioconversions was 2 mL, and different concentrations of 3-azabicyclo [3.3.0] octane were used (2, 5, 10, 20, 50, 100, 200, 300mM) in 1M phosphate buffer to maintain a constant pH during the bioconversions (pH 8). 0.2 g of LentiKats® with immobilized crude enzyme extract were also added to each well. Catalase was added to the bioconversion in order to avoid any build-up of H_2O_2, which could be toxic to the enzyme, as well as to make the bioconversion irreversible by removing a product.

The bioconversions were performed at 37°C and shaking was provided at 400 rpm with a Thermomixer Comfort shaker (Eppendorf, Cambridge, UK), (shaking diameter of 6 mm). Aliquots of 100 μL were taken at various time intervals. All experiments were performed in triplicate. The specific activities were determined as the amount of imine product 3-azabicyclo-[3,3,0]oct-2-ene formed per unit of time which was synthetized as described in (Rios et al., 2015) [10], and which was measured by gas chromatography and normalised by the units of enzyme used in the reaction. One unit of enzyme activity was defined as the amount of enzyme which catalysed the formation of 1 µmol of 3-azabicyclo-[3,3,0]oct-2-

ene for 1 min in a forward reaction at a 20 mM concentration at 37°C, 400 rpm and a pH of 8, using a 1 M phosphate buffer.

2.8. Analytics

The samples from the biotransformations were processed as follows: to 200 μL of the sample, 8 μL of 10 M NaOH and 1 mL of tert-butyl-methyl-ether were mixed. The sample was vortexed and centrifuged for 1 min at 13300 g. The organic phase was collected into a clean tube with 0.2 g sodium sulphate (non-aq.), before being vortexed and short spun. The organic phase was analysed by gas chromatography (GC).

Samples for kinetic analysis were processed as follows: 100 μL of samples were mixed with 10 μL of a 1 M NaOH solution. Then, 200 µL tert-butyl-methyl-ether was added to the aliquots, shaken at 37°C and 900 rpm for 30 min in a Thermomixer Comfort Shaker (Eppendorf, Cambridge, UK). The samples were centrifuged for 5 min at 3000 g and the organic phase was collected for gas chromatography analysis.

The concentration of created imine was measured using the GC Agilent Technologies 6890N Network GC System: column: CAM 0.25 μm, 30m x 0.32mm, 1.6 mL/min of hydrogen as a carrier gas with pressure 34.9 kPa, a 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. Volume of injection was 1 μL with split 1:50.

2.9. Activity calculation

Initial specific activity of MAO-N was calculated from the linear part of the biotransformations [9]. The activity of the enzyme was calculated as the change of the substrate amount in the time of the linear part according to the following equation:

$a = (n_1 - n_2) / (t_1 - t_2)$

The concentration of dry cell weight was calculated from the calibration curve y = 0.306x – 0.046 of the dependency of the concentration of the dry cell weight and OD_{600nm} of *E.coli*. The specific activity was calculated as the activity divided by the dry cell weight used for biotransformation:

$a_{sp} = a / m_{DCW}$

Respectively, the activity of the immobilised enzymes was divided by the amount of particles used for the biotransformation:

$a_{sp} = a/m_{immobilised\ enzyme}$

3. Results and discussion

As described previously [9], *E.coli* BL21(DE3) expressing MAO-N-D5 has a low productivity and the enzyme expression is not even inducible using IPTG. Therefore, a stable enzyme that may be repetitively used for biocatalysis is highly sought after.

3.1. Biomass production and whole cell biotransformation

Biomass production was made in batch mode in LB medium according to Zajkoska et al.'s (2014) procedures [9], with constant oxygen saturation (5%) achieved by an agitation cascade and without pH control. After 20 h of the fermentation process, the biomass reached 1.34 g/L 1.05–2.22 g/L of dry cell weight. The biomass at the end of the exponential phase (20 h) was

harvested using centrifugation (2750 g) and washed with 0.1 M of the phosphate buffer (pH=8) (Fig. 2) prior to use. Surprisingly, no improvement was achieved in biomass production by Fed batch or in enzyme induction for this expression system (data not shown).

– Insert Figure 2 –

To verify the biomass activity, the whole cell biotransformation with model substrate 3-Azabicyclo [3,3,0] octane HCl was performed. The biomass was able to convert 20 mM of the substrate within 15 hours (Fig. 3), corresponding to the initial specific activity 7.56 U/g_{DCW}. This also corresponds to findings in other research [3].

– Insert Figure 3 –

3.2. Crude enzyme extract preparation

Biomass disruption was performed in batch and continuous modes using a French press (Fisher Scientific) and Constant cell disruption systems (Constant Systems LTD), respectively. Batch crude enzyme preparation had almost half activity (0.56 U/mL) compared to the continuous one (1.05 U/mL). The continuous disruption is advantageous, since its protocol is easily scalable to litre amounts, which is an important outcome for potential applications of MAO in industry. For continuous cell disruption a slight drop of specific enzyme activity was observed (after the first cycle, 1.35 U/mL, and after the second cycle, 1.05 U/mL). Compared to whole cells, the enzyme retained 61.5% of the original activity (calculated using dry cell weight). Conversion of substrate with crude enzyme extract took approx. 10 hours (Fig. 3), which is comparable with the whole cell process and previously published data [3].

Due to the high cell density (0.058 g_{DCW}/mL) loaded to the continuous cell disruptors, the increase of specific activity of prepared MAO crude enzyme extract by the higher load of biomass was not possible. However, to increase the specific activity of the enzyme, crude lysate was concentrated by membrane ultrafiltration with 50 kDa cut off membrane. The concentration of crude enzyme to half volume resulted in a 1.4 times increase of the initial specific activity and a decrease of bioconversion duration to four hours (Fig. 3).

3.3. Immobilisation of crude enzyme extract

Concentrated enzyme was used for PVA hydrogel immobilization according to the laboratory immobilisation protocol (www.lentikats.eu) with the LentiPrinter® device. Crude enzyme extract was stabilised with a protease inhibitor (Complete EDTA-free, Roche) to prevent enzyme digest by proteases and activity decrease in long term storage and experiments. The initial specific activity for the 10% (w/v) load was 0.16 U/g.

3.4. Enzyme kinetics

As previously reported [8] (Rebros et al., 2006), immobilisation of enzymes influences various enzyme parameters, including the kinetics, pH and temperature profile. MAO-N-5 belongs to a family of enzymes that catalyse the oxidation of monoamines using FAD as a cofactor [11]. Previous studies have shown that the MAO-N-D5 kinetic profile has more resemblance to the human MAO-A, which follows a Ping-Pong bi-bi mechanism, rather than to the human MAO-B form, which follows a bi-bi ordered mechanism [12]. The mechanism and kinetic parameters of the whole cell MAO-N-D5 have previously been reported [10] based on high throughput, automated microwell experiments. In that work, a King-Altam scheme for the MAO-N-D5-mediated oxidation of amines was proposed which is shown in Fig. 4, and the corresponding rate model as in Equation 1. The same method was used here to investigate

the kinetics of the immobilised enzyme [13]. For kinetic parameter estimation, a programme using Matlab® software (MathWorks, Natick, MA, USA) was developed in order to automatically perform all of the nonlinear regressions and statistical analyses following the routines for kinetic parameter estimation published elsewhere[14].

– Insert Figure 4 –

$$\mathrm{v} = \frac{V_{max}([Amine][O_2])}{den} \qquad Eq.1$$

$$den = K_{O2}[Amine]\left(1 + \frac{[Amine]}{K_{i,amine}}\right) + K_{amine}[O_2]\left(1 + \frac{[O_2]}{K_{i,O2}}\right) + [Amine][O_2]$$
$$+ \frac{K_{Amine}}{K_{i,imine}}[O_2][Imine] + \frac{K_{amine}K_{iO2}}{K_{i,imine}}[Imine]$$

Where V_{max} is the maximum rate, K_{O2} and K_{amine} are the corresponding Michaelis-Menten constants for the amine and O_2, and K_{iamine}, K_{iO2} and K_{iimine} are the corresponding inhibition constants for the selected amine, O_2 and the resulting imine.

In order to determine the MAO LentiKats® kinetic parameters, we adapted a microscale methodology established in our previous work [14] to rapidly determine the kinetic constants of the immobilized biocatalyst. Briefly this required first obtaining the initial preliminary values of the Michaelis-Menten and rate constants through traditional initial rate experiments at low concentrations. This was followed with nonlinear regression methods applied to full

progress curves at high substrate concentrations to determine the inhibition constants, as well as helping refine the exact location of the solution, allowing to obtain the final value of all the parameters while reducing the number of experiments required. In this work, the initial values for the Michaelis-Menten constant and rate constant of 3-Azabicyclo [3,3,0] octane were obtained from literature, based on MAO-N whole cell kinetic studies [10]. Those were K_m = 6.4 mM and k_{cat} = 0.22 mM h^{-1} U^{-1}, which were used as initial values to perform nonlinear regressions to determine the rest of the kinetic parameters.

The pH was monitored using the SDR SensorDish of the microplates for all bioconversions, which showed the pH remained constant at 8 by using 1 M phosphate buffer. The DOT was also monitored and remained close to 100% saturation, demonstrating that the combination of liquid fill volume, well geometry and shaking diameter/frequency used were sufficient to ensure that oxygen mass transfer into the microwell never became rate limiting. Similar results were found for MAO-N whole cell form in 96 microplates [10].

The O_2 concentration could not be controlled using the microscale tools, so the Michaelis Menten plot varying O_2 concentration could not be experimentally obtained. For that reason the initial the Michaelis Menten constant of O_2 (Km_{o2}) was obtained from literature with a value of 0.7 mM [12]. Using air as the oxygen source, the O_2 saturation concentration that could be obtained in microplates was 0.21 mM at 37^{o}C [15], which was four times lower than the Km_{o2} obtained in the literature. Experimentally it was not possible to use pure oxygen with the microscale tools; therefore the kinetic parameters determined in this work are apparent kinetic parameters suitable when using air as an oxygen source. The final values of those kinetic parameters for the immobilized MAO-N-D5 are summarized in Table 1. For comparison the table also shows the parameters previously determined for the whole cell form of the enzyme [10].

-insert Table 1-

The k_{cat} of an immobilized enzyme is generally lower than the free form; nevertheless in this work the opposite was found where the k_{cat} for the MAO LentiKats® was almost two times bigger than the whole cell form (Table 1). In a previous work, the MAO-N-D5 whole cell form was found to be severely inhibited and deactivated by substrate 3-Azabicyclo [3,3,0] octane [10], therefore the immobilization method described in this work may have improved the stability of the biocatalyst, resulting in an apparent higher K_{cat} than then whole cell form. Those findings are in agreement with previous studies of the immobilized invertase into PVA gel using LentiKats® technique, showing a k_M 3.6 times higher than free enzyme and an increase in V_{max} of 17% [16]. At the other hand, the LentiKats® immobilized glucoamylase using maltose as a substrate presented a k_M 1.5 times lower than the free enzyme [8]. The substrate inhibition constant of the immobilized enzyme was almost 5 times smaller than the whole cell form, which means the MAO LentiKats® suffers from more inhibition than the whole cell, although the constants are both in the same order of magnitude. The immobilized MAO-N may have partially been found as lysate due to all the process steps to make the LentiKats®. This could translate that substrate diffusion limitation may be stronger in the whole cell than in the partially lysate immobilized MAO-N, therefore reducing the inhibition effect in the whole cell form. This phenomenon could also explain the higher K_{cat} observed for MAO-N LentiKats® in comparison to the whole cell form. In general the other kinetics parameters were not significantly altered between the immobilized MAO-N and the whole cell form. Imine and oxygen inhibition constants were found to be 2 orders of magnitude higher than substrate inhibition, therefore a bioreactor operation mode that could minimize substrate inhibition would be recommended. Substrate fed-batch addition could allow achieving higher space-time yields at larger scales. Similar to previous MAO-N kinetic studies [10,12], the kinetic model predicted than an increase in oxygen concentration (by

using pure oxygen or pressurizing the bioreactor) would have an important positive effect in the bioconversion kinetics. The high throughput microscale tools and kinetic model methodologies allowed identifying bottlenecks early on and guide bioreactor engineering using minimum resources.

3.5. pH and temperature kinetic profiles

One of the main parameters affected by immobilisation is the pH profile of immobilised enzymes. In all cases of enzyme immobilisations to PVA gel, there were more [8] or fewer [17] significant changes of pH profiles. As previously reported [3], MAO D5 has an optimum pH of 8 (Fig. 5). At a lower pH, there is a significant drop of relative enzyme activity, while at values higher than eight the decrease of activity is not that dramatic until pH levels of 9. Immobilisation of enzymes slightly changes the pH profile of MAO-N D5. The optimum was shifted to a more alkali level (pH 8.5–9). The drop of relative enzyme activity prior to the acidic level was less significant than it was for free enzymes, likely due to the protection of the enzyme by the PVA gel matrix.

– Insert Figure 5 –

The next parameter that is usually affected by immobilisation is the enzyme temperature profile. The maximum attained activity for free MAO-N D5 was 45°C. The enzyme was stable until 50°C, and after this point, the relative enzyme activity drops significantly (Fig. 6).

– Insert Figure 6 –

The optimum free enzyme activity of MAO-N D5 is quite an advantageous parameter for PVA immobilisation studies, since a temperature of 55°C is the maximum temperature where PVA hydrogel particles may be used [8, 16]. Remarkable improvement of MAO-N relative activities was achieved through immobilisation (Fig. 6). Besides the slight increase of

optimum temperature, enzyme activity significantly increased in the whole monitored range. This may be an advantage in immobilised enzyme application. From an energetic point of view, there is almost no difference in reaction conversion for 37 and 45°C, and compared to these conditions, conversion at 30°C reached just 10% lower conversion when the previous (at 37 and 45°C) were completed, even when the initial activity was almost half (Fig. 7). The initial specific activity of immobilised MAO–N was 0.15 U/g at 30°C, 0.25 U/g at 37°C and 0.3 U/g at 45°C.

– Insert Figure 7 –

3.6. Repetitive use of immobilised crude enzyme

Immobilised MAO crude enzyme extract was used for 12 repeated biotransformations to verify the possibility of the advantage of biocatalyst reuse. After 8–10 hours of the biotransformation (Fig. 8), the particles were washed and used for the next batch. To the best of our knowledge, this is the first report regarding the repetitive use of immobilised enzyme MAO-N.

– Insert Figure 8 –

After the fifth and ninth batches, the particles were stored for two days at 4°C. However, after repetitive use and even various storage situations (Fig. 8), the enzyme retained 80% of its initial activity, making the process industrially and economically interesting. Compared to whole cell immobilised biocatalysts [9], it is remarkably stable for an FAD dependent enzyme.

4. Conclusions

MAO-N D5 crude enzyme extract was successfully prepared using biomass produced in laboratory fermentors and continual cell disruptors for the disintegration of the cells. Crude enzyme retains 61.5% of the original whole cell activity. Prepared extract was concentrated and immobilised in a PVA gel, according to the optimised technique previously described [8, 17]. Immobilised MAO-N in the form of LentiKats® was tested for activity, stability and repetitive use. The full kinetic parameters of the immobilized MAO-N were obtained; highlighting an improved kinetic efficiency in comparison to whole cell MAO-N and also allowing early identification of bioconversion bottlenecks and guiding bioreactor design. Besides the significant stability of the immobilised enzyme in repetitive biotransformations, the pH and temperature profiles were also improved compared to the free enzyme. The main bottleneck of MAO-N enzyme production is a weak active biomass production, which needs to be improved prior to industrial application.

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Figure legends:

Fig. 1: Generalised reaction scheme for the enantioselective oxidation of a secondary amine to imine by MAO-N [3].

Fig. 2: Kinetics of *E.coli* growth and MAO-N D5 production during batch fermentation on LB medium in a 2L stirred-tank bioreactor.

Fig. 3: Comparison of bioconversion kinetics with different forms of the MAO-N-D5 biocatalyst: crude enzyme extract (CEE), concentrated crude enzyme extract (concentrated-CEE) and whole cells. Bioconversions performed in a Falcon tube (50 mL) with 20 mM substrate (3-Azabicyclo [3,3,0] octane HCl) in phosphate buffer (0.1M, pH=8), 37°C and orbital shaking at 250 rpm,.

Fig. 4: Proposed King-Altman scheme of the MAO-N-D5 mediated oxidation of amines following a Ping-Pong bi-bi mechanism. $EFAD_{ox}$: complex enzyme and FAD cofactor in the oxidised form, $EFAD_{red}$: complex enzyme and FAD cofactor in the reduced form. Reproduced from Rios-Solis et al (2015) [10].

Fig. 5: Effect of pH on the bioconversion of 3-Azabicyclo [3,3,0] octane HCl using free and immobilised MAO-N-D5.

Fig. 6: Effect of temperature on the bioconversion of 3-Azabicyclo [3,3,0] octane HCl using free and immobilised MAO-N-D5.

Fig. 7: Influence of temperature on bioconversion kinetics using immobilised MAO-N-D5. Experiments performed with 20 mM 3-Azabicyclo [3,3,0] octane HCl at 30, 37 and 45°C.

Fig. 8: Kinetics of repeated batch bioconversions using immobilised MAO-N-D5 biocatalyst. Immobilised biocatalyst prepared using concentrated MAO-N-D5 crude enzyme extract.

Table 1 Final values of the apparent kinetic parameters determined for MAO-N-D5 immobilised in PVA gel and the non-immobilised enzyme (in whole cell form). Data for non-immobilised enzyme taken from Rios-Solis et al (2015) [10].

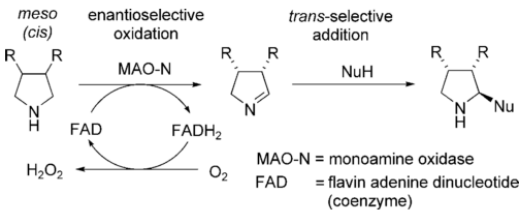

meso
(cis)
enantioselective
oxidation
trans-selective
addition
MAO-N
NuH
FAD
FADH2
H2O2
O2
Nu
MAO-N = monoamine oxidase
FAD = flavin adenine dinucleotide
(coenzyme)

Fig.1

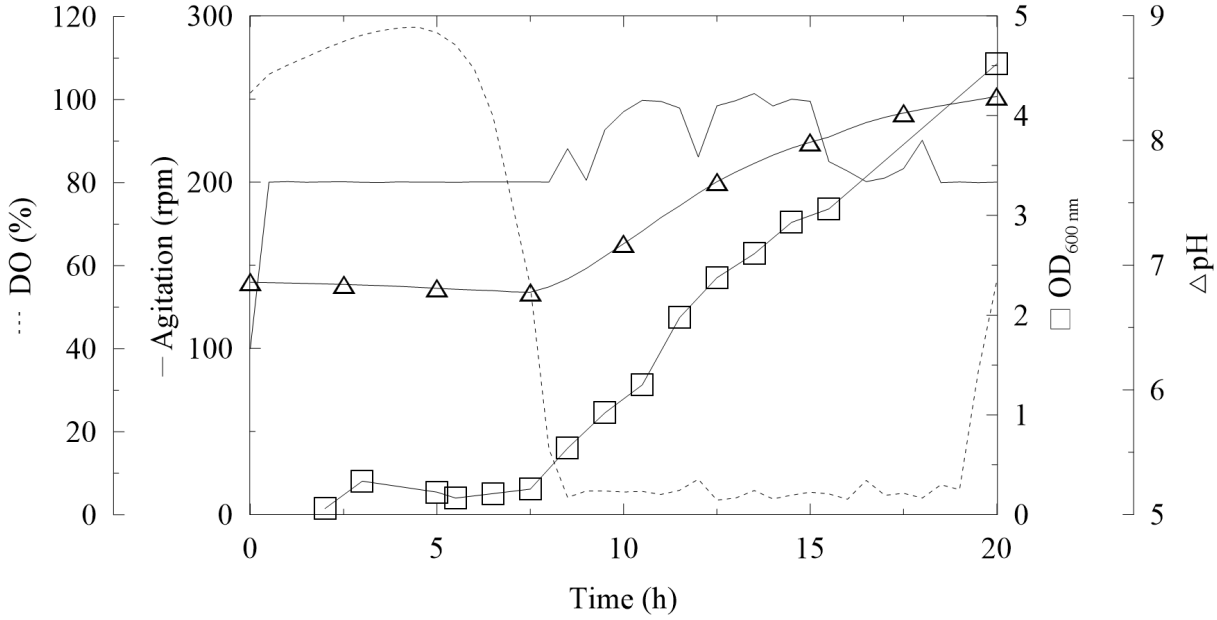

120
100
80
60
40
20
0
··· DO (%)
300
200
100
0
– Agitation (rpm)
0
5
10
15
20
Time (h)
5
4
3
2
1
0
□ OD600 nm
9
8
7
6
5
△pH

Fig.2

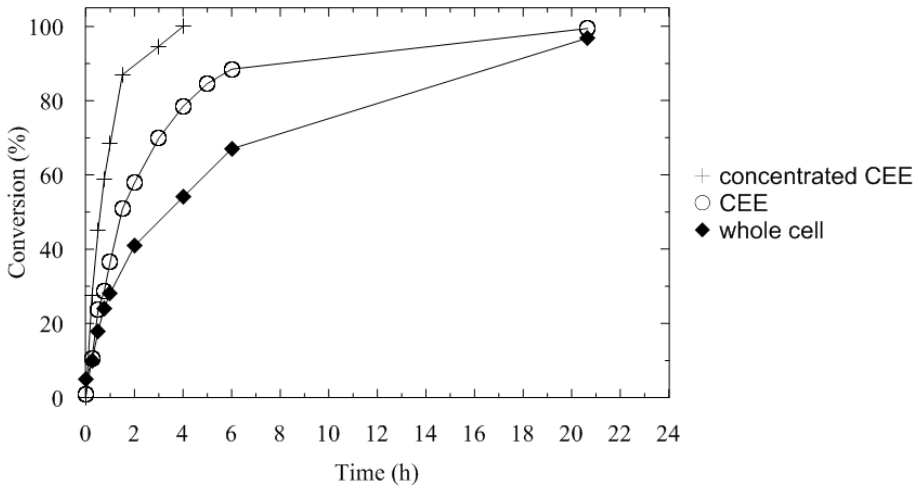
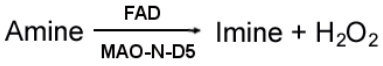


Fig.3

A) Overall reaction



B) King-Altman reaction scheme

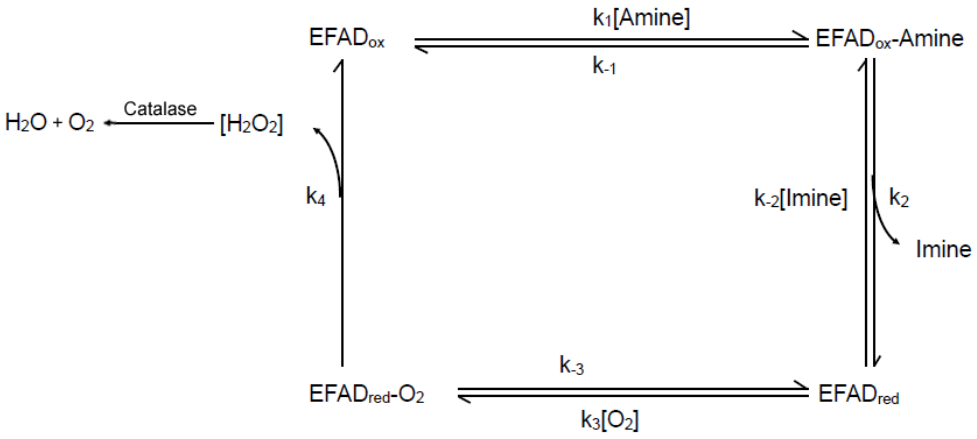


Fig.4

Fig.5

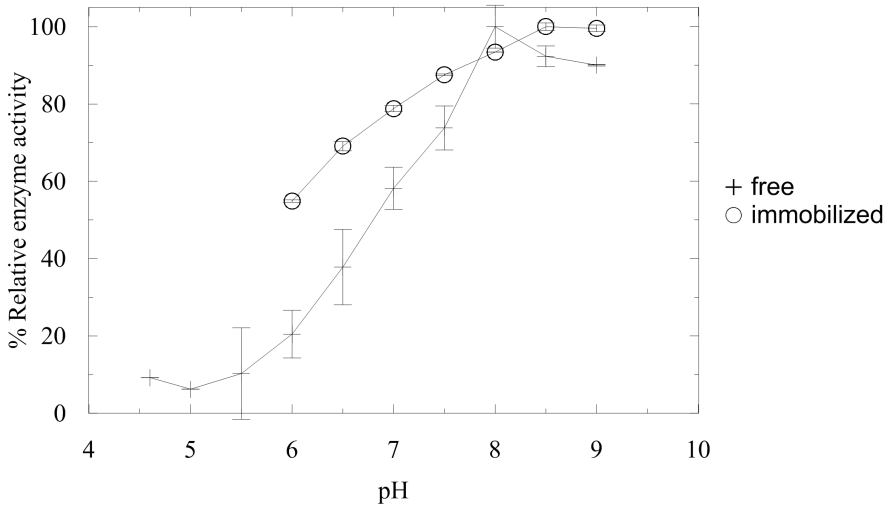
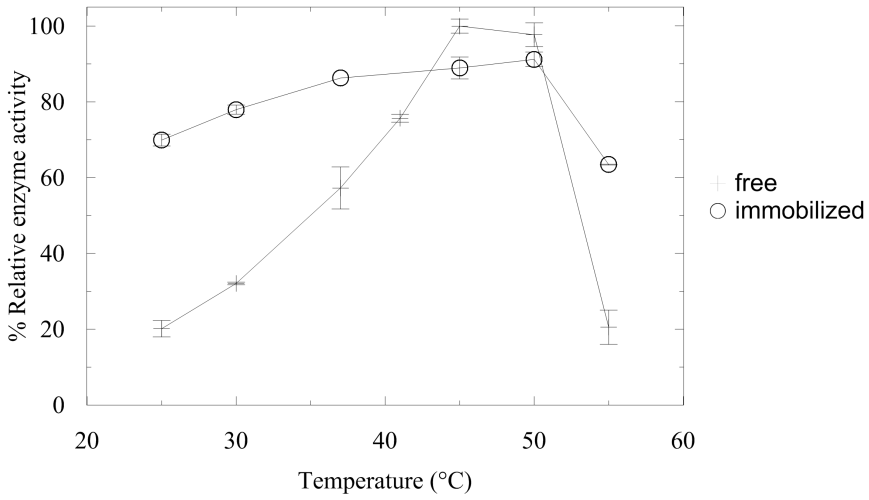

% Relative enzyme activity
100
80
60
40
20
0
4
5
6
7
8
9
10
pH
+ free
○ immobilized

Fig.6


% Relative enzyme activity
100
80
60
40
20
0
20
30
40
50
60
Temperature (°C)
free
immobilized

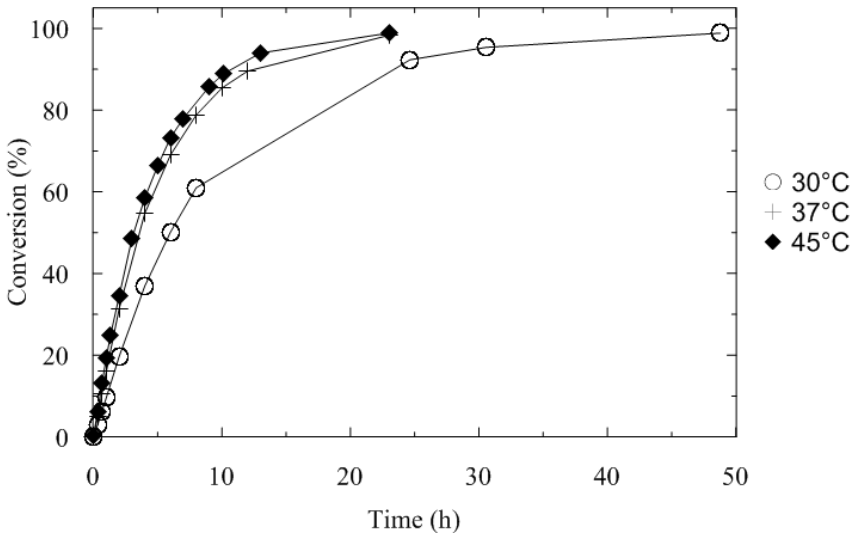


Fig.7

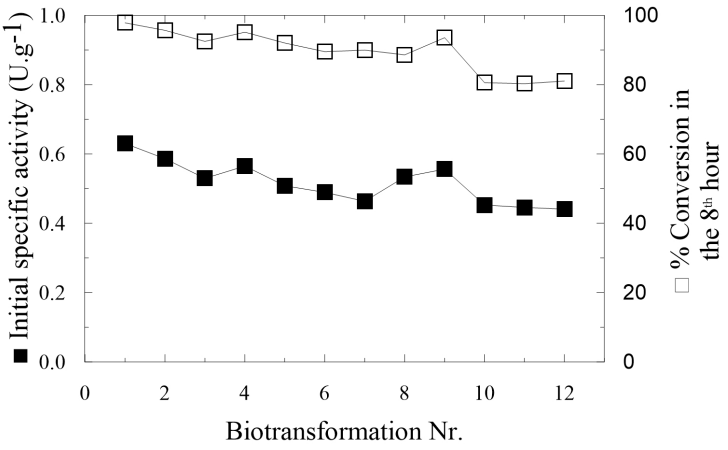
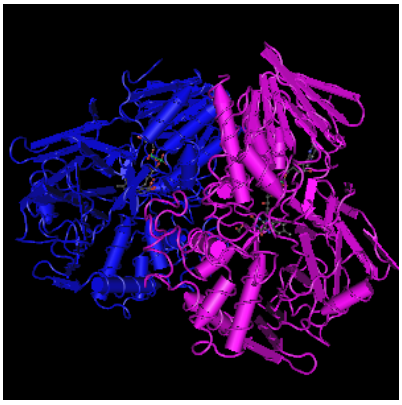

Initial specific activity (U.g-1)
1.0
0.8
0.6
0.4
0.2
0.0
100
80
60
40
20
0
% Conversion in the 8th hour
0
2
4
6
8
10
12
Biotransformation Nr.

Fig.8

Constant	Unit	Immobilised MAO-N-D5	Free MAO-N-D5
Rate constant (k_{cat})	mM hr^{-1} U^{-1}	0.42	0.22
Michaelis constant for Aza (K_{mAZA})	mM	7.31	6.4
Michaelis constant for O_2 (K_{mO2})	mM	1.1	1.2
Inhibition constant for Aza (K_{iAza})	mM	0.169	0.8
Inhibition constant for O_2 (K_{iO2})	mM	52.1	57.1
Inhibition constant for Imine (K_{iImine})	mM	65.4	54.5

Table 1

Graphical abstract:



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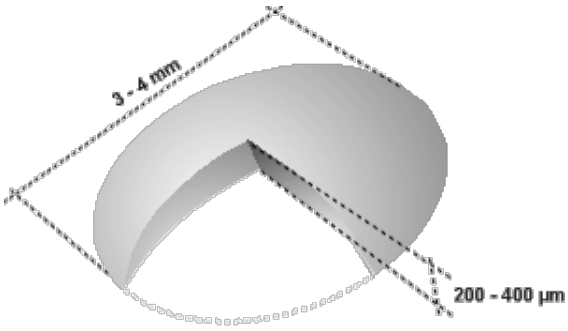

monoamine oxidase-N-D5

LentiKats®