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New ammonia lyases and amine transaminases: Standardization of production process and preparation of immobilized biocatalysts

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Abstract

Background: New enzymes for biotransformations can be obtained by different approaches including directed mutagenesis and *in vitro* evolution. These mutants have to be efficiently produced for laboratory research on bioreactions as well as for process development. In the framework of a European ERA-IB project, two different types of enzymes (ammonia lyases and aminotransferases) have been selected as biocatalysts for the synthesis of industrially relevant amines. New mutant enzymes have been obtained: a) aspartases able to recognize β-amino acids; b) ω-transaminases with improved activity. The objectives are to find out a common operational strategy applicable to different mutants expressed in *E. coli* with the same initial genetic background, the development of an integrated process for production and the preparation of stable useful biocatalysts.
Results: Mutant enzymes were expressed in *E. coli* BL21 under the control of isopropylthiogalactoside (IPTG) inducible promoter. The microorganisms were grown in a formulated defined medium and a high-cell density culture process was set up. Fed-batch operation at constant specific growth rate, employing an exponential addition profile allowed high biomass concentrations. The same operational strategy was applied for different mutants of both aspartase and transaminase enzymes, and the results have shown a common area of satisfactory operation for maximum production at low inducer concentration, around 2 μmol IPTG/g DCW. The operational strategy was validated with new mutants and high-cell density cultures were performed for efficient production. Suitable biocatalysts were prepared after recovery of the enzymes. The obtained aspartase was immobilized by covalent attachment on MANA-agarose, while ω-transaminase biocatalysts were prepared by entrapping whole cells and partially purified enzyme onto Lentikats (polyvinyl alcohol gel lens-shaped particles).
Conclusions: The possibility of expressing different mutant enzymes under similar operation conditions has been demonstrated. The process was standardized for production of new aspartases with β-amino acid selectivity and new ω-transaminases with improved substrate acceptance. A whole process including production, cell disruption and partial purification was set up. The partially purified enzymes were immobilized and employed as stable biocatalysts in the synthesis of chiral amines.

Keywords: amine transaminases, ammonia lyases, *E. coli*, fed-batch, immobilized biocatalysts.

INTRODUCTION

Biocatalytic synthesis is used to produce high-value chiral intermediates for the chemical and pharmaceutical industry, and fine and specialty chemicals. With many successfully implemented processes operating worldwide and the number of industrial biotransformations doubling every decade since 1960, unavailability of appropriate biocatalysts is thought to be a limiting fact for

biotransformation processes (Schmid et al. 2001; De Carvalho, 2011). For all the above applications, new enzymes are required as very often natural enzymes are not adapted to the demands of an industrial process. In many cases, the substrates are of non-natural identity or the reaction shall be conducted under non-natural conditions (like water-free conditions or the use of organic solvents). Innovative enzyme engineering approaches to optimize and adapt the properties of enzymes to the industrial process are needed. Protein engineering using computational techniques and site-directed mutagenesis, or directed (molecular) evolution techniques can be powerful tools to produce enzymes with optimized features such as activity, selectivity (enantio-, regio- and chemo-), stability, substrate specificity, and cofactor requirements, among others (Illanes et al. 2012). The new mutant enzymes have to be produced for research on bioreactions and also for integrated process development. It is then necessary to develop, if possible, common protocols to be employed for optimal enzyme production at laboratory level and easily scalable to a pilot and industrial scale.

In the framework of the ERA-IB (Europan Research Agency-Industrial Biotechnology) EngBiocat project, two groups of enzymes have been investigated as biocatalysts: ammonia lyases and transaminases. Both enzyme classes are already used in industrial processes, but a broader use is limited by the substrate spectrum these enzymes can accept (ERA-IB, 2009).

Ammonia lyases catalyze the addition of ammonium to simple double bonds yielding enantiopure high-value chiral amines. Unfortunately, the substrate spectrum of this enzyme class is very narrow and they produce mostly α-amino acids. Expanding this limited substrate acceptance to new molecules would broaden the industrial potential of these enzymes. In particular, L-aspartate ammonia-lyase or aspartase (EC 4.3.1.1) was chosen, as the α-carboxylate group is not mechanistically essential (Fibriansah et al. 2011), which could give rise to an expansion towards new molecules like β-amino acids and other secondary amines. Aspartase is mainly employed in the synthesis of the amino acid L-aspartate, one of the principal ingredients for manufacturing the low-calorie sweetener aspartame (L-aspartyl-L-phenylalanyl methyl ester) (Leuchtenberger et al. 2005). A thermostable aspartase from *Bacillus sp*. YM-55 (AspB) with high specific activity (Kawata et al. 1999) was selected as starting enzyme. The protein engineering work has been performed by c-LEcta GmbH in the framework of the mentioned project. The enzyme has been expressed in *E. coli*, exhibiting high specific activity and good thermal stability. Based on the published structure of AspB (Fujii et al. 2003), four residues were identified that bind the β-carboxylate group in the wild-type substrate. Those residues were exchanged in a combinational library to yield all 20 amino acids at each site. The theoretical complexity of the library is ≈ 1 million variants. The library was physically prepared and verified by sequencing of random members. After screening of nearly 300.000 clones, a single clone was obtained which expressed the desired activity: the variant AspB-C6 catalyzed the regio- and stereo selective amination of crotonic acid to β-D-aminobutyrate. The catalyzed reactions by the native and the mutant enzymes are presented in Figure 1a and 1b.

Transaminases are highly interesting enzymes for the synthesis of chiral amines from ketones (Panke et al. 2004; Constable et al. 2007; Nugent and El-Shazly, 2010). ω-Transaminases (ω-TA, EC 2.6.1.18) are pyridoxal-5'-phosphate (PLP) dependent enzymes that catalyze the transfer of an amine group from an amino-donor, usually an arylamine or amino acid, to a pro-chiral acceptor ketone, yielding a second chiral amine and a ketone by-product (Shin and Kim, 2002) (Figure 1c). The use of ω-TAs has gained attention because of its potential for the resolution of racemic amines as well as in the asymmetric synthesis of optically active amines (Shin et al. 2001; Koszelewski et al. 2010). Drawbacks in the synthetic application of transaminases are their narrow substrate acceptance, stereoselectivity and inefficient synthesis at high substrate concentrations. The strategy to broaden the substrate scope was to use structure-based enzyme variant library construction, starting from *Arthrobacter citreus* amine transferase cloned in *E. coli*. A large set of different enzyme mutants were identified by c-LEcta, possessing higher activities and allowing the synthesis of several chiral amines that could not be accessed with wild-type enzymes.

The main objective of the present work is to develop a common production operational strategy applicable to different mutant enzymes expressed in *E. coli* with the same initial genetic background. Significant amounts of each variant have to be obtained by using reliable production and downstream procedures for a proper evaluation as biocatalysts and research and development of new biotransformations. Optimum conditions for fed-batch IPTG induced fermentations at laboratory scale have to be set up for aspartase and transaminase enzymes, and validated with new mutants. In addition, the target enzymes are going to be partially purified and immobilized in order to get suitable biocatalysts.

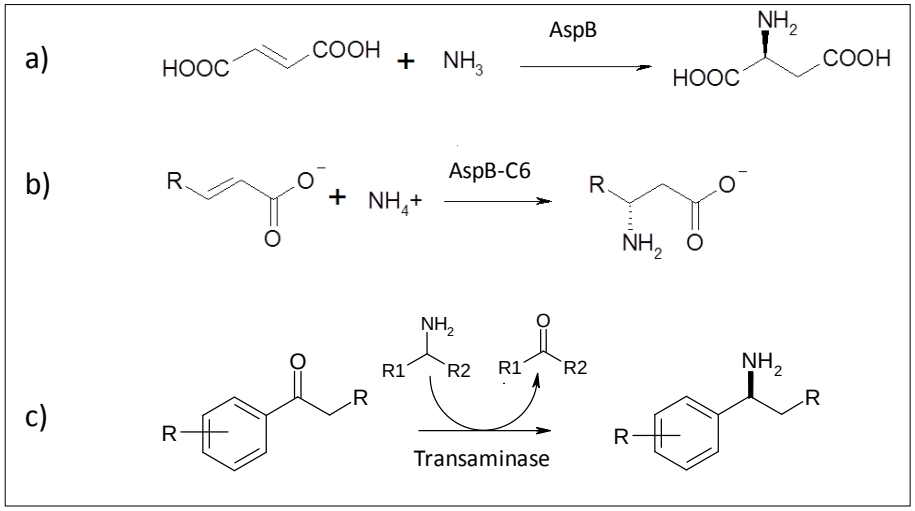

Fig. 1 Reactions catalyzed by (a) native AspB; (b) mutant AspB-C6; and (c) ω-TA.

MATERIALS AND METHODS

Strains

E. coli BL21 (DE3) strains harbouring plasmids pLE1-A10-BsAsp, pLE1-A10-BsAsp-C6 for aspartase expression and pLE1-A10-AcATA, pLE1-A10-AcATA-D4 and pLE1-A10-AcATA-44 for amino transferase were supplied by c-LEcta.

Reagents

A stock solution of kanamycin (Sigma, 100 mg x mL^{-1}) was prepared in milliQ water, filter sterilized and stored at 4ºC. IPTG (Isopropyl β-D-1-thiogalactopyranoside) was purchased from Sigma-Aldrich and a 100 mM stock solution was prepared using milliQ water as solvent, filter-sterilized and stored at -20ºC.

Culture media

Complex medium ZYM505 was used for pre-inoculum and inoculum preparation as well as for shake flask and some batch bioreactor cultures. It has the following composition: 10 g x L^{-1} tryptone, 5 g x L^{-1} yeast extract, 10 g x L^{-1} glycerol, 1 g x L^{-1} glucose, 7.10 g x L^{-1} Na_2HPO_4, 6.80 g x L^{-1} KH_2PO_4, 5.35 g L^{-1} NH_4Cl, 1.42 g L^{-1} Na_2SO_4 and 0.5 g L^{-1} $MgSO_4$ x $7H_2O$, and was supplemented with 0.05 g x L^{-1} kanamycin.

A defined mineral medium (DM), based on glucose as the sole carbon source, was used for both batch growth and initial batch phase in fed-batch bioreactor cultivation experiments. This medium was composed of 20 g x L^{-1} glucose, 11.9 g x L^{-1} K_2HPO_4, 2.4 g x L^{-1} KH_2PO_4, 1.8 g x L^{-1} NaCl, 3 g x L^{-1} $(NH_4)_2SO_4$, 0.1 g x L^{-1} $MgSO_4$ x $7H_2O$, 0.01 g x L^{-1} $FeCl_3$, 0.05 g x L kanamycin and 0.72 mL x L^{-1} of trace elements solution. The trace element solution composition contained (g x L^{-1}): 0.040 $AlCl_3$ x $6H_2O$, 1.74 $ZnSO_4$ x $7H_2O$, 0.16 $CoCl_2$ x $6H_2O$, 1.55 $CuSO_4$, 0.01 H_3BO_3, 1.42 $MnCl_2$ x $4H_2O$, 0.01 $NiCl_2$ x $6H_2O$, 0.02 Na_2MoO_4. This medium was also used for the pre-inoculum and inoculum preparation for the batch and fed-batch bioreactor cultures with defined medium.

The feed medium for high-cell-density fermentations consisted of 478 g L^{-1} glucose, 0.089 g L^{-1} $CaCl_2$, 9.56 g x L^{-1} $MgSO_4$, 0.49 g x L^{-1} $FeCl_3$ and 0.05 g x L^{-1} kanamycin. Phosphates were not included in the feeding solution in order to avoid co-precipitation with magnesium salts. Instead, a concentrated phosphate solution containing 500 g x L^{-1} K_2HPO_4 and 100 g x L^{-1} KH_2PO_4 was pulsed during the fed-batch phase to avoid their depletion when necessary.

Cultivation conditions

Inocula preparation. The different strains were grown at 30ºC on ZYM505 or DM agar plates supplemented with 50 µg x L^{-1} kanamycin. Colonies from agar plates were inoculated to 100 mL shaker flasks with 20 mL of ZYM505 or DM culture media and incubated at 30ºC in a rotary shaker at 130 rpm for 8 hrs. The required volume of these cultures was inoculated in 200 mL of ZYM505 or DM culture media into 1000 mL shaker flasks to get a 0.2 initial optical density at 600nm (OD_{600}). These cultures were incubated in a rotary shaker at 30ºC and 130 rpm for 16 hrs before inoculation into the bioreactor.

Batch cultures in bioreactor. Batch cultures were carried out using a Biostat® B bioreactor (Sartorius) equipped with a 2L fermentation vessel. The culture volume in batch cultures was 2L either of ZYM505 or DM culture medium. The pH was maintained at 7.00 ± 0.05 by adding 30% NH_4OH and 2M H_2SO_4 solutions to the reactor. The pO_2 value was maintained at 30% of air saturation by adapting the stirrer speed between 200 to 1100 rpm and supplying air at a flow rate of 1 vvm. In batch cultures of transaminase producer strains, temperature was kept at 30ºC while in batch cultures with aspartase producer strains, temperature was maintained at 37ºC until induction with IPTG, when it was changed to 30ºC to avoid formation of inclusion bodies.

In batch experiments, the required volume of inoculum was transferred to the reactor to get an initial OD_{600} value of 0.2. These cultures were induced with IPTG for protein production at the middle of exponential growth phase, when biomass concentration was in the range of 6 to 8 OD_{600} units.

Fed-batch cultures in bioreactor. Fed-batch cultures were carried out in a Biostat® B (Sartorius) 2L fermentation bioreactor, operating in the same conditions for pH control, oxygen supply and temperature as in batch cultures. DM culture medium was used in these experiments, with an initial culture volume of 1L plus the necessary volume of inoculum to get an initial OD_{600} value of 0.2.

An initial batch growth phase of 16 hrs was performed and, once the substrate was consumed, a fed-batch phase started supplying the feedstock solution with a peristaltic pump. In this fed-batch phase the specific growth rate (µ) was kept constant using discrete feed additions as an approximation to a predefined exponential feeding profile according to (Durany et al. 2005):

$$\Delta V = \frac{X_0 \cdot V_0}{S_f \cdot Y_{X/S\,ap}} \cdot \left(\exp\left(\mu_{set} \cdot t\right) - 1\right)$$

where: ΔV, volume to be added in the addition at time t; X_0 and V_0, biomass concentration and liquid volume in the reactor at time of the previous addition; S_f, glucose concentration in the feed solution; $Y_{X/S\ ap}$, apparent biomass/glucose yield; μ_{set}, desired specific growth rate; t, time since previous addition. In all fed-batch cultures $Y_{X/S\ ap}$ was assumed to be 0.35 g x g^{-1} and the value of μ_{set} used was 0.15 h^{-1}.

The exponential feeding profile was extended until biomass concentration reached a minimum of 100 OD_{600} units. At this moment induction of the cultures was carried out by an IPTG pulse at a determined value of the IPTG to biomass concentrations ratio, I/X (µmol IPTG x g^{-1} dry cell weight).

Analytical procedures

Samples were taken from culture broths at different time intervals for monitoring of growth parameters and enzyme production. Cell growth was monitored by optical density measurements at 600 nm (OD_{600}), after appropriate dilution to fit in the linear range of the spectrophotometer. The dry cell weight (DCW) of samples of known OD_{600nm} was measured by centrifugation of aliquots of the broth. The pellets were washed twice with deionised water and dried at 110ºC until constant weight. As a result, 1 OD_{600nm} was found to be equivalent to 0.303 g x L^{-1} DCW. This equivalence was used to determine the dry cell weight of cultures samples from OD_{600} measurements.

For the determination of glucose concentration in DM batch and fed-batch cultures, one millilitre of culture samples was centrifuged. The supernatant was then used for glucose and organic acids measurements. Glucose and organic acids were analyzed by HPLC (Dionex Ultimate 3000IR) on an Coregel 87H3 (Transgenomic) column at 40ºC with RI detector (HP 1047) using 15 mM H_2SO_4 (pH = 3.0) as eluent at a flow rate of 0.6 mL x min^{-1}.

To quantify the product concentration after induction of cultures, broth samples were withdrawn and centrifuged. The pellet was resuspended in the corresponding lysis buffers: 50 mM HEPES, 2 mM $MgCl_2$, 0.1 mg mL^{-1} DNase I, (pH = 7.0) for aspartase strains or 50 mM potassium phosphate, 0.1 mM PLP, 0.1 mg x mL^{-1} DNase I (pH = 7.4) for aminotransferase strains. Cell suspensions were disrupted in a 60/40/AA cell disruptor (Constant systems) with 2 disruption cycles at 1.4 kbar. Cellular debris was removed by centrifugation and the clear supernatant was collected for protein and activity analyses.

Protein concentration was determined by the Bradford method, using 1.25 mL of Coomassie® reagent purchased from Pierce (Rockford, IL, USA) and 50 µL of sample, the absorbance was read at 595 nm and concentration was calculated by using bovine serum albumin as standard.

Downstream processing

The fermentation broth was centrifuged at 6000 rpm for 20 min at 4ºC using a Beckman J2-21M/E centrifuge. Harvested cells were resuspended in the corresponding lysis buffer, as described in the analytical procedures, and disrupted in a 60/40/AA cell disruptor (Constant systems) with two disruption cycles at 1.4 kbar. Cellular debris was removed by centrifugation (8000 rpm at 4ºC for 40 min) and the clear supernatant was collected and kept at 4ºC for further purification steps.

Partial purification of AspB and AspB-C6. The cell lysate was collected, and 0.1 M L-aspartate and 11% (w/v) ammonium sulfate were added (for AspB-C6, the same concentration of crotonic acid was added instead of L-aspartate). Then the sample was heated at 72ºC for 5 min and immediately cooled in an ice bath. Precipitated proteins were removed by centrifugation (8500 rpm at 4ºC for 30 min). Finally 60% (w/v) ammonium sulphate was added to the supernatant and kept at mild stirring at 4ºC for 30 min. The precipitated enzyme was separated by centrifugation and kept at 4ºC as suspension in 60% (w/v) ammonium sulphate.

Partial purification of ω-TA. The cell lysate was collected, and the ω-TA enzyme was recovered by double precipitation with ammonium sulphate at 30 and 60% (w/v) of saturation. The precipitated enzyme was separated by centrifugation and kept at 4ºC as suspension in 60% (w/v) ammonium sulphate (8500 rpm at 4ºC for 30 min).

Enzyme activity assays

AspB. AspB activity was determined in buffer HEPES 50 mM pH 7 containing 0.1 M L-aspartate and in a final volume of 1 mL, according to the method previously described (Cárdenas-Fernández et al. 2012a). The activity was assayed in a Cary50 Bio UV-visible spectrophotometer (Varian, Palo Alto, CA, USA) by measuring the increase of absorption at 240 nm caused by the production of fumarate at 30ºC. One unit of AspB was defined as the amount of enzyme that produces 1 µmol of fumarate per min. The molar extinction coefficient of fumarate at 240 nm was 2.53 x 10^{-3} M^{-1} cm^{-1}.

AspB-C6. The activity of Asp-B6 was measured by the reaction between crotonic acid and NH_4Cl to yield β-aminobutyric acid. 25 µL of enzymatic sample were mixed with 48 µL of buffer HEPES-$MgCl_2$ pH 8, 12 µL of crotonic acid and 15 µL of NH_4Cl (final concentration: 0.1 M HEPES, 2 mM $MgCl_2$, 0.3 M crotonic acid and 0.2 de NH_4Cl) and the mixture was maintained at 350 rpm and 37ºC for 4 hrs (Thermoshaker HLC, Bovenden, Germany). A blank reaction was carried out, where 12 µL of buffer were added instead of crotonic acid. The reaction was stopped by increasing the temperature to 80ºC and holding it for 20 min. The sample was centrifuged at 13000 rpm for 10 min and the supernatant containing the product β-aminobutyric acid was recovered.

The concentration of β-aminobutyric acid was determined by derivatization with 2,4-dinitrofluorobenzene (DNFB). 25 µL of reaction sample was mixed with 10 µL of $NaHCO_3$ and 40 µL of DNFB (37.6 mM in acetone), and then incubated at 40ºC for 1.5 hrs. The reaction was stopped with 10

µL of 1 M HCl and 100 µL of 20% (v/v) acetonitrile. The derivatized β-aminobutyric acid was analyzed on a high-pressure liquid chromatography (HPLC) system (Dionex Ultimate 3000, Sunnyvale, CA, USA) fitted with a X-BridgeTM C18 5 µ m 4.6 mm x 250 mm column (Waters, Milford, MA, USA). The mobile phase A consisted of 0.1% trifluoroacetic acid in MiliQ water and the mobile phase B was 0.95% trifluoroacetic acid in 80% acetonitrile and 20% MiliQ water. The gradient elution was performed from 31.3 to 48.8% of phase B and run at 1 mL/min during 25 min. The eluted products were detected at 360 nm and quantitative analysis was performed from peak areas by the external standard method.

ω-TA. The activity of ω-TA was determined by spectrophotometry. 100 µL of sample were added to 900 µL of 50 mM buffer phosphate pH 7.4 containing 11 mM 1-phenylethylamine, 1.25% (v/v) dimethyl sulfoxide, 11 mM pyruvate and 0.1 mM PLP. The activity was assayed by measuring the increase of absorption at 240 nm caused by the production of acetophenone at 30ºC. The molar extinction coefficient of acetophenone at 240 nm is 0.28 mM^{-1} x cm^{-1}. One unit of activity was defined as the amount of enzyme that produces 1 µmol of acetophenone per minute.

Enzyme immobilization

Immobilization on Eupergit®. Beads of the epoxy support Eupergit® C or Eupergit® CM from Sigma-Aldrich (St Louis, MO, USA) were hydrated with deionized water at 4ºC for 24 hrs and filtered. The immobilization of AspB-C6 by covalent attachment on Eupergit® C was carried out by mixing 1 mL of wet support and 9 mL of buffered solution (1 M sodium bicarbonate, pH 9) containing 1.3 U of enzyme. The immobilization of ω-TA on Eupergit® CM was carried out by mixing 1 mL of wet support and 9 mL of buffered solution (sodium bicarbonate 1 M, pH 8) containing 40 U of enzyme. After 15 hrs at room temperature, the suspensions were filtered and the remaining epoxy groups in the matrix were blocked by incubation with 0.2 M 2-mercaptoethanol in HEPES buffer (0.1 M, pH 7) for 4 hrs. Finally, the suspensions were vacuum-filtered, washed with HEPES buffer (0.1 M, pH 8) containing 2 mM $MgCl_2$ for AspB-C6 and HEPES buffer (0.1 M, pH 7.5) containing 1 mM PLP for ω-TA, filtered again and stored at 4ºC. A blank without support was incubated under the same conditions and the enzymatic activity was measured as a control.

Immobilization on MANA-agarose. Monoaminoethyl-N-aminoethyl support (MANA-agarose) was prepared according to the method proposed by Fernández-Lafuente (Fernández-Lafuente et al. 1993). Aldehyde-agarose gels were obtained by etherification of agarose 10 BCL gels with glycidol. The surface density of aldehyde groups was measured by analyzing the consumed periodate with a solution of KI 10% saturated with $NaHCO_3$. MANA-agarose gels were obtained by activation of aldehyde-agarose gels with ethylenediamine 2 M in $NaHCO_3$ buffer (0.1 M, pH 10) and subsequent reduction of Schiff base with $NaBH_4$ (10 mg x mL^{-1}). The concentration of primary amine groups in MANA-agarose was estimated to be the same as the concentration of aldehyde groups in aldehyde-agarose gels (220 µ mol NH_2 x mL^{-1} of gel).

The covalent immobilization of AspB-C6 on MANA-agarose was carried out by mixing 1 mL of support with 9 mL of MES buffer (25 mM, pH 6) containing 1.5 U of enzyme for 15 min. After this time, 1.25 mL of supernatant were taken and replaced by the same volume of a fresh solution of CDI (200 mM) in MES buffer (25 mM, pH 6) to achieve a final concentration of 25 mM CDI. The suspension was mildly stirred for 2 hrs. Finally, to verify the desorption of the adsorbed but not attached enzyme, solid NaCl was added to a final concentration of 1 M and stirred for 1 hr. Finally, the suspension was vacuum-filtered, washed with HEPES buffer (0.1 M, pH 8) containing 2 mM $MgCl_2$, filtered again and stored at 4ºC. A blank without support was incubated under the same conditions and its activity with time was measured as a control.

Immobilization in LentiKats®. The immobilization of ω-TA by entrapment in LentiKats® was carried out according to the protocol from GeniaLab (LentiKats® tips and tricks, Braunschweig, Germany). LentiKats® gel was heated at 95ºC and then cooled to 35ºC. At this temperature 2 mL of enzymatic solution (32 U of ω-TA) in HEPES buffer (0.1 M, pH 7) were well mixed with 8 mL of LentiKats® liquid. The lens-shaped particles were formed by using the GeniaLab LentiKats® printer, which provided drops of approximately 3-4 mm in diameter, onto Petri dishes. The particles were kept at room temperature until evaporation of 70% of the water. Finally, the stabilizing solution, supplied by GeniaLab, was added and kept at room temperature for 2 hrs. After this time, the solution was filtered and the lens were collected, washed with HEPES buffer (0.1 M, pH7.5) containing 1 mM PLP and kept at 4ºC.

Immobilization process monitoring. All the immobilization processes were carried out under mild agitation conditions and at room temperature. In order to study the time course of the immobilization, aliquots of the supernatant and suspension, as well as samples of blank, were withdrawn periodically and enzymatic activity was tested. The retained activities (%) were calculated as the difference between activity of the suspension and the supernatant with respect to the activity of the initial suspension. The immobilization yields (%) were calculated as the differences between the initial suspension activity and the supernatant activity at the end of the process supernatant with respect to the activity of the initial suspension. Finally, at the end of the immobilization, the immobilized enzyme preparations were vacuum-filtered and their residual activity was measured.

RESULTS AND DISCUSSION

Lyases

As indicated in Introduction section, initial work started with *E. coli* BL21 strains harbouring a plasmid for the expression of thermostable aspartase from *Bacillus sp.* YM-55 (BsAsp) enzyme, supplied by c-LEcta (A10 strain). Expression and production has been performed in shake-flasks and batch stirred fermentor (2 L). Growth experiments in shake flasks in ZYM505 complex medium were performed to verify that strains presented the expected activities after either lactose or IPTG induction and to select the disruption procedure. One-shot mechanical disruption was found to be equivalent to the enzymatic lysis process in both cases and was selected for further experiments due to simplicity and easy scale-up. Complex medium batch fermentation in bioreactor yielded 9 g L^{-1} DCW of biomass and 21 kU x g^{-1} DCW.

Growth in a defined medium is the most suitable and economical for fed-batch high-cell density cultures. It permits a proper control of the addition strategy leading to higher productivities. A defined medium, derived from previously published ones (Vidal et al. 2003), was formulated with glucose as the limiting carbon source (see composition in Materials and Methods). A10 strain (AspB producer) was able to grow in this medium in a batch bioreactor and at 20 g x L^{-1} of glucose, 8.5 g x L^{-1} DCW were obtained. The maximum specific growth rate (μ) was 0.41 h^{-1}. Fed-batch operation with a predefined exponential addition profile was performed as explained in Materials and Methods section. After induction with an IPTG pulse of 100 μM (same concentration as in previous batchs), 18000 U x g^{-1} DCW and 900 kU x L^{-1} were obtained (50 g x L^{-1}).

The above medium and operational strategy constituted the basis for further production of different mutants coming from the enzyme engineering program for aspartase enzyme performed in order to obtain new mutants able to recognize β-amino acids. After screening of nearly 300,000 clones, only one, BsAspC6, was proven to exhibit the expected activity. A new activity unit definition was required and the bioconversion of crotonic acid to β-aminobutyric was employed in the activity test (see Materials and Methods). This new enzyme was produced in batch growth in the previously defined medium with a specific activity of 6 U x g^{-1} DCW and activity concentration of 32 x U L^{-1}.

The fed-batch operation developed for the parent strain was adapted for the mutants expressing the target enzyme. Previous works (Pinsach et al. 2008), dealing with the production of different enzymes in IPTG-inducible expression systems, have shown the direct dependency of the protein production on the inducer / biomass ratio (I/X, μmol IPTG g^{-1} DCW at the induction time). An optimum ratio is usually obtained in the region of low inducer concentration. Different fed-batch culture runs were performed and induced at different biomass concentration, employing the same IPTG pulse amount (see Materials and Methods).

The results of cultures induced at different I/X ratios in the range between 1 and 3.5 are presented in Figure 2. As can be seen, maximum specific activity and activity concentration were found for I/X ratio of 2.2 μmol IPTG g^{-1} DCW. At this induction ratio, the aspartase production was 7.96 U x g^{-1} DCW, yielding 68 g x L^{-1} DCW and 542 U x L^{-1}. Productivity in fed-batch was 11.3 U x L^{-1} h, compared with 1.3 U x L^{-1} x h^{-1} obtained in batch operation. These results indicate a slightly higher specific activity than in batch culture, which was induced at much higher I/X ratio and, obviously, higher volumetric production as a result of higher biomass concentration.

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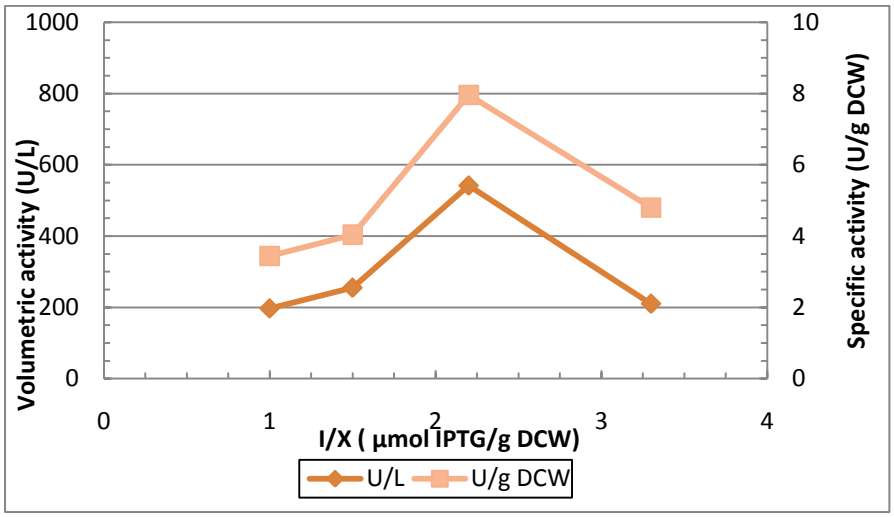

Fig. 2 Final AspB specific activity and volumetric activity for fed-batch cultures induced at different inducer-biomass ratio.

Under the above conditions, the mutant aspartase was successfully produced and recovered by the methodology described in Materials and Methods. The sequence includes a heat precipitation step to remove precipitated proteins and thermostable AspB-C6 recovery after ammonium sulphate precipitation. As can be seen in an example of laboratory scale purification presented in Table 1, a partially purified enzyme was obtained with around 60 U x g^{-1} protein.

The optimized operational process for ammonia lyase production will be employed for the production of further aspartase mutants and also translated to the other target enzymes.

Table 1. Example of AspB-C6 partial purification from culture lysates.

Purification step	Total activity (U)	Specific activity (U/g prot)	Activity recovered (cumulative) (%)	Purification factor
Cell extract	8.1	10.7	100	1
Heat precipitation[a]	5.7	31.7	70	3.0
Ammonium sulphate precipitation[b]	4.3	61.4	53	5.7

[a]Heat precipitation at 72ºC with $(NH_4)_2SO_4$ 11% (w/v) and crotonic acid 0.1 M for 5 min.
[b]Precipitation with $(NH_4)_2SO_4$ 60% (w/v) at 4ºC for 30 min.

Immobilization of partially purified AspB-C6 by covalent attachment was performed following the procedures previously optimized for the native enzyme AspB (Cárdenas-Fernández et al. 2012a), taking advantage of the amino and carboxylic groups of the amino acidic enzyme structure for the binding to functional groups of Eupergit® C (epoxy support) and MANA-agarose (amino support) respectively. Taking into account the hydrophobicity of Eupergit® C, the immobilization of AspB-C6 using this support was carried out at high ionic strength (Table 2). The pH value was selected considering basic conditions, in order to improve the reactivity of amino groups from lysine residues on the enzyme surface, but also considering the decrease of enzyme stability when increasing pH values. Despite immobilization yield was 85%, the enzymatic retention of AspB-C6 on Eupergit® C support scarcely reached 15%.

Table 2. Immobilization of AspB-C6 on Eupergit® C and MANA-agarose supports.

	Support	
	Eupergit® C	**MANA-agarose**
Activity (U/mL support)	1.3	1.5
pH	9	6
Buffer/Ionic strength	$NaHCO_3$ 1 M	MES 25 mM
Immobilization time (h)	19.0	2.3
Immobilization yield (%)	85	95
Retained activity (%)	15	45

On the other hand, the immobilization on the amino support MANA-agarose was carried out at acidic conditions to maximize the number of charged groups on both the enzyme surface and the support, as the first step of the immobilization process consists in the ionic adsorption. Once adsorbed, 25 mM carbodiimide was added to activate the carboxylic groups of the enzyme and promote covalent bond formation. The immobilization of the modified enzyme AspB-C6 on this support resulted in an enzyme retention of 45% while the immobilization yield reached 95%. Enzyme desorption was avoided by increasing ionic strength at the end of the immobilization process, this being indicative of the success of the covalent bond formation.

Retained activities of the modified AspB-C6 enzyme both in Eupergit® C and MANA-agarose support were lower than those obtained using AspB (24 and 45% respectively) (Cárdenas-Fernández et al. 2012a). Modifications of the amino acid sequence of the mutant enzyme AspB could be the responsible for the decrease of amino or carboxylic group content of the protein and, consequently, the enzyme retention values. Nevertheless, the MANA-agarose derivative resulted to be an appropriate biocatalyst for use in synthesis.

Amine transaminases

Expression and recovery of ω-TA cloned in *E. coli* in complex medium was performed in both shake-flasks and batch stirred fermentor (2L). A10 strains yielded 7-8 g L^{-1} DCW and 150 U x g^{-1} DCW. Production in defined medium was studied employing the improved strain ATAD4 (with higher activity), supplied by c-LEcta. In this case, batch growth yielded 200 U x g^{-1} DCW (1016 U L^{-1}), at a $\mu = 0.43\ h^{-1}$. Growth conditions were similar to those for the aspartase producing strain.

The fed-batch protocol for aspartase enzymes was adapted to the production of the transaminase enzyme. Experiments induced at different inducer / biomass (I/X) ratios were also performed, and the results are presented in Figure 3. As can be seen, there is a maximum for both specific and volumetric activity. Inducing at a ratio of 1.8 µmol IPTG x g^{-1} DCW, 452 x U g^{-1} DCW and 30376 U x L^{-1} (67 g x L^{-1} DCW) were obtained.

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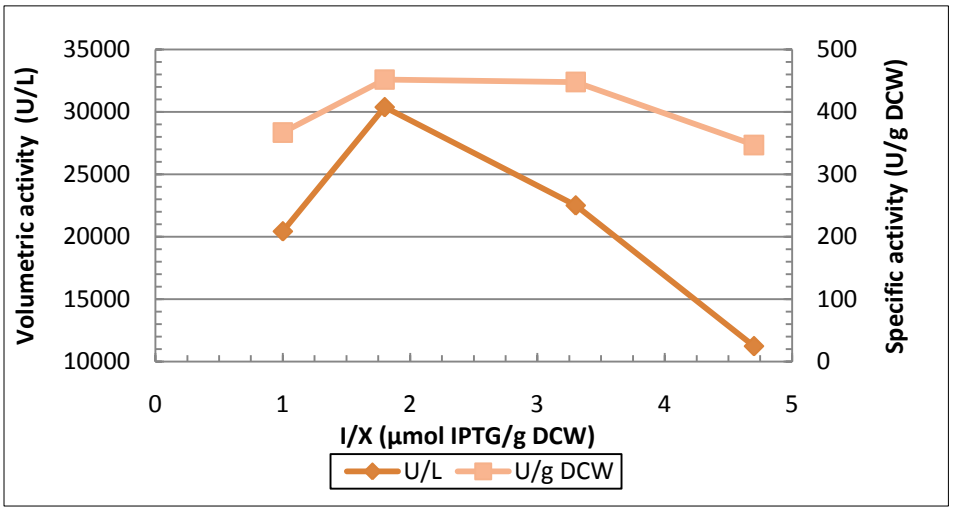

Fig. 3 Final transaminase specific activity and volumetric activity for fed-batch cultures induced at different inducer-biomass ratio.

In this case, fed-batch productivity was 1047 U x L^{-1} h^{-1}, compared to 42 x U L^{-1} x h^{-1} obtained in batch operation. Like in the case of aspartase, the improvement in fed-batch with respect to batch mode is in part due to higher biomass concentration. For transaminase, however, a significantly higher specific activity than in batch mode was reached (more than double).

Process similarities are very significant, as the optimal I/X ratio is in the same range for both enzyme classes, around 2 µmol IPTG x g^{-1} DCW, showing that a common operational procedure is possible.

The transaminase enzyme was recovered after two successive precipitation steps. Partially purified enzyme samples, with recovery yields around 80% have been employed to test the biocatalytic behaviour.

Assuming that common operational conditions for enzymes production in high cell density culture could be set up, the operational strategy was extended to a new improved mutant, namely ATAD44, supplied by cLEcTa. The results confirmed the assumption as fed-batch fermentation with induction at I/X ratio of 2 led to the expected biomass levels with a final activity of 483 U x g^{-1} DCW. Figure 4 shows the evolution with time of the different variables for the operation under the selected conditions. As can be seen, glucose concentration is maintained at levels near zero during fed-batch phase. After induction, there is expression of the target protein together with growth rate reduction and glucose accumulation due to metabolic burden.

The above results were considered a validation of the production process, which was standardized and ready to be applied to any other mutant as well as to scale up to pilot plant.

The purification of the D44 enzyme was also performed by two precipitation steps, and Table 3 shows an example of the high recoveries attained and the final specific activities. Recovery yields were higher than for AspB, being 80% as average.

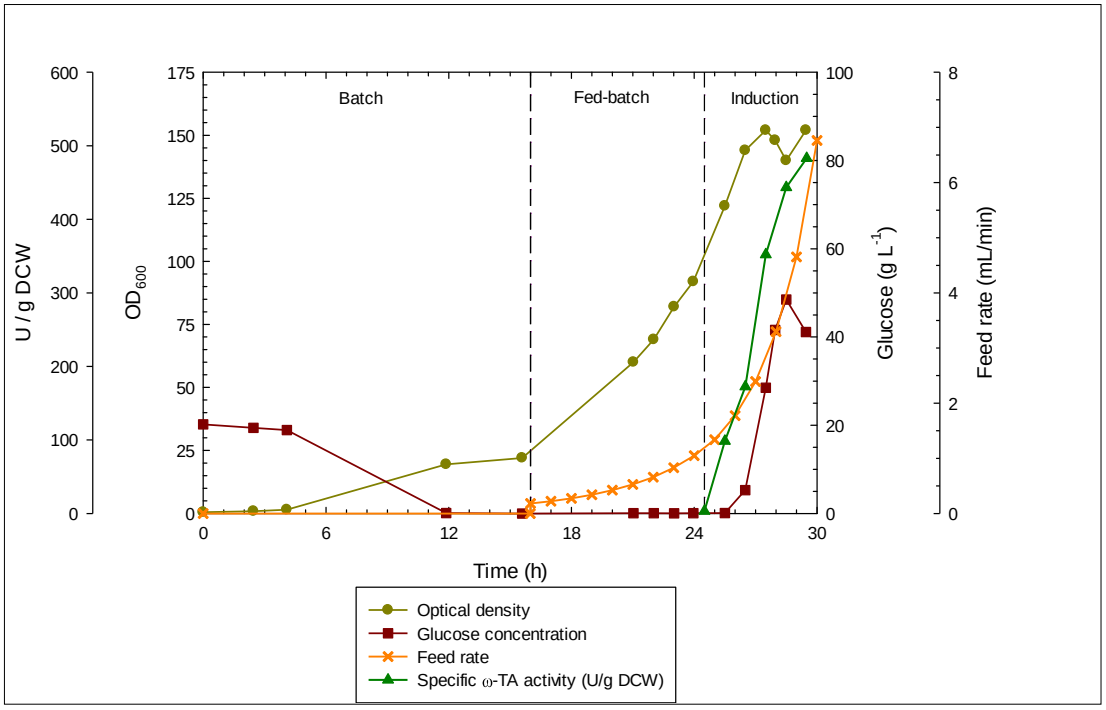

Fig. 4 Time evolution for fed-batch process with ATAD44 of optical density, glucose concentration, feed rate and specific ω-TA activity (U/g DCW).

Table 3. Example of ω-TA partial purification from culture lysates.

Purification step	Total activity	Specific activity	Activity recovered (cumulative)	Purification factor
	(U)	(U/g prot)	(%)	
Cell extract	863	600	100	1
Precipitation $(NH_4)_2SO_4$ 35%[a]	791	700	91.7	1.2
Precipitation $(NH_4)_2SO_4$ 60%[a]	706	1900	81.8	3.2

[a] Precipitations were carried out at 4ºC for 30 min under mild stirring.

Partially purified ω-TA was immobilized following two different strategies: covalent attachment in a hydrophobic epoxy support (Eupergit® CM) or entrapment on polyvinyl alcohol (LentiKats®) (Table 4). As ω-TA enzyme is unstable at high pH values, immobilization on Eupergit® CM was performed at a slightly alkaline pH and high ionic strength to promote hydrophobic interaction and covalent binding between epoxy groups of the support and amino, hydroxyl or sulphydryl groups of the enzyme. In spite of the high immobilization yield obtained, the retained activity of ω-TA at the end of the process was quite low (20%), probably because the possible stability improvement obtained by covalent bond formation cannot balance the loss of activity due to the high pH and immobilization time required.

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Table 4. Immobilization of ω-TA on Eupergit® CM and LentiKats® supports.

	Supports	
	Eupergit® CM	**LentiKats®**
Activity (U/mL support)	40	3
pH	8	7
Buffer / Ionic strength	$NaHCO_3$ 1 M	HEPES 0.1 M
Immobilization time (h)	72	3
Immobilization yield (%)	70	99
Retained activity (%)	20	52

Enzyme entrapment techniques do not require strong pH, temperature or ionic strength conditions, so that these can be selected in order to ensure the stability of the enzyme. As drawback, enzyme leakage is likely to occur. ω-TA was immobilized by entrapment using LentiKats® at mild conditions, obtaining 52% of activity retention and immobilization yield of almost 100%. The difference between both values could be due to a loss of activity caused by conformational changes during immobilization, or a decrease of the observed reaction rate caused by mass transfer limitations. Enzyme leakage was not observed during the immobilization and stabilization process.

LentiKats® immobilized ω-transaminase enzyme and whole cells were employed as biocatalysts for the asymmetric synthesis of aromatic chiral amines (Cárdenas-Fernández, 2012b).

CONCLUDING REMARKS

The present work has demonstrated the possibility of developing an efficient common production process for different mutant enzymes expressed in *E. coli*. Similar operation conditions in fed-batch mode have been specified, employing an exponential addition profile and pulse IPTG induction. Optimum productivities were found at low inducer biomass ratio (2 μmol IPTG x g^{-1} DCW). An integrated process including fermentation and partial purification has been standardized for production of new aspartases and new ω-transaminases with improved synthetic capabilities. Finally, the enzymes have been immobilized in order to obtain suitable biocatalysts to be used in the synthesis of chiral amines.

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