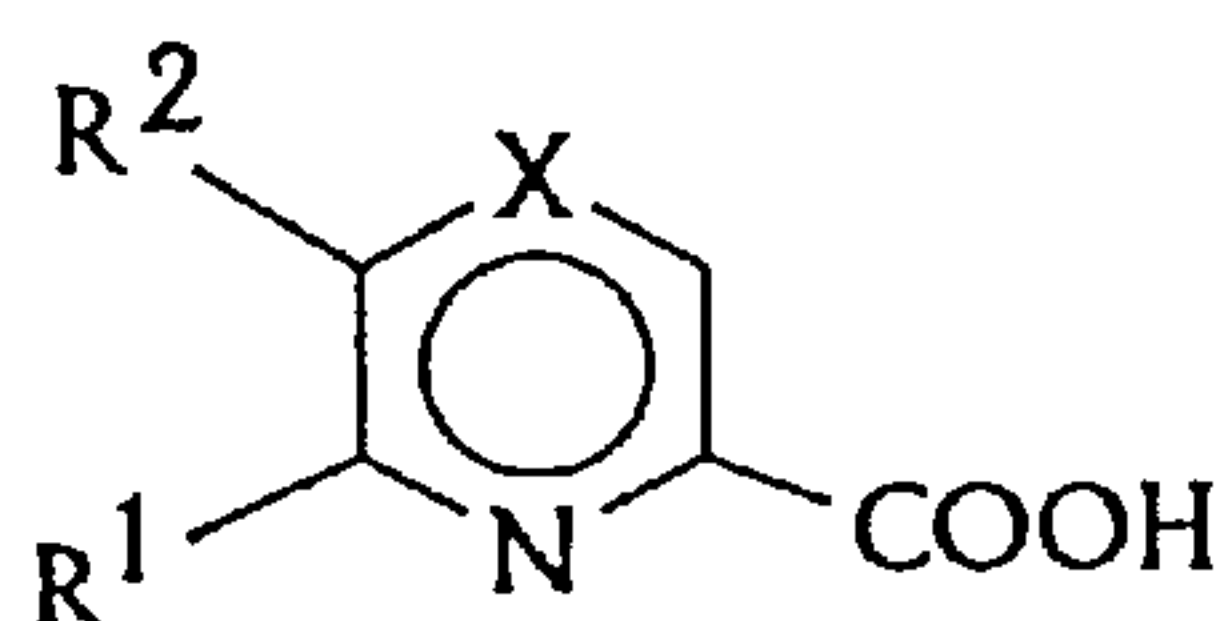




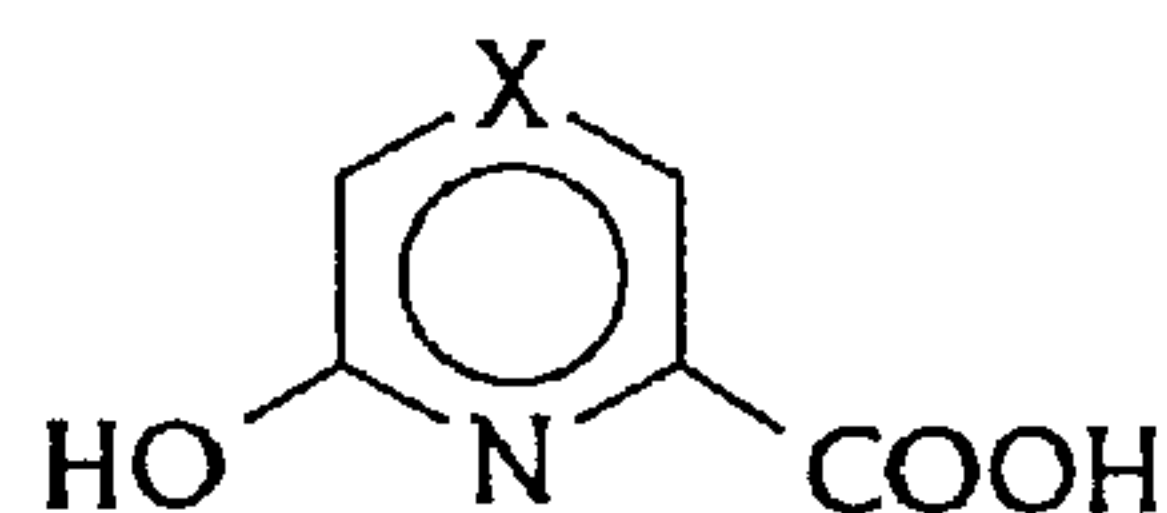
(22) Date de dépôt/Filing Date: 1996/05/29
(41) Mise à la disp. pub./Open to Public Insp.: 1996/12/08
(45) Date de délivrance/Issue Date: 2008/01/22
(30) Priorités/Priorities: 1995/06/07 (CH1664/95);
1995/06/13 (CH1733/95)

(51) Cl.Int./Int.Cl. *C12P 17/12* (2006.01)
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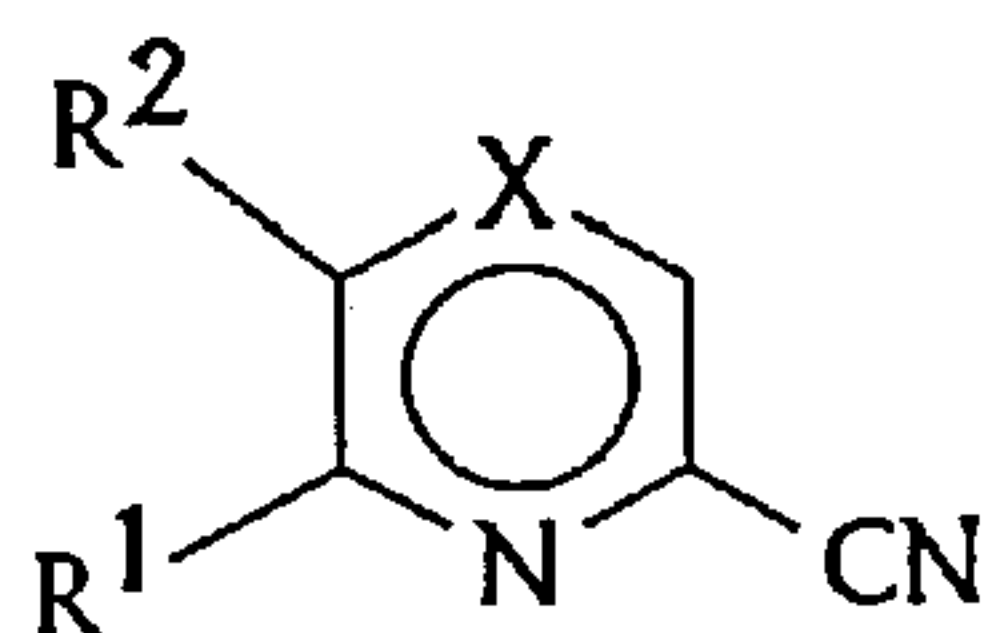
(54) Titre : METHODE MICROBIOLOGIQUE POUR LA PREPARATION D'ACIDES CARBOXYLIQUES
HETEROAROMATIQUES, A L'AIDE DE MICROORGANISMES DU GENRE ALCALIGENES
(54) Title: MICROBIOLOGICAL PROCESS FOR THE PREPARATION OF HETEROAROMATIC CARBOXYLIC ACIDS
USING MICROORGANISMS OF THE GENUS ALCALIGENES



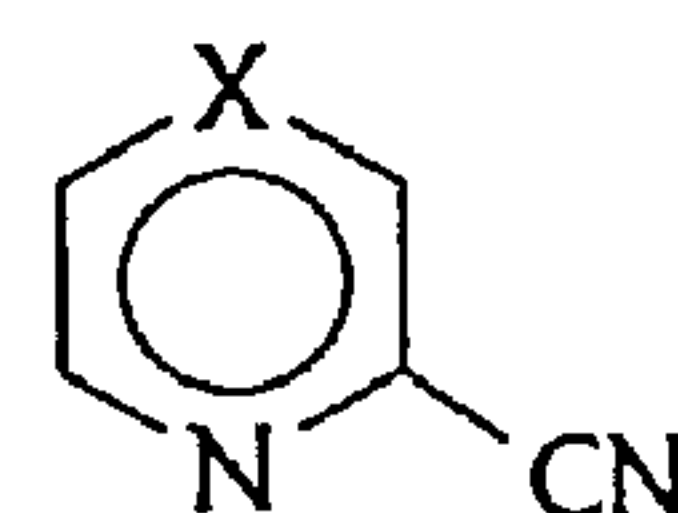
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II



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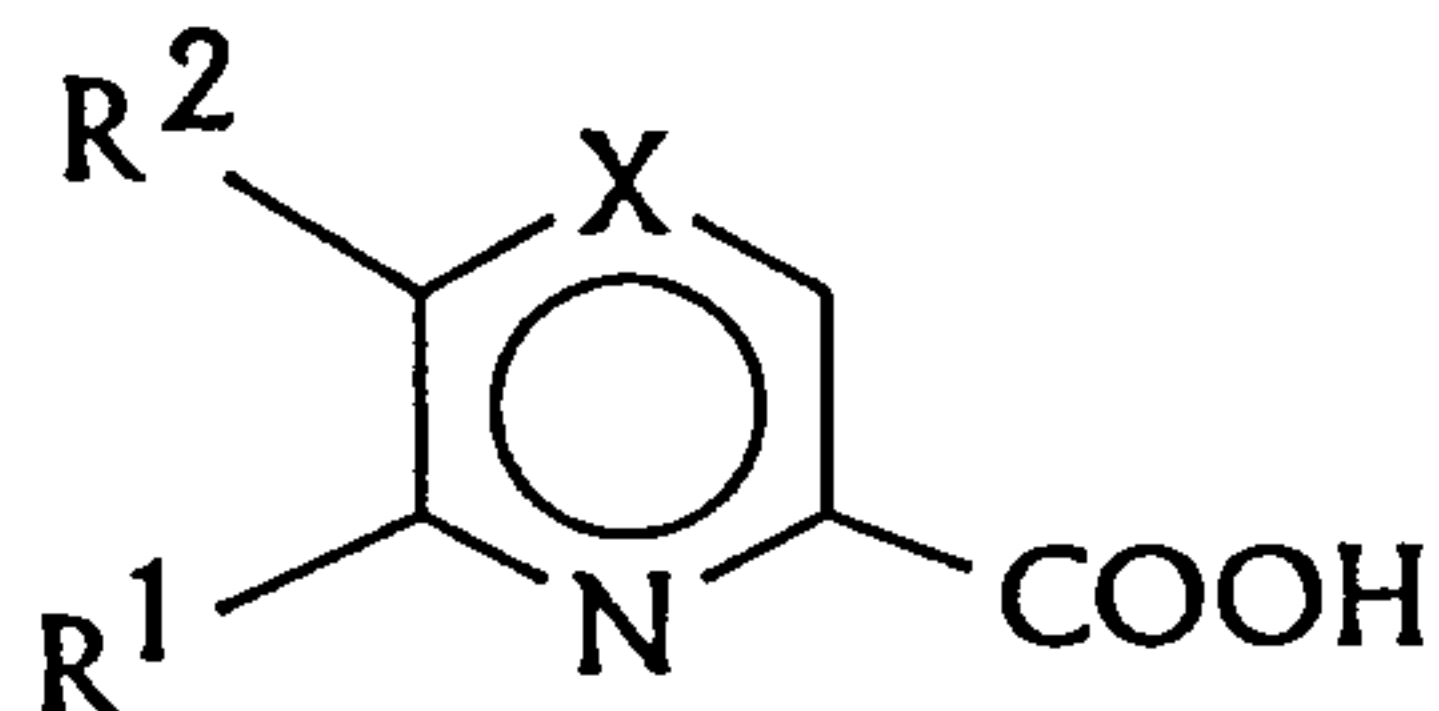
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(57) Abrégé/Abstract:

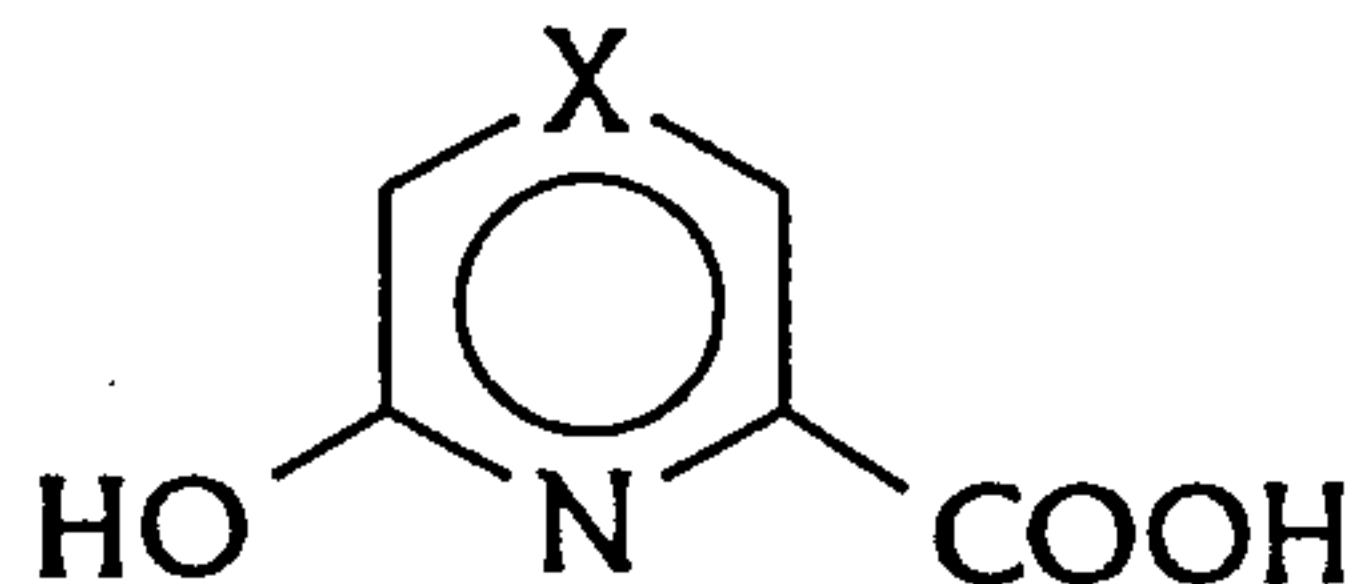
A microbiological process for the preparation of heteroaromatic carboxylic acids or physiologically tolerated salts thereof, of the general formulae: (see formula I) (see formula II) in which R¹ and R² are identical or different and denote a hydrogen or halogen atom and X denotes a nitrogen atom or -CH-, is described herein. The conversion is carried out in such a way that heteroaromatic nitriles of the general formulae: (see formula III) (see formula IV) in which R¹, R² and X are as previously defined, are converted as substrate, using 2-cyanopyridine-utilizing microorganisms of the genus *Alcaligenes* which have been cultured before the biotransformation in the presence of a dicarboxylic acid, tricarboxylic acid or a carbohydrate, into the corresponding carboxylic acid, and the latter is converted where appropriate into a physiologically tolerated salt thereof.

ABSTRACT OF THE DISCLOSURE

A microbiological process for the preparation of heteroaromatic carboxylic acids or physiologically tolerated salts thereof, of the general formulae:

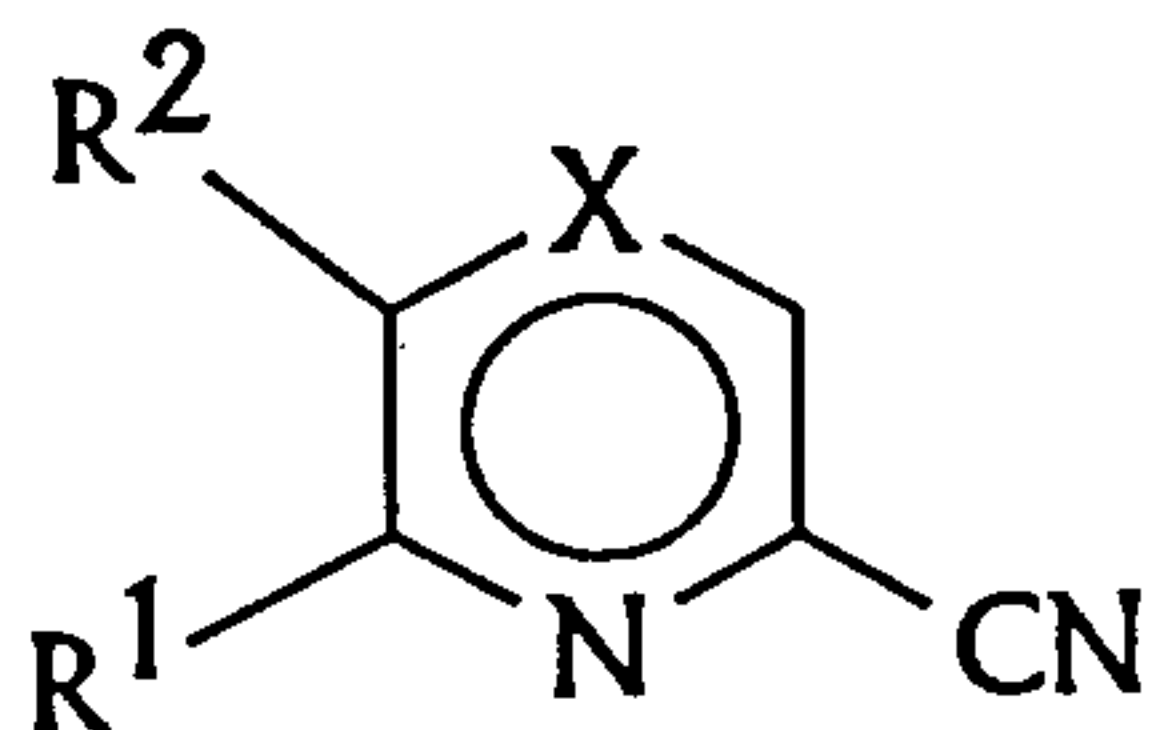


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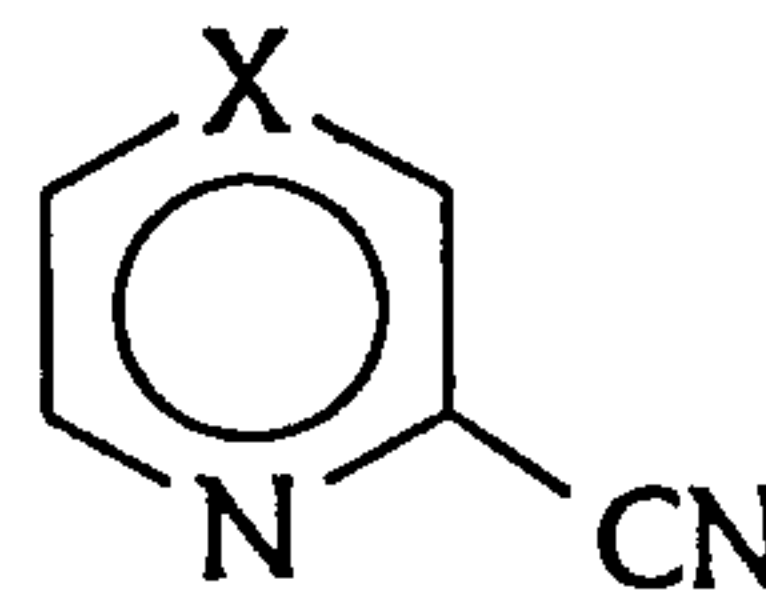


II

in which R^1 and R^2 are identical or different and denote a hydrogen or halogen atom and X denotes a nitrogen atom or -CH-, is described herein. The conversion is carried out in such a way that heteroaromatic nitriles of the general formulae:



III



IV

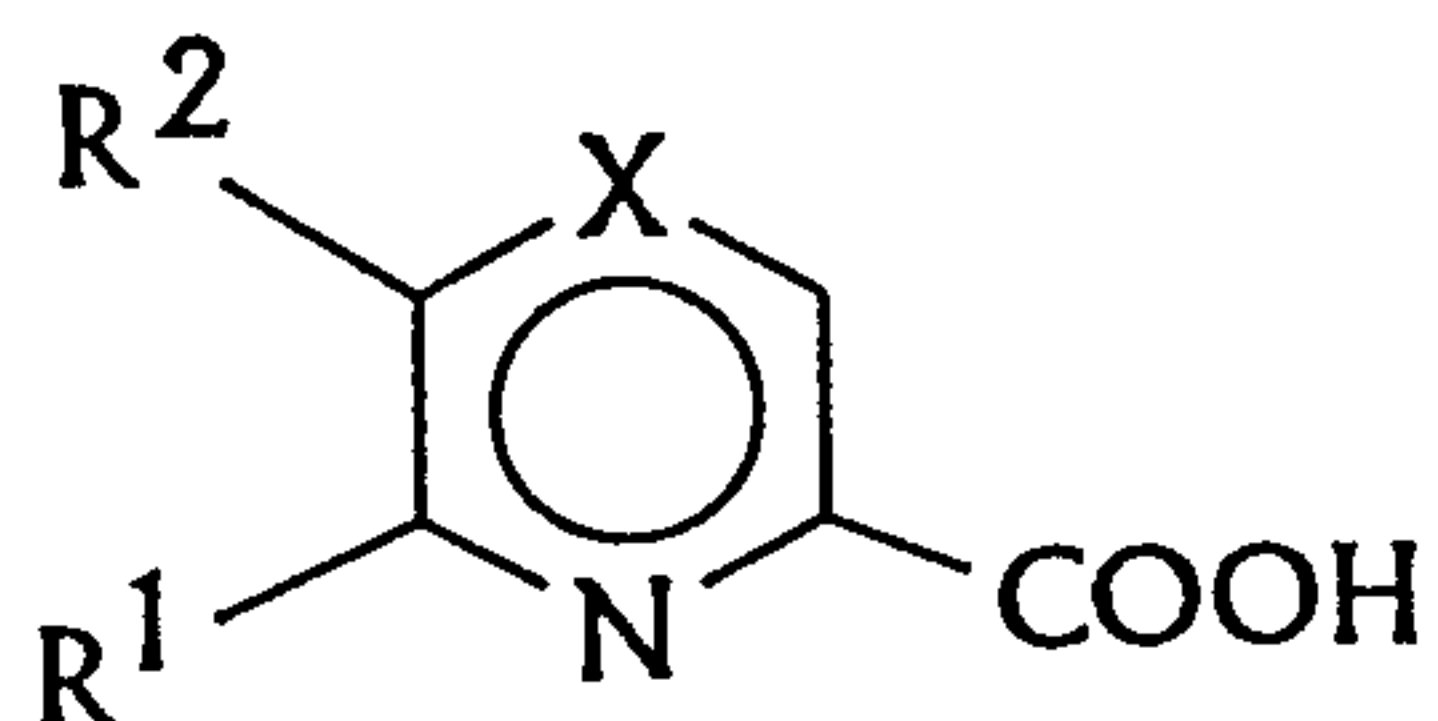
in which R^1 , R^2 and X are as previously defined, are converted as substrate, using 2-cyanopyridine-utilizing microorganisms of the genus *Alcaligenes* which have been cultured before the biotransformation in the presence of a

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dicarboxylic acid, tricarboxylic acid or a carbohydrate, into the corresponding carboxylic acid, and the latter is converted where appropriate into a physiologically tolerated salt thereof.

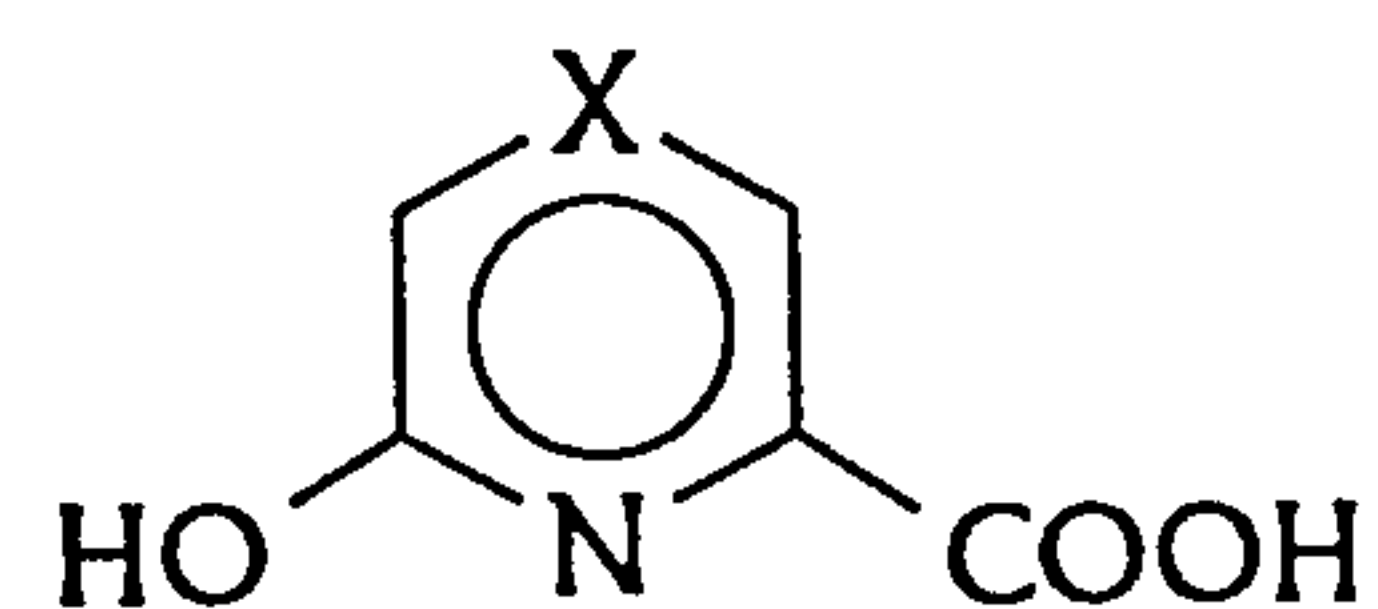
The invention relates to a novel microbiological process for the preparation of a heteroaromatic carboxylic acid of general formula I or II, or a physiologically tolerated salt thereof,

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I



II

in which R¹ and R² are identical or different and denote a hydrogen or halogen atom and X denotes a nitrogen atom or -CH-.

15

Heteroaromatic carboxylic acids, for example, 6-hydroxypicolinic acid, are important intermediate products for the preparation of pharmaceuticals, such as for the preparation of 2-oxypyrimidine (Berichte der Deutschen Chemischen Gesellschaft 1912, 45, pages 2456-2467) or for

20

the preparation of herbicides (EP-A 0 447 004). It is known in general that microorganisms containing nitrile hydratases and amidases or nitrilases convert nitriles into the corresponding acids. For example, EP-A 0 187 680 describes a microbiological process for the

25

preparation of organic acids such as, for example nicotinic acid using microorganisms of the genus *Corynebacterium*, *Nocardia*, *Bacillus*, *Bacteridium*, *Micrococcus* and

Brevibacterium. It is obligatory to carry out this reaction in the presence of light energy. EP-A 0 444 640 discloses a microbiological process for the preparation of organic acids, such as nicotinic acid, using microorganisms of the
5 genus *Rhodococcus*. It is obligatory to carry out this reaction in the presence of a lactam.

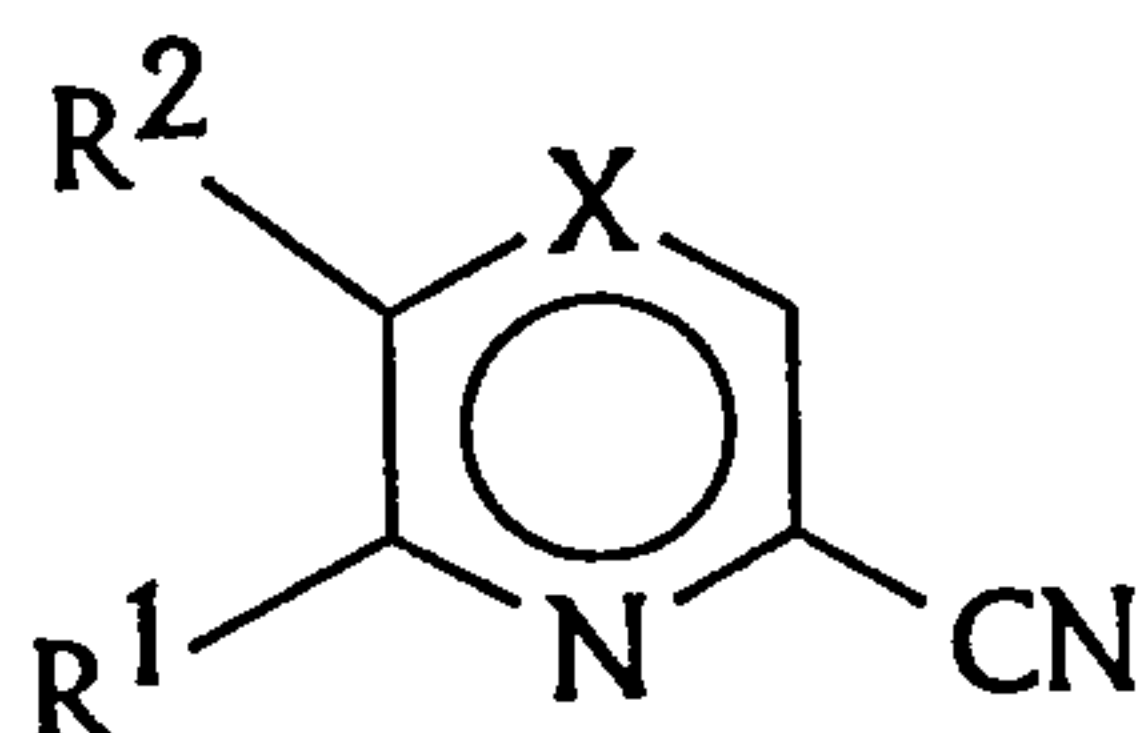
It is furthermore known that microorganisms of the species *Rhodococcus rhodochrous* J1 convert, for example, 2-cyanopyrazine to pyrazinecarboxylic acid (Kobayashi et al.,
10 J. of Antibiotics, Vol. 43, No. 10, 1990, pages 1316-1320). However, these microorganisms are unable to convert 2-cyanopyridine into picolinic acid (Mathew et al., Appl. Environmental Microbiology, Vol. 54, No. 4, 1988, pages 1030-1032).

15 It is also known that 2-cyanopyridine-utilizing microorganisms of the genus *Alcaligenes* convert 2-cyanopyridine into 6-hydroxypicolinic acid (EP-A 0 504 818). It is a disadvantage of this process that the 6-hydroxypicolinic acid is formed only in moderate yield.

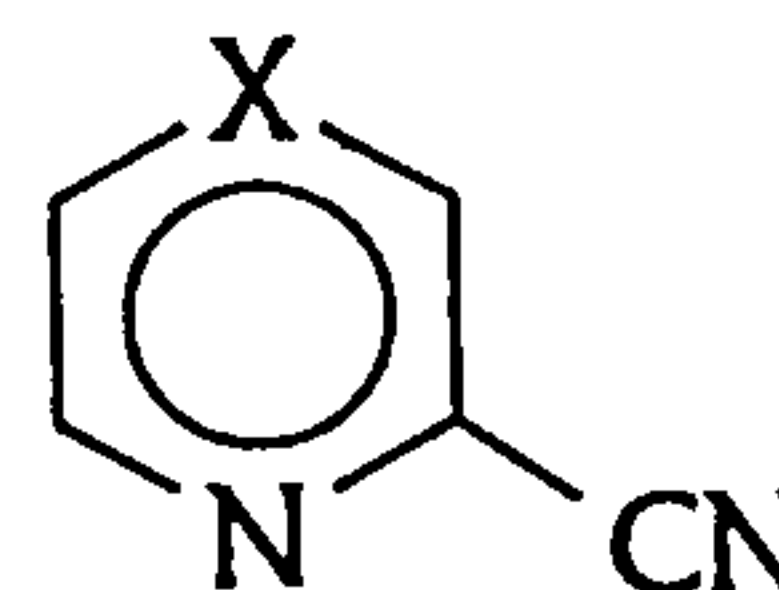
20 It was an object of the present invention to provide a more economic microbiological process for the preparation of heteroaromatic carboxylic acids or their physiologically tolerated salts such as pyrazinecarboxylic acid, picolinic acid or chromium picolinate using micro-
25 organisms of the genus *Alcaligenes*, wherein the formed carboxylic acids or their physiologically tolerated salts are formed in good yield.

This object has been achieved with the process outlined below.

The process is carried out according to the invention in such a way that a heteroaromatic nitrile of the
5 general formula III or IV,



III



IV

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in which X, R¹ and R² have the previously stated meaning, is converted as substrate, using 2-cyanopyridine-utilizing
15 microorganisms of the genus *Alcaligenes* which have been cultured before the biotransformation in the presence of a dicarboxylic acid, tricarboxylic acid or of a carbohydrate, into a heteroaromatic carboxylic acid according to formula I or II. The heteroaromatic carboxylic acid is then
20 converted where appropriate into a physiologically tolerated salt thereof. Physiologically tolerated salts of these carboxylic acids means hereinafter, for example, chromium, calcium or ammonium salts.

Before the actual biotransformation, the micro-
25 organisms of the genus *Alcaligenes* used for the process are normally cultivated (cultured) and their effective enzymes are expediently induced with 2-cyanopyridine. 2-Cyano-

pyridine can be used for culture and induction at a concentration of 0.01 to 20% by weight, preferably at a concentration of 0.1 to 1% by weight.

A dicarboxylic acid means hereinafter fumaric acid, succinic acid, maleic acid, glutaric acid, malonic acid or their salts and derivatives such as esters.

A tricarboxylic acid means hereinafter citric acid, isocitric acid or their salts and derivatives such as esters. Salts and derivatives of these dicarboxylic acids and tricarboxylic acids which can be used are fumarate, malate, malonate, oxalacetate, citrate, aconitate, isocitrate, 2-oxoglutarate, succinate or succinyl-CoA. Fumarate, malonate or succinate is preferably used.

Carbohydrates mean hereinafter monosaccharides such as glucose, disaccharides such as sucrose, trehalose or maltose, trisaccharides such as raffinose, sugar alcohols such as glycerol. Glycerol is preferably used as carbohydrate.

The dicarboxylic acid, tricarboxylic acid or the carbohydrate is expediently used at a concentration of 0.1 to 20% by weight, preferably at a concentration of 0.5 to 5% by weight.

It is possible to use as culture medium the media customary among those skilled in the art, such as, for example, the mineral salt medium of Kulla et al. (Arch. Microbiol., 135, 1-7, 1983), low molarity phosphate buffer

or a medium as shown in Table 1. The medium described in Table 1 is preferably used.

After the culture phase and before the actual addition of the substrate, either the microorganisms are
5 harvested by conventional separation processes, or the substrate is added directly to the microorganisms.

The substrates used for the biotransformation, the heteroaromatic nitriles of the formulae III and IV, such as, for example, 2-cyanopyridine, are purchasable compounds.

10 X in the general formulae I to IV denotes a nitrogen atom or -CH-, preferably -CH-. The radicals R¹ and R² are identical or different and denote hydrogen or halogen such as fluorine, chlorine, bromine or iodine. Possible substrates are, accordingly, 2-cyanopyridine, 6-chloro-2-
15 cyanopyridine, 5,6-dichloro-2-cyanopyridine, 2-cyanopyrazine, 6-chloro-2-cyanopyrazine, 5-bromo-6-chloro-2-cyanopyrazine, etc. 2-Cyanopyridine, 2-cyanopyrazine or 6-chloro-2-cyanopyridine is expediently used as a substrate.

The substrate can be added all at once or
20 continuously for the biotransformation. The substrate is advantageously added in such a way that the substrate concentration in the medium does not exceed 20% by weight, preferably in such a way that the substrate concentration does not exceed 10% by weight.

25 The biotransformation, which is normally carried out with stationary cells, is advantageously carried out using the 2-cyanopyridine-utilizing microorganisms of the

species *Alcaligenes faecalis* which are disclosed in EP-A 0
504 818 and are designated DSM 6335, or using their
functionally equivalent variants or mutants. These micro-
organisms were deposited on 03.01.1991 at the Deutsche
5 Sammlung von Mikroorganismen und Zellkulturen GmbH,
Mascheroder Weg 1b, D-38124 Braunschweig, in accordance with
the Budapest treaty.

"Functionally equivalent variants or mutants"
means microorganisms which have essentially the same
10 properties and functions as the original microorganisms.
Variants and mutants of this type can be formed for example
by UV irradiation.

The same media can be used for the bio-
transformation as for the culturing of the microorganisms.
15 The biotransformation can also take place in the presence or
absence of the dicarboxylic acids, tricarboxylic acids or
carbohydrates described above.

The pH is expediently in the range from 4 to 10,
preferably in the range from 5 to 9. The biotransformation
20 can be carried out at a temperature from 10 to 50°C,
preferably at a temperature from 20 to 40°C.

After a usual conversion time of 6 to 100 hours,
the appropriate carboxylic acids according to formula I or
II can then be obtained by customary working up methods such
25 as, for example, by acidification. The carboxylic acids can
also be isolated in the form of salts such as, for example,
ammonium or chromium salt.

If the prepared heteroaromatic carboxylic acids are heteroaromatic carboxylic acids hydroxylated in position 6 (general formula II), the biotransformation is expediently carried out under aerobic conditions. If, however, a non-
5 hydroxylated heteroaromatic carboxylic acid such as, for example, picolinic acid is prepared, the biotransformation is expediently carried out under anaerobic conditions.

Examples

10

Example 1

Preparation of 6-hydroxypicolinic acid

The conditions chosen for the preparation of 6-hydroxypicolinic acid using the strain *Alcaligenes faecalis*
15 DSM 6335 were as follows. A 7.5 l fermenter with an operating volume of 5 l was used. *Alcaligenes faecalis* DSM 6335 was cultured in a mineral salt medium (Table 1) with sodium fumarate as the sole source of carbon and energy, and 2-cyanopyridine as inducer at 30°C, 600 rpm and a pH of 7.0.
20 The aeration rate was about 3 l/minute during this. Addition of the sodium fumarate took place under pO₂ control when the pO₂ was >30%. A 20% strength stock solution of sodium fumarate, to which 0.5% 2-cyanopyridine was added, was used. The cells were cultured until the optical
25 density, measured at 650 nm (OD₆₅₀), was 16 over the course of 23 hours before the biotransformation was started. For

the growth phase, about 160 g of sodium fumarate in the form of a 20% strength solution (about 800 ml) was used.

No source of carbon or energy was added during the aerobic biotransformation of 2-cyanopyridine to 6-hydroxy-
5 picolinic acid. The biotransformation took place with stationary cells.

The addition of the 2-cyanopyridine took place by means of a pump with limitation. The pump rate was monitored "online" by means of HPLC. The concentration of
10 the intermediate picolinic acid, for which the rate of formation is about 2.5 times greater than the rate of conversion of picolinic acid into 6-hydroxypicolinic acid (10 g/l per hour versus 4 g/l per hour), was limited to values <2 g/l, since otherwise the conversion of picolinic
15 acid into 6-hydroxypicolinic acid was inhibited.

Since 2-cyanopyridine is a solid at room temperature, it was necessary to heat the reservoir containing 2-cyanopyridine to 50°C, making it possible to add 2-cyanopyridine in liquid form. It was possible with
20 this process to prepare 75 g/l 6-hydroxypicolinic acid within 31 hours. The intermediate picolinic acid was no longer detectable at the end of the biotransformation.

To isolate the 6-hydroxypicolinic acid, the cells were removed by filtration. The cell-free solution was then
25 heated to 60°C and acidified with concentrated sulphuric acid to a pH of 2-2.5. At this pH, the 6-hydroxypicolinic acid was precipitated from the solution.

It was then slowly cooled, with stirring, to 4°C and filtered, and the residue was washed with demineralized water and dried (100 mbar, 55°C). About 2 g/l 6-hydroxypicolinic acid remained in the mother liquor from this. The yield was 87% based on the quantity of 2-cyanopyridine used.

Table 1

Composition	Concentration (g/l)
Disodium fumarate	10
Yeast extract	1
MgCl ₂ · 6H ₂ O	0.8
Na ₂ SO ₄	0.25
(NH ₄) ₂ SO ₄	1.0
NH ₄ Cl	2.33
NaCl	0.2
CaCl ₂ · 2H ₂ O	0.16
MnSO ₄	1.8x10 ⁻²
H ₃ BO ₃	3x10 ⁻²
NiCl ₂	2x10 ⁻³
NaMoO ₄	3x10 ⁻³
FeSO ₄ · 7H ₂ O	0.3
Na ₂ EDTA · 2H ₂ O	0.75
2-Cyanopyridine	1
KH ₂ PO ₄	0.4
Na ₂ HPO ₄	0.96

Example 2**Preparation of picolinic acid**

The biomass was cultured as described in Example 1. The formation of picolinic acid took place under strictly anaerobic conditions. A 500 ml glass bottle with a rubber septum was charged with 400 ml of biomass of $OD_{650}=20$ and was used for the biotransformation. Incubation took place at 30°C. Before the biotransformation was started, the mixture was made anaerobic with pure nitrogen. For this purpose, nitrogen (50 mbar gauge pressure) was passed through a needle into the stirred mixture for about 30 minutes in order to drive out the oxygen quantitatively. In order to prevent oxygen entering during the biotransformation or on addition of the 2-cyanopyridine, the gas introduction was maintained during the biotransformation (about 10 mbar gauge pressure). 2-Cyanopyridine was added in 12 steps each of 10 g/l, in each case after one hour had elapsed. The addition can, however, also take place continuously. HPLC was used to check whether 2-cyanopyridine had been completely converted into picolinic acid before adding another portion. During the biotransformation there was no detectable formation of picolinamide. It was possible with this process to prepare about 150 g/l picolinic acid within 26 hours. 6-Hydroxypicolinic acid was not formed during this.

For isolation, the cell-free picolinic acid solution was precipitated with $CaCl_2/H_2SO_4$. For this purpose,

the cell-free picolinic acid solution from Example 2 was diluted 3-fold and, while stirring, 0.5 equivalent of CaCl_2 per equivalent of picolinic acid was added after the cell-free fermentation solution had been heated to 90°C . The resulting calcium/picolinic acid complex precipitated immediately. The resulting complex was cooled to 4°C with stirring, filtered off on a glass frit (porosity 3) and washed with demineralized water. The filter cake was suspended in demineralized water and acidified to a pH of 2.5 with concentrated sulphuric acid. During this the picolinic acid was dissolved out of the complex and, at the same time, insoluble calcium sulphate was formed. Since free picolinic acid is very soluble in water it was possible to remove calcium sulphate by filtration. The picolinic acid solution was evaporated to dryness and analyzed. The crude yield was about 70% with a purity of 86% by titration. The water content was 0.7% measured by the Karl-Fischer method.

20 **Example 3**

Preparation of chromium(III) picolinate

Aqueous chromium trichloride hexahydrate solution (23.95 g, 0.09 mol of Cr in 63 ml of water) was added dropwise over a period of 3.5 hours to an ammonium picolinate solution (271.4 g; 0.325 mol; 16.8%), pH 7.1, 73°C in a 500 ml flask. The resulting violet solution was stirred for a further 1 hour and then slowly cooled to 3°C .

After the red solid which had formed had settled out, the upper blue phase was decanted off. The solid was suspended in 100 ml of water for 30 minutes, and decantation was repeated. After a second suspension in 50 ml of water (30
5 minutes), the solid was filtered off with suction and dried at 50°C in vacuo. 33.64 g of dark red crystals were obtained (90% yield).

Example 4

10 **Culturing of *Alcaligenes faecalis* DSM 6335 with various sources of carbon**

300 ml conical flasks containing 100 ml of A+N medium (Table 1 without disodium fumarate) were used for culturing *Alcaligenes faecalis* (DSM 6335) in addition, 2 g/l
15 2-cyanopyridine and 10 g/l of the following sources of carbon were added to the medium:

disodium fumarate

glycerol

disodium malonate

20 disodium succinate.

Incubation took place in a shaker at 30°C. After growth for 16 hours, the cells were spun down and resuspended in fresh A+N medium (without a source of carbon) containing 10 g/l 2-cyanopyridine. The optical density of
25 the cell suspension, measured at 650 nm (OD_{650}), was 10. The cell suspensions (total volume 10-20 ml) were then incubated again at 30°C. The formation of 6-hydroxypicolinic acid was followed by spectrophotometry, measuring the absorption of

the cell-free solution at 308 nm. The following average productivities were determined for the formation of 6-hydroxypicolinic acid:

Carbon	Productivity (in g/l per hour)
Disodium fumarate	2.4
Glycerol	2.0
Disodium malonate	4.2
Disodium succinate	0.14

Example 5

Preparation of 6-hydroxypyrazinecarboxylic acid

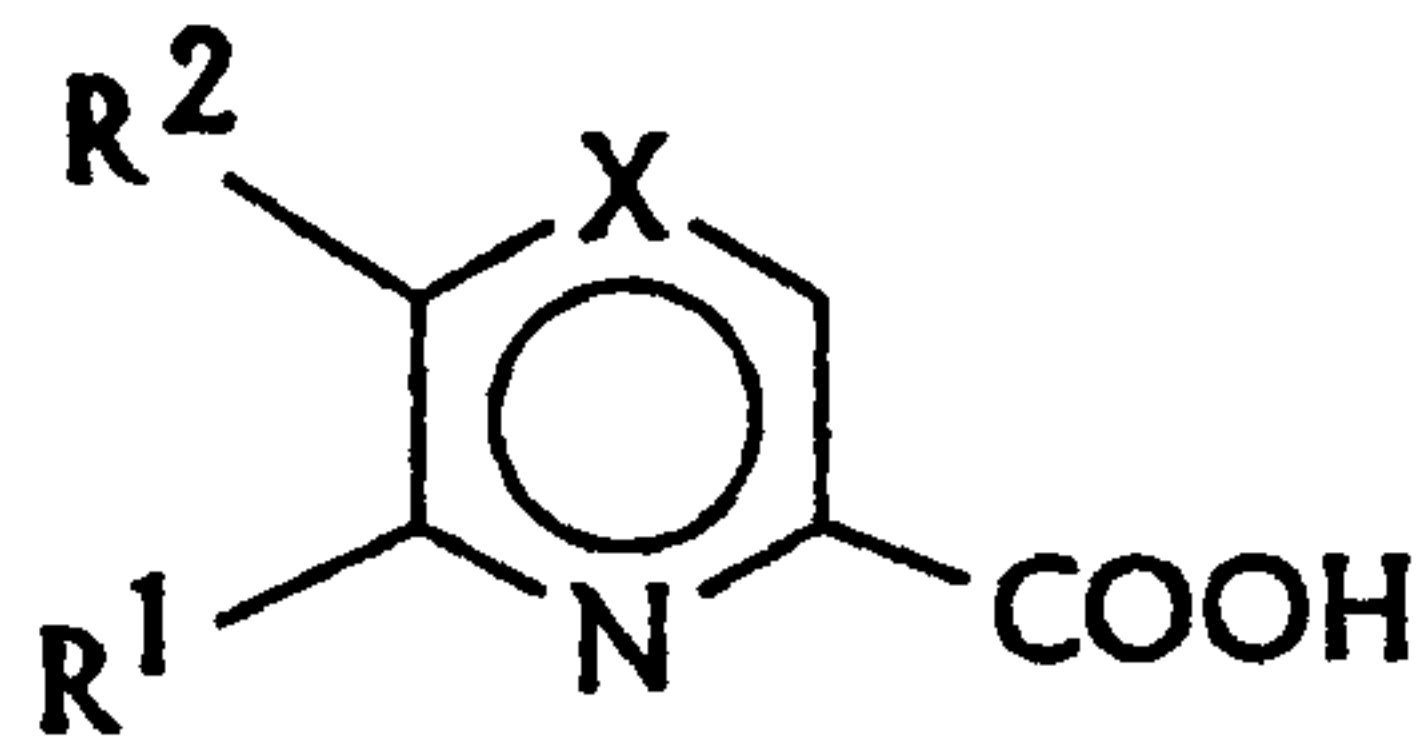
Alcaligenes faecalis (DSM 6335) was cultured as in Example 4 with fumaric acid as the source of carbon. The washed cells were resuspended in A+N medium containing 10 g/l 2-cyanopyrazine ($OD_{650}=10$) and incubated at 30°C. The formation of 6-hydroxypyrazinecarboxylic acid was followed by spectrophotometry, measuring the absorption of the cell-free solution at 320 nm. It was possible to determine the decrease in the concentration of 2-cyanopyrazine (substrate) by measuring the absorption at 270 nm. The total amount of 2-cyanopyrazine used had been converted into 6-hydroxypyrazinecarboxylic acid after 7 hours.

Example 6**Preparation of 6-chloropicolinic acid and pyrazinecarboxylic acid**

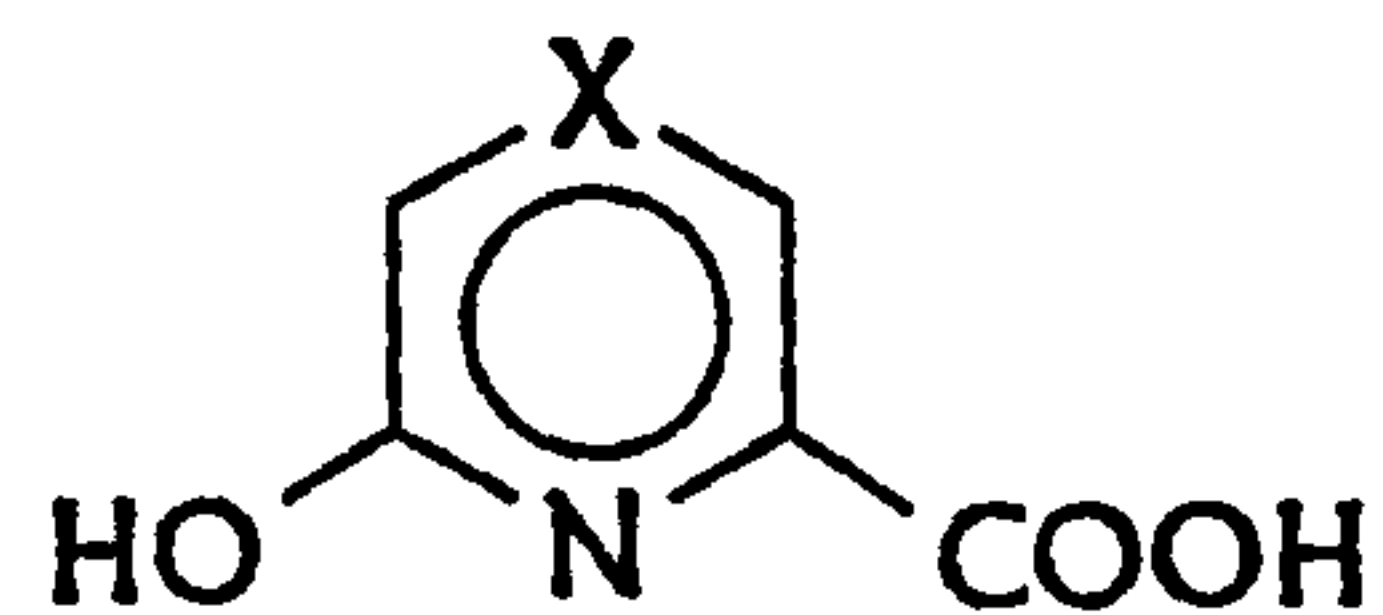
Alcaligenes faecalis (DSM 6335) was cultured as in
5 Example 4 with fumaric acid as a source of carbon. The
washed cells were resuspended in A+N medium in glass vessels
(OD₆₅₀=10) which could be closed with rubber stoppers, and
nitrogen was introduced through needles in order to remove
dissolved oxygen. Then 2-cyanopyrazine or 6-chloro-2-cyano-
10 pyridine was added as a substrate to the cell suspensions,
until the final concentration was 10 g/l, and incubated at
30°C. After 3 hours, the starting substances had been
converted quantitatively into the corresponding acids
[detection by thin-layer chromatography; silica gel 60 with
15 fluorescence indicator, mobile phase: chloroform 30/ethanol
55/NH₄OH (25%) 10/H₂O 5].

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

1. A microbiological process for the preparation of a heteroaromatic carboxylic acid of the general formula I or II, or a physiologically tolerated salt thereof,



I

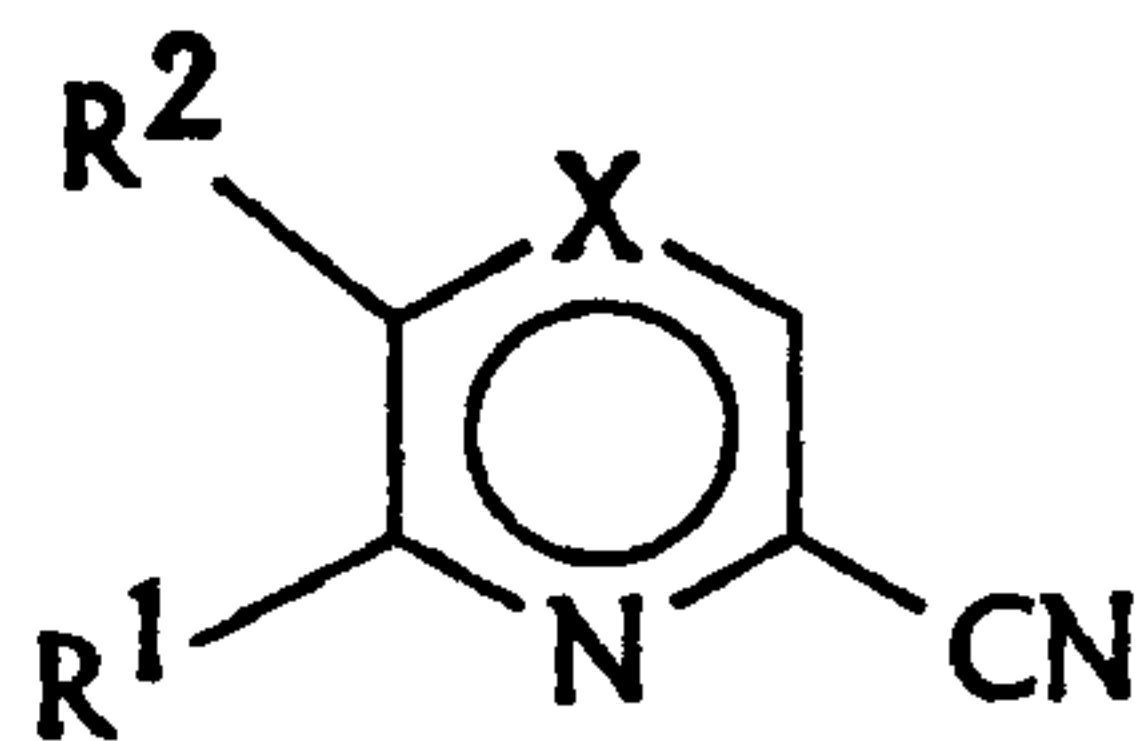


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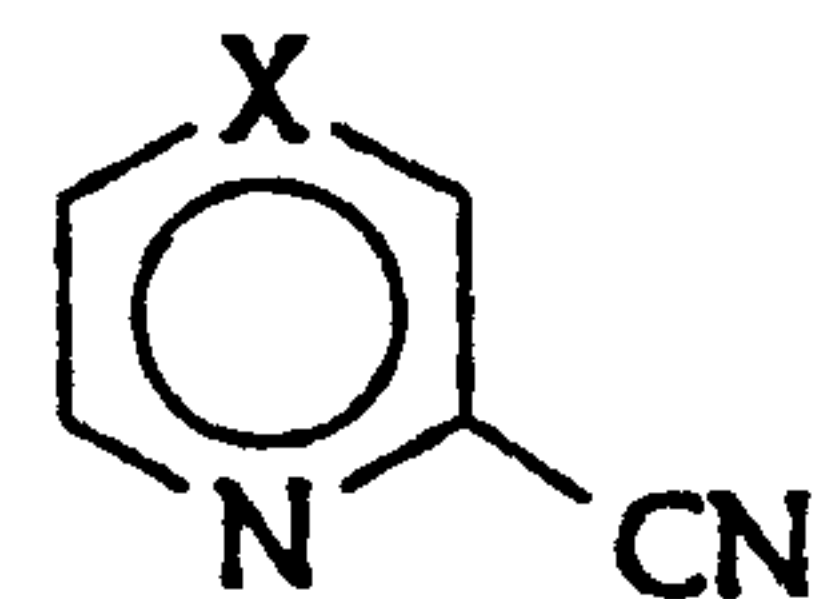
wherein

R¹ and R² are identical or different and denote a hydrogen or halogen atom and

X denotes a nitrogen atom or -CH-; in which process in a biotransformation, a heteroaromatic nitrile of the general formula III or IV,



III



IV

wherein

R^1 , R^2 and X are as defined previously, is converted as substrate, using 2-cyanopyridine-utilizing microorganisms of the genus *Alcaligenes* which have been cultured before the biotransformation in the presence of a dicarboxylic acid, tricarboxylic acid or a carbohydrate at a concentration of from 0.1 to 20% by weight, into the corresponding carboxylic acid.

2. The process according to claim 1, wherein the corresponding carboxylic acid is converted into a physiologically tolerated salt thereof.

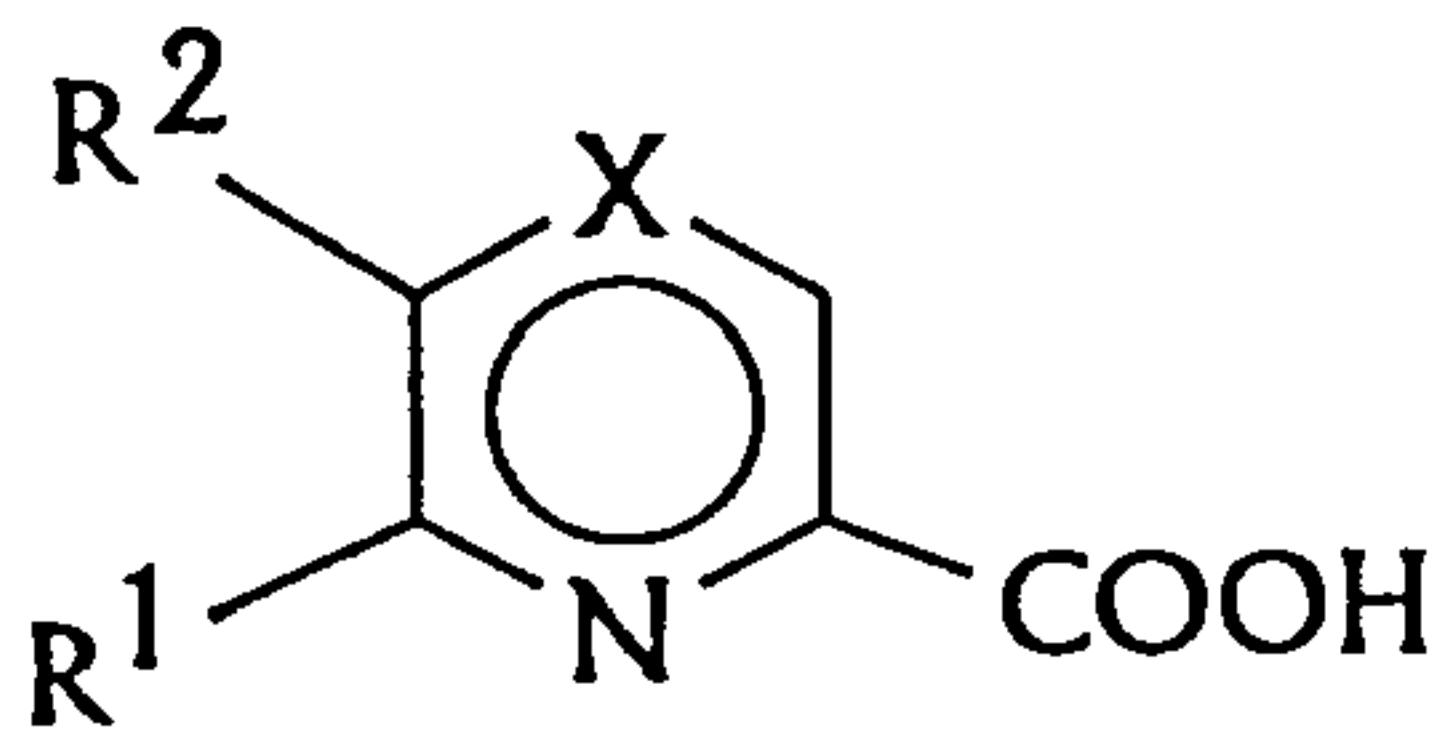
3. The process according to claim 1 or 2, wherein the biotransformation is carried out with microorganisms of the species *Alcaligenes faecalis* with the designation DSM 6335, or with their functionally equivalent variants or mutants.

4. The process according to any one of claims 1 to 3, wherein the biotransformation is carried out at a pH of 4 to 10 and a temperature of 10 to 50°C.

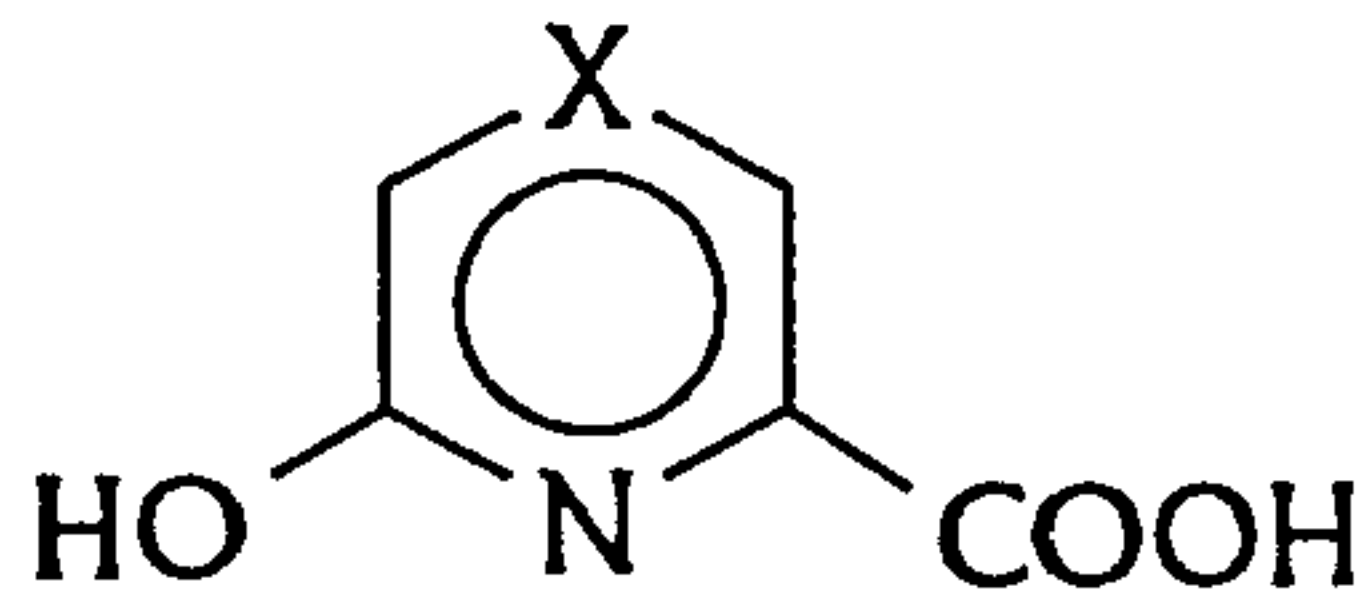
5. A process for the preparation of picolinic acid or a physiologically tolerated salt thereof, wherein 2-cyanopyridine as substrate is converted under anaerobic conditions, using 2-cyanopyridine-utilizing microorganisms of the genus *Alcaligenes* which have been cultured before the

biotransformation in the presence of a dicarboxylic acid, into picolinic acid.

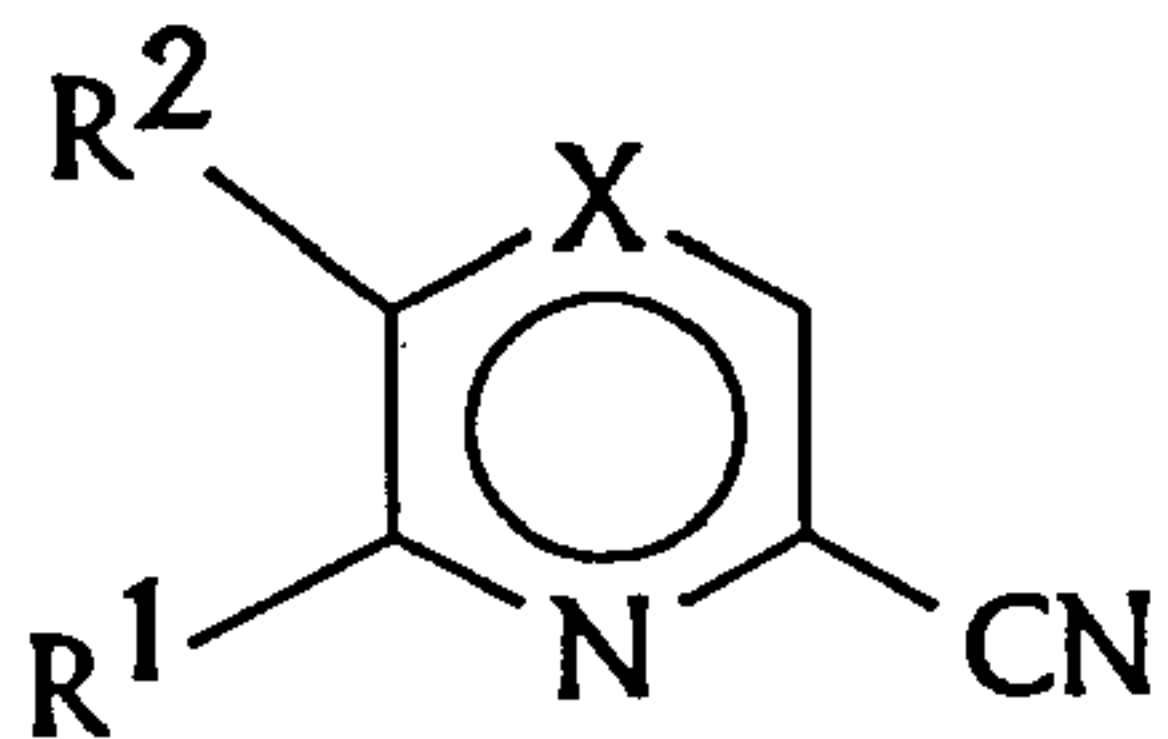
6. The process according to claim 5, wherein the picolinic acid is converted into a physiologically tolerated salt thereof.



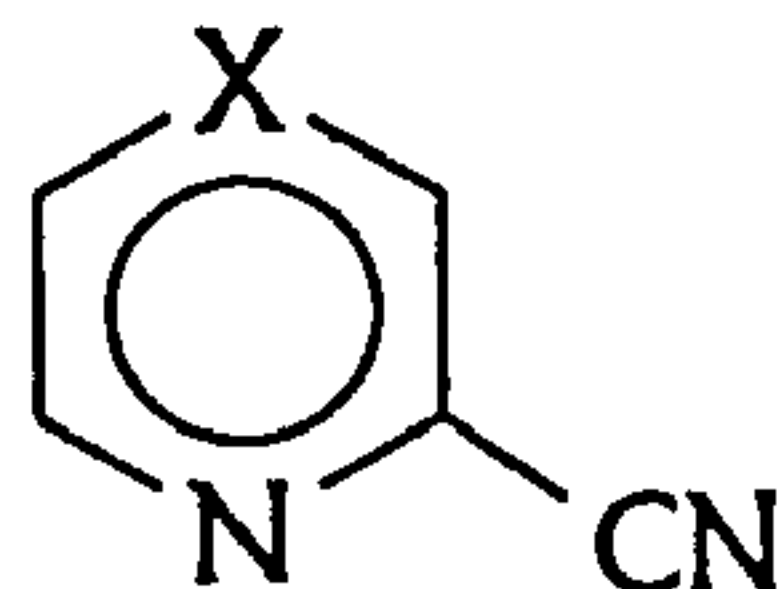
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