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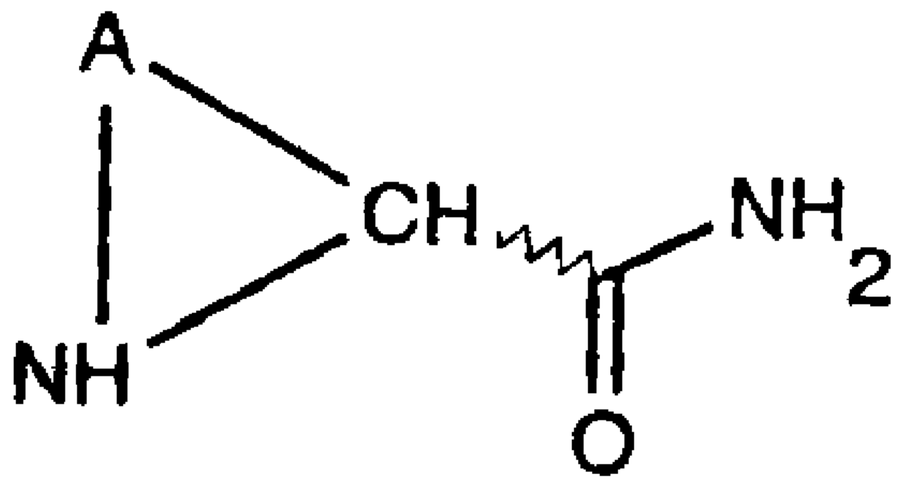
(54) Titre : PROCEDE BIOTECHNOLOGIQUE DE PREPARATION D'ACIDES S-α-AMINOCARBOXYLIQUES ET R-α -AMINOCARBOXAMIDES CYCLIQUES

(54) Title: BIOTECHNOLOGICAL PROCESS FOR THE PREPARATION OF CYCLIC S-α-AMINO CARBOXYLIC ACIDS AND R-α-AMINO CARBOXAMIDES



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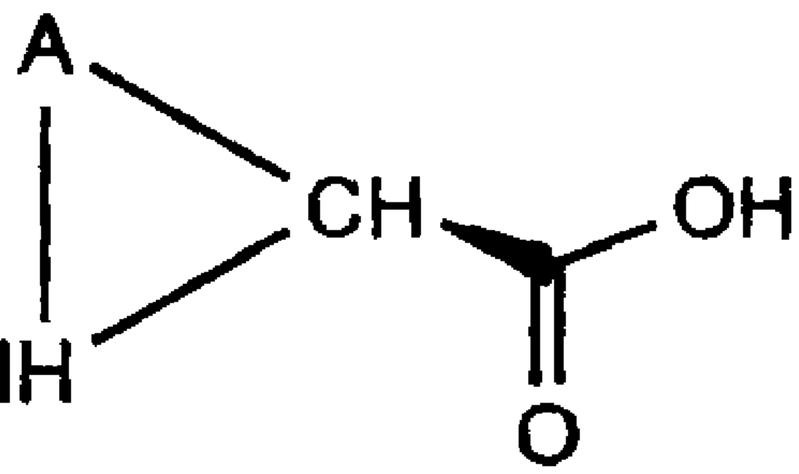

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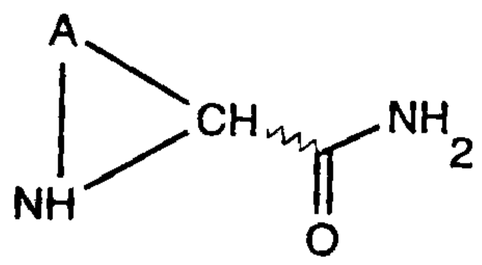
(57) Abrégé/Abstract:
Novel microorganisms which are capable of utilizing an α-imino carboxamide, in the form of the racemate or an optically active isomer thereof, of the general formula: (see formula I) in which A, together with -NH- and -CH-, forms an optionally substituted 5- or 6-membered saturated heterocyclic ring, as sole nitrogen source, and converting the (RS)-α-imino carboxamide of formula I into an S-α-imino carboxylic acid of the general formula: (see formula II) in which A has the above meaning.

ABSTRACT OF THE DISCLOSURE

Novel microorganisms which are capable of utilizing an α-imino carboxamide, in the form of the racemate or an optically active isomer thereof, of the general formula:



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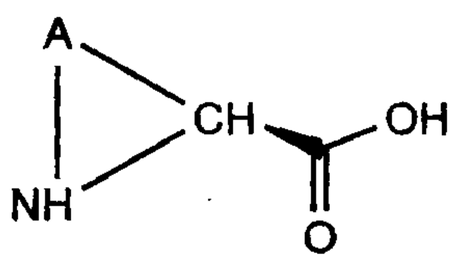
in which A, together with -NH- and -CH-, forms an optionally substituted 5- or 6-membered saturated heterocyclic ring, as sole nitrogen source, and converting the (RS)-α-imino carboxamide of formula I into an S-α-imino carboxylic acid of the general formula:

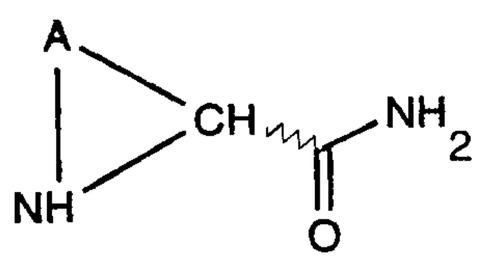


II

in which A has the above meaning.

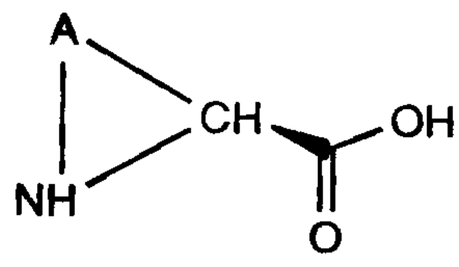
Biotechnological Process For The Preparation of Cyclic S-α-imino Carboxylic Acids And R-α-imino Carboxamides

This invention relates to novel microorganisms which are capable of utilizing α-imino carboxamides, in the form of the racemate or an optically active isomer, of the general formula:



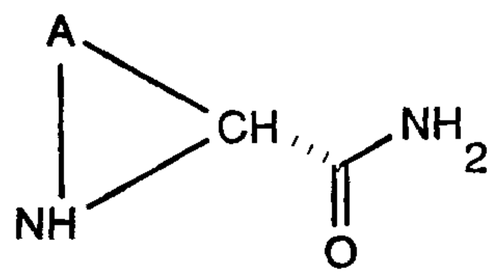
I

in which A, together with -NH- and -CH-, forms an optionally substituted 5- or 6-membered saturated heterocyclic ring, as sole nitrogen source, and converting the (RS)-α-imino carboxamide of general formula I into an S-α-imino carboxylic acid of the general formula:



II

in which A has the above meaning. These microorganisms and their cell-fee enzymes are used in a novel process for the preparation of S-α-imino carboxylic acids of formula II and/or for the preparation of R-α-imino carboxamides of the general formula:



III

in which A has the above meaning.

S-α-imino carboxylic acids of formula II, such as, for example, S-α-pipecolic acid, are important intermediates for the preparation of numerous bioactive compounds, such as, for example, thioridazine or pipradol (Ng-Youn-Chen et al., J. Org. Chem., Vol. 59, No. 8, 1994).

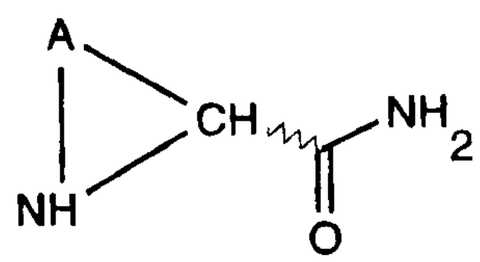
Besides numerous chemical racemate resolutions of (RS)-pipecolic acid and its derivatives, biotechnological racemate resolutions are also known. Thus, for example, Huh et al. (Biosci., Biotech. Biochem., 56(12), 2081 - 2082) describe the racemate resolution of (RS)-pipecolic acid using an R-amino-acid oxidase. This entails specific oxidation of the R-isomer to Δ^1-piperidine-2-carboxylic acid, resulting in S-pipecolic acid. After chemical reduction of the Δ^1-piperidine-2-carboxylic acid to (RS)-pipecolic acid, the action of the R-amino-acid oxidase then results in S-pipecolic acid again. This involves a continual decrease in the content of R-pipecolic acid. An analogous process for preparing S-proline is described in J. Ferm. Bioeng., 74, 189 - 190, 1992. However, these two processes have the disadvantage that they are not practicable on the industrial scale. Another disadvantage is that purified R-amino-acid oxidase must be used.

It is furthermore known that racemic pipecolic esters are converted under the action of a lipase from *Aspergillus niger* into S-pipecolic acid and R-pipecolic esters (Ng-Youn-Chen et al., 1994, ibid). However, this

process has the disadvantage that S-pipecolic acid is obtained with an ee (enantiomeric excess) purity of only 93%.

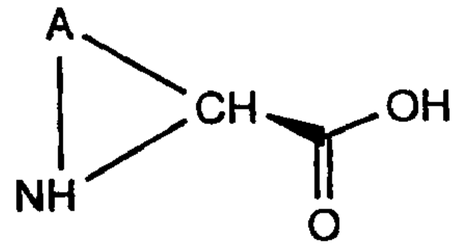
It is an object of the present invention to provide a simple and industrially practicable bio-technological process for the preparation of cyclic S-α-imino carboxylic acids, isolatable in good enantiomeric purity.

According to the invention, there is provided a microorganism capable of utilizing an α-imino carboxamide, in the form of the racemate or an optically active isomer thereof, of the general formula:



I

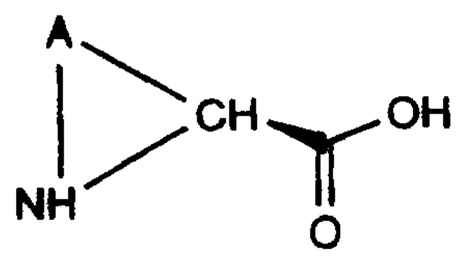
in which A, together with -NH- and -CH-, forms an optionally substituted 5- or 6-membered saturated heterocyclic ring, as sole nitrogen source, and converting the (RS)-α-imino carboxamide of formula I as defined into an S-α-imino carboxylic acid of the general formula:



II

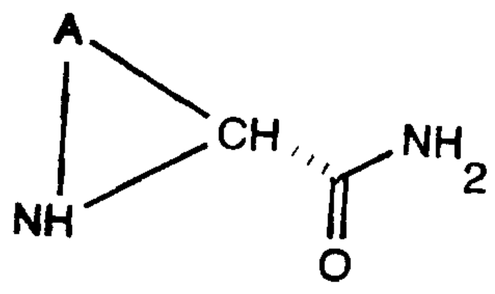
in which A has the above meaning, the microorganism being one selected from the group consisting of *Pseudomonas putida*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Klebsiella terrigena*, and functionally equivalent variants or mutants thereof.

Another aspect of the invention provides a process for the preparation of an S-α-imino carboxylic acid of the general formula:



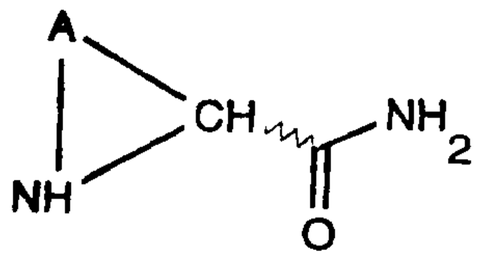
II

in which A has the above meaning, an R-α-imino carboxamide of the general formula:



III

in which A has the above meaning, or both thereof, the process comprising the steps of: a) biotransforming S-α-imino carboxamide in an (RS)-α-imino carboxamide of the general formula:



I

in which A has the above meaning, using a microorganism as defined above, or cell-free enzymes from such microorganism, into the corresponding S-α-imino carboxylic

acid (II), b) isolating the S-α-imino carboxylic acid produced in the biotransformation, and, optionally, c) isolating the residual R-α-imino carboxamide (III).

The microorganisms according to the invention can be isolated from soil samples, sludge or waste water with the assistance of conventional microbiological techniques. These microorganisms are isolated according to the invention by:

a) cultivating them in a medium with an α-imino carboxamide (formula I) in the form of the racemate or an optically active isomer thereof as sole nitrogen source and with a suitable carbon source in a conventional manner, and

b) selecting from the culture obtained by cultivation those microorganisms which are stable and are capable of converting an (RS)-α-imino carboxamide (formula I) into an S-α-imino carboxylic acid (formula II).

It is accordingly possible to use all microorganisms which specifically contain these S-amino-acid amidases. It is expedient to select microorganisms which utilize piperazinecarboxamide or pipecolamide, in the form of the racemate or of the optically active isomers, as sole nitrogen source. It is preferable to select those which utilize S-piperazinecarboxamide or S-pipecolamide as sole nitrogen source.

The microorganisms are able to utilize as carbon source, for example, sugars, sugar alcohols, carboxylic

acids or alcohols as growth substrate. Sugars which can be used include hexoses such as, for example, glucose or pentose. Carboxylic acids which can be used include di- or tricarboxylic acids or the salts thereof, such as, for example, citric acid or a succinate. It is possible to use as alcohol a trihydric alcohol such as, for example, glycerol. Preferably, a trihydric alcohol such as glycerol is used as carbon source.

The selection medium and culture medium can be those normally used by skilled workers, such as, for example, the mineral salt medium of Kulla et al. (Arch. Microbiol., 135, 1 - 7, 1983) or that described in Table 1. Preferably, the medium described in Table 1 is used.

The active enzymes of the microorganisms are expediently induced during cultivation and selection. It is possible to use as enzyme inducer, for example, piperazine-carboxamide, pipecolamide or acetamide.

The cultivation and selection expediently take place at a temperature of 15 to 50°C, preferably of 20 to 45°C, and at a pH between pH 5 and pH 10, preferably between pH 6 and pH 9.

Preferred microorganisms with specific S-amino-acid amidase activity are piperazinecarboxamide-utilizing microorganisms of the genus *Klebsiella*, in particular of the species *Klebsiella pneumoniae* with the number DSM 9175 and 9176, or of the species *Klebsiella terrigena* with the number DSM 9174, and their functionally equivalent variants and

mutants. These microorganisms were deposited on April 25, 1994 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-38124 Braunschweig, in accordance with the Budapest Treaty.

Further suitable microorganisms are pipecolamide-utilizing microorganisms of the genus *Pseudomonas*, in particular of the species *Pseudomonas putida* with the number DSM 9923, or of the species *Pseudomonas fluorescens* with the number DSM 9924, and their functionally equivalent variants and mutants. These microorganisms were deposited on April 20, 1995 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-38124 Braunschweig, in accordance with the Budapest Treaty.

By "functionally equivalent variants and mutants", are intended microorganisms which have essentially the same properties and functions as the original microorganisms. Variants and mutants of this type can be produced by chance, for example, by UV irradiation.

The scientific description of the microorganism DSM 9175 identified as *Klebsiella pneumoniae* is as follows:

Properties of the strain

Cell form	Rods
width µm	0.8 - 1.0
length µm	1.0 - 3.0
Motility	-
Gram reaction	-
Lysis by 3% KOH	+

Aminopeptidase (Cerny)	+
Spores	-
Oxidase	-
Catalase	+
Growth	
anaerobic	+
Acid from (OF test)	
glucose aerobic	+
glucose anaerobic	+
Gas from glucose	+
Acid from (ASA)	
glucose	+
fructose	+
xylose	+
erythritol	-
adonitol	+
D-mannose	+
L-rhamnose	+
dulcitol	-
inositol	+
sorbitol	+
α-methyl-D-glucoside	+
cellobiose	+
maltose	+
lactose	+
L-sorbose	+
L-fucose	-
D-arabitol	+
ONPG	+
ADH	-
LDC	+
ODC	-

VP	+
indole	-
H_2S production	-
Simmons citrate	+
phenylalanine deaminase	-
urease	-
hydrolysis of gelatin	-
DNA	-

Abbreviations:

ASA = acetylsalicylic acid

OF = oxidation fermentation

ONPG = O-nitrophenyl galactosidase

ADH = Alcohol dehydrogenase

VP = Voges Proskauer

The scientific description of the microorganism DSM 9176 identified as *Klebsiella pneumoniae* is as follows:

Properties of the strain

Cell form	Rods
width µm	0.8 - 1.0
length µm	1.0 - 3.0
Motility	-
Gram reaction	-
Lysis by 3% KOH	+
Aminopeptidase (Cerny)	+
Spores	-
Oxidase	-
Catalase	+
Growth	
anaerobic	+

Acid from (OF test)	
glucose aerobic	+
glucose anaerobic	+
Gas from glucose	+
Acid from (ASA)	
glucose	+
fructose	+
xylose	+
erythritol	-
adonitol	+
D-mannose	+
L-rhamnose	+
dulcitol	-
inositol	+
sorbitol	+
α-methyl-D-glucoside	+
cellobiose	+
maltose	+
lactose	+
L-sorbose	-
L-fucose	+
D-arabitol	+
ONPG	+
ADH	-
LDC	+
ODC	-
VP	+
indole	-
H_2S production	-
Simmons citrate	+
phenylalanine deaminase	-
urease	-

hydrolysis of gelatin	-
DNA	-

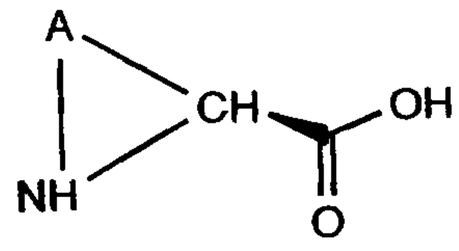
The scientific description of the microorganism DSM 9174 identified as *Klebsiella terrigena* is as follows:

Properties of the strain

Cell form	Rods
width µm	0.8 - 1.0
length µm	1.0 - 2.0
Motility	-
Gram reaction	-
Lysis by 3% KOH	+
Aminopeptidase (Cerny)	+
Spores	-
Oxidase	-
Catalase	+
Growth	
anaerobic	+
Acid from (OF test)	
glucose aerobic	+
glucose anaerobic	+
Gas from glucose	+
Acid from (ASA)	
glucose	+
fructose	+
xylose	+
erythritol	-
adonitol	+
D-mannose	+
L-rhamnose	+

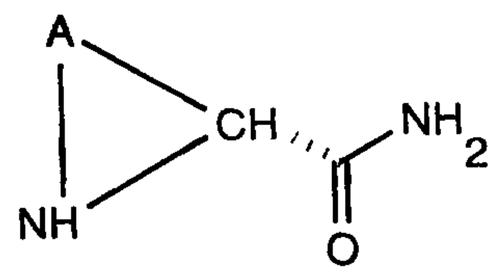
dulcitol	-
inositol	+
sorbitol	+
α-methyl-D-glucoside	+
cellobiose	+
maltose	+
lactose	+
L-sorbose	+
L-fucose	+
D-arabitol	+
5-ketogluconate	+
ONPG	+
ADH	-
LDC	+
ODC	+
VP	+
indole	-
H_2S production	-
Simmons citrate	+
phenylalanine deaminase	-
urease	-
hydrolysis of gelatin	-
DNA	-

The process according to the invention for the preparation of S-α-imino carboxylic acids of the general formula:



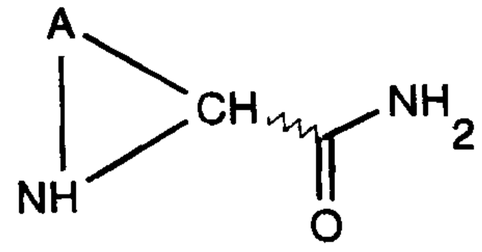
II

in which A has the above meaning, and/or of R-α-imino carboxamides of the general formula:



III

in which A has the above meaning, takes place by converting the S-α-imino carboxamide in the (RS)-α-imino carboxamide of the general formula:



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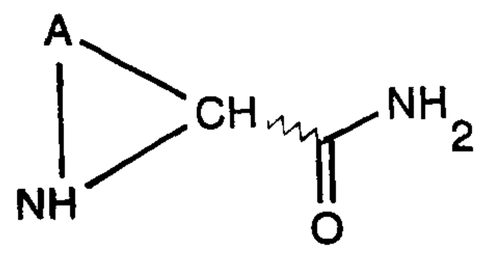
in which A has the above meaning, using the specific microorganisms which have already been described, or using cell-free enzymes from these microorganisms, whereby to form the S-α-imino carboxylic acid and isolating it, the biotransformation resulting not only in the S-α-imino carboxylic acid (II) but also the R-α-imino carboxamide (III), which is isolated where appropriate.

The precursors, namely the (RS)-α-imino carboxamides of the general formula:



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in which A, together with -NH- and -CH-, forms a 5- or 6-membered saturated heterocyclic ring, optionally substituted, can be obtained from the corresponding aromatic amides by a hydrogenation reaction familiar to the skilled worker.

Imino carboxamides of formula I with a 5-membered saturated heterocyclic ring which can be used include optionally substituted prolinamide, pyrazolidinecarboxamide, imidazolidinecarboxamide, oxazolidinecarboxamide, isoxazolidinecarboxamide, thiazolidinecarboxamide or triazolidinecarboxamide. Indolinamide can be used, for example, as substituted prolinamide.

Imino carboxamides of formula I with a 6-membered saturated heterocyclic ring which can be used include piperazinecarboxamide, pipecolamide, morpholinecarboxamide, perhydroquinolinecarboxamide (quinolinancarboxamide), perhydroisoquinolinecarboxamide (isoquinolinancarboxamide), perhydroquinoxalinecarboxamide (quinoxalinancarboxamide), which are likewise optionally substituted. Representatives of imino carboxamides with a substituted 6-membered saturated heterocyclic ring can be C_1-C_4-alkyl-substituted such as, for example, 4-methylpipecolamide, H_2N-CH_2-substituted such as, for example, 4-aminomethylpipecolamide

or CN-substituted such as 4-cyanopipecolamide. Piperazine-carboxamide, pipecolamide or 4-methylpipecolamide is preferably used.

The enzymes for the cell-free system can be obtained by disruption of the microorganisms in a manner familiar to the skilled worker. It is possible to use for this purpose, for example, the ultrasound, French press or lysozyme method. These cell-free enzymes can also be immobilized on a suitable carrier material.

Particularly suitable for the process are the previously described specific microorganisms of the species *Klebsiella terrigena* DSM 9174, *Klebsiella pneumoniae* DSM 9175 and DSM 9176, of the species *Pseudomonas putida* DSM 9923 and *Pseudomonas fluorescens* DSM 9924 and their cell-free enzymes. Preferably, the process is carried out with the microorganism *Klebsiella terrigena* DSM 9174, or *Klebsiella pneumoniae* DSM 9175 or DSM 9176. Likewise suitable are the functionally equivalent variants and mutants of the microorganisms described.

The biotransformation can be carried out after conventional cultivation of the microorganisms using dormant cells (cells which are not growing and no longer require a carbon or energy source) or using growing cells.

It is possible to use as medium for the process with dormant cells those familiar to the skilled worker, such as, for example, the mineral salt medium of Kulla et al., 1983 (ibid) described above, low-molar phosphate

buffer, HEPES buffer or the medium described in Table 1. Normally a medium containing a carbon source and nitrogen source, such as, for example, commercially available media or the medium shown in Table 1, is used for the process with growing cells. Preferably, the process is carried out in the medium shown in Table 1.

The biotransformation is expediently carried out with a single or continuous addition of (RS)-α-**imino** carboxamide in such a way that the concentration of (RS)-α-**imino** carboxamide does not exceed 20% by weight, preferably 10% by weight.

The pH of the medium can be within a range from pH 5 to pH 11, preferably from pH 7 to pH 10.

The biotransformation is expediently carried out at a temperature of 25 to 65°C, preferably of 30 to 60°C.

After a usual reaction time of 1 to 100 hours, the S-α-**imino** carboxamide of the formula I is completely converted into the S-α-**imino** carboxylic acid (II), with R-α-**imino** carboxamide (III) also resulting.

The S-α-**imino** carboxylic acid and/or the R-α-**imino** carboxamide obtained in this way can be isolated by conventional working-up methods such as, for example, by acidification, chromatography or extraction.

The following Examples illustrate the isolation of microorganisms and the performance of the process according to the invention:

Example 1

a) **Isolation of microorganisms capable of utilizing racemic piperazinecarboxamide as sole nitrogen source:**

The A medium whose composition is given in Table 1 was used to isolate microorganisms which are capable of utilizing racemic piperazinecarboxamide as sole nitrogen source. 100 mℓ of this medium were placed in a 300 mℓ Erlenmeyer flask, and various soil samples (2 g) from the area of the LONZA AG works in Visp, Switzerland were added. The flasks were incubated without agitation at 30°C for 5 days. Then 1 mℓ of this A medium was used to inoculate a fresh flask containing the same medium. This flask was in turn incubated under the same conditions. This enrichment cycle was repeated a total of 5 times. The enrichments were then streaked onto agar medium (A medium with the addition of 16 $g\ell^{-1}$ agar) to give single colonies.

The isolated microorganisms were investigated in the following qualitative test system for stereoselective amidases. Single colonies were used to inoculate 100 mℓ of A medium in 300 mℓ Erlenmeyer flasks. These flasks were incubated on a shaker at 30°C for 3 days, and the cultures were fully developed after only one day. The cell-free culture supernatants were then investigated by thin-layer chromatography (silica gel, mobile phase: 11 parts of ethanol, 6 parts of $CHCl_3$, 6 parts of NH_4OH (25%), detection

with ninhydrin) for the content of piperazinecarboxylic acid and piperazinecarboxamide. Microorganisms which had converted about half the amount of (RS)-piperazine-carboxamide used were used for biochemical investigations in order to establish which strains contained S-specific amidases.

b) **Biochemical investigations to identify micro-organisms with S-specific amidases:**

To prepare crude protein extract, the cells were grown in 1 ℓ of A medium at 30°C and then harvested and washed. 5 g of cells (wet weight) were resuspended in 10 mℓ of 69 mM phosphate buffer, pH 7.0, and disrupted using a FRENCH® press. The crude extract was centrifuged at 40,000 x g for 2 hours and then frozen in portions at -20°C. To determine the stereoselectivity, the hydrolysis rates for R-prolinamide and S-prolinamide were compared. The following enzyme assay was used for this purpose: assay volume 1 mℓ, containing 69 mM phosphate buffer, pH 7.0, 100 - 800 µg crude protein extract, 2 mg of S- or R-prolinamide·HCl, incubation time 1 - 24 hours, incubation temperature 30°C, detection with ninhydrin after thin-layer chromatography (see above). These amidases of the strains DSM 9174, DSM 9175 and DSM 9176 show very slow hydrolysis of R-prolinamide. These strains were used to prepare optically active cyclic α-amino-acid derivatives.

The crude extracts of the strains DSM 9175 and DSM 9176 showed under the same conditions hydrolysis of (RS)-piperazinecarboxamide and (RS)-pipecolamide. By changing the incubation temperature and the pH of the assay solution, it was found that the specific activity of the amidases was maximal between a temperature of 30 - 60°C and a pH of 7 - 10.

Table 1

A medium:

To form this medium, the minimal medium described below was additionally mixed with 2gℓ^{-1} (RS)-piperazine-carboxamide and 10 g of 10 gℓ^{-1} glycerol.

B medium:

To form this medium, the minimal described below was additionally mixed with 1 gℓ^{-1} (RS)-pipecolamide and 4 gℓ^{-1} glucose.

Minimal medium:

Composition	Concentration (mg/ℓ)
Yeast extract	500
Na_2SO_4	100
Na_2HPO_4	2000
KH_2PO_4	1000
NaCl	3000
$MgCl_2x6H_2O$	400
$CaCl_2x2H_2O$	14.5
$FeCl_3x6H_2O$	0.8
$ZnSO_4x7H_2O$	$100x10^{-3}$
$MnCl_2x4H_2O$	$90x10^{-3}$
H_3BO_3	$300x10^{-3}$
$CoCl_2x6H_2O$	$200x10^{-3}$
$CuCl_2x2H_2O$	$10x10^{-3}$
$NiCl_2x6H_2O$	$20x10^{-3}$
$NaMoO_4x2H_2O$	$30x10^{-3}$
EDTA Na_2x2H_2O	5
$FeSO_4x7H_2O$	2

Examples 2 to 4

Preparation of S-piperazinecarboxylic acid:

The following conditions were selected to prepare S-piperazinecarboxylic acid using the strains DSM 9174, DSM 9175 and DSM 9176. A 1.5 ℓ fermenter equipped with a pH control unit and with a working volume of 1 ℓ was used for the biotransformations. For fermentations in A medium, the amount of glycerol was increased to 30 $g\ell^{-1}$ and the amount of (RS)-piperazinecarboxamide was increased to 20 $g\ell^{-1}$. The cells were grown at pH 7.0, a temperature of 30°C and an aeration rate of 0.5 ℓmin^{-1}.

In one case (Example 4) the cells were initially grown under these conditions for 16 hours and then the temperature was increased to 40°C and the pH of the medium was increased to 8.0. After defined times, the amount of S-piperazinecarboxylic acid formed was estimated by thin-layer chromatography, and the fermentations were stopped after 36 - 72 hours, as soon as approximately half of the piperazine-carboxamide used had reacted. At this time, the optical densities of the cell suspension at 650 nm were between 6 and 10. To isolate S-piperazinecarboxylic acid, the cell-free solution was concentrated to 100 mℓ under reduced pressure. The solution was acidified to pH 1.0 with concentrated HCl in order to precipitate the acid as dihydrochloride. The isolated acid was recrystallized in 0.1 M HCl and dried. To determine the ee (enantiomeric

excess) of the acid formed, the acid was first derivatized with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and analysed by capillary electrophoresis (see Table 2 for the capillary electrophoresis conditions). The results are given in Table 3.

Table 2

Capillary electrophoresis conditions

CE apparatus	Hewlett-Packard HP ^{3D}CE
Detector	Hewlett-Packard diode array detector
Buffer	10 mM disodium hydrogen phosphate, 10 mM boric acid, 150 mM sodium dodecyl sulphate, pH 9.0
Electrolyte	900 mℓ of buffer plus 100 mℓ of methanol
Capillary	HP G1600-61211
Electric field	20 kV
Current	about 24 - 30 μA
Oven temperature	20°C
Detector setting	210 nm (bandwidth 5 nm)
Migration time	about 17.1 min (S acid) about 17.7 min (R acid)

Table 3

Example Strain	Crude product	Recrystallized	Yield	ee value
No. 2 DSM 9175	12.82 g	10.74 g	68.3%	99.6
No. 3 DSM 9174	15.53 g	13.1 g	83.3%	99.4
No. 4 DSM 9176	21.23 g	11.32 g	72.0%	99.6

Example 5

Preparation of S-pipecolic acid using *Klebsiella*:

Dormant cells of the *Klebsiella pneumoniae* strains DSM 9175 and DSM 9176 converted 20 $g\ell^{-1}$ (RS)-pipecolamide at 47°C and a pH of 8.0 into the (S)-acid within 6 hours.

When this conversion was carried out with *Klebsiella pneumoniae* DSM 9175, S-pipecolic acid with an ee of 96.5% was obtained.

Example 6

Preparation of S-4-methylpipecolic acid:

For this conversion, racemic 4-methylpipecolamide (substrate) was converted using the crude protein extract from *Klebsiella pneumoniae* DSM 9175 or *Klebsiella terrigena* DSM 9174 in analogy to Example 1b. After incubation at pH 8.0 and 47°C for 24 hours, about 50% of the amide used in a

0.2% strength substrate solution were converted (measured by TLC analysis).

Example 7

Isolation of microorganisms which utilize pipecolamide as sole nitrogen source:

100 mℓ of B medium were placed in 300 mℓ Erlenmeyer flasks, and approximately 2 g soil samples were added. The flasks were incubated without agitation at room temperature for 3 days. Subsequently fresh flasks containing the same medium were treated with 2 mℓ from the previous cultures and incubated without agitation at 30°C for 4 days. This enrichment cycle was repeated a total of 3 times, carrying out the two last steps under sterile conditions. The last step was additionally carried out on a shaker at 140 rpm. Subsequently the enrichments were streaked onto B medium with addition of 16 g/ℓ agar to give single colonies. Then those colonies with stable properties for converting (RS)-pipecolamide into pipecolic acid were selected. For this purpose, the cultures were streaked onto nutrient agar and, after growing for a few days, again streaked from the nutrient agar plate onto pipecolamide /glucose plates. The two strains which showed the required stable properties were identified after Gram staining and oxidase test with 20 NE API strips. Two *Pseudomonas*

species, namely *Pseudomonas putida* DSM 9923 and *Pseudomonas fluorescens* DSM 9924, were identified.

Example 8

Preparation of S-pipecolic acid:

The strains isolated in Example 7 were used to carry out 1% pipecolamide biotransformations (substrate concentration 1%) in 0.1 nM phosphate buffer at pH 7.0, 30°C and 130 rpm. The biomass from the precultures was concentrated and taken up in 0.1 M phosphate buffer, pH 7.0, in order to obtain a biomass concentration of $OD_{650\ nm}$ = 10. The mixtures were incubated on a shaker at 130 rpm and 30°C, and samples were taken at various times. The cell-free supernatants were investigated by thin-layer chromatography (silica gel, mobile phase: 11 parts of ethanol, 6 parts of $CHCl_3$, 6 parts of NH_4OH 25%) for the content of remaining amide and pipecolic acid formed. Subsequently the cell-free supernatants were subjected to HPLC investigation (isothiocyanate derivatization) in order to check the enantiomeric purity of the pipecolic acid formed. The results showed that *Pseudomonas putida* and *Pseudomonas fluorescens* produced S-pipecolic acid with an optical purity of more than 90%. The optimal conditions for the biotransformation with *Pseudomonas putida* were at pH 8.0 and a temperature of 30°C and for *Pseudomonas fluorescens* were pH 8.0 and 50°C. When the biotransformation was carried out with *Pseudomonas*

putida, S-pipecolic acid was obtained with an ee of 95.0%; when the biotransformation was carried out with *Pseudomonas fluorescens*, S-pipecolic acid with an ee of 97.3% was obtained.

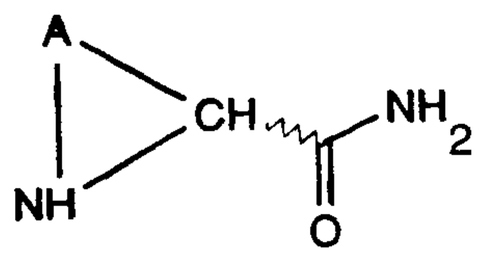
Example 9

Preparation of S-piperazinecarboxylic acid:

The two isolated strains described in Example 7 for pipecolamide were used to carry out a 1% piperazinecarboxamide biotransformation at pH 7.0, 30°C and 130 rpm. The cell-free supernatants were likewise investigated for the content of remaining piperazinecarboxamide and piperazinecarboxylic acid formed by thin-layer chromatography. HPLC analysis allowed checking of the optical purity of the piperazinecarboxylic acid formed. When the biotransformation was carried out with *Pseudomonas putida*, S-piperazinecarboxylic acid with an ee of 73.9% was obtained; when the biotransformation was carried out with *Pseudomonas fluorescens*, S-piperazinecarboxylic acid with an ee of 59.5% was obtained.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A microorganism capable of utilizing as sole nitrogen source an α-imino carboxamide, in the form of the racemate or an optically active isomer thereof, of the general formula:



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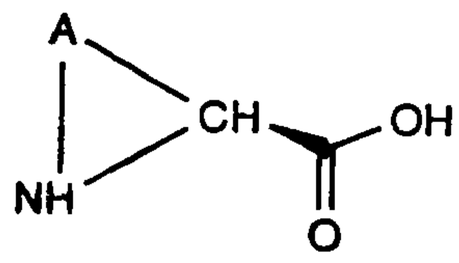
in which A, together with -NH- and -CH-, forms an optionally substituted 5- or 6-membered saturated heterocyclic ring, and converting the (RS)-α-imino carboxamide of formula I as defined into an S-α-imino carboxylic acid of the general formula:



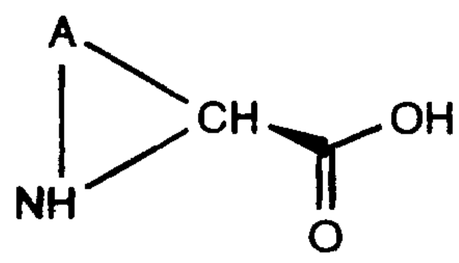
II

in which A has the above meaning, said microorganism being one selected from the group consisting of *Pseudomonas putida*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Klebsiella terrigena*, and functionally equivalent variants or mutants thereof.

2. A microorganism according to claim 1, which is capable of utilizing as sole nitrogen source pipecolamide or piperazine-carboxamide, in the form of the racemate or an optically active isomer thereof.

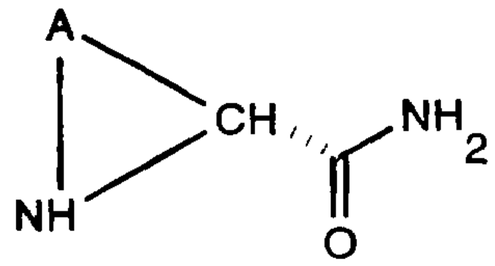
3. A microorganism according to claim 1 or 2 of the species *Pseudomonas putida* DSM 9923, *Pseudomonas fluorescens* DSM 9924, *Klebsiella terrigena* DSM 9174, or *Klebsiella pneumoniae* DSM 9175 or DSM 9176, or a functionally equivalent variant or mutant thereof.

4. A process for the preparation of an S-α-imino carboxylic acid of the general formula:



II

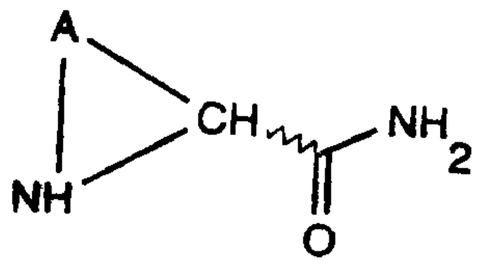
in which A has the meaning as defined in claim 1, an R-α-imino carboxamide of the general formula:



III

in which A has the above meaning, or both thereof, the process comprising the steps of a) biotransforming S-α-imino

carboxamide in an (RS)-α-imino carboxamide of the general formula:



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in which A has the above meaning, using a microorganism as defined in any one of claims 1 to 3, or cell-free enzymes from such microorganism, into the corresponding S-α-imino carboxylic acid (II), b) isolating the S-α-imino carboxylic acid produced in the biotransformation, and, optionally, c) isolating the residual R-α-imino carboxamide (III).

5. A process according to claim 4, wherein the biotransformation is carried out using a microorganism from the group consisting of the species *Klebsiella terrigena* DSM 9174, *Klebsiella pneumoniae* DSM 9175, *Klebsiella pneumoniae* DSM 9176, and functionally equivalent variants or mutants thereof, or cell-free enzymes from any of said microorganisms.

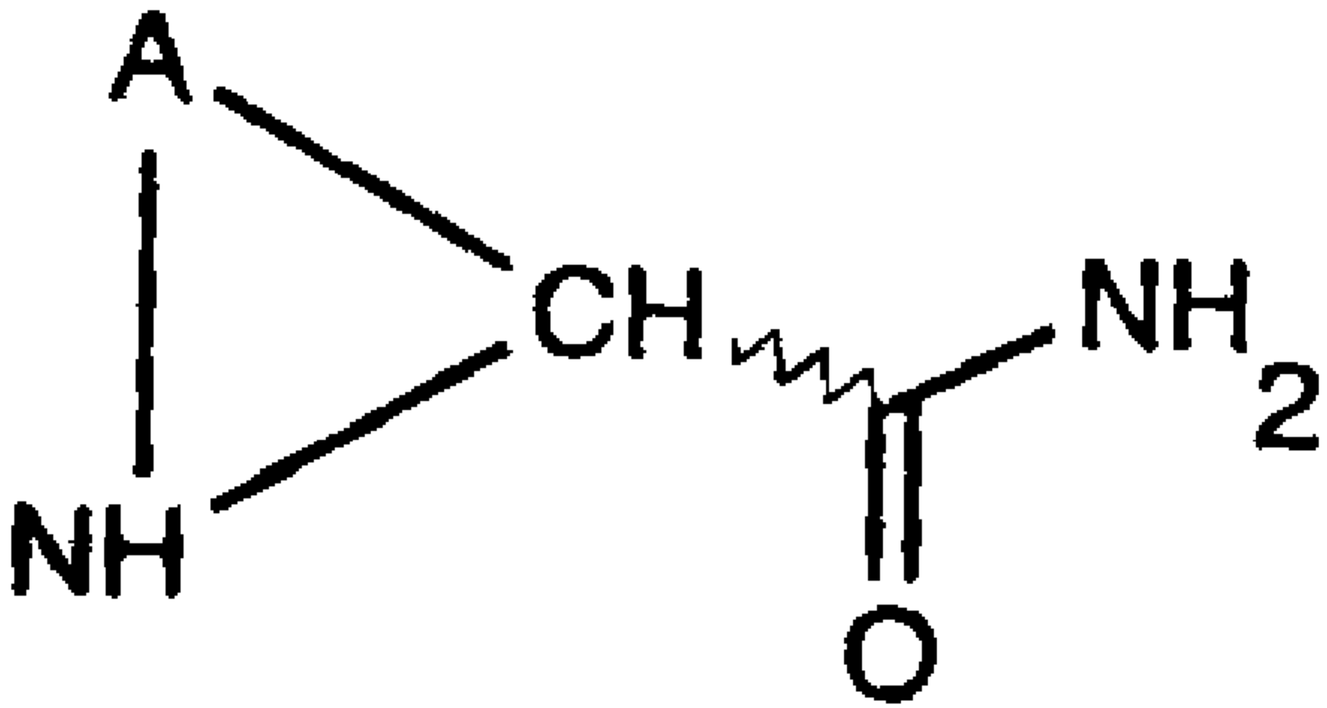
6. A process according to claim 4 or 5, wherein (RS)-pipecolamide, (RS)-piperazinecarboxamide, (RS)-4-methylpipecolamide or (RS)-prolinamide is used as the (RS)-α-imino carboxamide.

7. A process according to any one of claims 4 to 6, wherein the biotransformation is carried out with addition of the (RS)-α-imino carboxamide in such a way that the concentration of (RS)-α-imino carboxamide in the culture medium does not exceed about 20% by weight.

8. A process according to claim 7, wherein the (RS)-α-imino carboxamide is supplied in a single dose.

9. A process according to claim 7, wherein the (RS)-α-imino carboxamide is supplied continuously.

10. A process according to any one of claim 4 to 9, wherein the biotransformation is carried out at a pH of 7 to 10 and at a temperature of 30 to 60° C.



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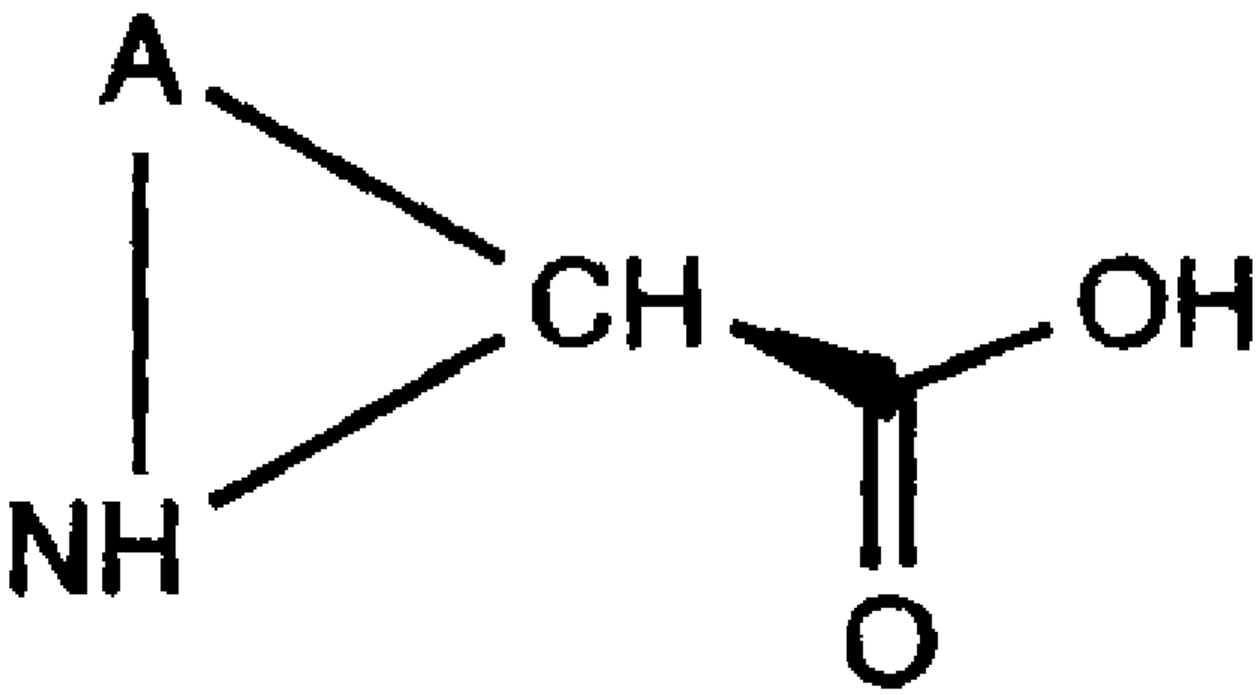

II