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Enzymatic dynamic kinetic resolution of racemic N-formyl- and N-carbamoyl-amino acids using immobilized L-N-carbamoylase and N-succinyl-amino acid racemase.

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Abstract:	Taking advantage of the catalytic promiscuity of L-carbamoylase from Geobacillus stearothermophilus CECT43 (BsLcar) and N-succinyl-amino acid racemase from Geobacillus kaustophilus CECT4264 (GkNSAAR), we have evaluated the production of different optically pure L-α-amino acids starting from different racemic N-formyl- and N-carbamoyl-amino acids using a dynamic kinetic resolution approach. The enzymes were immobilized on two different solid supports, resulting in improved stability of the enzymes in terms of thermostability and storage when compared to the enzymes in solution. The bienzymatic system retained up to 80% conversion efficiency after 20 weeks at 4 ºC, and up to 90% after one week at 45 ºC. The immobilization process also resulted in a great enhancement of the activity of BsLcar towards N-formyl-tryptophan, showing for the first time that substrate specificity of L-carbamoylases can be influenced by this approach. The system was effective for the biosynthesis of natural and unnatural L-amino acids (e.e. 100%), such as L-methionine, L-alanine, L-tryptophan, L-homophenylalanine, L-aminobutyric acid and L-norleucine, with a higher performance toward N-formyl-α-amino acid substrates. Biocatalyst reuse was studied, and after 10 reaction cycles over 75% activity remained.
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	Prof. Dr. rer. nat. Christoph Syldatk has a broad experience in L-carbamoylases and in the production of optically pure D- and L-amino acids. He has has used L-carbamoylases in the past for KR and DKR of amino acids using the "hydantoinase process"
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	Hemraj S Nandanwar CSIR-Institute of Microbial Technology (India) hemraj@imtech.res.in Dr. Nandanwar has recently published studies on Bacillus L-carbamoylases, including their immobilization.
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Dear Editor,

Please find in the attached file the manuscript entitled "*Enzymatic dynamic kinetic resolution of racemic N-formyl- and N-carbamoyl-amino acids using immobilized L-N-carbamoylase and N-succinyl-amino acid racemase*" for submission in "*Applied Microbiology and Biotechnology*".

Taking advantage of the catalytic promiscuity of L-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) and *N*-succinyl-amino acid racemase from *Geobacillus kaustophilus* CECT4264 (GkNSAAR), we have evaluated the production of different optically pure L-α-amino acids starting from different racemic *N*-formyl- and *N*-carbamoyl-amino acids using a dynamic kinetic resolution approach. The enzymes were immobilized on two different solid supports, resulting in improved stability of the enzymes in terms of thermostability and storage when compared to the enzymes in solution. The bienzymatic system retained up to 80% conversion efficiency after 20 weeks at 4 ºC, and up to 90% after one week at 45 ºC. The immobilization process also resulted in a great enhancement of the activity of BsLcar towards *N*-formyl-tryptophan, showing for the first time that substrate specificity of L-carbamoylases can be influenced by this approach. The system was effective for the biosynthesis of natural and unnatural L-amino acids (e.e. 100%), such as L-methionine, L-alanine, L-tryptophan, L-homophenylalanine, L-aminobutyric acid and L-norleucine, with a higher performance toward *N*-formyl-α-amino acid substrates. Biocatalyst reuse was studied, and after 10 reaction cycles over 75% activity remained.

Yours sincerely,

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Enzymatic dynamic kinetic resolution of racemic *N*-formyl- and *N*-carbamoyl-amino acids using immobilized L-*N*-carbamoylase and *N*-succinyl-amino acid racemase.

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Abstract

Taking advantage of the catalytic promiscuity of L-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) and *N*-succinyl-amino acid racemase from *Geobacillus kaustophilus* CECT4264 (GkNSAAR), we have evaluated the production of different optically pure L-α-amino acids starting from different racemic *N*-formyl- and *N*-carbamoyl-amino acids using a dynamic kinetic resolution approach. The enzymes were immobilized on two different solid supports, resulting in improved stability of the enzymes in terms of thermostability and storage when compared to the enzymes in solution. The bienzymatic system retained up to 80% conversion efficiency after 20 weeks at 4 ºC, and up to 90% after one week at 45 ºC. The immobilization process also resulted in a great enhancement of the activity of BsLcar towards *N*-formyl-tryptophan, showing for the first time that substrate specificity of L-carbamoylases can be influenced by this approach. The system was effective for the biosynthesis of natural and unnatural L-amino acids (e.e. 100%), such as L-methionine, L-alanine, L-tryptophan, L-homophenylalanine, L-aminobutyric acid and L-norleucine, with a higher performance toward *N*-formyl-α-amino acid substrates. Biocatalyst reuse was studied, and after 10 reaction cycles over 75% activity remained.

Keywords: L-*N*-carbamoylase; *N*-succinyl-amino acid racemase; *N*-acetyl-amino acid racemase; L-norleucine; L-homophenylalanine; L-2-aminobutyric acid A; L-tryptophan.

Introduction

Optically pure amino acids are of great interest for the chemical industry and of considerable economic importance because of the broad spectrum of their industrial applications (Leuchtenberger et al., 2005). The increasing interest in unnatural amino acids is giving rise to a new discipline of study, as can be inferred from the appearance of a complete book on this topic (Pollegioni and Servi, 2012). By way of example, L-homophenylalanine is a precursor for the preparation of angiotensin-converting enzyme (ACE) and renin inhibitors, such as enalapril, lisinopril, quinapril, ramipril, trandolapril and benazepril, among others (Johnson et al., 1985). L-α-aminobutyric acid (L-ABA, also known as L-homoalanine), is a key chiral intermediate for the synthesis of several important drugs, such as levetiracetam or brivaracetam (antiepileptic drugs) and ethambutol (antituberculosis drug) (Zhang et al., 2010). L-norleucine (2-aminohexanoic acid) has recently been shown to present cytotoxic activity against human tumor cell lines (Fazary et al., 2011), and it is also a key chiral intermediate for the synthesis of several new lead compounds (Gutierrez et al., 2013; Simmons, 2008). Despite the direct commercial applications of unnatural amino acids, in recent years, the possibility to incorporate unnatural amino acids site-specifically into proteins has had a major impact on protein engineering (Liu and Schultz, 2010; Zhang et al., 2013). By removing the constraints imposed by nature on the number and properties of genetically encoded amino acids, this technique has proven very valuable to probe protein function, and to improve protein function for medical and industrial applications (Liu and Schultz, 2010; Zhang et al., 2013).

Biocatalysis has become an established technology for the industrial manufacture of fine chemicals (Breuer et al., 2004), and optically pure amino acid production is no

exception (Leuchtenberger et al., 2005). Different methods have been described for the enzymatic production of L-amino acids, such as the Hydantoinase (Altenbuchner et al., 2001), Amidase (Asano, 2007) or Acylase processes (May et al., 2002). Given the progress made in this field, it is clear that biocatalysts were not merely a trend of the last decade, rather their continues to grow (Kircher, 2012; Gotor and Flitsch, 2011).

Several of the above-described biotechnological methods rely on the use of at least one enatiospecific or highly enantioselective enzyme to achieve enantiopure (or highly enantiomeric enriched) amino acids, turning the process into a kinetic resolution (Huerta et al., 2001). Furthermore, since in most cases racemic mixtures are the starting point for these processes, an additional chemical or enzymatic racemization step of the non-hydrolyzed isomer turns the process into a dynamic kinetic resolution (DKR), allowing total conversion of the racemic substrates used in each case (Huerta et al., 2001; Altenbuchner et al., 2001; Asano, 2007; May et al., 2002).

In previous works, we confirmed the substrate promiscuity of an L-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) and an *N*-succinylamino acid racemase from *Geobacillus kaustophilus* CECT4264 (GkNSAAR) toward different *N*-substituted amino acids (Pozo-Dengra et al., 2009 and 2010). We further confirmed the effectiveness of coupling BsLcar and GkNSAAR as a DKR-biotool using racemic mixtures of different *N*-acetyl-, *N*-carbamoyl- and *N*-formyl-amino acids (Soriano et al., 2014). This bienzymatic system presented the highest efficiency for *N*-formyl-amino acids, and allowed the production of different enantiopure L-α-amino acids (e.e. 100%) due to the strict enantiospecificity of L-carbamoylases (Martínez-Rodríguez et al., 2010). In the present work, we aimed to enhance further the performance of the

BsLcar/GkNSAAR tandem by using an immobilization approach, as this technique is known to alter properties such as catalytic activity or thermal stability (Garcia-Galan et al., 2011; Rodrigues et al., 2013). DKR has been carried out with the two fastest substrates (*N*-formyl and *N*-carbamoyl-amino acids). In order to establish whether this approach might be of economic interest, reusage of the bienzymatic system has also been assayed.

Materials and methods

General protocols and reagents

Racemic mixtures and optically pure D- and L-amino acids were purchased from Sigma-Aldrich (Madrid, Spain). Sepabeads EC-HFA/S (Resindion, Rome, Italy) and Immobeads IB-A161 (ChrialVision, Leiden, The Netherlands) matrixes were used for enzyme immobilization. The *N*-carbamoyl- and *N*-formyl-amino acids used in this work were synthesized according to previous works (Boyd, 1933; Hussenet, 2001; Soriano-Maldonado et al., 2014).

Expresion and purification of BsLcar and GkNSAAR enzymes.

Procedures for the purification and preparation of the enzymes were the same as those previously described using cobalt affinity chromatography (Clontech Laboratories Inc.) (Soriano-Maldonado et al., 2014). The purified enzymes were concentrated using vivaspin concentrators (Sartorius), dialyzed against 100 mM borate-HCl pH 8.0 buffer and stored at 4 ºC until use. Protein concentrations were determined from the absorbance of coefficient extinction of tyrosine residues (ε=43,430 and 58,900 M^{-1} cm^{-1} for BsLcar and GkNSAAR respectively).

Preparation of immobilized enzymes

Immobilization of the purified enzymes was performed by mixing 0.1 g of Sepabeads EC-HFA/S **(**Resindion SRL) with 400 µl of BsLcar (0.5-0.7 mg mL^{-1}) or GkNSAAR (7 mg mL^{-1}) separately in 100 mM borate-HCl pH 8.0 buffer, following the manufacturer's instructions, with slight modifications. The mixture was placed in a thermo shaker (TS-100, BOECO, Germany) for 12 h at 700 rpm and 20 ºC. The matrixes with the immobilized enzymes were decanted and rinsed thoroughly with 400 µl of distilled water, and treated with the same volume of 3 M Glycine pH 8.2 for a further 12 h. The immobilized enzymes were then rinsed again with water and resuspended in 400 µl of the aforementioned borate-HCl buffer. The protocol for immobilization onto Immobead IB-A161 was very similar, except that the 12-hour treatment with 3 M Glycine pH 8.2 was not necessary for this matrix.

The concentration of immobilized enzymes was determined from the equation:

$$(\text{mg Enzyme})_0 - (\text{mg Enzyme})_f \ / \ \text{mg solid support}$$

Where $(\text{mg Enzyme})_0$ and $(\text{mg Enzyme})_f$ correspond to the amount of enzyme in solution before and after the immobilization process, respectively. A medium-scale immobilization process was scaled up after optimization of the enzyme concentration used. For this purpose, both enzymes were separately immobilized on 1g of matrix with 4 ml of enzyme at the previously established concentration and following the steps described above, but using 4 mL of distilled water, 3 M Glycine pH 8.2 and 100 mM borate-HCl buffer.

Characterization of the immobilized enzymes

Based on the calculated specific activities of the enzymes under the immobilization conditions, we assayed different mixtures of immobilized BsLcar:GkNSAAR (ratios 1:1 to 1:20), following a standard reaction, as follows: the immobilized enzymes were mixed with 7.5 mM substrate in 100mM borate-HCl pH 8.0, in the presence of 1 mM $CoCl_2$ (reaction volume 1010 µl). The reaction mixture was incubated at 45-55 ºC with shaking (850 rpm), and aliquots of 75 µl were retrieved at different intervals. The reaction was stopped by addition of 675 µl of 1% H_3PO_4. After centrifugation (13000xg), the resulting supernatants were analyzed by high performance liquid chromatography (HPLC) (see below). After selection of a fixed ratio of enzymes (10 mg BsLcar plus 20 mg GkNSAAR for EC-HFA/S; 25 mg BsLcar plus 50 mg GkNSAAR for IB-A161), pH and temperature studies were carried out following the standard reaction.

pH studies were conducted using *N*-carbamoyl and *N*-formyl-D,L-methionine as substrates, using the standard reaction described above. A pH range of 5.0 to 10.0 was assayed (sodium citrate pH 5.0-6.0; sodium cacodylate pH 6.5-7.5; borate-HCl 8.0-9.0; borate-NaOH 9.5-10.0). The effect of temperature on the conversion rate of the bienzymatic system was evaluated from 30 to 80 ºC. The thermal stability of the two immobilized systems was also measured using two approaches, both in 100 mM borate-HCl pH 8.0, following the standard reaction; residual activity of the bienzymatic system was measured a) after 18-hour preincubation at temperatures ranging from 30 to 80 ºC and b) after preincubation at 4 and 45 ºC for several days.

Substrate specificity studies were performed with each different *N*-formyl- and *N*-carbamoyl-D,L-amino acid (7.5 mM) dissolved in 100 mM borate-HCl pH 8.0, 1 mM $CoCl_2$ together with the immobilized enzymes (10 mg BsLcar plus 20 mg GkNSAAR for EC-HFA/S; 25 mg BsLcar plus 50 mg GkNSAAR for IB-A161). Different temperatures were used according to the optimal parameters found with the different reaction systems. Larger-scale reactions were carried out with *N*-formyl-D,L-norleucine at 160 mM (25 mg IB-A161-BsLcar plus 50 mg IB-A161-GkNSAAR) at pH 8.0 and 45 ºC. In both the small and large-scale reactions, 75 µl aliquots were retrieved at different reaction times, and the reaction was stopped by addition of 675 µl of 1% H_3PO_4. After centrifugation, the resulting supernatants were analyzed by HPLC as described below.

Chromatographic analysis

To determine the enantiomeric excess (e.e.) of different amino acids produced by the enzymatic tandem, chiral separations were carried out using the same HPLC system equipped with a chirobiotic T column (250 × 4.6 mm, 5 µm, Astec, Sigma-Aldrich). The mobile phase was 70: 29.5: 0.05 methanol:10 mM ammonium acetate: acetic acid, pumped at a flow rate of 0.3 ml min^{-1} and measured at 203 nm (Soriano-Maldonado et al., 2014). On the other hand, an HPLC system (LC2000Plus HPLC System, Jasco) equipped with a Zorbax C_{18} column (3.0 x 250 mm, Agilent Inc.) was used to detect *N*-formyl-amino acids, *N*-carbamoyl-amino acids, and the corresponding L-amino acids, as previously described (Soriano-Maldonado et al., 2014). The mobile phases contained H_3PO_4 20 mM pH 3.2 and methanol at different proportions (50:50 to 95:5, depending on the substrate), pumped at a flow rate of 0.2 to 1.0 ml min^{-1} and measured at 200-203 nm.

Reutilization of the immobilized enzymes

Reuse of the immobilized enzymes was assayed using mixtures of the different solid supports (10 mg BsLcar plus 20 mg GkNSAAR for EC-HFA/S; 50 mg BsLcar plus 100 mg GkNSAAR for IB-A161). The solid supports were incubated at 45 ºC with 1 mL of 7.5 mM *N*-formyl-D,L-methionine in 100 mM borate-HCl pH 8.0 supplemented with 1mM $CoCl_2$. The supernatant was removed on completion of the reaction and analyzed by HPLC to quantify the amount of L-methionine produced. The solid supports were washed once with 1 mL of 100 mM borate-HCl pH 8.0, and 1 mL of 7.5 mM *N*-formyl-D,L-methionine in 100 mM borate-HCl pH 8.0 supplemented with 1mM $CoCl_2$ was added to a 2-mL tube containing the immobilized enzymes, and incubated again at 45 ºC. The same process was repeated for several cycles.

Results

Immobilization process

The amounts of BsLcar and GkNSAAR immobilized onto the EC-HFA/S support (HFA-BsLcar and HFA-GkNSAAR) were 0.4±0.0 and 8.0±0.7 μg prot·mg^{-1} beads respectively, whereas 2.6±0.2 and 15.0±1.2 μg prot·mg^{-1} beads were obtained for the immobilization of BsLcar and GkNSAAR onto the IB-A161 support (A161-BsLcar and A161-GkNSAAR). These results show that IB-A161 proved more efficient in terms of the amount of immobilized enzyme. Under these immobilization conditions, both HFA-GkNSAAR and A161-GkNSAAR showed activities in the range of 0.4±0.2 μmol·mg $prot^{-1}$·min^{-1} with *N*-formyl-L-methionine as substrate, *i.e.* slightly below the activity found previously with GkNSAAR in solution (1.6±0.1 μmol·mg $prot^{-1}$·min^{-1}; Soriano-Maldonado et al., 2014). On the other hand, HFA-BsLcar and A161-BsLcar showed activity values of 14.0±4.0 and 8.5±3.5 μmol·mg $prot^{-1}$·min^{-1}, respectively, with *N*-

formyl-L-methionine as substrate, which is very similar to the value found in solution (12.0±5.5 μmol·mg prot^{-1}·min^{-1}; Soriano-Maldonado et al., 2014). Thus, our results show that the immobilization process did not substantially alter the specific activity of the enzymes.

Effect of pH and temperature

Based on the specific activities of the enzymes under the immobilization conditions, we assayed different mixtures of immobilized BsLcar:GkNSAAR. When using the HFA-BsLcar / HFA-GkNSAAR mixture, total consumption was achieved after 420 min for a ratio 1:20, whereas it took 540 min for a ratio 1:2 (data not shown). The 1:2 ratio was chosen for subsequent experiments (10 mg HFA-BsLcar:20 mg HFA-GkNSAAR), as the decrease in time in total consumption of the substrate did not outweigh the higher amounts of GkNSAAR used. When assaying the A161-BsLcar / A161-GkNSAAR mixture, the minimum amount of support to be used was conditioned by the size of the solid support, which is larger and more difficult to handle[1]. Based on our results, a 1:2 ratio was also selected (25 mg A161-BsLcar:50 mg A161-GkNSAAR).

Studies of pH and temperature dependence for the production of L-methinonine when using both immobilized bienzymatic systems were similar to those previously described for the enzymes in solution (Soriano-Maldonado et al., 2014): The highest conversion rate for the biocatalyst proved to be 7.5-8.0 for both *N*-formyl- and *N*-carbamoyl-amino acid substrates. Temperature ranges with the fastest conversion were 40-55 ºC for *N*-formyl-amino acids and 55-70 ºC for *N*-carbamoyl-derivatives when using the HFA support (Fig. 1 Sup. Mat.). The results with the A-161 support varied slightly, and the

[1]25 mg of the A161-matrix was selected as the minimum amount to be used reproducibly.

highest conversion rates were obtained at 45-75 ºC with *N*-formyl-amino acids and 65 ºC with *N*-carbamoyl-derivatives. Thus, immobilization on the A-161 support increased the temperature at which reactions could be carried out. On the other hand, these results suggest that the immobilization process did not substantially alter the enzymatic behavior when compared to the enzymes in solution (Soriano-Maldonado et al., 2014). Thermal stability of the system was studied by preincubation of the immobilized enzymes together for 18 hours in 100 mM borate-HCl pH 8.0, at temperatures ranging from 30 to 80 ºC. Conversion rates decreased when the immobilized enzymes were incubated at temperatures of over 55 °C, and were lost completely at over 70 ºC for the HFA-BsLcar / HFA-GkNSAAR system (Fig. 1). Similar results were obtained when using the A161-BsLcar / A161-GkNSAAR tandem (Fig. 1), although this immobilized system still presented 30% activity at 70 ºC after 18 hours of incubation. This value is slightly higher than that found with the enzymes in solution, when no conversion was observed after 10 hours of incubation of the bienzymatic system at 70 ºC (Soriano-Maldonado et al., 2014), thus showing that the A161 support slightly increased the thermal stability of the bienzymatic tandem. On the other hand, in view of the loss of activity at over 55º C for both HFA- and A161-immobilized tandems (Fig. 1), it was decided to carry out all the subsequent reactions at temperatures ≤ 55 ºC.

Further studies of the system's stability were carried out over prolonged times of storage/incubation.The immobilized BsLcar/GkNSAAR enzymes were found to maintain full activity after 4 weeks at 4ºC, retaining up to 80% after 20 weeks at this temperature. These values are much higher than those obtained with the enzymes in solution, when 50% of activity was lost after 4 weeks at the same temperature. Furthermore, the immobilized enzymes maintained 90% and 50% of their activity after

7 and 14 days of incubation at 45 ºC, respectively, which also improves on the results obtained previously for the enzymes in solution. The immobilization procedure therefore resulted in greater enzyme stability than using enzymes in solution, as has been observed for other enzymes immobilized on solid supports (Sheldon 2007; Garcia-Galan et al., 2011; Rodrigues et al., 2013).

Bienzymatic production of enantiopure L-α-amino acids

HFA- and A161-immobilized tandems were assayed with different *N*-formyl- and *N*-carbamoyl-amino acid racemates. Both systems were effective for production of methionine, alanine, norleucine, norvaline, ABA, homophenylalanine, phenylglycine and ethionine (Tables 1 and 2), obtaining an e.e. of 100% in all cases. On the other hand, they were not effective for *tert*-leucine production. The A-161 tandem was also effective for the biosynthesis of L-tryptophan under the conditions assayed (Table 2). Interestingly enough, whereas the HFA-immobilized tandem showed a negligible conversion toward L-tryptophan (Table 1), similar to the results observed with the enzymes in solution, the conversion rate with *N*-formyl-L-tryptophan was greatly enhanced when using the A-161 tandem (more than 500 times faster; Table 2). These results arose from an improvement on the hydrolysis of the substrate by BsLcar, which might be due to BsLcar structure distortion altering the catalytic centre environment of the enzyme, as has been observed for other enzymes (Garcia-Galan et al., 2011; Rodrigues et al., 2013). The L-enantiospecific character of the immobilized BsLcar was maintained after the immobilization process, allowing the production of these L-amino acids with an e.e. value of 100%, as was observed previously for the enzymatic tandem in solution (Soriano-Maldonado et al., 2014). By way of example, the enantiomeric

purity of homophenylalanine produced by the immobilized tandem is shown in Figure 2.

Operational stability of the immobilized enzymes and large-scale reaction

The relative activity of the A161-BsLcar / A161-GkNSAAR tandem decreased only slightly after each cycle, with an average of 3% activity loss per cycle (Fig. 3), maintaining over 75% of the initial activity after 10 cycles. However, the relative activity of the HFA-BsLcar / HFA-GkNSAAR tandem was almost totally lost after 4 cycles (Fig. 3). SDS-PAGE gels of the supernatant fractions of the different reaction cycles showed that protein was partially removed from the matrix after each reaction cycle (data not shown). The same experiment was carried out under the same conditions of pH and temperature, but in the absence of shaking, and it was observed that no protein was lost by SDS-PAGE. Furthermore, this effect was seen to be time- and shake-dependent, thus allowing us to conclude that the mechanical force was responsible for the loss of activity, as has been described for other immobilization processes (Garcia-Galan et al., 2011). This drawback considerably diminishes the likelihood of a putative industrial application of the HFA-BsLcar / HFA-GkNSAAR tandem. On the other hand, since our results showed that recycling of the A161-BsLcar / A161-GkNSAAR tandem was possible, it was decided to test the process on a larger scale, as its implementation at industrial level depends on several factors, such as whether high concentrations of substrate/products inhibit the enzymatic reaction. The A161-BsLcar / A161-GkNSAAR tandem was therefore assayed for L-norleucine production using 160 mM of *N*-formyl-D,L-norleucine in 100 mM borate-HCl pH 8.0 at 45 ºC. Total conversion was achieved in 11 hours (Fig. 4), with a productivity of 16

mmol norLeu·L^{-1}·h^{-1}. The nature of the product (L-norleucine) was confirmed by RMN (Fig. 2 SM) and its enantiomeric purity was checked by chiral HPLC.

Discussion

Optically pure amino acids are of great interest, since they are important building blocks in pharmaceutical and other industrial sectors. A wide range of these compounds have been synthesized by different enzymatic methods, such the Hydantoinase (Altenbuchner et al., 2001), Amidase (Asano, 2007) or Acylase processes (May et al., 2002). Based on the Acylase process, an alternative method to synthesize L-homophenylalanine using an L-carbamoylase and an *N*-succinyl-amino acid racemase was proposed by Hsu et al. (Hsu et al. 2007). We have further confirmed that BsLcar and GkNSAAR are highly promiscuous enzymes, and can accept different *N*-substituted-amino acids (Pozo-Dengra et al., 2009 and 2010), allowing to couple both enzymes for the biosynthesis of different optically pure natural and non-natural amino acids (Soriano-Maldonado et al. 2014). Our results suggested that the industrially-used "Acylase process" could be named "Amidohydrolase process" in a more general way, since substitution of L-acylase by L-carbamoylase increases the substrate spectrum to be used with this enzymatic process.

Enzyme immobilization has arisen as one of the common strategies to be used to increase enzymatic performance (Sheldon, 2007; Garcia-Galan et al., 2011; Rodrigues et al., 2013). Whereas the immobilization process often results in enzymes with higher stability, it also often results in lower enzymatic activity if the proper support is not selected (Rodrigues et al., 2013). In this work we have shown that the immobilization of BsLcar/GkNSAAR tandem resulted into conversion levels comparable to the enzymes

in solution, but with enhanced stability, and allowing enzyme reuse. The system was more effective for the degradation of *N*-formyl-derivatives (Tables 1 and 2), as was observed previously with the enzymes in solution (Soriano-Maldonado et al., 2014). When comparing the performance of both systems, the HFA-tandem might in principle seem slower than the A161-tandem (Tables 1 and 2). However, the effective amount of the enzymes in the reaction is different in both systems as a result of: a) the different immobilization effectiveness for both supports (see above), and b) the different amount of support used for each system. Indeed, the A161-tandem uses four times the amount of GkNSAAR used by the HFA-tandem. Since it has previously been shown that the limiting step of the reaction in this process is the GkNSAAR enzyme (Soriano-Maldonado et al., 2014), reactions carried out with the A161-tandem should prove approximately 4 times faster than with the HFA-tandem, and Tables 1 and 2 show that this is the case. Reuse of the HFA-BsLcar / HFA-GkNSAAR showed that enzymatic activity was almost totally lost after 4 cycles (Fig. 3). This drawback considerably diminishes the likelihood of a putative industrial application of this support. On the other hand, our results showed that recycling of the A161-BsLcar / A161-GkNSAAR tandem was possible, retaining more than 75% of its conversion capability after 10 reaction cycles.

The immobilization of GkNSAAR resulted in approximately a 4-fold decrease in its specific activity when compared to the enzyme in solution, but with an increased stability in terms of half-life. Similar results were obtained when *Deinococcus radiodurans* NSAAR (DraNSAAR) was immobilized, resulting in a 90% decrease of its specific activity (Yen et al., 2010). Regarding L-carbamoylases, previous studies have shown that immobilization of the enzymes from *Arthrobacer* and *Bacillus* genera has

resulted in a substantial decrease in their specific activity (Ragnitz et al. 2001a and b; Yen et al., 2010; Nandanwar et al. 2013), except in the case of a recombinant $(Asp)_6$-tagged L-carbamoylase from *Arthrobacter* (AauBLcar), which maintained similar activities to the enzyme in solution (Ragnitz et al. 2001 enzandmicrobtech). BsLcar could also be reused for several cycles as other *Bacillus* L-carbamoylases, maintaining similar significant half-lifes (Nandawar et al. 2013; Yen et al. 2010). Unexpectedly, BsLcar activity towards *N*-formyl-L-tryptophan was greatly enhanced (aprox. 500 times; Table 2) when using the A-161 support. Whereas this phenomenon has been observed for other biotechnologically-relevant enzymes such as lipases (Rodrigues et al., 2013), this is the first evidence showing that substrate spectrum of L-carbamoylases can be altered via immobilization. Thus, and although additional studies are mandatory to ascertain if this property can be generalized to other L-carbamoylases, our results suggest that substrate spectrum of L-carbamoylases could be also enhanced by an immobilization procedure.

Whereas alternative L-carbamoylase/NSAAR biocatalysts have been used only for the synthesis of L-homophenylalanine (Hsu et al. 2007; Yen et al. 2010; Nandawar et al. 2013), the BsLcar/GkNSAAR tandem is the first L-carbamoylase/NSAAR biocatalyst shown to produce different optically pure amino acids (e.e. 100%) starting from different racemic *N*-substituted amino acids (Tables 1 and 2). The immobilized system had a productivity of 16 mmol L-norLeu$\cdot L^{-1}\cdot h^{-1}$ (yield >99%; e.e. 100%), showing no inhibition at high concentrations of substrate or product (Fig. 4), which is a pre-requisite to scale it up to analytical or industrial scale. This productivity is higher than that reported for the other L-carbamoylase/NSAAR systems (Hsu et al. 2007; Yen et al. 2010). Furthermore, BsLcar/GkNSAAR tandem presents higher yields and

enantiomeric excess than other methods used for L-homophenylalanine biosynthesis (Ahmad 2010; Vaidya et al., 2012). L-ABA production also proved more simple and with similar or even higher productivity when compared to other methods using enzymatic cascades such as L-threonine deaminase/L-leucine dehydrogenase/formate dehydrogenase (Tao et al., 2013) or L-threonine deaminase/aromatic aminotransferase/acetolactate synthase/alanine racemase/D-amino acid oxidase (Zhu et al. 2011).

Whereas the economic aspects of the immobilization process need to be studied, in this work we show an effective immobilized bienzymatic system for the biosynthesis of optically pure (e.e. 100%) unnatural L-α-amino acids such as L-phenylglycine, L-homophenylalanine, L-aminobutyric acid and L-norleucine, among others. Interestingly, the immobilization process resulted in a great enhancement of the activity of BsLcar towards *N*-formyl-tryptophan, enhancing the substrate spectrum to be used with this system. The shelf life of both enzymes was greatly enhanced when immobilized. Biocatalyst reuse was studied, and over 75% of activity remained after 10 reaction cycles.

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Substrate	Transformed substrate (%)	Reaction time (hours)
N-Formyl-D,L-methionine	>95	5
N-Carbamoyl-D,L-methionine	92	48
N-Formyl-D,L-aminobutyric acid	>95	28
N-Carbamoyl-D,L-aminobutyric acid	60	48
N-Formyl-D,L-norvaline	>95	7
N-Carbamoyl-D,L-norvaline	>95	48
N-Formyl-D,L-norleucine	>95	5
N-Carbamoyl-D,L-norleucine	90	48
N-Formyl-D,L-ethionine	>95	18
N-Formyl-D,L-homophenylalanine	>95	9
N-Formyl-D,L-phenylglycine	70	48
N-Formyl-D,L-alanine	88	48
N-Formyl-D,L-tryptophan	< 0.1	48
N-Formyl-D,L-pheanylalanine	12	48
N-Formyl-D,L-*tert*-leucine	N.D.	48

Table 1. Substrate specificity of the immobilized HFA-BsLcar/GkNSAAR tandem. Reactions were carried out in triplicate with 7.5 mM of the different substrates in 100 mM borate-HCl pH 8.0, 1 mM $CoCl_2$ at 45 °C, using 10 and 20 mg of immobilized enzymes, respectively. Enantiomeric excess (e.e.) of the amino acid produced was 100 % in all cases, as measured by chiral HPLC.

Substrate	Transformed substrate (%)	Reaction time (hours)
N-Formyl-D,L-methionine	>95	2
N-Carbamoyl-D,L-methionine	>95	9
N-Formyl-D,L-aminobutyric acid	>95	3
N-Carbamoyl-D,L-aminobutyric acid	>95	11
N-Formyl-D,L-norvaline	>95	3
N-Carbamoyl-D,L-norvaline	>95	7
N-Formyl-D,L-norleucine	>95	2
N-Carbamoyl-D,L-norleucine	>95	9
N-Formyl-D,L-ethionine	>95	5
N-Formyl-D,L-homophenylalanine	>95	3
N-Carbamoyl-D,L-homophenylalanine	54	18
N-Formyl-D,L-phenylglycine	60	18
N-Formyl-D,L-alanine	90	18
N-Formyl-D,L-tryptophan	63	18
N-Formyl-D,L-phenylalanine	40	18
N-Formyl-D,L-*tert*-leucine	N.D.	18

Table 2. Substrate specificity of the immobilized A161-BsLcar/GkNSAAR tandem. Reactions were carried out in triplicate with 7.5 mM of different substrates in 100 mM borate-HCl pH 8.0, 1 mM $CoCl_2$ at 45 °C, using 25 and 50 mg of immobilized enzymes, respectively. Enantiomeric excess (e.e.) of the amino acid produced was 100 % in all cases, as measured by chiral HPLC.

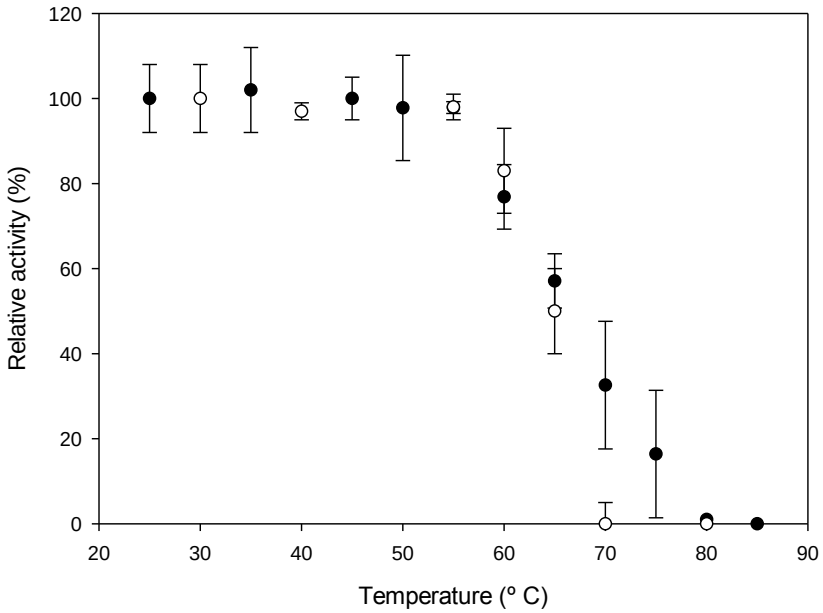

Figure 1. Remaining relative activity of the HFA-BsLcar/GkNSAAR (○) and A161-BsLcar/GkNSAAR (●) bienzymatic systems after 18-hour pre-incubation at different temperatures, followed by the standard enzymatic assay. Reactions were carried out in triplicate.

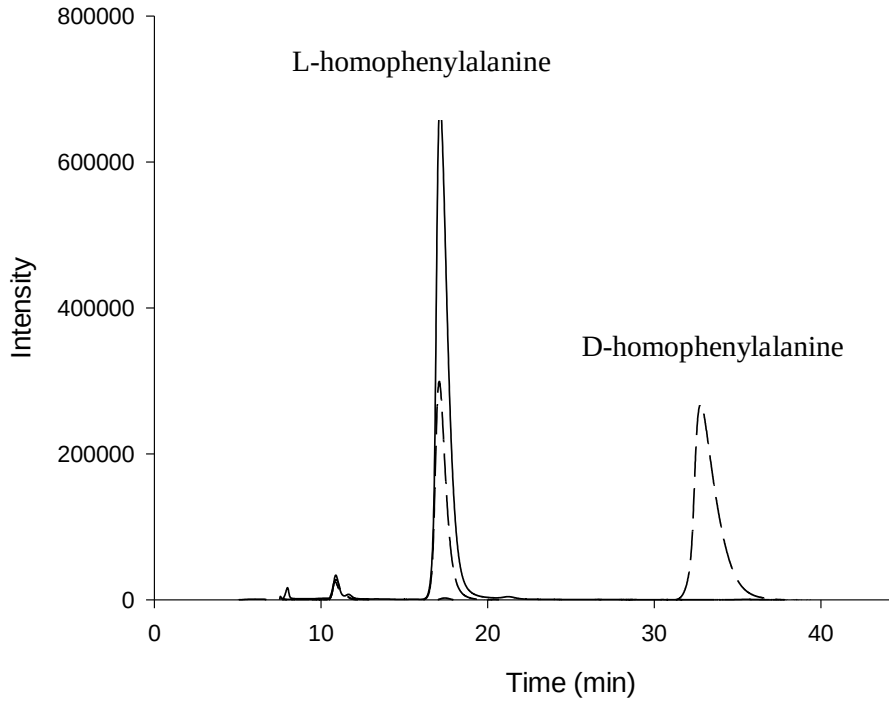

Figure 2. Chiral-HPLC chromatogram showing commercial D,L-homophenylalanine (dashed line) and a sample of a finished standard reaction of the bienzymatic system (continuous line), using racemic *N*-formyl-homophenylalanine as substrate. Optically pure L-homophenylalanine was produced by the enzymatic tandem (>95% yield, e.e. 100%).

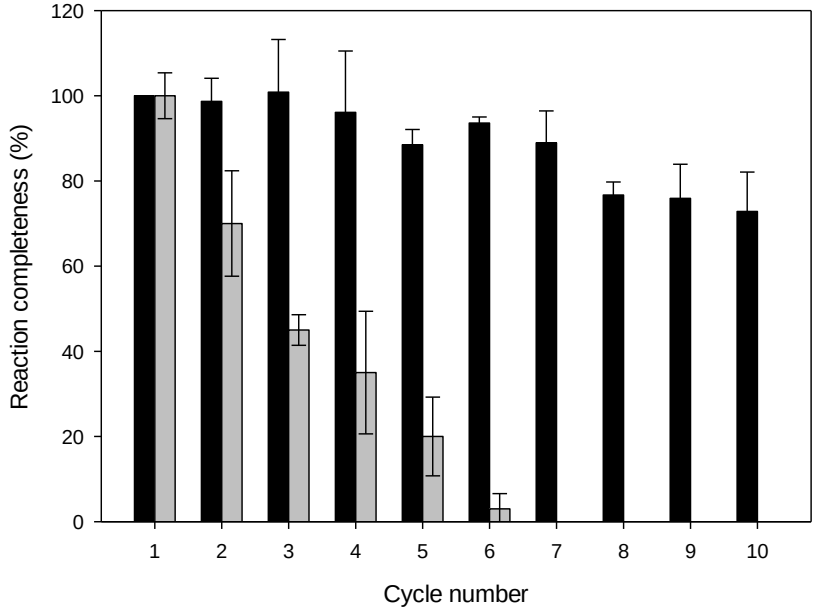

Figure 3. Achieved reaction completeness when reusing the immobilized HFA-BsLcar/GkNSAAR (grey bars) and A161-BsLcar/GkNSAAR (black bars) bienzymatic systems. Reactions were carried out in triplicate as described in material and methods.

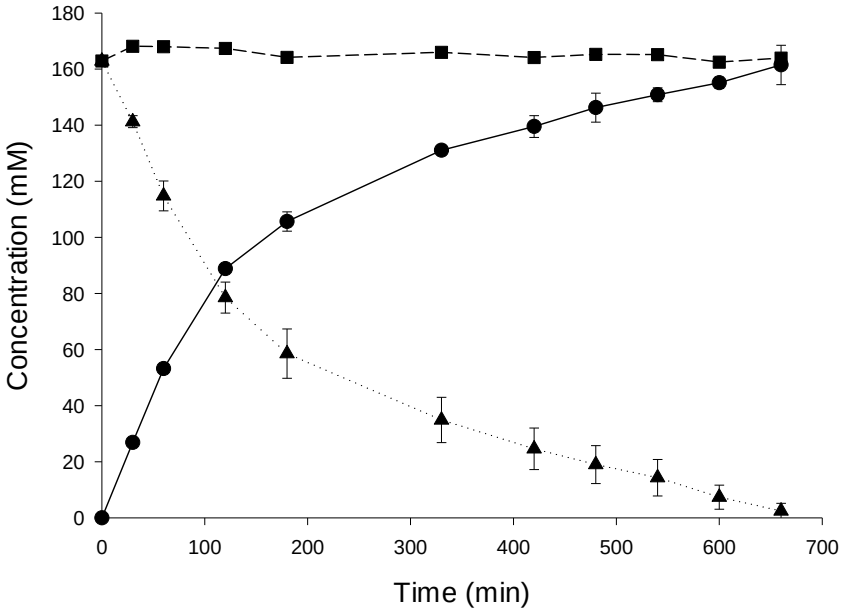

Figure 4. Profile of conversion of 160 mM *N*-formyl-D,L-norleucine (▲) into L-norleucine (●) by the immobilized A161-BsLcar/GkNSAAR tandem. The sum of both compounds is also shown (■). Reactions were carried out in triplicate in 100 mM borate-HCl pH 8.0, 1 mM $CoCl_2$ at 45 °C, using 25 and 50 mg of immobilized BsLcar and GkNSAAR enzymes, respectively.

Supplementary Material

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