Novel microorganisms, their use and process for the production of L-α-amino acids

Description

The invention relates to novel microorganisms which have high specific activities and possess the ability to convert D-, L- or D,L-5-monosubstituted hydantoins or D-, L- or D,L-N-carbamoyl-α-amino acids to the corresponding enantiomerically pure L-α-amino acids.

Numerous methods for the fermentative or enzymatic conversion of N-5-monosubstituted hydantoins to the enantiomerically pure L-α-amino acid have hitherto been described in the literature or patented (see Syldatk, C., Müller, R., Pietzsch, M., Wagner, F., MICROBIAL AND ENZYMATIC PRODUCTION OF L-AMINO ACIDS FROM D,L-5-MONOSUBSTITUTED HYDANTOINS in: Biocatalytic production of amino acids and derivatives (Rozell, J.D. and Wagner, F., eds.), Hanser Verlag, Munich, 1992, pp. 129 - 176).

Yokozeki, K., Sano, K., Eguchi, C., Iwagami, H. and Mitsugi, K. (1986): OPTIMAL CONDITIONS FOR THE ENZYMATIC PRODUCTION OF L-AROMATIC AMINO ACIDS FROM THE CORRESPONDING 5-SUBSTITUTED HYDANTOINS, Agric. Biol. Chem. 51, 729 - 736, and Yokozeki, K., Hirose, Y. and Kubota, K. (1986): MECHANISM OF ASYMMETRIC PRODUCTION OF L-AROMATIC AMINO ACIDS FROM THE CORRESPONDING HYDANTOINS BY FLAVOBACTERIUM SP., Agric. Biol. Chem., 51, 737 - 746, describes a reaction mechanism for the hydrolysis of 5-arylalkylhydantoins, the example given being 5-benzylhydantoin, with a purified hydantoinase from Flavobacter sp. AJ-3912. The relative rates of the forward and reverse reactions were determined and it was shown that the forward reaction of D-5-benzylhydantoin to N-carbamoyl-D-phenylalanine proceeded markedly slower than the

hydrolysis of L-5-benzylhydantoin to N-carbamoyl-L-phenylalanine. No statement was made about the mechanism of the enantioselective hydrolysis of 5-alkylhydantoins because D,L-5- methylthioethylhydantoin could only be converted to N-carbamoyl-L-methionine in trace amounts.

The object of the present invention is to provide novel microorganisms which are easy to cultivate and produce enzymes which are capable of producing L-α-amino acids from D-, L- and/or D,L-5-monosubstituted hydantoin and/or from a D-, L- and/or D,L-N-carbamoyl-α-amino acid in comparatively large amounts or at comparatively fast rates. Further objects are a corresponding process for the production of L-α-amino acids and the use of the microorganisms.

This object is achieved with the microorganisms DSM 7329 and DSM 7330. These microorganisms were deposited on 17.11.1992 in the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), Mascheroder Weg 1 B, D-3300 Brunswick; appropriate certificates are enclosed with the description.

The newly isolated microorganisms deposited in the German Collection of Microorganisms represent novel varieties of the species Arthrobacter.

In the process for the production of L-α-amino acids by the enzymatic conversion of a D-, L- and/or D,L-5-monosubstituted hydantoin and/or a D-, L- and/or D,L-N-carbamoyl-α-amino acid, the conversion is carried out by means of the microorganism or microorganisms DSM 7329 and/or DSM 7330 and/or by means of enzymes produced by these microorganisms.

If the process is carried out with the microorganisms themselves, this is preferably done with dormant or killed

microorganisms so that they do not themselves utilize the substrate.

If the process is carried out by means of the enzymes produced by these microorganisms, this can be done with a mash of the microorganisms (obtained *e.g.* by means of a French press or glass bead mill), with a crude extract or with more or less purified enzymes of the microorganisms.

The present invention further relates to the use of the microorganism DSM 7329 and/or the microorganism DSM 7330 for the culture of mutants or variants of these microorganisms,

or for the preparation of a gene coding for a carbamoylase or hydantoinase and/or hydantoin racemase,

or for the insertion of a gene coding for a carbamoylase and/or hydantoinase and/or hydantoin racemase into a microorganism or a cell,

or for the production of an L-α-amino acid.

The culture of mutants or variants of these microorganisms can be carried out *e.g.* by the selection of spontaneously occurring mutations. Other possibilities are *e.g.* the mutations by the action of chemical substances and/or radioactive radiation and/or UV light.

The preparation of one or more genes from the microorganisms is used *e.g.* for sequence analysis of the genes for the enzymes, or for insertion into a microorganism: selection of the microorganism into which the gene is inserted makes it possible to obtain a microorganism which produces larger amounts of the enzyme or enzymes which are decisive for the process described above. Under certain circumstances, the gene can also be

incorporated into an animal or plant cell, said incorporation of the gene being carried out by the methods conventionally used in microbiology, *e.g. via* a vector.

The novel microorganisms described produce a number of enzymes in amounts such that the cell mass has a very high activity for the processes described above. No strains with a higher activity for these processes have been disclosed hitherto.

The above-described process for the production of L-α-amino acids proceeds practically stereospecifically with the microorganisms of the present invention or their enzymes, it being possible to convert a very wide variety of 5-substituted hydantoins. The corresponding amino acids are obtained in accordance with the reaction scheme given below. According to the present invention, the radical in the hydantoin can be an alkyl radical and can be branched, cyclic, substituted by one or more heteroatoms, aromatic, heteroaromatic, *etc.* Difficulties were created only by 5-methylhydantoin, 5,5-disubstituted hydantoins, polysubstituted phenylhydantoins such as 5-(3'-methyl-4'-aminomethylphenyl)hydantoin, bulky substituents directly on the hydantoin, such as 5-t-butylhydantoin, and radicals with charged substituents near the hydantoin, such as *e.g.* 5-ß-carboxyethylhydantoin, 5-γ-aminopropylhydantoin and 5-δ-aminobutylhydantoin. On the other hand, a large number of compounds could be converted, such as *e.g.* 5-substituted hydantoins with the substituent isopropyl, N-propyl, N-butyl, methylthioethyl, isobutyl, indolylmethyl, cyclohexylmethyl, benzyl, naphthylmethyl or phenyl, a very wide variety of substituents being possible, *e.g.* p-fluoro, p-chloro, p-amino, p-methoxy or p-carboxy on the benzyl. Thus the 5-substituent on the hydantoin or the N-carbamoylamino acid is not subject to any fundamental restrictions since, by virtue of the large number of possible substituents and the large number of possible

functionalizations of the substituents or the amino acid residues, basically all typical 5-substituted hydantoins or corresponding N-carbamoylamino acids are suitable substrates.

European patent 0 159 866 B1 describes a bacterium of the genus Arthrobacter sp. DK-200, which is capable of forming L-α-amino acids from the corresponding 5-arylalkylhydantoins and/or from the appropriate N-carbamoylamino acid. Said patent only describes the synthesis of L-phenylalanine, L- tryptophan and L-tyrosine. No information is given on specific enzyme activities or the enzymatic mechanism.
Arthrobacter sp. DK-200 has obvious differences from the novel microorganisms Arthrobacter sp. DSM 7329 and DSM 7330:
Arthrobacter sp. DK-200 cannot cleave starch hydrolytically; this is a decisive physiological characteristic for the identification of the species within the genus Arthrobacter. Furthermore, the strain shows no urease activity and the strain is classified as positive in respect of denitrification. Also, growth of the strain Arthrobacter DK-200 can no longer be observed at a temperature above 33.2°C. In addition, there are differences in physiological characteristics, for instance in the utilization of lactose as a carbon source and in the formation of acids from sugars (L-arabinose negative and glycerol positive), as shown in Table 1.

Table 1:

Physiological and bacteriological properties	DSM 7329	DSM 7330	DK 200
Denitrification	-	-	-
Hydrolytic cleavage of starch	+	+	-
Urease	+	+	-
Growth at	33°C	35°C	17.8°C
not at	>34°C	>36°C	>33.3°C
Growth at [% NaCl]	2%	5%	2%
not at	>3%	>6%	>5%
Formation of acids from sugar			
L-Arabinose	+	+	-
Glycerol	-	-	+
Utilization of carbon sources			
Lactose	+	+	-

Patent specification DE 37 12 539 C2 describes 3 microorganisms, namely DW 3 (DSM 3745), CW 4 (DSM 3746) and CW 5 (DSM 3747), which are to be classified in the group of the saprophytic aerobic corynebacteria. These microorganisms are capable of liberating the appropriate aromatic L-α-amino acid from the corresponding 5-arylalkylhydantoins and N-carbamoyl-α-amino acids. The conversion of D,L-5-methylthioethylhydantoin to L-methionine *via* D-N-carbamoylmethionine is also described. No specific productivities for the organisms used are given for these conversions. The conversions of D-N-carbamoylamino acids to L-amino acids is accounted for by the presence of a racemase at the stage of the N-carbamoyl intermediates (DE 37 12 539, p. 2, lines 42 - 44). The most recent publication on the strain DSM 3747 does not include participation of a D-N-carbamoylamino acid in the preparation of L-amino acids from the corresponding D,L-5-

monosubstituted hydantoins (*cf.* Syldatk, C., Müller, R., Pietzsch, M., Wagner, F., MICROBIAL AND ENZYMATIC PRODUCTION OF L-AMINO ACIDS FROM D,L-5-MONOSUBSTITUTED HYDANTOINS in: Biocatalytic production of amino acids and derivatives (Rozell, J.D. and Wagner, F., eds.), Hanser Verlag, Munich, 1992, pp. 129 - 176). The distinction between the microorganisms DSM 3745 to DSM 3747, described in patent specification DE 37 11 539, and the two microorganisms DSM 7329 and DSM 7330 is obvious at the physiological level, as shown by the comparison in Table 2.

Table 2:

Physiological and bacteriological properties	DSM 7329	DSM 7330	DSM 3745	DSM 3746	DSM 3747
Gram staining	+	+	+/-	-	-
Oxidase	-	-	+	-	-
No growth above	34	36	37	35	35
Formation of acids from sugar					
L-Arabinose	+	+	+	-	+
D-Sorbitol	+	+	-	-	-
Utilization of carbon sources					
Glycerol	-	-	+	+	+
Starch	-	-	+	+	+
Citric acid	-	-	+	+	+

A number of processes are known for the production of aliphatic L-α-amino acids from 5-alkylhydantoins or the corresponding N-carbamoylamino acids.

Patent specification DE 37 02 384 A1 (US patent 5 071 752) describes the formation of L-amino acids from the

corresponding 5-substituted hydantoins, the examples given being L-leucine, L-valine and L-cysteine, with the microorganism Nocardia spec. DSM 3306. However, said patent neither indicated the specific activity nor proposed a mechanism for the hydrolysis of the hydantoins.

According to Japanese patent Sho 55-29678, a Corynebacter sepedonicum is allowed to act on L- or D,L-N-carbamoyl-methionine and the L-N-carbamoylmethionine present in the substrate mixture is converted to L-methionine. However, this process only enables the L-enantiomeric substrate to be converted to L-methionine, the D-N-carbamoylmethionine remaining unconverted. Likewise, Japanese patent specification JP-B-29678/80 describes a process in which L-methionine is formed from D,L-N-carbamoylmethionine with a microbial enzyme. The L-methionine has to be separated from the unreacted D-N-carbamoylmethionine after the conversion.

Guivarch, M., Gillonnier, C. and Brunie, J.-C. (1980): OBTENTION D'AMINO ACIDES OPTIQUEMENT ACTIFS A L'AIDE D'HYDANTOINASES (PREPARATION OF OPTICALLY ACTIVE AMINO ACIDS USING HYDANTOINASES), Bull. Soc. Chim. Fr. No. 1-2, 91 - 95, describes the formation of L-methionine from D,L-5-methylthioethylhydantoin with Arthrobacter ureafaciens, it being assumed here that racemization takes place at the intermediate stage of D- and L-N-carbamoylmethionine. This reaction pathway does not exist in the novel microorganisms DSM 7329 and DSM 7330.

Japanese patent (B 2) Hei 4-39316 describes two novel Arthrobacter spec., DP-B-1001 and DP-B-1002, which form the appropriate L-amino acids from 5-substituted hydantoins and from N-carbamoylamino acids, irrespective of whether they are in the D, L or D,L form or in the form of a mixture. Said patent specification makes no statement about a reaction mechanism. However, these novel microorganisms

differ essentially from the organisms DSM 7329 and DSM 7330 in terms of their physiological characteristics, as can be seen in Table 3. Moreover, there are marked differences in the specific activities for the conversion of D-N-carbamoylmethionine to L-methionine, as shown in Table 4.

Table 3:

Physiological and bacteriological properties	DSM 7329	DSM 7330	1001	1002
Hydrolytic cleavage of starch	+	+	-	-
Growth at [°C]	33	35	30	30
not above [°C]	34	36	37	37
Growth at [% NaCl]	2%	5%	2%	2%
not over	3%	6%	5%	5%
Nutrient requirement	-	-	biot.	biot.
Utilization of malonate	+	+	+	-
Formation of acid from sugar				
D-Sorbitol	+	+	-	-
Inositol	+	+	-	-
Glycerol	-	-	+	+
Utilization of carbon sources				
D-Sorbitol	+	+	-	-
Glycerol	-	-	+	+
Adonitol	-	+/-	-	-
Ethanol	+	+	-	-
ß-Alanine	+	-	+	-
L-Ornithine	+	+	-	-
L-Threonine	-	+	+	+
L-Valine	+	-	-	-
Acetic acid	-	-	+	+
D,L-Lactic acid	-	-	+	+

Physiological and bacteriological properties	DSM 7329	DSM 7330	1001	1002
Propionic acid	-	-	+	+
meso-Tartaric acid	-	+	-	-
m-Hydroxybenzoic acid	-	-	+	+
p-Hydroxybenzoic acid	-	-	+	+
Malonic acid	+	+	+	-
Succinic acid	-	-	+	+
Citric acid	-	-	+	+
Gluconic acid	-	-	+	+
Fumaric acid	+	-	+	+
Uric acid	-	+	-	-

The specific activities of whole cells were calculated using the following equation:

$$A_{spec.} = \frac{\text{mmol or g substrate converted}}{\text{g WBM} \cdot \text{h}}$$

Table 4:

According to JP (B 2) Hei 4-39316, the Japanese strain DP-B-1001 possesses the following specific activities for D-N-carbamoylmethionine:

$$A_{spec.} = \frac{2.0 \text{ (mmol)}}{10 \text{ (g WBM)} \cdot 48 \text{ (h)}} = 0.00417 \text{ (mmol/g WBM x h)}$$

Under identical conditions, the strain DSM 7330 possesses the following specific activities for D-N-carbamoylmethionine:

$$A_{spec.} = \frac{2.6 \text{ (mmol)}}{10 \text{ (g WBM)} \cdot 21 \text{ (h)}} = 0.0124 \text{ (mmol/g WBM x h)}$$

i.e. an increase in the specific activities for D-CM by a factor of 3.3 based on (g/g WBM x h), under the conditions indicated in Example 1 of the JP-B cited above.
Using a different buffer (0.1 M Na carbonate buffer, pH: 8.5) and adding twice the amount of substrate, a spec. activity of:

$$A_{spec.} = \frac{5.2 \text{ (mmol)}}{10 \text{ (g WBM)} \cdot 21 \text{ (h)}} = 0.0248 \text{ (mmol/g WBM x h)}$$

was found, *i.e.* a further increase to a factor of 6.5.
Under modified conditions (0.2 M Tris/HCl buffer, pH: 8.0, 30°C, no introduction of gas and no other additives), the following specific activity was found:

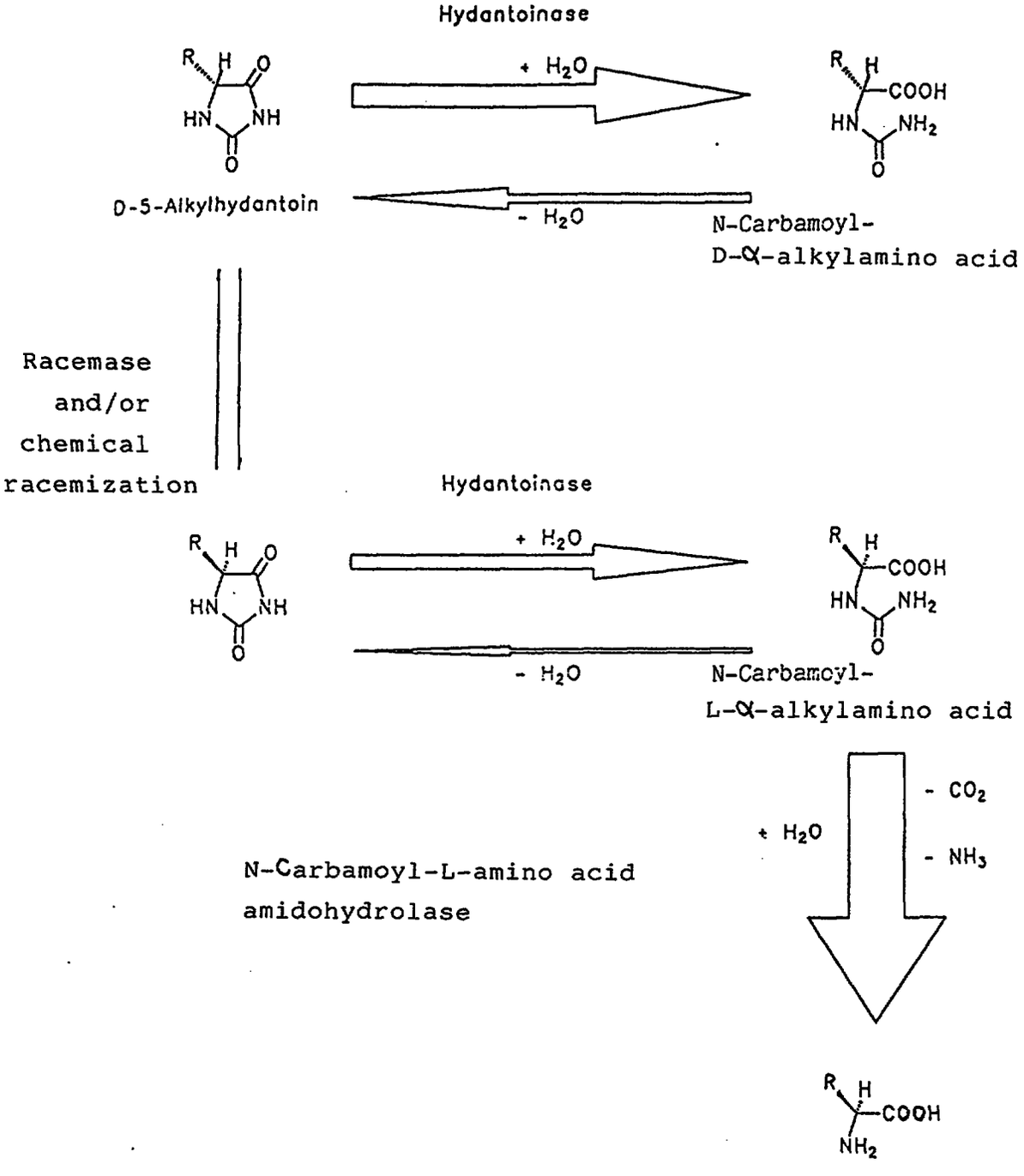
$$A_{spec.} = \frac{4.24 \text{ (mmol)}}{10 \text{ (g WBM)} \cdot 9.33 \text{ (h)}} = 0.045 \text{ (mmol/g WBM x h)}$$

i.e. a further increase to a factor of 12!

WBM: wet bacterial mass;
D-CM: D-N-carbamoylmethionine.

The present invention was used to isolate two novel Arthrobacter spec., namely DSM 7329 and DSM 7330, which have a markedly increased productivity compared with the state of the art and produce predominantly aliphatic L-amino acids, *e.g.* L-methionine, from D- and/or L- and/or D,L-5-monosubstituted hydantoin, and/or from the corresponding N-carbamoylamino acid, and/or from a mixture of both the classes of substances mentioned, according to

the newly elucidated reaction mechanism illustrated in Scheme 1. "Alkyl" is only given here as an example of the abovementioned range of substrates. The rates found are represented by the size of the arrow.


Hydantoinase
+ H2O
D-5-Alkylhydantoin
- H2O
N-Carbamoyl-
D-α-alkylamino acid
Racemase
and/or
chemical
racemization
Hydantoinase
+ H2O
- H2O
N-Carbamoyl-
L-α-alkylamino acid
N-Carbamoyl-L-amino acid
amidohydrolase
+ H2O
- CO2
- NH3
L-α-Alkylamino acid

The invention is explained in greater detail below with the aid of Examples.

Example 1: Isolation of the microorganisms

In each case 50 ml of a synthetic medium containing D,L-5-methylthioethylhydantoin as the only nitrogen source, of the composition

KH_2PO_4	1.0	g/l
K_2HPO_4	1.816	g/l
$MgSO_4 \cdot 7H_2O$	0.123	g/l
$CaCl_2 \cdot 2H_2O$	0.017	g/l
$FeSO_4 \cdot 7H_2O$	0.006	g/l
Glucose	5.0	g/l
D,L-Methylthioethylhydantoin	1.0	g/l
Trace element solution	10	ml/l

pH: 7.0,

the trace element solution having the following composition:

Ethylenediaminetetraacetate (Na^+)	500	mg/l
$FeSO_4 \cdot 7H_2O$	200	mg/l
$ZnSO_4 \cdot 7H_2O$	10	mg/l
$MnCl_2 \cdot 4H_2O$	3	mg/l
H_3BO_3	30	mg/l
$CoCl_2 \cdot 4H_2O$	1	mg/l
$CuCl_2 \cdot 2H_2O$	1	mg/l
$NiCl_2 \cdot 6H_2O$	1	mg/l
$Na_2MoO_4 \cdot 2H_2O$	3	mg/l,

are incubated for 4 days with 1 g of a soil sample from a very wide diversity of locations, in a 500 ml conical flask with baffles at 30°C, pH 7.0 and 100 rpm on a rotary shaker (Braun, Melsungen). 1 ml of each of these suspensions is then inoculated in fresh sterile of the abovementioned

nutrient solution and incubated again for 4 days at 30°C, pH 7.0 and 100 rpm on the rotary shaker. After 10 cultivation passes, each suspension culture is streaked out on a complex medium on agar plates *via* serial dilution until individual colonies have grown after incubation at 30°C for 3 days. A pure culture is prepared from the individual colonies in known manner.

Composition of the complex medium for the individual micro-organism strains:

Glucose	10.0 g/l
Technical-grade yeast extract	0.5 g/l
Citric acid	0.5 g/l
$MgSO_4 \cdot 7H_2O$	0.1 g/l
$CaCl_2 \cdot 2H_2O$	0.05 g/l
KH_2PO_4	1.0 g/l
K_2HPO_4	1.816 g/l
Trace element solution	10.0 ml/l
Agar	20.0 g/l

pH: 7.0

To test for the enzymatic activity for the preparation of L-α-amino acids from D,L-5-monosubstituted hydantoins, the resulting pure cultures are grown in submersion cultures as follows:
In a 500 ml conical flask with baffles, 100 ml of sterile nutrient medium I are inoculated with a rinse from a freshly prepared pure culture and incubated at 30°C, pH 7.0 and 100 rpm on the rotary shaker for 24 h. From the resulting suspension culture, 500 ml of sterile nutrient medium II are inoculated with 10 ml of the preculture in a 2000 ml conical flask with baffles. Further cultivation is carried out for 36 h at 30°C, pH 7.2 and 100 rpm on the rotary shaker. The cells are then centrifuged off and washed twice with 0.9% NaCl solution. The resulting wet cell mass can be either tested immediately for the

enzymatic activity or stored at -18°C for up to several weeks.

Nutrient medium I has the following composition:

Glucose	10.0 g/l
$(NH_4)_2SO_4$	6.50 g/l
Citric acid	0.32 g/l
$MgSO_4 \cdot 7H_2O$	0.20 g/l
$CaCl_2 \cdot 2H_2O$	0.02 g/l
$MnCl_2 \cdot 4H_2O$	0.02 g/l
$FeSO_4 \cdot 7H_2O$	0.02 g/l
KH_2PO_4	4.54 g/l
K_2HPO_4	7.61 g/l

Nutrient medium II has the following composition:

Glucose	15.0 g/l
Technical-grade yeast extract	1.0 g/l
$(NH_4)_2SO_4$	6.50 g/l
Citric acid	0.62 g/l
$MgSO_4 \cdot 7H_2O$	0.40 g/l
$CaCl_2 \cdot 2H_2O$	0.04 g/l
$MnCl_2 \cdot 4H_2O$	0.04 g/l
$FeSO_4 \cdot 7H_2O$	0.04 g/l
KH_2PO_4	3.40 g/l
$Na_2HPO_4 \cdot 2H_2O$	4.42 g/l
D,L-5-Indolylmethyl-N-3-methylhydantoin	0.25 g/l

The enzymatic activity of the resulting wet cell mass (WBM) is tested comparatively under the following reaction conditions:
50 mg of D,L-5-methylthioethylhydantoin and 2.5 g of WBM are added to 50 ml of 0.2 molar Tris/HCl buffer of pH 8.0 and incubated for 24 h at 30°C. The cell suspension is

then centrifuged and the clear supernatant is examined by HPLC analysis.

Two strains, which are deposited in the German Collection of Microorganisms (DSM) under the numbers DSM 7329 and DSM 7330, were found to be particularly active. They have the following physiological and bacteriological properties, which are collated in Table 5.

Table 5:

Physiological and bacteriological properties	DSM 7329	DSM 7330
Properties		
Cell size (L·ϕ)	(0.3 - 0.6) · (0.1 - 0.2) μm	
Cell shape	short rods	
Pleomorphism	+	+
Mobility	-	-
Cilia	-	-
Gram staining	+	+
Spores	-	-
Acid resistance	-	-
Nitrate reduction	-	-
Denitrification	-	-
MR test	-	-
VP test	-	-
Hydrolytic cleavage of starch	+	+
Utilization of inorganic nitrogen sources	+	+
Stain formation	-	-
Urease	+	+
Oxidase	-	-
Catalase	+	+
Growth at [°C]	33	35
not above [°C]	34	36

Physiological and bacteriological properties	DSM 7329	DSM 7330
Growth at [% NaCl]	2%	5%
not over	3%	6%
Behaviour towards oxygen (aerobic +)	+	+
Nutrient requirement	-	-
Gluconic acid oxidation	-	-
Utilization of malonate	+	+
Formation of acid from sugar		
L-Arabinose	+	+
D-Xylose	+	+
D-Glucose	+	+
D-Mannose	+	+
D-Fructose	+	+
D-Galactose	+	+
Maltose	+	+
Sucrose	+	+
Lactose	+	+
Trehalose	+	+
D-Sorbitol	+	+
D-Mannitol	+	+
Inositol	+	+
Glycerol	-	-
Starch	-	-
Raffinose	+	+
Adonitol	-	-
Rhamnose	+	+
Utilization of carbon sources		
L-Arabinose	+	+
D-Xylose	+	+
D-Glucose	+	+

Physiological and bacteriological properties	DSM 7329	DSM 7330
D-Mannose	+	+
D-Fructose	+	+
D-Galactose	+	+
Maltose	+	+
Sucrose	+	+
Lactose	+	+
Trehalose	+	+
D-Sorbitol	+	+
D-Mannitol	+	+
Inositol	+	+
Glycerol	-	-
Starch	-	-
Raffinose	+	+
Adonitol	-	+/-
Rhamnose	+	+
Ethanol	+	+
ß-Alanine	+	-
D,L-Arginine	+	+
L-Histidine	+	+
L-Ornithine	+	+
L-Threonine	-	+
L-Valine	+	-
Formic acid	-	-
Acetic acid	-	-
Butyrate	-	-
D,L-Lactic acid	-	-
Propionic acid	-	-
meso-Tartaric acid	-	+
D-(-)-Tartaric acid	-	-
m-Hydroxybenzoic acid	-	-
p-Hydroxybenzoic acid	-	-
Glycolic acid	-	-

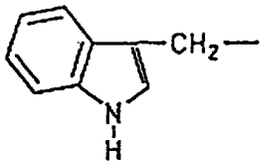
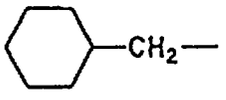
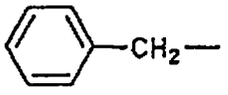
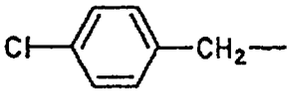
Physiological and bacteriological properties	DSM 7329	DSM 7330
Malonic acid	+	+
Succinic acid	-	-
Citric acid	-	-
α-Ketoglutarate	+	+
Pyruvate	+	+
Glyoxylate	-	-
Gluconic acid	-	-
Fumaric acid	+	-
Pimelic acid	-	-
Uric acid	-	+
Hippuric acid	+	+
Adipic acid	-	-
Glutaric acid	-	-
Pantothenic acid	-	-
Levulinic acid	-	-
Citraconic acid	-	-
Azelaic acid	-	-
Itaconic acid	-	-
Betaine	+	+
L-Methionine	-	-
L-Tryptophan	-	-
L-Phenylalanine	-	-

Mutants or variants with increased and/or more stable enzyme activities can be accumulated from these microorganisms by chemical and/or physical mutation or by selection in continuous culture.

Table 6 shows examples of the conversions of D,L-5-monosubstituted hydantoins which can be hydrolyzed with the microorganisms according to the invention *via* the N-carbamoyl-α-amino acids to L-α-amino acids according to the indicated reaction mechanism; within the limits of

experimental error, the same results are found with both strains.

Table 6:

R =	Amount introduced [mmol]	D,L-Hydantoin [mmol]	N-Carbamoyl derivative [mmol]	L-α-Amino acid [mmol]
$CH_3-S-CH_2-CH_2-$	11.5	-	-	11.4
$CH_3-CH_2-\underset{CH_3}{CH}-$	12.8	0.64	3.84	8.3
	8.7	-	-	8.6
	10.2	1.02	3.06	6.12
	10.5	-	-	10.4
	8.9	-	-	8.8

-: not detectable

Reaction conditions: The indicated substrate concentration is incubated with 1.5 g of WBM at 30°C for 24 h in 50 ml of 0.1 M sodium carbonate buffer of pH 8.5.

Example 2: Culture of the biomass

A 500 ml conical flask containing 100 ml of sterile nutrient medium I is inoculated with a platinum loop of the strain DSM 7330 from a slant agar culture and cultivated on the rotary shaker at 100 rpm and 30°C. After an incubation time of 24 h, this suspension culture is used as the inoculum for the 2nd preculture. Ten 2000 ml Erlenmeyer flasks containing 500 ml of sterile nutrient medium I are each inoculated with 10 ml of the 1st preculture and incubated again for 24 h at 100 rpm and 30°C. After the incubation period, this preculture II is used as the inoculum for a 50 l bioreactor. This is equipped with an Intensor system and, filled with 45 l of nutrient medium corresponding to the composition of nutrient medium II, is sterilized for 30 min at pH 6.5, a temperature of 121°C and an overpressure of *ca.* 1 bar. After cooling to 30°C, the contents are adjusted to pH 7.0 with 10% NaOH and then inoculated with the suspension culture from preculture II. Cultivation is carried out at 30°C, a stirrer speed of 400 rpm and an aeration rate of 0.8 v/v/m. After 18 h the wet cell mass is centrifuged off and then washed twice with 0.9% sodium chloride solution. The resulting wet cell mass can be used for the enzymatic reaction either immediately or at a later time after storage at -18°C. 1.725 kg of wet cell mass are obtained.

The strain DSM 7329 is cultivated analogously to give 1.69 kg of wet cell mass, corresponding to 0.34 kg of dry cell mass.

Example 3:

83 g of wet cell mass of DSM 7330 and 20 g (115 mmol) of D,L-methylthioethylhydantoin are suspended in 1000 ml of 0.1 M Soerensen buffer of pH 8.0 and incubated for 28 h in a 2 l stirring reactor at 35°C with the introduction of N_2

gas and with stirring. The cells are then centrifuged off and the supernatant is analyzed by high pressure liquid chromatography (HPLC). 16.4 g (110 mmol) of L-methionine were found in the supernatant.
Yield = 95.6% of theory.

Example 4:

100 g of wet cell mass of DSM 7330 and 10 g (52 mmol) of D-carbamoylmethionine are suspended in 1000 ml of 0.2 M Tris/ HCl buffer of pH 8.0 at 30°C and incubated for 9 h in a 2 l stirring reactor without the introduction of gas and with stirring. After centrifugation, 6.6 g (44 mmol) of L-methionine are found in the supernatant.
Yield = 84.6% of theory.

Example 5:

100 g of wet cell mass of DSM 7329 and 10 g (52 mmol) of L-carbamoylmethionine are suspended in 1000 ml of 0.2 M Tris/ HCl buffer of pH 8.0 at 30°C and incubated for 3.5 h in a 2 l stirring reactor without the introduction of gas and with stirring. After centrifugation, 7.68 g (51.5 mmol) of L-methionine are found in the supernatant.
Yield = 99% of theory.

Example 6:

100 g of wet cell mass of DSM 7330 and 10 g (52 mmol) of D,L-carbamoylmethionine are suspended in 1000 ml of 0.2 M Tris/HCl buffer of pH 8.0 at 30°C and incubated for 9 h in a 2 l stirring reactor without the introduction of gas and with stirring. After centrifugation, 7.16 g (48 mmol) of L-methionine are found in the supernatant.
Yield = 92% of theory.

Example 7:

100 g of wet cell mass of DSM 7330 and 10 g of technical-grade hydantoin, consisting of 10.4 mmol of D,L-carbamoyl-methionine and 41.6 mmol of D,L-methylthioethylhydantoin, are suspended in 1000 ml of 0.2 M Tris/HCl buffer of pH 8.0 at 30°C and incubated for 15 h in a 2 l stirring reactor without the introduction of gas and with stirring. After centrifugation, 7.01 g (47 mmol) of L-methionine are found in the supernatant.
Yield = 90.3% of theory.

Example 8: Preparation of a crude extract and purified enzyme solution

The microorganisms DSM 7329 and 7330 are ground in a glass bead mill for *ca.* 15 min and the biomass is then separated off by centrifugation. The supernatant is the crude enzyme extract and contains *ca.* 21 g/l of protein.

The crude extract is worked up by conventional methods to isolate the hydantoinase contained therein. In the present case, this is done by progressive fractional precipitation beginning with 1.7 M ammonium sulphate followed by separation with protamine sulphate (3 g/l) and then by precipitation of the protein with 2.5 M ammonium sulphate. The prepurified product obtained in this way is purified further by column chromatography using successively butylsepharose, phenylsepharose and anion exchange twice (pH 7.8, pH 6.6). The strain DSM 7329 has a protein content of 0.12 g/l after the 1st anion exchange chromatography and the strain DSM 7330 has a protein content of 0.1 g/l after the 2nd anion exchange chromatography (Bredford's method). The protein purity is determined by gel electrophoresis.

The crude enzyme extract (21 g/l) and the purified enzyme solution (0.1 g/l of hydantoinase) are used in the following Examples.

Example 9:

0.00575 mmol of D-methylthioethylhydantoin was incubated for 1 h with 100 l of enzyme in 900 l of 0.1 M Tris/HCl buffer of pH 8.5 at 42°C. When the activity test was complete, 0.00351 mmol of D-carbamoylmethionine was measured in the supernatant, corresponding to a conversion of 61 mol%.

Example 10:

0.00575 mmol of L-methylthioethylhydantoin was incubated for 1 h with 100 l of enzyme in 900 l of 0.1 M Tris/HCl buffer of pH 8.5 at 42°C. When the activity test was complete, 0.000863 mmol of L-carbamoylmethionine was measured in the supernatant, corresponding to a conversion of 15 mol%.

Example 11:

0.00521 mmol of D-carbamoylmethionine was incubated for 1 h with 100 l of enzyme in 900 l of 0.1 M Tris/HCl buffer of pH 8.5 at 42°C. When the activity test was complete, 0.000162 mmol of D-methylthioethylhydantoin was measured in the supernatant, corresponding to a conversion of 3.1 mol%.

Example 12:

0.00521 mmol of L-carbamoylmethionine was incubated for 1 h with 100 l of enzyme in 900 l of 0.1 M Tris/HCl buffer of pH 8.5 at 42°C. When the activity test was complete, 0.0000521 mmol of L-methylthioethylhydantoin was measured

in the supernatant, corresponding to a conversion of 1.0 mol%.

Example 13:

0.00521 mmol of L-carbamoylmethionine was incubated for 0.5 h with 100 l of crude extract in 900 l of 0.1 M Tris/HCl buffer of pH 8.5 at 42°C. When the activity test was complete, 0.00521 mmol of L-methionine was measured in the supernatant.

Claims

1. The microorganism deposited under DSM 7329 and DSM 7330.

2. A process for the production of L-α-amino acids by the enzymatic conversion of a D-, L- and/or D,L-5-monosubstituted hydantoin and/or a D-, L- and/or D,L-N-carbamoyl-α-amino acid, characterized in that the conversion is carried out by means of the microorganism or microorganisms DSM 7329 and/or DSM 7330 and/or by means of enzymes produced by these microorganisms.

3. A process according to Claim 2, characterized in that the conversion is carried out with dormant micro-organisms.

4. Use of the microorganism or microorganisms DSM 7329 and/or DSM 7330 for the culture of mutants or variants of these microorganisms,

or for the preparation of a gene coding for a carbamoylase and/or hydantoinase and/or hydantoin racemase,

or for the insertion of a gene coding for a carbamoylase and/or hydantoinase and/or hydantoin racemase into a microorganism or a cell.

* C L M S *