Optically Pure α-Amino Acids Production by the "Hydantoinase Process"

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Abstract: Optically pure D- or L-amino acids are used as intermediates in several industries. D-amino acids are involved in the synthesis of antibiotics, pesticides, sweeteners and other biologically active peptides. L-amino acids are used as feed and food additives, as intermediates for pharmaceuticals, cosmetics, pesticides and as chiral synthons in organic synthesis. The specific activity of these optically pure amino acids depends on their structure, chirality and purity. There are two main approaches to obtain optically pure amino acids, namely chemical and enzymatic synthesis. Chemical synthesis gives racemic mixtures of amino acids of low yield and is not environment friendly. One of the most widely-used enzymatic method is the "Hydantoinase Process". In this cascade of reactions, the chemically synthesized D,L-5-monosubstituted hydantoin ring is first hydrolyzed by a stereoselective hydantoinase enzyme to give the corresponding *N*-carbamoyl α-amino acid that is hydrolyzed by highly enantiospecific *N*-carbamoyl α-amino acid amidohydrolase (*N*-carbamoylase) to yield the free amino acid. At the same time, the remaining non-hydrolyzed 5-monosubstituted hydantoin is racemized by the hydantion racemase enzyme. This process has evolved over the years from the isolation of microorganisms with one or several of these enzymes to the construction of recombinant systems for industrial application.

Keywords: D- and L-amino acids, "Hydantoinase Process", hydantoinase, D- and L-*N*-carbamoyl-amidohydolase, hydantoin racemase, polycistronic messenger, fermentation, recombinant system.

INTRODUCTION

In recent years, pharmaceutical and fine chemicals companies have focused on the production of molecules that act as building blocks for pharmaceuticals, agrochemicals, active ingredients, antibiotics, hormones or enzyme receptors and food ingredients such as sweeteners and vitamins. For these compounds chirality plays a crucial role in the desired interactions. D- and L-amino acids are important intermediates in the manufacture of these products, and in many cases the production of these precursors has been patented, but only by a few companies. The methods of production have evolved along with the technology, from chemical synthesis at first to the current application of enzyme engineering.

L-Amino acids are used as feed and food additives, as intermediates for pharmaceuticals, cosmetics, pesticides and as chiral synthons in organic synthesis. Of the 20 standard protein amino acids, the 9 essential ones (L-valine, L-leucine, L-sioleucine, L-lysine, L-theronine, L-methionine, L-histidine, L-phenylalanine, and L-tryptophan) occupy a key position in that they are not synthesized in animals and humans, but must be ingested with feed or food [1]. Among the many examples of the use of these L-amino acids in the food industry, L-lysine has been used as a supplement of wheat-based food, enhancing protein quality and resulting in improved growth and tissue synthesis [2]; L-aspartic acid and L-phenylalanine are starting materials for the peptide sweetener L-aspartyl L-phenylalanyl methyl ester (Aspartame). Non-natural L-amino acids are able to imitate the structure of natural amino acids in a type of molecular mimicry and thereby modulate the natural reaction. They are therefore used as constituents of many drugs as protease inhibitors for preventing HIV infection, peptide ligands for the fibrinogen receptor, effective tachykinin antagonists, and neuronal receptor ligands [3]. Other non-natural L-amino acids are widely used as pharmaceutical intermediates, such as the anti-hypertensives ramipril, enalapril, benazapril, and prinivil, all based on L-homophenylalanine [4].

On the other hand, D-amino acids are usually called non-natural amino acids and they are not used as frequently as L-amino acids. However, they are becoming increasingly important, especially in the field of fine chemicals. These compounds are valuable intermediates for the preparation of semi-synthetic antibiotics, pesticides, and other products of interest for the pharmaceutical, food and agrochemical industries. To name only a few of the most valuable intermediates, D-(phenyl or substituted phenyl)glycines are available intermediates of antibiotics such as semi-synthetic penicillins and cephalosporins; D-valine is used for the synthesis of the insecticide fluvanilate; D-alanine for the production of the dietetic sweetening agent Alitam®; D-citrulline and D-homo-citrulline are used in the potent LH-RH antagonist [5].

Four methods have been described for the production of amino acids: protein hydrolysis and fractional extraction, chemical synthesis, fermentation, and enzymatic catalysis. Extraction of L-amino acids from protein hydrolysate is limited to obtaining some, but not all, natural amino acids. In the chemical synthesis of amino acids, unless asymmetrical starting compounds are used, a mixture of the D and L stereoisomers is obtained in equal proportions. The racemic mixture is therefore optically inactive and the stereo-isomers must be separated. The separation of the enantiomers by classical disteroisomeric crystallization of salts is the most costly production step, and in any case this method can only yield 50% of the desired enantiomer [6]. In addition, the reactions require drastic conditions, such as high temperatures [7].

The chemo-enzymatic process or entire biocatalytic synthesis solves these problems, providing purity of the optical compound and 100% yield, as well as the production of both natural and non-natural compounds in environment friendly and mild reaction conditions. It can be used in both simple and complex transformations without the need for the tedious blocking and deblocking steps that are common in enantioselective organic synthesis. The first step is to search for microorganisms which could enzymatically produce amino acids and the screening method itself is often patented. Of the bacteria, molds, yeasts, actinomycetes and imperfect fungi which pass the screenings, bacteria provide the best results. Suitable strains have been isolated from soil, vegetal material, sea water and from public collections, thanks to their ability to grow on D,L-5-monosubstituted hydantoins supplied as the sole carbon and/or nitrogen source [8]. In the beginning, whole-cell systems of microorganisms and tissue extracts were used to transform the substrates, and it was believed that only one enzyme was involved in the "Hydantoinase Process". The first indication that the "Hydantoinase Process" was a multi-enzymatic system appeared in 1978, when one of the intermediaries in the hydrolysis

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from hydantoin to amino acids was the object of the patent US4094741 [9]. Only the D-isomer of the carbamoyl amino acid was produced, with the subsequent chemical transformation to optically pure D-amino acid. Researchers of Ajinomoto Co. Inc. tried to immobilize the "enzyme" produced by microorganisms and they noted that there was more than one enzyme participating in the conversion [10]. These two facts indicated that several steps were involved in the reaction.

The "Hydantoinase Process" was elucidated and described as a multi-enzymatic pathway composed of three enzymes [11]. In this cascade of reactions, the chemically synthesized D,L-5-monosubstituted hydantoin ring is first hydrolyzed by a stereoselective hydantoinase enzyme. Further hydrolysis of the resulting enantiospecific *N*-carbamoyl α-amino acid to the corresponding free D- or L-amino acid is catalyzed by highly enantiospecific *N*-carbamoyl α-amino acid amidohydrolase (*N*-carbamoylase). At the same time as the hydantoinase hydrolyzes the enantiospecific 5-monosubstituted hydantoin in the first step, the remaining non-hydrolyzed 5-monosubstituted hydantoin is racemized by the hydantoin racemase enzyme. To date the biological function of these enzymes and their natural substrates remain unclear, although some D-hydantoinases have shown dihydropyrimidinase activity [12], the enzyme involved in the reductive catabolism of pyrimidines [13]. The microorganisms probably use this pathway to obtain carbon or nitrogen form substances similar to hydantoins which are present in vegetal material in decomposition from secondary metabolism [14]. This pathway may be activated only when the easily assimilable sources are used up, and the microorganisms with this capacity produce carbon and nitrogen which is assimilable by the rest of the population present in the environment. It is not easy to find microorganisms that produce D-amino acids, and the system must be induced because the enzymes are produced only in extreme circumstances.

MICROORGANISMS

The first articles on the enzymatic hydrolysis of hydantoins were by Gaebler and Keltch in 1926 and by Sobotka *et al.* in 1932 [15,16], reporting the enzymatic hydrolysis of 5-phenyl-5-ethylhydantoin by *Aspergillus* sp. In the 1960's, the Ajinomoto Co. Inc. (Tokyo, Japan) was the first company to patent the production of amino acids from hydantoinpropionic acid by biochemical hydrolysis. They used living cells corresponding to several microorganism strains such as *Bacillus*, *Pseudomonas*, *Micrococcus*, *Aerobacter*, *Escherichia* and *Achromobacter* to produce L-glutamic acid. This invention was first patented in Japan in 1963, and subsequently in the United Kingdom and the United States [17].

Degen *et al.* from Snamprogetti S.p.A. [18] described the test for the selection of microorganisms which use hydantoin as nitrogen source. They isolated bacterial strains from soil, plants, debris of various kinds, etc., as well as strains from bacterial collections. All of them were inoculated in culture media with meat peptone (10 gr/l), yeast extract (10 gr/l), glucose (5 gr/l), NaCl (3 gr/l) and D,L-5-methylhydantoin (1 gr/l). After 20 - 24 hours of incubation, 5 ml of the culture was inoculated to fresh medium and cultured for an additional 18 to 22 hours. An enzymatic reaction was carried out using D,L-5-phenylhydantoin as substrate, and the quantity of carbamoyl was determined. Ajinomoto Co. Inc. [10] described a method to isolate microorganisms that produce the D-isomer. The microorganisms were selected because they grew with the D-isomer of the 5-substituted-hydantoin as the sole nitrogen source, but could not grow significantly in environments which utilize the L-isomer. The medium contained glucose, yeast extract, KH_2PO_4, K_2HPO_4, $MgSO_4*7H_2O$, $FeSO_4*H_2O$, $MnSO_4*4H_2O$ and D or L-5-substituted hydantoins. A low pH was maintained to prevent racemization of the D- or L-isomer of the hydantoins. These authors were the first to demonstrate that enzyme production was higher when the culture medium contained a small amount of inducer, such as 5-substituted hydantoins [10], D,L-5-methylhydantoin, D,L-5-(2-methylthiothyl)hydantoin [19], uracil, cytocine and tymine, and their derivatives such as dihydrouracil and dihydrotymine [20]. The amount of inducer added to the culture medium is 0.05% - 0.3% by weight, because it has been demonstrated that higher concentrations inhibit the microorganisms' growth. This induction/inhibition mechanism is a handicap for the industrial scale-up of the wild system.

Arthrobacter aurescens is one of the few isolated microorganisms capable of converting 5-monosubstituted hydantoins to L-amino acids. In 1992, the Rütgerswerke Company patented the method for microorganism isolation [21]. They prepared 50 ml of a synthetic nutrient medium with 3-methyleneindolyl-5-hydantoin (1 gr/l) as the single carbon source of the composition, mixed with 0.2 - 0.6 g of soil or water samples from various sites and incubated at 27 ºC for 3 to 5 days. 1 ml of each culture was transferred into fresh nutrient solution and incubated again. After 10 cultivation cycles, each culture was spread on agar plates on a complex medium. The individual colonies were then transferred into pure cultures using conventional procedures and the enzymatic activity was verified. The cell lines obtained were filed with the Deutsche Sammlung Für Mikroorganismen (German Collection of Microorganisms and Cell Cultures GmbH, DSM) as DSM 3745, 3746 and 3747. Four years later, the Degussa Company described a novel invention exemplified by DSM 7329 and DSM 7330, two new specimens of *Arthrobacter* species, detailing a method of cultivating mutants or variants of microorganisms. These microorganisms were isolated in synthetic medium containing D,L-5-methyl thioethyl hydantoin as the sole source of nitrogen with the same method as in the US5516660 patent [22]. In 1998, Degussa mutated *Arthrobacter aurescens* DSM 7330 to obtain DSM 9771, which exhibits substantial advantages such as higher specific activity and accelerated cell growth due to the absence of inducer to produce the enzymes [23]. This strain is able to produce 99.8% enantiomerically pure L-methionine and was the source of hydantoinase, hydantoin racemase and carbamoylase genes for the subsequent patents of this company.

At first only the L-form was used industrially, but due to the broad applications of D-amino acids in the pharmaceutical industry as intermediates for the preparation of antibiotics and the difficulty to obtain them from natural sources companies started to isolate microorganisms for their production. Two strategies have been employed: developing screening methods to isolate new microorganisms, or testing the capability of the strains deposited in the public or private culture collections. Snamprogetti Co. isolated 17 colonies of *Pseudomonas* sp. and 2 of *Pseudomonas fluorescens*, comparing their activity with 5 *Pseudomonas* strains from different public collections. Although all of the isolated strains were capable of using hydantoin as single nitrogen source, only two, 942 and 945, gave an acceptable yield of conversion [18]. Thus, these authors showed that an activity assay is the only reliable method to detect the enzymes' presence, not the microorganism's capacity to survive using hydantoin as the only source of nitrogen or carbon.

Some efforts have been made to isolate thermophile microorganisms able to live at high temperature. These microorganisms produce heat-resistant enzymes that improve the hydrolytic process, as it can be conducted at comparatively high temperatures (40 - 60ºC) and allows the use of high concentrations of hydantoin which have poor solubility at room temperature. To isolate heat resistant microorganisms Snamprogetti inoculated samples from several sources such as soil, compost or vegetables at 50ºC in culture medium with meat peptone, yeast extract and NaCl and 5-D,L-methylhydantoin. After 10 - 20 h 2 ml were inoculated in fresh medium and grown for a further 18 - 20 h. Two strains were isolated and classified as *Bacillus stearothermophilus* 1286 (NRRL B-11079) and *Bacillus brevis* 1287 (NRRL B-11080) [24]. BASF Company used another method and source for isolating thermophile

Table 1. Microorganisms with High Capacity of Hydrolysis and Broad Substrate Spectra that have been Described, Used or Isolated in Patents Published in the United States Patent and Trademark Office

Microorganisms involved in L-amino acids production		
Patent	**Microorganism**	**Company**
US4016037	*Flavobacterium aminogenes* AJ3912 (FERM-P3133)= *Microbacterium liquefaciens* AJ3912 (FERM-P3133)	Ajinomoto Co. In. [25]
US5108914	*Arthrobacter aurescens* DSM 3745, 3746 and 3747	Rütgerswerke AG [21]
US5516660	*Arthrobacter aurescens* DSM 7329, 7330	Degussa AG [22]
Microorganisms involved in D-amino acids production		
Patent	**Microorganism**	**Company**
US4111749	*Pseudomonas* sp. 942 (CBS154.75), 945 (CBS146.75)	Snamprogetti, S.p.A. [18]
US4248967	*Bacillus stearothermophilus* 1286 (NRRL B-11079) *Bacillus brevis* 1287 (NRRL B-11080)	Snamprogetti, S.p.A.[24]
US4312948	*Agrobacterium radiobacter* 1302 (NRRL B-11291)	Snamprogetti, S.p.A. [26]
US4912044	*Baciliaceae* family CBS 303.80, 363.80	BASF AG [27]
US5068187	*Hansenula ciferri* *Hansenula henricii* *Hansenula nonfermentans* *Hansenula polymorpha*	Mitsui Toatsu Chemicals Inc [28]
US5283182	*Bacillus brevis* IFO 12333	Beecham Group PLC [29]
US5523224	*Bacillus thermoglucosidasius*	Boehringer Mannheim GmbH [30]
US5565344	*Agrobacterium radiobacter* KNK 712 (FERM BP-1900) *Pseudomonas* sp. KNK 003A (FERM BP-3181)	Kanegafuchi Kagaku Kogyo Kabushiki Kaisha [31]
US5858759	*Agrobacterium* 80/44-2A	SmithKline Becham p.l.c. [32]
US5863785	*Comamonas* sp E 222 *Blastobacter* sp. A17 p-4	Kanegafuchi Chemical Industry Co. Ltd. [33]
US6087136	*Pseudomonas* sp. NCIM 5070 (ATCC 55940)	National Chemical Laboratory [34]
US6121024	*Pseudomonas* sp. M1 (NCIM 5109)	National Chemical Laboratory [35]
US6403357	*Bacillus circulans*	National Science Council [36]
US6800464	*Arthrobacter crystallopoietes* DSM 20117	Degussa AG [37]
US7060485	*Flavobacterium* sp AJ11199 (FERM-P4229 or BP-8063)	Ajinomoto Co. Inc. [38]
US7098019	*Pasteurella pneumotropica* AJ11221 (FERM-P4348 or BP8064)	Ajinomoto Co. Inc. [39]

strains. They filtered water from hot springs in Iceland and Yellowstone Park (USA) through a membrane. The filters were then placed on a nutrient agar and incubated at 60ºC. The colonies which grew on the membrane filter were transfered to a pure culture. The test was made with methylthioethylhydantoin at 60 ºC for 24 hours. Two strains, both belonging to the *Bacillaceae* family, were selected and deposited in Centraal Bureau Uuor Schinmmelcultures (CBS) under numbers 303.80 and 363.80 [27] Table **1**. The isolation of thermophile microorganisms has been the objective of several companies, and a few microorganisms of the same family have been isolated: *Bacillus thermoglucosidasius* [30], *Bacillus brevis* IFO 12333 [29] or *Bacillus circulans* [36]. These strains have the handicap of low cellular density and protein production, but they are excellent gene donors for current recombinant systems due to the heat resistance of their enzymes. The highest cellular density in a culture is obtained by yeast strains. There are not many examples of eukaryotic microorganisms which are able to hydrolyze hydantoins. Only the Mitsui Toatsu Chemical company described the isolation of several yeasts, *Hansenula ciferri*, *Hansenula nonfermentans* and *Hansenula polymorpha* in US Patent US5068187 [28]. The advantage is that the higher cellular density of the culture offsets the low expression of the "Hydantoinase Process" enzymes in wild type strains.

EVOLUTION OF THE ART

Ajinomoto Co. Inc. and Kyowa Hakko Kogyo Co. were the first to patent the use of whole cells of microorganisms or crude extract to obtain optically pure D- or L- amino acids based on the

"Hydantoinase Process". They related the process for the production of L-glutamic acid and L-lysine using whole-cell systems of microorganisms [2,17,40]. These first systems were only able to produce one product. The pharmaceutical demand for non-natural D- or L-amino acids with aliphatic and aromatic substituents led the companies to develop systems which were able to hydrolyze a wide range of substrates. The Kanegafuchi Kagaku Kogyo Kabushiki Kaisha company from Japan described a process for obtaining D-*N*-carbamoyl-2(phenyl or substituted phenyl)glycines by subjecting 5-(phenyl or substituted phenyl)hydantoins to the action of a culture broth of microorganisms [9]. This initial process of fermentation has several drawbacks: sometimes the micro-organisms cannot supply the amount of enzyme required for production; the yield may decrease due to the microorganisms' use of the product; when two or more enzymes are involved in the transformation the permeability of the cell membrane for the substrates determines the reaction velocity; and the organism may exhibit undesirable enzyme activity in certain circumstances. The expression levels of the enzymes involved in the hydrolysis can be enhanced using an efficient inducer. However, using an inducer leads to an increase in production costs, variability in enzyme yields and a decrease in microorganism growth. These drawbacks for scaling up the system for industrial purposes meant that companies needed to develop new ways, namely modification by mutagenesis using chemical or physical methods which give a significantly higher yield, and over-expression of the genes responsible for the enzymes involved in the process [41]. Both methods have been used for the production of optically pure amino acids, although the latter has proved more successful.

In 1980, the Ajinomoto Company tried to purify and immobilize the "enzyme" that hydrolyzes hydantoins to optically pure amino acids and they noted that more than one enzyme was involved. Thereafter, there were several patent applications informing of the production of D- or L-*N*-carbamoyl-α-amino acids [9, 19, 20] which indicated that the reaction takes place in two steps. In 1982 Olivieri *et al*. described how D-amino acids are produced from the corresponding hydantoins catalyzed by enzymatic complexes as shown in Fig. (**1**). In the initial hydantoin molecule, R could be an aliphatic or aromatic radical, either substituted or unsubstituted [26].

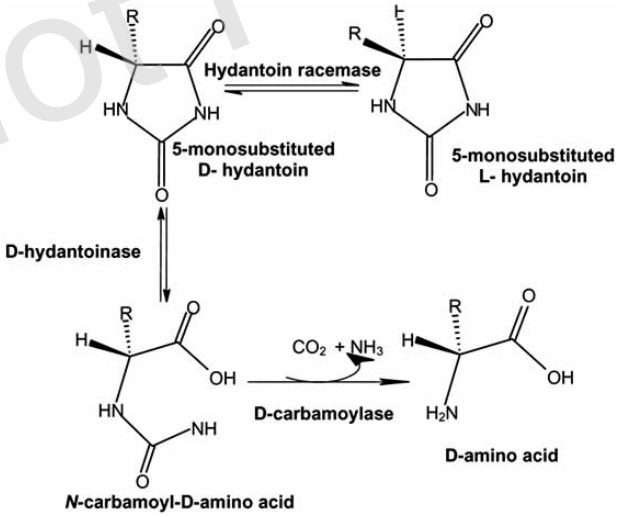

Fig. (1). "Hydantoinase process". Optically pure D-amino acid production by hydrolysis of D,L-5-monosubstituted hydantoins.

In 1998, Grifantini *et al.* [42] isolated both hydantoinase and carbamoylase genes from *Agrobacterium tumefaciens*, and inserted them into an expression plasmid in such a way that a polycistronic expression cassette was created. The constitutive expression of both enzymes in *Escherichia coli* was more efficient than the natural expression in *A. tumefaciens*. The authors revealed the gene disposition in the wild strain in an operon structure. They harboured a plasmid with a 4000 pb insert from *Agrobacterium tumefaciens*, and the sequence analysis revealed an open read frame (ORF1) encoding a polypeptide of 304 amino acids (GenBank accession number X91070), preceded by a putative ribosome-binding site (RBS1) and an upstream sequence similar to the -10 and -35 regions of the E. coli σ^{70} promoter. The sequence showed 43% homology with the *Comamonas* carbamoylase. Further sequence analysis of the 4000 pb fragment allowed the identification of an additional ORF2, going backward from ORF1. This fragment was also preceded by a putative RBS2 and by a σ70-like promoter sequence. The ORF2 showed homology to enzymes belonging to the amidohydrolase family. These authors informed that the hydantoinase and carbamoylase genes are clustered, and that their expression might be regulated by a mechanism which utilizes overlapping promoters. Fig. (**2**).

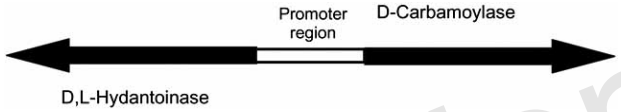

Fig. (2). Disposition of the genes of D,L-hydantoinase and D-carbamoylase in an operon system as described by Grifantini *et al.* in 1998 [42].

Using this model of two enzymes it was possible to hydrolyze a racemic mixture of hydantoins to optically pure D-amino acids, but the conversion velocity was too slow for industrial application. Chemical racemization of the remaining non-hydrolyzed enantiomer is strongly dependent on the bulkiness and electronic factors of the substituent in 5-position. Table **2** shows some examples of spontaneous racemization of hydantoins [43]. High racemization velocities are only observed for *p*-hydroxyphenylhydantoin, phenylhydantoin and hydroxymethylhydantoin. For the remaining compounds chemical racemization is only carried out under strong reaction conditions of alkaline pH and high temperature. Researchers found that some microorganisms were able to develop the conversion of the remaining enantiomer via enzymatic racemization due to the presence of a third enzyme named hydantoin racemase. Fig. (**1**). In this way 100% conversion was obtained, the velocity of hydrolysis was increased and accumulation of one of the isomers was avoided. Fig (**3**). The use of hydantoin racemase started with the discovery of the enzyme in *Arthrobacter aurescens* DSM 3747 in 2001 [14], although its presence had already been described in 1992 in *Pseudomonas* sp. NS671 [44]. This bacterium was able to hydrolyze the 5-monosubstituted hydantoins to their corresponding L-amino acids, and the genes involved in the process were encoded in the 172-kb native plasmid of *Pseudomonas* sp. NS671. One fragment of 12.77 kb which contained 5 ORFs from this natural plasmid was cloned and sequenced. The D,L-hydantoinase enzyme was encoded by both *hyu*A and *hyu*B genes, and downstream from these fragments three ORFs appeared, the first corresponding to the *N*-carbamoyl-L-amino acid amido hydrolase (*hyu*C), the second, of unknown function, named ORF5, and finally the hydantoin racemase gene (hyuE). A similar structure was shown for the genes present in *Arthrobacter aurescens* DSM 3747. The hydantoin racemase, hydantoinase and carbamoylase were all arranged in the same order as in *Pseudomonas* sp. NS671. Fig. (**4**). However, the genes involved in the "Hydantoinase process" do not always have the same organization. The location of the genes in *Agrobacterium* sp. IP I-671 and in *Arthrobacter aurescens* DSM 3747 were described simultaneosly. In 1998, Grifantini *et al*. [42] had reported the divergent orientation of the carbamoylase and hydantoinase genes in *Agrobacterium*, but in 2001 Hils *et al.* [45] were the first to describe the operon structure

Table 2. Racemization Rate Constants (k_{rac}) and the Corresponding Half-Life Times ($t_{1/2,rac}$) for Various Hydantoins. Data Obtained from Pietzsch and Syldatk (2002) [43]

5-Substituted Hydantoins	Corresponding Amino Acid	k_{rac} (h^{-1})	$t_{1/2,rac}$ (h)
p-Hydroxyphenylhydantoin	p-Hydroxy-phenylglycine	2.26	0.12
Phenylhydantoin	Phenylglycine	2.59	0.27
Hydroxymethylhydantoin	Serine	0.43	1.60
Phenylethylhydantoin	Homo-phenylalanine	0.144	4.8
Benzylhydantoin	Phenylalanine	0.14	5.00
Methylthioethylhydantoin	Methionine	0.12	5.82
Neopentylhydantoin	γ-Methyleucine	0.11	6.4
1-Hydroxyethylhydantoin	Alothreonine	0.067	6.41
Trimethylsilylmethylhydantoin	l-3-Trimethylsilyl-alanine	0.049	10.4
3´-Ureidopropylhydantoin	Citruline	0.044	14.26
1´-Methylethylhydantoin	Aloisoleucine	0.043	15.84
Imidazolylmethylhydantoin	Histidine	0.032	16.09
Isobutylhydantoin	Leucine	0.020	21.42
Methylhydantoin	Alanine	0.012	33.98
Ethylhydantoin	2-Aminobutyric acid	0.018	38.51
Isopropylhydantoin	Valine	0.0108	55.90

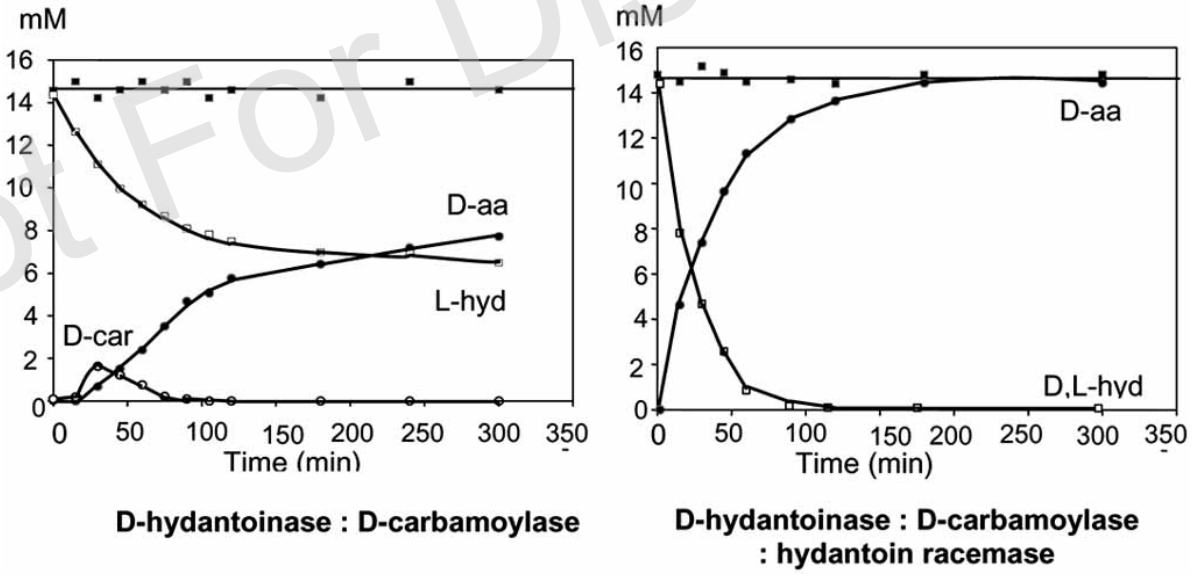

Fig. (3). Conversion of methylthioethyl-hydantoin with a polycistronic system with D,L-hydantoinase:D-carbamoylase and the same reaction with triple polycistronic system D,L-hydantoinase:D-carbamoylase:hydantoin racemase. With the double system only 50% of the initial racemic mixture is converted, but the triple system ensures 100% conversion in 100 minutes [46].

for the three genes in this bacterial family. Fig (**4**). They developed a genomic phage library of *Agrobacterium* sp. IP I-671 selecting a plasmid containing a fragment of 7125 pb. In this fragment, located in the natural 190 kb plasmid of the bacteria, the D-carbamoylase gene was detected using as template the D-carbamoylse (*hyu*C) gene amplified by PCR and DIG-labeled. At the C-terminal end of *hyu*C, and in the same orientation, two ORFs were found encoding a putative protein similar to the small subunit of a D-amino acid dehydrogenase (*hyu*D) and a hydantoin racemase (*hyu*A). At a distance of 470 bp upstream from *hyu*C, and in opposite orientation a putative D-hydantoinase (*hyu*H) was found. Fig. (**4**). These authors describe hydantoin racemase as the enzyme able to speed up hydantoin racemization; the bottleneck of amino acid production.

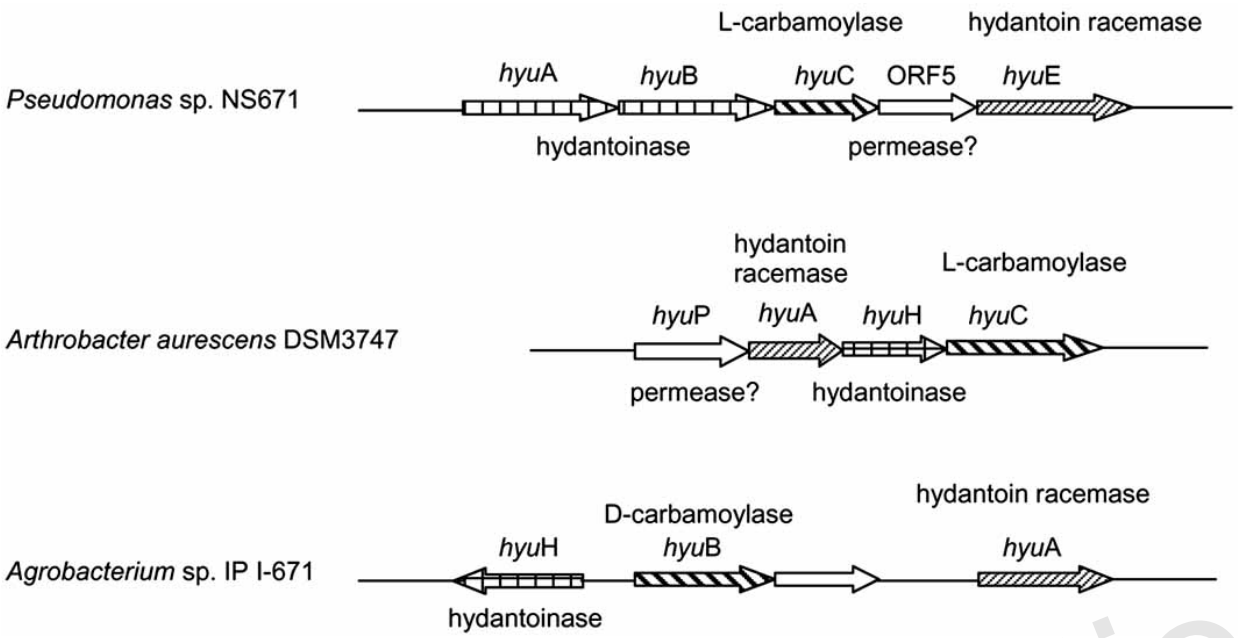

Fig. (4). Genetic organization of the genes involved in the "Hydantoinase Process".

Hydantoin racemase activity has been found in several lower organisms although its function is not completely clear. The corresponding encoding genes have been found together with a hydantoinase, a carbamoylase, and in some cases with a putative hydantoin transporter enzyme [14]. Hydantoin and *N*-carbamoyl-amino acid substrates are not very common in nature nowadays. However, carbamoyl-amino acids rather than free amino acids could have been present in early life stages [47, 48]. The presence of *N*-carbamoyl-amino acids suggests that these enzymes were necessary in earlier organisms to obtain different amino acids. In this environment, the hydantoinase enzyme would have had two functions, as it is able to cycle the *N*-carbamoyl-amino acids to their corresponding 5-monosubstituted hydantoins and hydrolyze the latter to the linear form, the second function predominating. The D- or L- carbamoylases would be the enzymes determining the enantiomer of the amino acid produced. 5-monosubstituted hydantoins can racemize spontaneously [43], but taking into account the supposed prebiotic conditions of the primitive earth (T = 25 ºC, pH = 8), the presence of the hydantoin racemase enzyme seems neccesary to obtain both enantiomers of the monosubstituted hydantoins. Sequence studies seem to indicate that no homologous genes or proteins appear in upper organisms, and might indicate that this enzyme is an ancient remnant of the Bacteria Kingdom. At present, this enzyme is responsible for the fast racemization of the substrates, which makes it the key enzyme for the scale-up of the process. Not many hydantoin racemases have been characterized, the available genes coming from: *Pseudomonas* sp. NS671 [44], *Arthrobacter aurescens* DSM 3747 [14], *Microbacterium liquefaciens* AJ 3912 [49] and *Sinorhizobium meliloti* CECT 4114 [50], and two from *Agrobacterium tumefaciens* C58 [51,52] (Table **3**). All of them have been used in patents due to their advantage over the system with only two of the three enzymes in the "Hydantoinase Process".

The hydantoin racemases from *Pseudomonas* sp. NS671, *Agrobacterium tumefaciens* C58 1 and 2 and *Sinorhizobium meliloti* CECT4114, have shown better rates of racemization for hydantoins with aliphatic susbstituents, whereas *Arthrobacter aurescens* DSM 3747 hydantoin racemase racemizes aromatic ones faster. In the absence of structural information about hydantoin racemases, site-active mutants of hydantoin racemase enzyme from *Sinohrizobium meliloti* CECT4114 (SmeHyuA) were obtained by Martinez-Rodriguez *et al.* in 2006 [53]. Sequence homology of this enzyme with several glutamate and aspartate racemases showed two highly conserved cysteine residues involved in the catalytic activity. The importance of these two cysteines in the recognition and isomerization of 5-monosubstituted hydantoins was proved. SmeHyuA Cys76 and Cys181 were mutated to alanine and serine, and biochemical analysis, isothermal titration calorimetry, circular dichroism and fluorescence studies were carried out to assess the implication of each residue in activity or substrate recognition. Results showed that Cys76 is responsible for recognition and proton retrieval of the D-isomer, while Cys181 is responsible for L-isomer recognition and racemization [54]. A model was obtained for hydantoin racemase from *Sinorhizobium meliloti* CECT 4114 using the previously reported structure of the aspartate racemase from *Pyrococcus horikoshii* [55]. The model showed that both cysteines are located opposite one another, supporting the hypothesis of a "two-base mechanism" for the reaction. Fig. (**5**). When a D-isomer of a 5-monosubstituted hydantoin is available, Cys76 (in thiolate form) acts as a base and retrieves a proton. Cys181 acts as an acid, inserting a proton in the opposite side of the substrate, thus producing L-monosubstituted hydantoin. On the other hand, the isomerization of the L-isomer of the substrate is carried out by the binding to Cys181 and the retrieval of a proton. In this case, this residue acts as the base, and Cys76 donates a proton to the putative intermediate formed. This hypothetical racemase mechanism may be the basis for modifications of the amino acids involved in the catalytic pocket with a view to enhancing its activity.

RECOMBINANT SYSTEM

The development of Molecular Biology techniques and the "Genome Project" of many microorganisms have been responsible for the increase in the number of patents since 1990. Fig. (**6**). Until that date the industry had screened for microorganisms with hydantoinase or carbamoylase activity to isolate and use them in fermentation processes. However, low yield was obtained since the expression and activity of the native enzymes is very poor and induction of the system is necessary. The development of recombinant systems has overcome these problems. Cloning of the enzymes was patented for the preparation of microorganisms which contain one or several enzymes.

Table 3. Properties of the Hydantoin Racemases Described

Strain	System Chirality	MW Monomer	Number of Subunits	Optimal pH	Optimal T
Pseudomonas sp. NS671	L	32 KDa	6	9.5	45 ºc
Arthrobacter aurescens DSM 3747	L	32.1 KDa	6, 7, 8	8.5	55 ºc
Agrobacterium tumefaciens C58 1	D	32 KDa	4	7.5	55 ºc
Agrobacterium tumefaciens C58 2	D	27 KDa	4	7.5	55 ºc
Microbacterium liquefaciens AJ 3912	L	27 KDa	4	8.2	55 ºc
Sinorhizobium meliloti CECT 4114	D	31 KDa	4	8.5	40 ºc

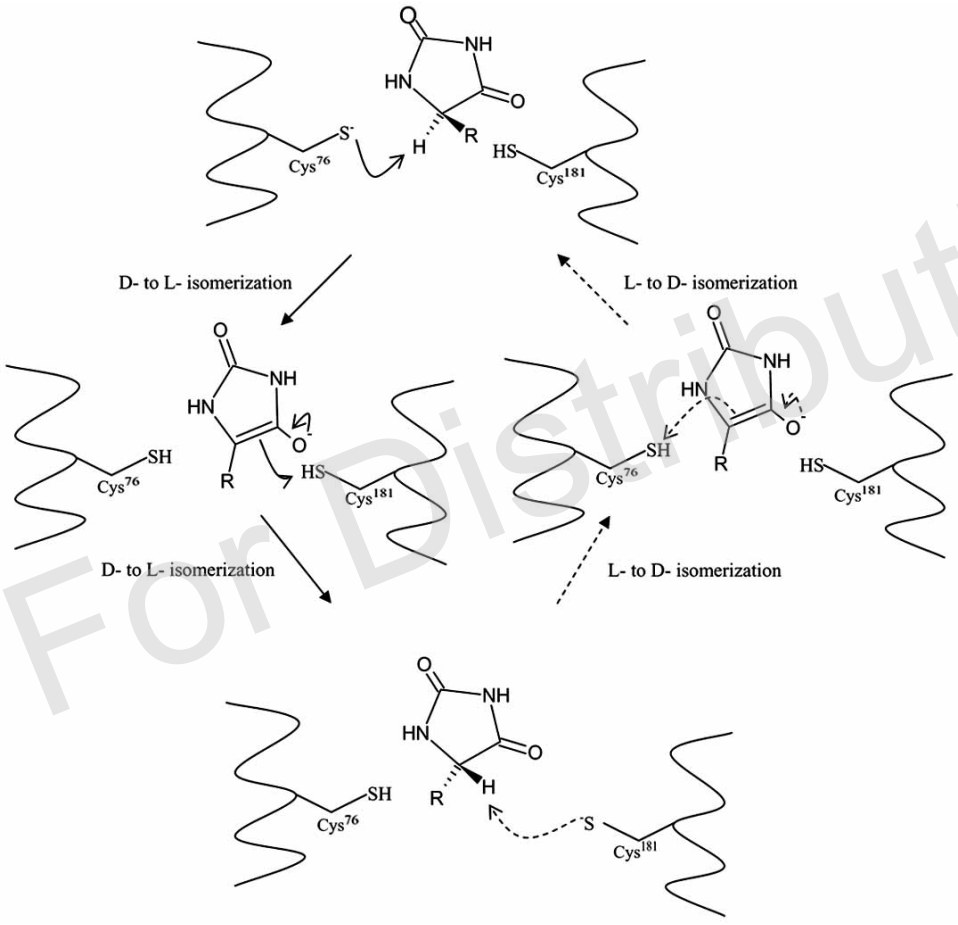

Fig. (5). Proposed racemization mechanism of hydantoin racemase using a two-base mechanism.

Hydantoinase was the first enzyme cloned, and the DNA donor microorganism was *Thermus* sp. [41]. The DNA was cleaved with restriction endonucleases and the fragments were integrated by the action of a DNA ligase in a vector which was cut with the same enzymes. The plasmid was then transferred into a bacterium for expression of the genes contained in the fragments. The hydantoinase-producing clones were then isolated. For this screening, it was necessary to obtain antibodies. The enzyme was purified from the strain CBS 303.80 and the N-terminal end was sequenced. Three rabbits were injected with increasing concentrations of the enzyme preparation for 10 weeks. The IgG fraction of the rabbit antiserum was obtained by chromatography on protein A-Sepharose. The clones were identified immunologically and by activity using D,L-methylthioethylhydantoin as substrate. The fragment was subcloned to obtain the open reading frame of the enzyme in a plasmid which was introduced into *Escherichia coli* to express the enzymatically active hydantoinase. Other authors have isolated enzymes from several sources following this strategy, but at present the amplification of the genes by Polymerase Chain Reaction (PCR) technology has many advantages as it is less time-consuming and avoids the preparation and screening of a genomic library. The cloning is made in one step and the protein sequence can be modified to improve the enzyme characteristics such as high solubility, activity, chirality and temperature stability. There are several examples of enzymes amplified by PCR for cloning [30, 56, 36, 57], and others in which PCR has been used to provide new characteristics, such as in the World patent 00/58449 [58], where

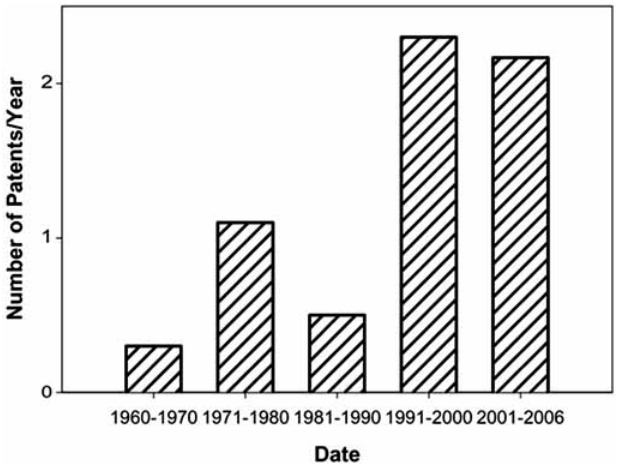

Fig. (6). Number of patents related with the "Hydantoinase process" published per year. The number of patents published in the United States Patent and Trademark Office in each period of time was divided by the number of years, 10 years in all cases except for the last period which only covers 6 years until 2006.

the authors changed the enantioselectivity of the hydantoinase from *Arthrobacter* sp. DSM 9771. To do so, they amplified it by PCR and cloned it in a plasmid, subjecting it to random mutagenesis using error-prone PCR. This technique is also known as directed evolution and was described by Kuchner and Arnold in 1997 [59]. They described how *Taq*DNA polymerase can cause an error in gene synthesis under reaction conditions such as high concentrations of $MgCl_2$ or extremely low concentrations of one of the PCR compounds. From random amplification, approximately 10,000 clones were generated and separated into single colonies using a replicating robot. These were then sown on micro-titter plates containing LB medium supplemented with ampicillin and rhamnose as inducer. The plates were incubated for 48 hours at 30ºC and at a constant shaking of 250 r.p.m. The clones were screened by colorimetric activity assay using D- or L-methyl-ethylhydantoin as substrate. The activity was measured following the variation of absorbency at 580 nm of the acid-base agent cresol red included in the reaction medium. From this first screening 2% showed significantly higher activity than the wild-type, and the mutant 11DH7 was chosen as the parent for a second round of random mutagenesis. The mutations in the amino acids sequence in 11DH7 with respect to the wild type were Ile95Leu+Gln251Arg. From this second round the mutant 22CG2 was obtained and it included a third mutation in the position 180 (Ile95Leu+ Val180Ala+Gln 251Arg). The 22CG2 mutant was then subjected to saturation mutagenesis in order to introduce all 20 different amino acids into position 95, and they found that the highest L-selectivity was obtained in the identified mutant Q2H4 (Ile95Phe+Val 180Ala+Gln251Arg).

D- or L-carbamoyl-α-amino acid amidohydrolase, or D-, L-carbamoylase, has also been cloned for over-production. Since the first assays with pure enzyme D-carbamoylase has shown several limitations. This enzyme is unstable and sensitive to oxidative phenomena and temperature. It also shows a tendency to aggregate due to hydrophobic and/or covalent bonds. Instability limits the reutilization of the enzyme, which pushes up production costs, and so many attempts have been made to eliminate one or several of the handicaps of this enzyme by introducing random mutations in the gene sequence. Treatment with mutagenic agents such as hydroxylamine hydrochloride and sodium nitrite or random mutation by PCR have been used to introduce mutations into the sequence of *Agrobacterium* sp. KNK712 (FERM-BP1900) carbamoylase. Three sites were found to be related to thermostability: histidine 57, proline 203 and valine 236. Several assays were made substituting these three sites with several amino acids, mutating one or several positions at a time. The most stable enzyme was obtained when histidine 57 was substituted with tyrosine, proline 203 with glutanamine and valine 236 with alanine, improving thermostability from 61.8ºC to 80ºC [60]. Other examples have been described for D-carbamoylase from *Agrobacterium radiobacter* NRRL B-11291 [61,62], where the substitution of threonine 212 and 262 by alanine increased the enzyme's thermostability by 13ºC: from 61ºC to 74ºC. Finally, the quantity of the improved carbamoylases has been increased by incorporating a strong promoter upstream from the gene.

Hydantoin racemase was the last enzyme of the "Hydantoinase Process" to be discovered and characterized, and all researchers have highlighted its importance [63-65]. The screening process for hydantoin racemases was patented in WO 2004/111227 [66]. The authors described the use of an L-enantiomer of a 5-monosubstituted hydantoin as substrate and a D-selective hydantoinase. The racemization rate and hence the activity of the hydantoin racemase can be measured easily by the formation of the *N*-carbamoyl-D-amino acid by HPLC or colorimetric methods. When D-hydantoins are used, corresponding L-selective hydantoinases have to be used in the screening process. These authors claimed the screening process for hydantoin racemases, the nucleic acid sequence coding for the hydantoin racemase from *Arthrobacter aurescens* DSM3747, the mutagenic gene to increase the enzymatic activity and a whole-cell catalyst system. Now the complete system consists of three enzymes, and there are several possibilities to re-design it. The three enzymes can be produced together in a single host or independently. The handicap of producing the enzymes separately is that three parallel fermentations are required, and if the reactions are carried out with complete cells the cellular membrane is a barrier for the substrates. On the other hand, the activity rate of the three enzymes is different, and so to avoid substrate accumulation it is necessary to match the expression of each one to its respective conversion rate. When they are obtained independently, it is possible to combine them, thus increasing the proportion of protein with lower velocity of hydrolysis. Several authors have cloned the three enzymes in different plasmids and expressed them separately [39,67]. In the Spanish patent ES2241394, the authors cloned the genes hydantoinase and D-carbamoylase from *Agrobacterium radiobacter* NRRL B-11291 generating plasmids pSER11 and pJMC38, respectively, and the hydantoin racemase gene from *Agrobacterium tumefaciens* C58, giving plasmid pSER14. *Escherichia coli* BL21 was transformed with each plasmid and cultured separately. Due to the differential activity rate of each enzyme, the best yield was obtained when the cellular extracts of each recombinant host were mixed in the ratio 2:2:1 hydantoinase:carbamoylase:racemase.

The alternative is to express the three enzymes in one host, which reduces production costs, cellular rupture and purification. This can be carried out by expressing the enzymes in plasmidic systems or by chromosomal integration in the host under a well-known promoter. Whatever the system used, to adapt the turnover rate of all enzymes expressed several methods can be employed: a) Expressing the three enzymes with different promoters. The gene with the lowest activity is directed by the strongest promoter. The disadvantage of this method is that each promoter needs a different inducer, which increases production costs. b) Expressing the three genes with one promoter in a polycistronic messenger. In this case, the translation ratios are directed by changing the ribosome binding site, which determines the efficiency of protein synthesis, or by changing the order of the genes, cloning the enzyme with the worst catalytic rate just behind the promoter. c) Changing the activity of each enzyme by either random or directed mutation. Random

mutation is very time-consuming, and directed mutation requires exhaustive knowledge of the protein, which to date is not available. d) Inserting several copies of the gene whose enzyme has the lowest activity rate to increase the quantity, or using plasmid with a different number of copies.

In World Patent WO04111227 [66], the authors develop a system with two plasmids, one with the genes for the hydantoinase and carbamoylase for both L- and D-amino acid production (pOM22 for L-production and pJAVIER16 for D-production), and the other one with the hydantoin racemase gene (pOM21-BB5). Plasmids pOM22 and pJAVIER16 confer ampicillin resistance to the bacteria, and pOM21-BB5 confers chloramphenicol resistance. *E. coli* JM109 transformed with both pOM21-BB5 and pOM22 is able to produce optically pure L-amino and the cells that contain pOM21-BB5 and pJAVIER16 D-amino acids. In both cases the total yield of the amino acids was >60%. This strategy involves the use of two antibiotics, increasing production costs and reducing the growth kinetics.

In US Patent US6713288 [68], the authors carried out several constructions which avoid the use of inducers or antibiotics to maintain the plasmids. One of these consists of inserting the hydantoinase gene in the *E. coli* chromosome without an inducible promoter [11,68] and inserting the other two enzymes in a plasmid under a rhamnose inducible promoter. The results obtained were not so good as when they used a system of two plasmids, one with L-*N*-carbamoylase and hydantoin racemase and the other with hydantoinase. In the former system, total conversion of the D,L-5-(3-indolylmethyl)-hydantoin was not possible, but when the two-plasmid system was used, L-tryptophan was obtained in four hours. Using a similar strategy [69] hydantoinase and D-*N*-carbamoylase from *Flavobacterium* sp. AJ11199 were expressed in one vector and hydantoin racemase from *Microbacterium liquefaciens* AJ3912 in another, the three enzymes regulated by the trp promoter. They tested the activity of the system with several hydantoins, obtaining 99% conversion in 48 hours for D-phenylalanine, D-tyrosine, O-benzyl-D-serine, D-leucine, D-norvaline and D-norleucine, but only 70% conversion for D-tryptophan and 88% for D-valine. These authors suggest that the slow rates and yields are due to *N*-carbamoyl-D-valine accumulation, since this substrate chiefly depends on the specificity of the D-*N*-carbamoylase from *Flavobacterium* sp. AJ11199 (FERM-P4229).

The expression of the three enzymes in a polycistronic messenger has been developed by Martinez-Gomez *et al*. in 2007 [46]. They cloned the D-hydantoinase and D-carbamoylase enzymes from *Agrobacterium tumefaciens* BQL9 and hydantoin racemase 1 or 2 from *Agrobacterium tumefaciens* C58. The three genes were inserted in the pBSK vector in the order D-*N*-carbamoylase-hydantoinase-hydantoin racemase. This strategy reduces the high selective pressure of the two-plasmid system, and produces the coexpression of the three genes with only one inducer, isopropyl-β-D-thiogalactopyranoside (IPTG). Both aliphatic and aromatic hydantoins substituted in C-5 were hydrolyzed to the corresponding optically pure D-amino acids (Table **4**) without the accumulation of intermediates due to the high overexpression of D-carbamoylase. Fig. (**3**). Both systems showed higher reaction rates for the conversion of aliphatic amino acids than for aromatic ones. System 1 was able to hydrolyze the 5-monosubstitued hydantoins faster than System 2. The same authors had previously determined that hydantoin racemase 1 showed the highest substrate affinity and racemization velocity [51,52]. For industrial application the stability of the plasmid was tested for System 1, and it was found to be stable for 90 generations without ampicillin pressure after the initial inoculation. Taking advantage of the escape synthesis of pBSK plasmid, the hydrolysis velocity was measured, finding that total conversion of D-L-methyl-thioethylhydantoin was obtained after 32 h, as opposed to 5 h with the inducer.

OWNERSHIP OF THE INVENTION. WHERE IS THE "HYDANTOINASE PROCESS" PANTENTED?

"Hydantoinase Process" related patents are only present in a few countries, where the companies which produce D- or L- amino acids or their derivates are established. As Fig. (**7**) shows, the United State Patents and Trademark Office (USPTO) has the highest numbers of patents, followed by the Japanese Office.

The first USPTO patent for the use of the "Hydantoinase Process" was made in 1967 by the Ajinomoto Co. Inc. (Tokyo, Japan) [17]. Thereafter the number of patents increased and three new companies appeared; two from Japan and one from Italy. From 1980 the number of patents by these firms continued to increase, in the 1990's new companies appeared: 4 from Germany and one each from Japan, Italy and the United Kingdom. In the last decade, two Indian companies, one more from Germany and two from the

Table 4. Conversion Time in Minutes of Several D,L-5-Monosubstituted Hydantoins by Systems 1 and 2. For Tyrosine, p-Hydroxy-Phenylglycine, Valine and Tryptophan the Conversion does not reach 100%

SUBSTRATE	D-AMINO ACID	PSYS1	PSYS2
D,L-5-Butylhydantoin	Leucine	105	120
D,L-5-Isobutylhydantoin	Norleucine	120	150
D,L-5-Propylhydantoin	Norvaline	120	150
D,L-5-Methyl-thioethylhydantoin	Methionine	150	210
D,L-5-Benzylhydantoin	Phenyl-alanine	300	360
D,L-5-Ethylhydantoin	Aminobutyric acid	340	360
D,L-5-Phenylhydantoin	Phenyl-glycine	360	360
D,L-5-p-Hydroxy-benzylhydantoin	Tyrosine	720 (97%)	720 (95%)
D,L-5-p-Hydroxy-phenylhydantoin	p-Hydroxy-phenylglycine	720 (95%)	720 (93%)
D,L-5-Isopropylhydantoin	Valine	720 (93%)	720 (83%)
D,L-5-Indolylmethylhydantoin	Tryptophan	720 (80%)	720 (70%)

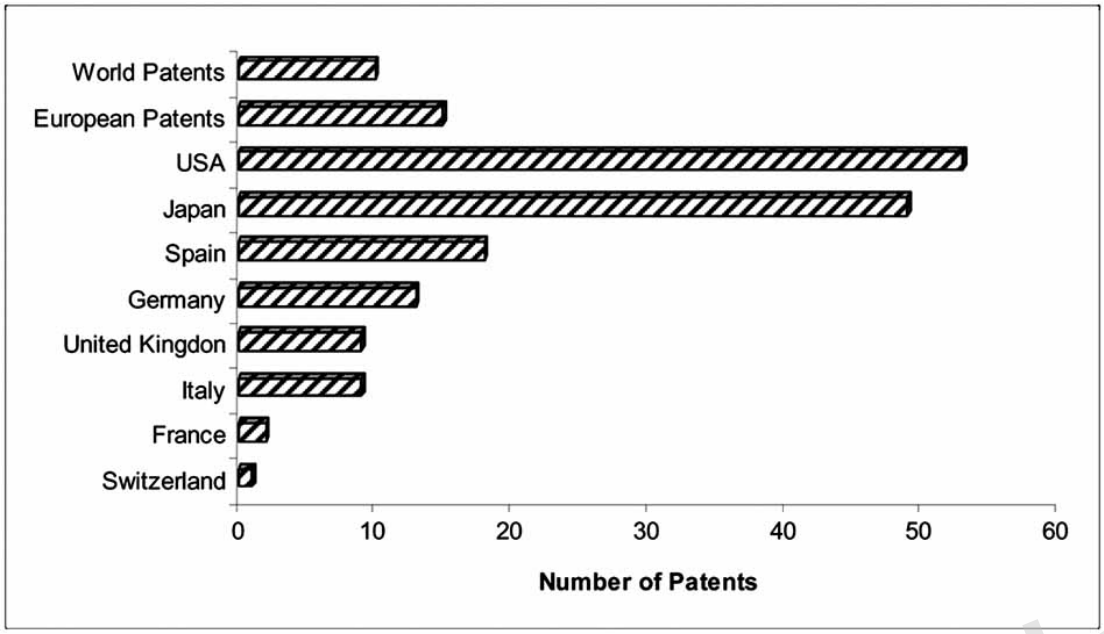

Fig. (7). Number of patents published related to the "Hydantoinase Process" since 1960.

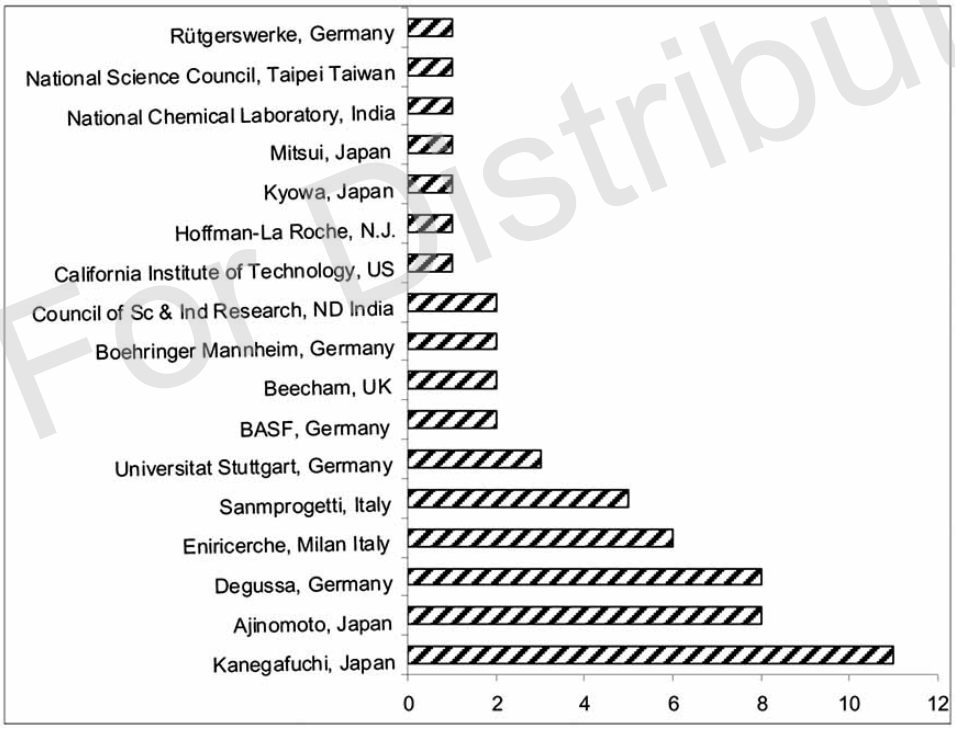

Fig. (8). Number of United States patents obtained by each company.

United States have patented their inventions, and for the first time educational establishments such as universities, have also done so. Of the 53 American Patents, approximately half are the property of three companies: 20% are assigned to Kanegafuchi Kagaku Kogyo Kabushiki Kaisaha (Osaka, Japan), 15% to Ajinomoto Co. Inc. (Tokyo, Japan) and 15% to Degussa AG from Germany. The remainder belong to 14 different companies or state institutions. Fig. (**8**). The "Hydantoinase Process" is an invention which has been developed and applied to the manufacturing process owing to the high value of the products, and knowledge of the process is dominated by private companies, with academia and government institutions playing a collaborating role.

CURRENT & FUTURE DEVELOPMENTS

Optically pure D- or L-amino acids are used as intermediates in several industries, and their specific activity depends on their chirality and purity. There are two main approaches to obtaining optically pure amino acids, namely chemical and enzymatic synthesis. Chemical synthesis gives racemic mixtures of amino acids of low yield and it is not environment friendly. Enzymatic synthesis based on the overproduction of a specific amino acid by fermentation of microorganisms is only useful for a few natural amino acids, and depends on the microorganisms. Many chemical companies have embraced biocatalysis for the manufacture of

enantiomerically pure amino acids, developing new methods that include: a) amino acylase, b) amidase and c) hydantoinase. The Hydantoinase Process is an economically attractive method for the production of many non-natural amino acids, which are components of potential pharmaceuticals [70].

The number of patents in the "Hydantoinase Process" has increased greatly since the system was elucidated. In the last decade patents have focused on building recombinant plasmids with these three enzymes. The enzymes produced by recombinant DNA technology increase the competitiveness of the "Hydantoinase Process", as the expression and purification of the enzymes are improved. The future starts with the heterologous expression systems. Cloning and manipulation of the genes allow random mutagenesis and directed evolution, used to improve enzyme properties such as activity, stability, stereoselectivity and yield. The optimized enzymes can then be employed *in vitro* in enzyme cascades, or *in vivo* combined in whole-cell catalysts, as has already been demonstrated in several patents. The use of whole-cell systems of recombinant microorganisms harboring these plasmids is currently being incorporated into the manufacturing process, showing great potential. Finally, recent advances in protein crystallization are contributing to our understanding of the natural enzymes, helping to predict the key residues for enzyme enhancement.

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