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Recombinant Polycistronic Structure of Hydantoinase Process Genes in *Escherichia coli* for the Production of Optically Pure D-Amino Acids▽†

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Two recombinant reaction systems for the production of optically pure D-amino acids from different D,L-5-monosubstituted hydantoins were constructed. Each system contained three enzymes, two of which were D-hydantoinase and D-carbamoylase from *Agrobacterium tumefaciens* BQL9. The third enzyme was hydantoin racemase 1 for the first system and hydantoin racemase 2 for the second system, both from *A. tumefaciens* C58. Each system was formed by using a recombinant *Escherichia coli* strain with one plasmid harboring three genes coexpressed with one promoter in a polycistronic structure. The D-carbamoylase gene was cloned closest to the promoter in order to obtain the highest level of synthesis of the enzyme, thus avoiding intermediate accumulation, which decreases the reaction rate. Both systems were able to produce 100% conversion and 100% optically pure D-methionine, D-leucine, D-norleucine, D-norvaline, D-aminobutyric acid, D-valine, D-phenylalanine, D-tyrosine, and D-tryptophan from the corresponding hydantoin racemic mixture. For the production of almost all D-amino acids studied in this work, system 1 hydrolyzed the 5-monosubstituted hydantoins faster than system 2.

Optically pure D-amino acids are valuable intermediates for the preparation of semisynthetic antibiotics, pesticides, and other products of interest for the pharmaceutical, food, and agrochemical industries (22, 25). The enzymatic method to synthesize them potentially allows any optically pure amino acid to be obtained from a wide spectrum of D,L-5-monosubstituted hydantoins used as the substrate. This method is both cheaper and less contaminating than the chemoenzymatic process (10). In this cascade of reactions, named the hydantoin process (1), firstly, the chemically synthesized D,L-5-monosubstituted hydantoin ring is hydrolyzed by a stereoselective hydantoinase enzyme (D-hydantoinase). Further hydrolysis of the resulting *N*-carbamoyl-D-amino acid to yield the free D-amino acid is catalyzed by highly enantiospecific *N*-carbamoyl-D-amino acid aminohydrolase (D-carbamoylase). At the same time that D-hydantoinase hydrolyzes the enantiospecific D-5-monosubstituted hydantoin, the chemical and/or enzymatic racemization of L-5-monosubstituted hydantoin starts. Chemical racemization of the 5-monosubstituted hydantoins proceeds via keto-enol tautomerism under alkaline conditions (23) and is usually a very slow process (20). High velocities of chemical racemization have been observed only for D,L-5-phenyl- and D,L-5-*p*-hydroxy-phenylhydantoin because of the resonance stabilization by the 5-substituent, while all other hydantoins take many hours to racemize (13). Consequently, although the hydantoinase process has been successfully used to produce 100% D-phenyl- and D-*p*-hydroxy-phenylglycine (4, 8, 19), only 50% of the remaining hydantoins are converted to the corresponding amino acid, while the other 50% correspond to L-hydantoin, which is not hydrolyzed by D-hydantoinase. For these hydantoins, faster racemization is possible via an enzymatic reaction incorporating a third enzyme named hydantoin racemase together with D-hydantoinase and D-carbamoylase enzymes.

Our group has characterized several recombinant hydantoin racemases from different microorganisms in order to study their biochemical properties and substrate specificities (12, 15, 17). We have also developed a three-step enzymatic reaction for D-amino acid production using a recombinant whole-cell biocatalyst after the separate expression of D-hydantoinase, D-carbamoylase, and hydantoin racemase from *Agrobacterium* species in three different *Escherichia coli* strains (16). However, enzyme production requires three individual cultivations, and transport of the reaction intermediate might be a limiting factor. Alternatively, the three enzymes can be coexpressed in the same host cell and directly used as biocatalysts. The first recombinant system coexpressing these three genes in *E. coli* was employed to produce L-amino acids cloning hydantoin racemase together with L-hydantoinase and L-carbamoylase, all from *Arthrobacter aurescens* (24). More recently, D-hydantoinase and D-carbamoylase from *Flavobacterium* sp. and hydantoin racemase from *Microbacterium liquefaciens* have been coexpressed in *E. coli* (18). These two systems coexpress the three genes after cloning in plasmids with different antibiotic resistance genes. This strategy involves adding several antibiotics to the medium, resulting in high selective pressure on the growth of the recombinant cells. Additionally, both of

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these recombinant systems have shown intermediate accumulation as a result of weak D-carbamoylase activity, which has been described as a limiting factor in the overall process (19).

In order to overcome these two problems, we have designed a biocatalyst for the production of optically pure D-amino acids from D,L-5-monosubstituted hydantoins by coexpressing the three genes in one plasmid in a polycistronic structure. We have developed two whole-cell recombinant systems, both of which contain two common genes, the D-hydantoinase and D-carbamoylase genes from *Agrobacterium tumefaciens* BQL9. The third gene is the hydantoin racemase 1 gene ($hyuA1_{At}$) (12) for the first system and the hydantoin racemase 2 gene ($hyuA2_{At}$) (15) for the second system, both from *A. tumefaciens* C58. We have then compared the potentials of the recombinant strains for the biocatalytic production of different optically pure D-amino acids.

MATERIALS AND METHODS

General protocols and reagents. Standard methods were used for the cloning and expression of the different genes (2, 21). Restriction enzymes, T4 DNA ligase, and the thermostable *Pwo* polymerase together with primers for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). The 5-monosubstituted hydantoins used in this work, D,L-5-methylthioethylhydantoin (D,L-MTEH), D,L-5-ethylhydantoin, D,L-5-propylhydantoin, D,L-5-isopropylhydantoin, D,L-5-butylhydantoin, D,L-5-isobutylhydantoin, D,L-5-benzylhydantoin, D,L-5-*p*-hydroxybenzylhydantoin, D,L-5-*p*-hydroxyphenylhydantoin, D,L-5-phenylhydantoin, and D,L-5-indolylmethylhydantoin, were synthesized as described previously in the literature (7). The resulting *N*-carbamoyl-D-amino acids were synthesized as described in previous works (3). The optically pure D-amino acids were purchased from Sigma Aldrich Quimica S.A. (Madrid, Spain).

Microbes and culture conditions. *A. tumefaciens* BQL9 was used as the donor of the D-carbamoylase gene. This strain was cultivated at 30°C for 20 h in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2). Three *E. coli* BL21 strains were grown as previously described (5, 11, 14). One harbored pBSH1, expressing the D-hydantoinase gene from *A. tumefaciens* BQL9, while the others harbored pSER12 and pSER22, expressing hydantoin racemase genes 1 ($hyuA1_{At}$) and 2 ($hyuA2_{At}$) from *A. tumefaciens* C58, respectively. *E. coli* strains DH5α, JM109, TOP10F, and BL21, used to clone and express the multienzymatic systems, were routinely grown in LB broth at 37°C on rotary shakers at 250 rpm.

Cloning of D-hydantoinase, D-carbamoylase, and hydantoin racemase genes in *E. coli*. The recombinant plasmid pSER12 (12) was used as the template for PCR amplification of the $hyuA1_{At}$ gene (723 bp; GenBank accession no. AY436503). The primers used were 5′-AA*CTGCAG*G**CAGGAAA**GCTATT**ATG**CGTGCGA TGCAT-3′ (the PstI site is in italics, and the ribosome-binding site and start codon are in bold) and 5′-GC*CCTCGAG***TTA**GGCGCAGGCGA-3′ (the XhoI site is in italics, and the stop codon is in bold). Amplification was performed in a PerkinElmer Thermocycler 2400 programmed as follows: initial denaturation at 94°C for 5 min; 35 cycles of 30 s at 94°C for denaturing, 30 s at 57°C for annealing, and 1 min at 72°C for synthesis; and a final extension step of 5 min at 72°C. The PCR product was purified from agarose gel by using QIAquick (QIAGEN), treated with PstI and XhoI, and then ligated into the pBluescript II SK(+) plasmid (pBSK; Stratagene Cloning Systems), which was cut with the same enzymes to create plasmid pSER14. After cloning, the plasmid was transferred into *E. coli* DH5α to verify the presence of the insert. The sequence was analyzed at least twice using standard T3 and T7 primers. Sequencing analysis was carried out using the dye dideoxy nucleotide sequencing method with an ABI 377 DNA sequencer (Applied Biosystems).

The D-carbamoylase gene was cloned in pSER14 in a fashion similar to that described for $hyuA1_{At}$. As no recombinant plasmid with this gene was available in our laboratory, the gene was isolated directly by PCR from the *A. tumefaciens* BQL9 strain. The strain's genomic DNA was isolated as described previously (6), and the primers were designed based on the sequence with GenBank accession number X91070 (8). The forward primer was 5′-GC*TCTAGA*GTGA**CAGGAA AG**CTTT**ATG**ACACGTCAGATGATACTTGC-3′, with an XbaI site (in italics), a ribosome-binding site and a start codon (in bold), and a stop codon (underlined). The reverse primer was 5′-TT*CTGCAG***TTA**GAATTCCGCGAT CAGACCG-3′, with a PstI site (in italics) and a stop codon (in bold). The PCR product and the recombinant plasmid pSER14 were cleaved with XbaI and PstI to allow subcloning of the D-carbamoylase gene upstream from $hyuA1_{At}$. The resulting construct harboring the D-carbamoylase and $hyuA1_{At}$ genes was named pSER15 and transferred into *E. coli* DH5α.

The last enzyme gene cloned was the D-hydantoinase gene from *A. tumefaciens* BQL9, and in this case the recombinant plasmid pSBH1 (5) harboring this gene was used as the template for PCR amplification. The primers used were 5′-AA *CTCGAG*G**CAGGAAA**GCTTT**ATG**GATATCATCATC-3′ (the XhoI site is in italics, and the ribosome-binding site and start codon are in bold) and 5′-AA*G GTACC***TTA**TTGCTTGTATTTGCGGCG-3′ (the KpnI site is in italics, and the stop codon is in bold). The gel-purified PCR product was double digested with XhoI and KpnI and ligated with pSER15, which had been treated identically. The resulting plasmid (pSER42) containing the D-hydantoinase, D-carbamoylase, and $hyuA1_{At}$ genes was transferred into *E. coli* DH5α and named system 1.

For the construction of system 2, $hyuA1_{At}$ was replaced in the pSER42 plasmid by $hyuA2_{At}$. The primers 5′-*CTGCAG*G**CAGGAAA**GCTATT**ATG**CGCATCCT CGTC-3′ (the PstI site is in italics, and the ribosome-binding site and start codon are in bold) and 5′-*CTCGAG***TTA**CGCCGTCATGCGGTCC-3′ (the XhoI site is in italics, and the stop codon is in bold) were employed to amplify the gene $hyuA2_{At}$ (744 bp; GenBank accession no. AY436504) from the recombinant plasmid pSER22 (15), which was used as the template for PCR. The gel-purified PCR product was double digested with PstI and XhoI and ligated with similarly treated pSER42. The resulting plasmid (pAMG3) containing the D-hydantoinase, D-carbamoylase, and $hyuA2_{At}$ genes was transferred into *E. coli* DH5α and named system 2.

The stability of pSER42 and pAMG3 in the BL21 strain of *E. coli* was determined by the replica method described previously (9). Plasmid stability was quantified according to the linear relationship between the logarithm of the proportion of plasmid-containing cells and the number of generations. The number of generations was obtained from serial subcultures of cells in flasks of 10 ml of LB without ampicillin at 37°C. To maintain bacterial growth in exponential phase, each subculture was grown for 4 h and an appropriate volume was transferred into a new medium so that the initial optical density at 600 nm was 0.1. The proportion of plasmid-containing cells was calculated as the ratio of the number of cells forming on LB plates with ampicillin pressure to the total number of cells (including plasmid-free and plasmid-containing cells) grown on LB plates without ampicillin. Cell samples were diluted to obtain colony counts within the range of 30 to 300. All final plate counts were taken from the average of counts of at least three replicates.

Expression of the recombinant systems. Cells of *E. coli* strains carrying the recombinant plasmids were grown in LB medium supplemented with 100 μg ml^{-1} of ampicillin. A single colony was transferred into 10 ml of LB medium with ampicillin at the above-mentioned concentration in a 100-ml flask. This culture was incubated overnight at 37°C with shaking. In a 2-liter flask, 500 ml of LB with the appropriate concentration of ampicillin was inoculated with 5 ml of the overnight culture. After 4 h of incubation at 37°C with vigorous shaking (250 rpm), the optical density at 600 nm of the resulting culture was 0.3 to 0.5. For the induction of gene expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0 to 0.4 mM, with induction temperatures from 28 to 37°C for 0 to 10 additional hours. The cells were collected by centrifugation (Beckman JA2-21 centrifuge; 7,000 × *g* at 4°C for 10 min). The pellets were then stored at −20°C until activity was measured. For determination of the dry mass of cells, aliquots (15 ml) of cells were taken, centrifuged, and dried at 60°C for 24 h. The dried cells were then weighed until the weight became stable.

With a view to verifying the active fraction of the recombinant cells, 2-ml aliquots were previously disrupted in ice by sonication using a UP 200 S ultrasonic processor (Dr. Hielscher GmbH, Germany) for 6 periods of 30 s at pulse mode 0.5 and with sonic power at 60%. The supernatant was obtained after centrifugation (Beckman JA2-21 centrifuge; 10,000 × *g* at 4°C for 20 min) and separated from the pellet, which was then resupended in the same buffer. The molecular mass of the monomeric form of each enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by using the Laemmli method (11) with a low-molecular-weight marker kit (Amersham Biosciences, Barcelona, Spain).

Whole-cell transformation assay. The cells were suspended to a concentration of 1 g/ml in 100 mM borate-HCl buffer (pH 8). For the reaction, 300 μl of the cells was added to 600 μl of prewarmed (55°C) D,L-MTEH solution (end concentration of 15 mM) and supplemented with 300 μl of 100 mM borate-HCl buffer (pH 8). The reaction mixture was incubated at 55°C, aliquots were taken during the reaction, and the reaction was stopped by the addition of four times the reaction mixture volume of 1% H_3PO_4. Each reaction mixture was made in triplicate. After centrifuging, the resulting supernatants were analyzed by high-performance liquid chromatography (HPLC). The HPLC system (Breeze HPLC

system; Waters, Barcelona, Spain) equipped with a Luna C_{18} column (4.6 by 250 mm; Phenomenex, Madrid, Spain) was used to determine the concentrations of D,L-5-monosubstituted hydantoins, *N*-carbamoyl-D-amino acids, and D-amino acids. The mobile phase used in the analysis (20 mM phosphoric acid, pH 3.2 [85%], and methanol [15%]) was pumped at a flow rate of 0.75 ml min^{-1}. The UV detector was fixed at 210 nm, and D,L-MTEH, *N*-carbamoyl-D-methionine, and D-methionine were detected and quantified.

For larger-scale reactions, the necessary cells were obtained as described above but using 12 2-liter flasks containing 500 ml of LB supplemented with ampicillin. *E. coli* BL21(pSER42) strains were both induced with 0.1 mM IPTG and left uninduced at 34°C for 8 h. Reactions were conducted in 1-liter Erlenmeyer flasks containing 300 ml of working volume. The reaction mixture volume contained 300 mM D,L-MTEH (52.3 g/liter) and 0.25 g of cells/ml in 1 M sodium phosphate buffer, pH 8. The flasks were incubated with shaking (150 rpm) in a water bath (Memmert WB 22) at 55°C. All reactions were carried out in triplicate and monitored by HPLC analysis of the aliquots as described above.

RESULTS AND DISCUSSION

Cloning and expression of the multienzymatic systems in *E. coli*. Two different multienzymatic systems were developed for D-amino acid production from D,L-5-monosubstituted hydantoins. System 1 was constructed with the D-hydantoinase and D-carbamoylase genes from *A. tumefaciens* BQL9 (5) and the hydantoin racemase 1 gene from *A. tumefaciens* C58 ($hyuA1_{At}$) (12) in the pBSK plasmid. System 2 was constructed from system 1 by replacing $hyuA1_{At}$ with $hyuA2_{At}$. Both constructions have a polycistronic structure of three genes preceded by the *lac* promoter for their expression (Fig. S1 in the supplemental material). This strategy reduces the high selective pressure on the growth of the recombinant cells compared with the method of coexpression of the three genes after cloning in three plasmids with different antibiotic resistance genes. The growth of the recombinant cells developed in this work is both faster and cheaper, since only one antibiotic is added to the medium.

Previous works have suggested that the D-carbamoylase enzyme step limits the overall process and consequently causes high accumulation of the intermediate (*N*-carbamoyl-D-amino acid) in the reaction (14, 19). For this reason, the D-carbamoylase gene was cloned closest to the promoter in order to obtain the highest synthesis of D-carbamoylase among the three enzymes. This strategy was made possible by adding a stop codon upstream from the ribosome-binding site sequence and the beginning of the D-carbamoylase gene in the forward primer, thus avoiding a fusion protein with this gene product and the N-terminal end of the β-galactosidase expressed in pBSK.

The coexpression of the three genes in both systems using standard induction conditions (0.2 mM IPTG at 37°C for 4 h) allowed the total hydrolysis of D,L-MTEH to the corresponding D-amino acid methionine, although system 1 was faster. The expression levels of the genes in both systems were also evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. S2 in the supplemental material), with larger amounts of the D-carbamoylase enzyme in both systems and in the soluble fraction.

Optimal host cell strain and induction conditions for D-amino acid production. In order to obtain the highest quantity of optically pure D-amino acid in the shortest possible time, the induction conditions for the polycistronic structures were optimized and several *E. coli* strains were analyzed as hosts to the recombinant plasmids. The induction studies comprised experiments with different concentrations of the inductor (IPTG), ranging from 0 to 0.4 mM, and with the induction temperatures increasing from 28 to 37°C and the induction times increasing from 0 to 10 additional hours. These studies were made for both systems in *E. coli* strain JM109 [JM109(pSER42) and JM109(pAMG3)]. Optimal conditions for induction were determined by identifying the conditions corresponding to the fastest conversion of D,L-MTEH to D-methionine (Table S1 in the supplemental material). Both constructions showed the highest activity after 8 h of induction in the culture. Likewise, both systems produced 100% D-methionine in the shortest time at an induction temperature of 34°C. However, the optimal inductor concentrations for D-methionine production were different, 0.1 mM IPTG for system 1 and 0.2 mM for system 2. These results provided the optimal induction conditions for the subsequent experiments. Four *E. coli* strains (DH5α, JM109, TOP10F, and BL21) were studied as hosts of the recombinant systems for D-methionine production, and for both systems *E. coli* strain BL21 proved to be the best (Table S1 in the supplemental material). Stability studies of both plasmids in this strain, as described in Materials and Methods, demonstrated that the recombinant cells had grown for at least 90 generations without ampicillin pressure after the initial inoculation.

Optimization of the reaction conditions for D-amino acid production. Temperature and pH dependency are known to affect the kinetic properties of the conversion of D,L-5-monosubstituted hydantoins into the corresponding D-amino acids. The activities of the enzymes belonging to the whole-cell system are strongly influenced by the pH and the temperature of the reaction mixture, considerably influencing the amount of D-amino acid produced. For this reason, both parameters were studied with both multienzymatic systems. Previous studies have described different optimal pHs for D-hydantoinase and D-carbamoylase enzymes (4, 10, 19). The optimal pH described for both hydantoin racemases is 7.5 (12, 15). To determine the optimal pH for each whole-cell reaction, D-methionine conversion over a range of pH 6 to 10.5 was studied. Three different buffers were used, 200 mM potassium phosphate buffer from pH 6.5 to 8, 100 mM borate-HCl buffer from pH 8 to 9, and 100 mM borate-NaOH buffer at pH 9.5 and 10.5. Both whole-cell systems showed the fastest conversion at pH 8 (Fig. 1). This is an intermediate value, considering that the optimum for D-hydantoinase ranges from pH 8.8 to 9.7 (5), that for D-carbamoylase is pH 7, and that for both hydantoin racemases is pH 7.5 (11, 14). Likewise, pH 8 was found to be optimal for a previous whole-cell biocatalyst containing separately expressed D-hydantoinase, D-carbamoylase, and hydantoin racemase developed in our laboratory (15) and for a recent system coexpressing the three enzymes in *E. coli*, in which the optimum pH ranged from 7.5 to 8 (18).

The optimum temperature for both whole-cell systems was around 55°C, at which the systems reached 100% conversion from D,L-MTEH to D-methionine in 100 min at pH 8 (Fig. 2). The conversion percentages increased as temperatures increased up to 55°C, but at higher temperatures the percentage of D-methionine conversion decreased sharply. Similar results have been reported for recombinant systems with D-hydantoinase and D-carbamoylase enzymes whose thermostability was analyzed (4, 19) and for the system also including the hydantoin racemase enzyme expressed separately (16).

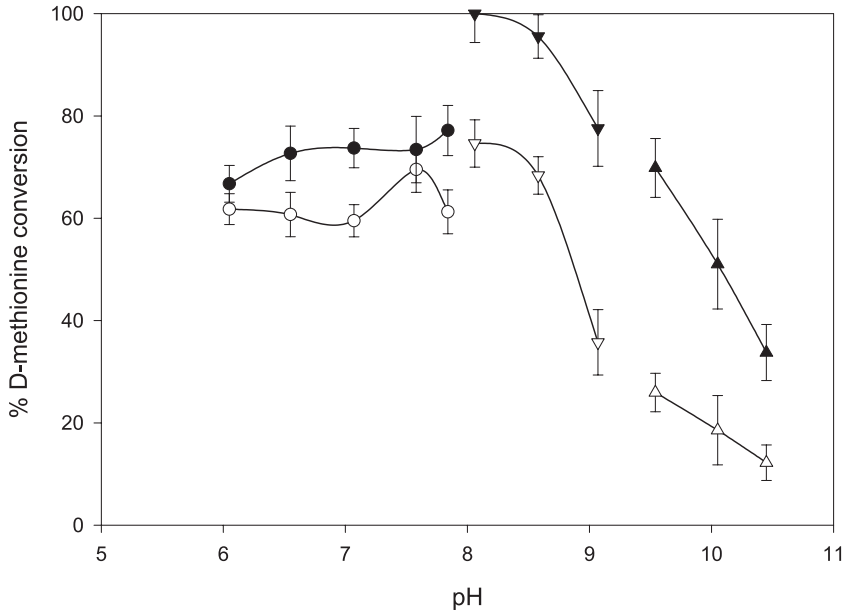

FIG. 1. Effect of pH on systems 1 (black) and 2 (white) to produce D-methionine from D,L-MTEH. The buffers used were 200 mM potassium phosphate buffer from pH 6.5 to 8 (circles), 100 mM borate-HCl buffer from pH 8 to 9 (inverted triangles), and 100 mM borate-NaOH buffer at pH 9.5 and 10.5 (right-side-up triangles). Reactions were performed in triplicate, and error bars represent the standard errors of the means.

Substrate specificity of the multienzymatic systems for D-amino acid production. Whole-cell systems coexpressing the three enzymes for optically pure D-amino acid production were evaluated using different racemic mixtures of hydantoins. The polycistronic construction in one plasmid avoids the problem of high selective pressure due to double antibiotic selection on the growth of recombinant cells. This leads to a combination of higher cell density and higher recombinant enzyme levels, which enhance the potential of the biocatalyst. A reaction profile for D-methionine production from D,L-MTEH for both systems is shown in Fig. 3. System 1, containing $HyuA1_{At}$ together with D-hydantoinase and D-carbamoylase enzymes, was able to hydrolyze all the substrate and produce total conversion to obtain optically pure D-methionine in about 100 min (Fig. 3A). System 2 was slower, taking about 200 min to produce 100% conversion of the substrate to the product (Fig.

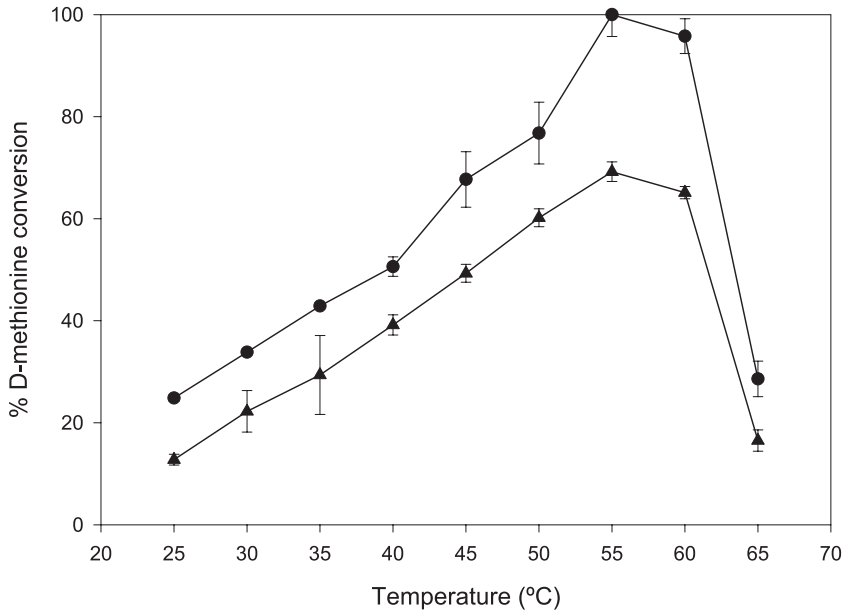

FIG. 2. Effect of temperature on systems 1 (●) and 2 (▲) to produce D-methionine from D,L-MTEH. Activity was measured at different temperatures by using 0.25 g of recombinant cells/ml in 100 mM borate-HCl buffer (pH 8) and 15 mM D,L-MTEH. Reactions were performed in triplicate, and error bars represent the standard errors of the means.

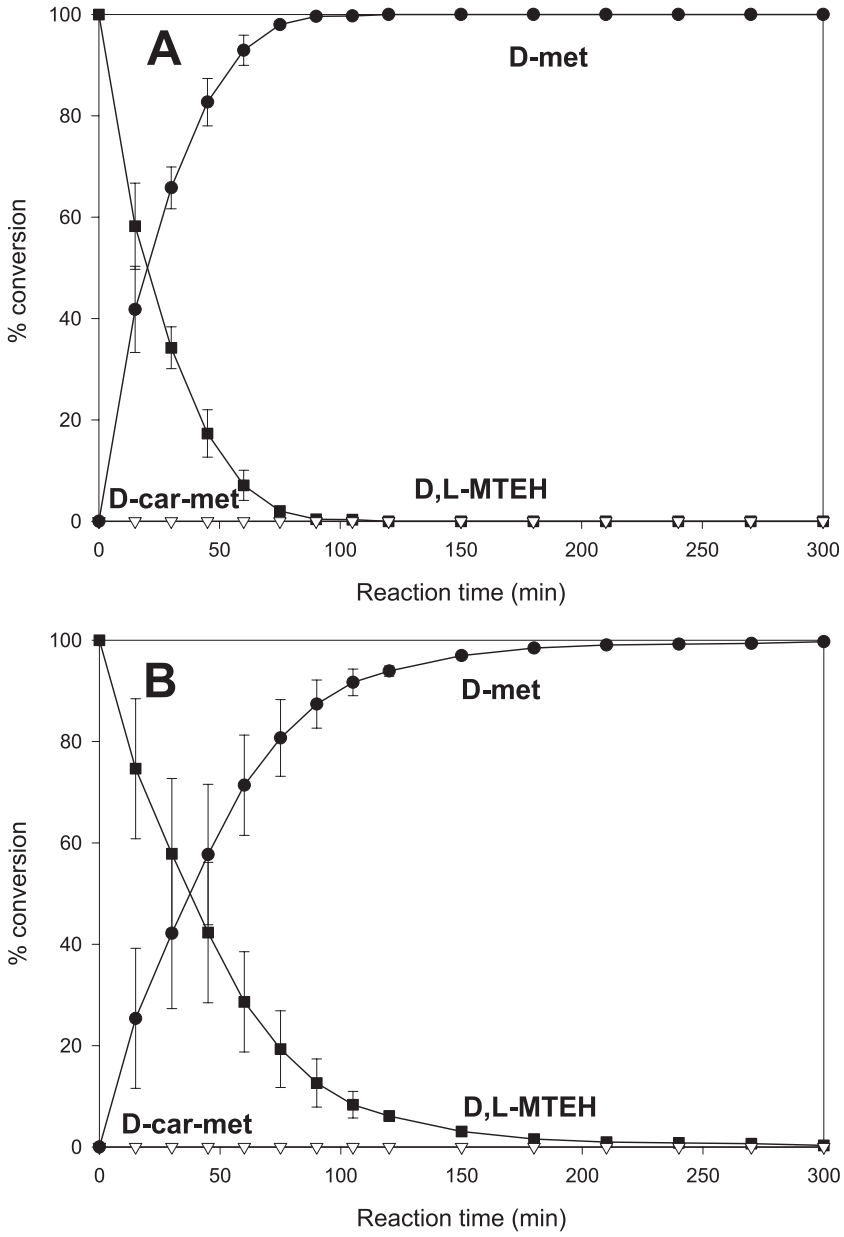

FIG. 3. Comparison of reaction profiles of D-methionine (D-met) production from 15 mM D,L-MTEH without D-carbamoyl methionine (D-car-met) accumulation by using pSER42 (A) and pAMG3 (B) in *E. coli* strain BL21. Reactions and measurements were carried out in triplicate as described in Materials and Methods.

3B). Other hydantoins, with both aliphatic and aromatic substituents in C-5, were hydrolyzed to the corresponding optically pure D-amino acid (Fig. 4). All the substrates studied in this work, whether with slow or fast chemical racemization (20), have been converted by both systems to the corresponding D-amino acids without the accumulation of the intermediate *N*-carbamoyl-D-amino acid. This is the first time that this bottleneck has been overcome. There is no substrate inhibition due to the higher overexpression of D-carbamoylase than of the other two enzymes involved in this biotransformation.

Both systems showed higher reaction rates for the conversion of aliphatic amino acids than for aromatic ones. These results are similar to those obtained recently in a three-gene coexpression system for D-amino acid production in which the aliphatic D-amino acids were produced more efficiently than the aromatic ones (18). The highest initial reaction rate has been for D-leucine production, with 1.70 and 1.01 U/mg of cells (dry weight) for systems 1 and 2, respectively (Fig. 4). The substrates with aromatic substituents were hydrolyzed more slowly, with system 1 reaction rates of about 0.07 and 0.13 U/mg of cells (dry weight) for tryptophan and tyrosine, respectively. The rate of D-valine production was very low, similar to that of the production of aromatic D-amino acids. However, this was not due to an accumulation of the intermediate, unlike in previous studies in which the low production of this D-amino acid was due to D-carbamoylase substrate specificity (18). For

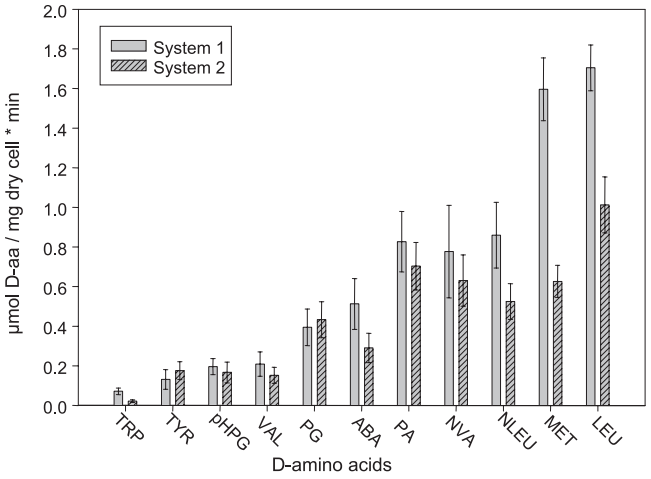

FIG. 4. Initial reaction rates for the production of different optically pure D-amino acids from 5-monosubstituted hydantoins using systems 1 and 2. The reactions were performed as described in Materials and Methods at pH 8 and 55°C. TRP, D-tryptophan; TYR, D-tyrosine; pHPG, D-*p*-hydroxyphenyl glycine; VAL, D-valine; PG, D-phenylglycine; ABA, D-aminobutyric acid; PA, D-phenylalanine; NVA, D-norvaline; NLEU, D-norleucine; MET, D-methionine; LEU, D-leucine; D-aa, D-amino acid. Reactions were performed in triplicate, and error bars represent the standard errors of the means.

the systems used in our work, the low D-valine production may depend chiefly on the substrate specificity of the D-hydantoinase enzyme. Surprisingly, the isopropyl group in the hydantoin, the substrate of D-valine, has the same behavior as more voluminous substituents such as the aromatic ones, while propyl (D-norvaline), butyl (D-norleucine), and isobutyl (D-leucine) groups in the hydantoins have been clearly hydrolyzed.

For the production of almost all D-amino acids studied in this work, system 1 was able to hydrolyze the 5-monosubstituted hydantoins faster than system 2. System 1 was slightly slower than system 2 only in the production of the aromatic amino acids D-tyrosine and D-phenylglycine. This finding is in agreement with previously described results showing that the $HyuA1_{At}$ enzyme (included in system 1) is more viable for industrial application than $HyuA2_{At}$ (included in system 2) due to its higher substrate affinity and racemization velocity (15).

The behavior of the recombinant biocatalyst on a larger scale is of considerable economic interest. For this reason, we have analyzed D-methionine production from 300 mM D,L-MTEH (52.3 g/liter) in a reaction mixture volume 300 times that used at the laboratory level (300 ml). When system 1 was induced under optimal conditions, the yield of D-methionine reached 100% in 6 h at an initial reaction rate similar to that obtained at the laboratory level (1.59 U/mg of cells [dry weight]), and there was no D-carbamoyl-methionine accumulation (Fig. 5A). However, when IPTG was not added to the mixture, this system showed less activity than the induced system (0.56 U/mg of cells [dry weight]), giving total conversion at approximately 32 h (Fig. 5B).

The development of this multienzymatic system for the production of D-amino acids from any D,L-5-monosubstituted hydantoin allows the hydantoinase process to produce not only two amino acids, such as D-phenylglycine and D-*p*-hydroxyphenylglycine (this paper; 4, 19), but also many unnatural

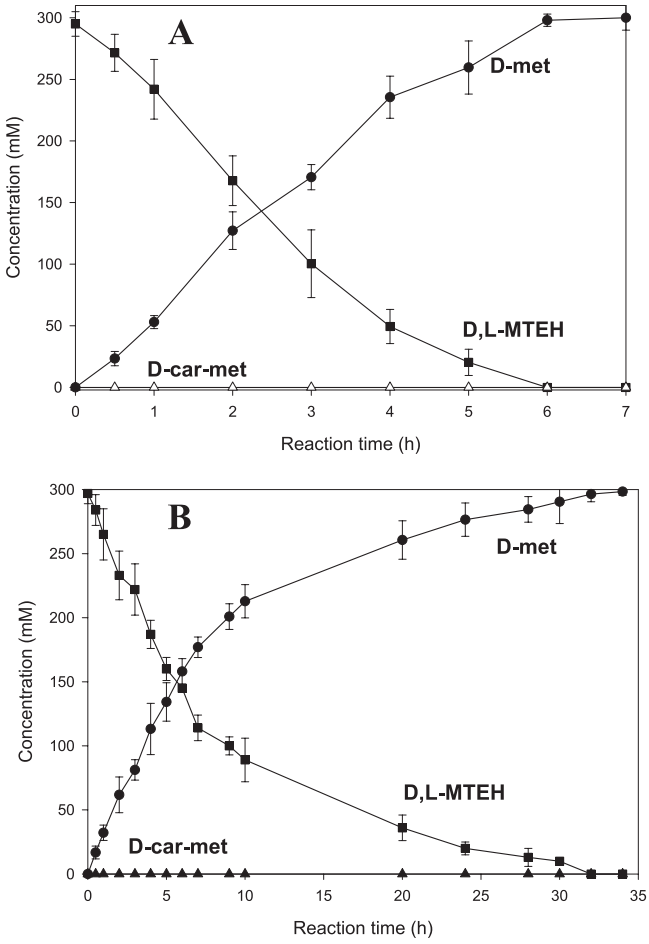

FIG. 5. Profile of D-methionine (D-met) production from 300 mM D,L-MTEH without D-carbamoyl methionine (D-car-met) accumulation by using pSER42 in *E. coli* strain BL21 induced with 0.1 mM IPTG (A) and uninduced (B) in a large-scale reaction with an end volume of 300 ml. Reactions and measurements were carried out in triplicate as described in Materials and Methods. Error bars represent the standard errors of the means.

D-amino acids that could be components of potential pharmaceuticals.

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