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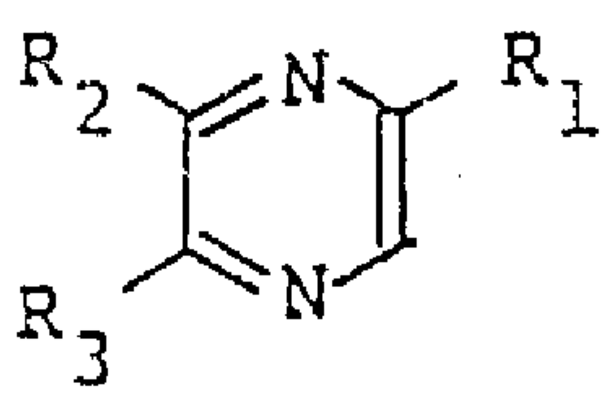
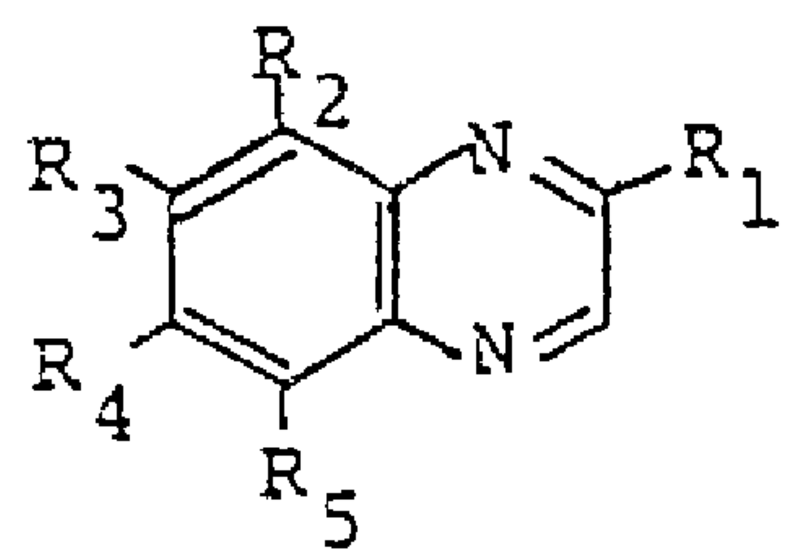
(30) 1990/09/25 (3091/90) CH

(54) **METHODE MICROBIOLOGIQUE POUR LA PREPARATION D'HETEROCYCLES HYDROXYLES**

(54) **MICROBIOLOGICAL PROCESS FOR THE PRODUCTION OF HYDROXYLATED HETEROCYCLES**



I



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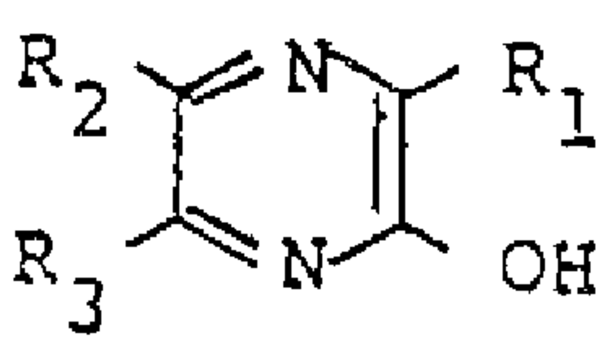

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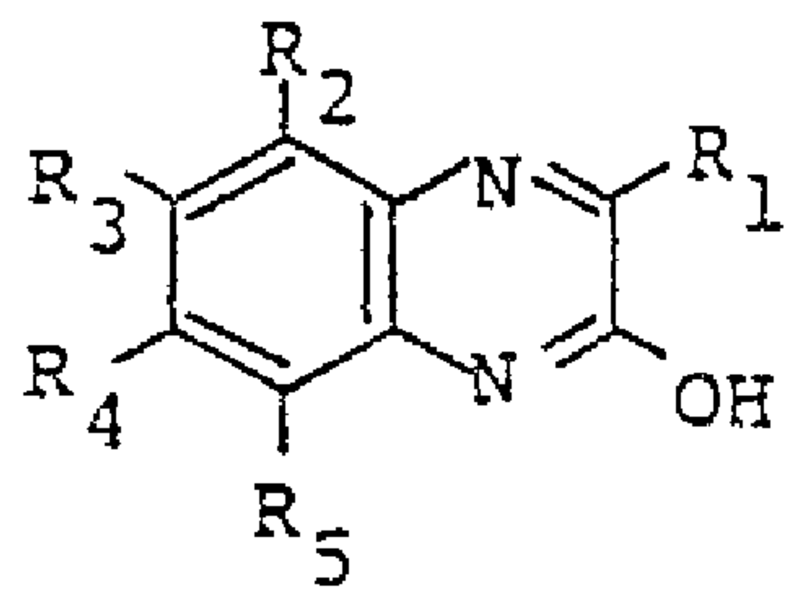
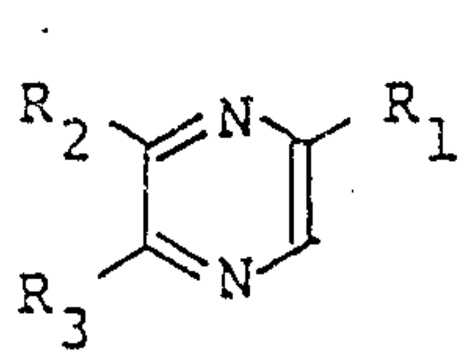


IV

(57) Novel microorganisms are disclosed which are capable of growing with 2,5-dimethylpyrazine as the sole carbon, nitrogen and energy source. These microorganisms hydroxylate heterocycles of the general formula: (see above formulas I or II) to form heterocycles of general formula: (see above formulas III or IV) The latter compounds are accumulated in the growth medium.

ABSTRACT OF THE DISCLOSURE

Novel microorganisms are disclosed which are capable of growing with 2,5-dimethylpyrazine as the sole carbon, nitrogen and energy source. These microorganisms hydroxylate heterocycles of the general formula:



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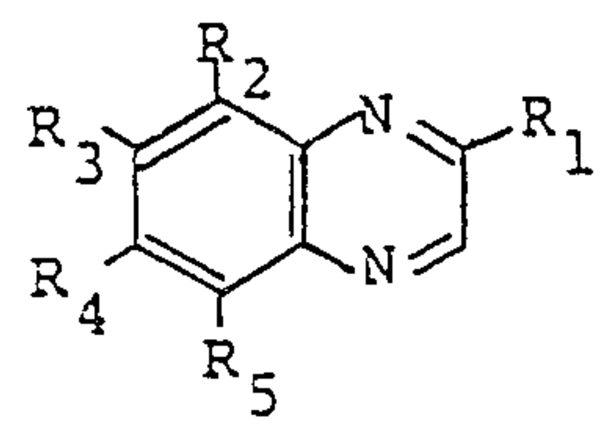
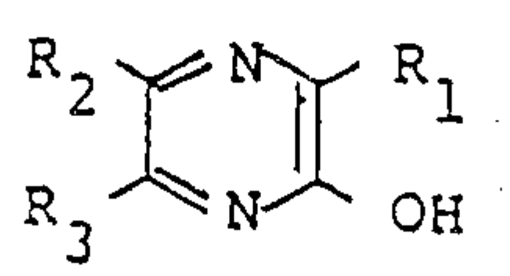


I

II

to form heterocycles of general formula:



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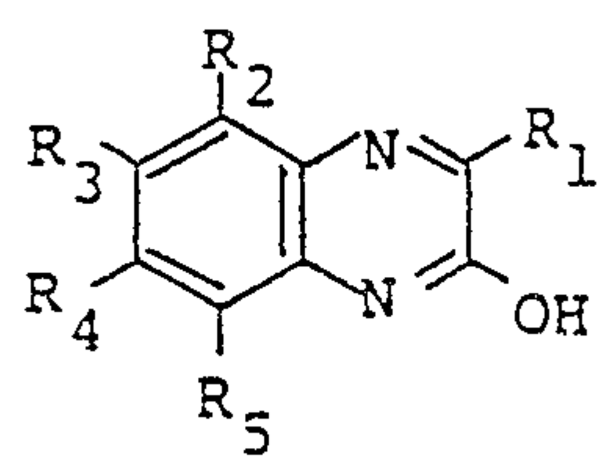
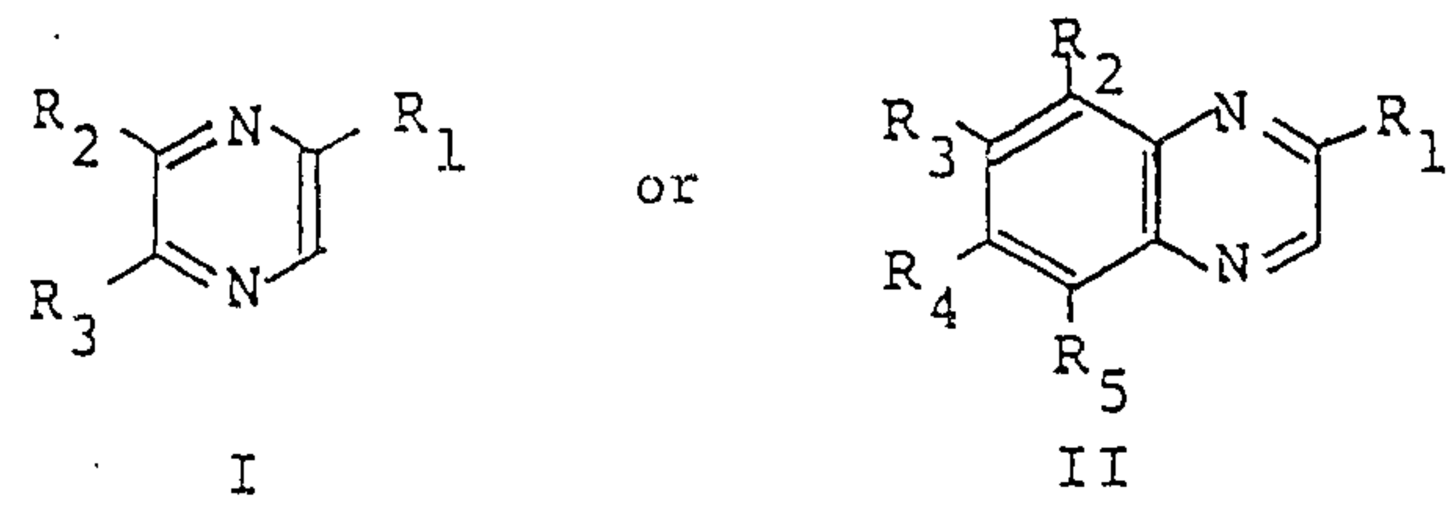


III

IV

The latter compounds are accumulated in the growth medium.

This invention relates to novel microorganisms, which grow with 2,5-dimethylpyrazine, and hydroxylate pyrazines or quinoxalines of general formula:



as well as to a process for the production of hydroxylated pyrazines or quinoxalines.

Hydroxylated pyrazines are, for example, important intermediate products for the production of methoxyalkylpyrazines. Methoxyalkylpyrazines are essential components of aromatic substances [Maga and Sizer, J. Agric. Food Chem., 21, (1973), pp. 22 to 30]. Hydroxylated quinoxalines are, for example, important pharmaceutical intermediate products (U.S. Patent No. 4,814,444).

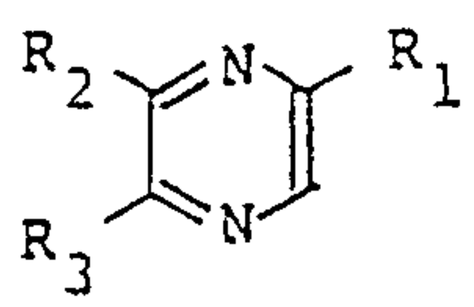
Thus far, only chemical processes are known for the production of hydroxylated quinoxalines and hydroxylated pyrazines. For example, U.S. Patent No. 4,814,444 describes a process in which 6-chloro-2-hydroxyquinoxaline-4-oxide is reduced in the presence of a catalyst to 6-chloro-2-hydroxyquinoxaline. But this process has the drawback that it is currently not feasible on an industrial scale.

A chemical process for the production of hydroxylated pyrazines is described, for example, in Karmas and Spoerri, J. Amer. Chem., 74, (1952), pp. 1580 to 1584, in which, for example, 2-hydroxy-5-methylpyrazine is synthesized starting from methylglyoxal and glycinamide hydrochloride. However, this process has the drawback that the product is highly contaminated.

Studies on the biological catabolism of 2-hydroxypyrazine in Matley and Harle, Biochem. Soc. Trans., 4, (1976), pp. 492 to 493, and studies on 2-pyrazinecarboxyamide in Soini and Pakarinen, FEMS Microbiol. Lett., (1985), pp. 167 to 171, have also been described. But no microorganisms have become known which hydroxylate pyrazines or quinoxalines of general formula I or II (set out herein) and accumulate the latter in a growth medium.

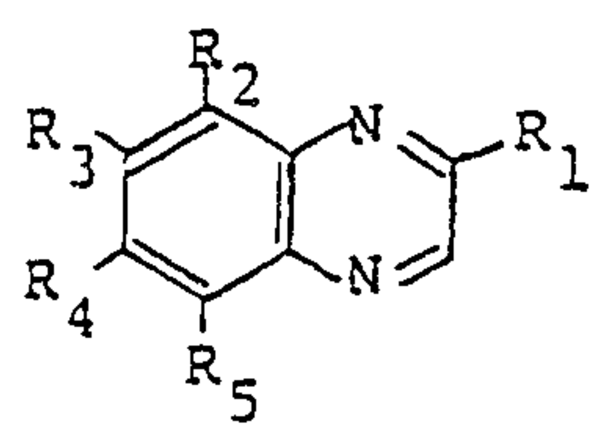
The main object of the invention is to find and provide a new type of microorganism, which is capable in a simple way of hydroxylated regiospecifically-substituted pyrazines or quinoxalines of general formula I or II, as well as providing a process for the production of hydroxylated pyrazines and quinoxalines.

The microorganisms of the invention are capable of growing with 2,5-dimethylpyrazine as a sole carbon, nitrogen and energy source, and of converting pyrazines of general formula I as substrate:



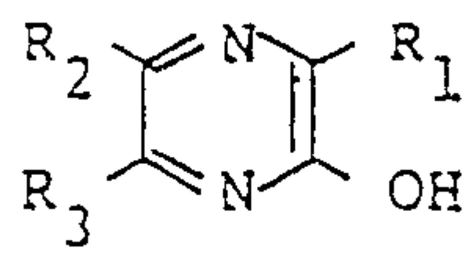
(I)

or quinoxalines of general formula II as substrate:



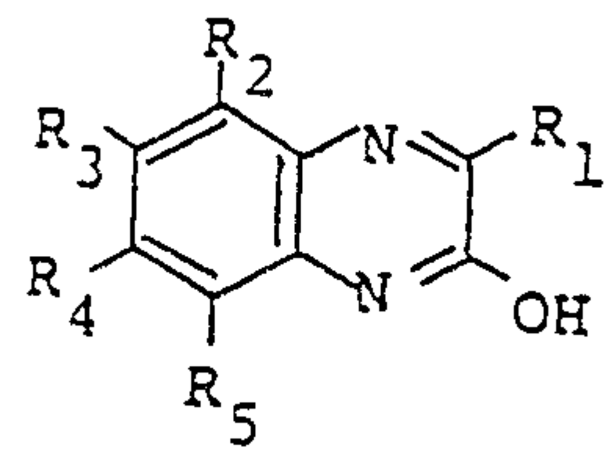
(II)

wherein R_1 is a C_1-C_4 alkyl group or a halogen atom and R_2, R_3, R_4 and R_5 are the same or different and each is a hydrogen atom, a C_1-C_4 alkyl group or a halogen atom, to a hydroxylated pyrazine of general formula:



(III)

or to a hydroxylated quinoxaline of general formula:



(IV)

respectively, wherein R_1, R_2, R_3, R_4 and R_5 have the above-stated meaning, and the latter is accumulated in the growth medium.

The invention also includes biologically pure and substantially biologically pure cultures of the microorganisms of the invention.

The invention further includes a process of for producing hydroxylated pyrazines and quinoxalines using the microorganisms of the invention.

As studies with soil samples from sewage treatment plants, earth, anthills and compost piles have shown, microorganisms, which catabolize 2,5-dimethylpyrazine, are capable of hydroxylating the pyrazines or quinoxalines of general formula I or II. According to the invention, all of these strains that use 2,5-dimethylpyrazine as the sole carbon, nitrogen and energy source are suitable for this hydroxylation, and are selected according to the usual microbiological techniques. Suitably, all gram-positive and gram-negative microorganisms can be used which catabolize 2,5-dimethylpyrazine and hydroxylate the heterocycles of general formula I or II as a substrate to form heterocycles of general formula III or IV which are accumulated in the growth medium. The preferred microorganisms are Rhodococcus erythropolis having deposit number DSM (German Collection of Microorganisms) No. 6138 and Arthrobacter sp. having deposit number DSM No. 6137.

Since the precise identification of the microorganisms with DSM No. 6138 and DSM No. 6137 did not take place until after the priority date of this application, the characterization of the microorganism with the previous designation Rhodococcus equi Heida (DSM No. 6138) now designated as Rhodococcus erythropolis (DSM No. 6138) and the microorganism with the previous designation Micrococcus sp. YVG (DSM No. 6137) which is now designated as Arthrobacter sp. (DSM No. 6137) will be given. These strains were deposited on September 7, 1990 in the German Collection of Microorganisms (DSM) and Zellkulturen (Cell Cultures) GmbH, Mascherodeweg 1b, 3300 Braunschweig/FRG.

A scientific description of Arthrobacter sp. (DSM No. 6137) is as follows:

Characterization: In young cultures pleomorphous rods, in older cultures coccoid to cocci; gram-positive; strictly aerobic, no acid formation from glucose

mobility	-
spores	-
catalase	+
mesodiaminopimelic acid in the cell wall:	no

Peptidoglycan type: A3alpha, Lys-Ala_{2-3}; the alpha-carboxyl group of the D-glutamic acid of the peptide subunit is substituted by a glycine radical.

A scientific description of Rhodococcus erythropolis (DSM No. 6138) is as follows:

Amino acid of the peptidoglycan	
diaminopimelic acid (DAP)	+
Sugar from whole-cell hydrolyzates:	
arabinose (ARA)	+
glactose (GAL)	+
madurose (MAD)	-
xylose (XYL)	-
glucose (GLU)	+
ribose (RIB)	+

Fatty acids:

Unbranched saturated and unsaturated fatty acids plus tuberculostearic acid present

15 to 30 percent

Mycolic acids:

Mycolic acids with a chain length of C_{35}-C_{40} present Menaquinones:

Type MK-8 (H_2) 93 percent

Physiological tests for species-identification of microorganisms containing mycolic acid:

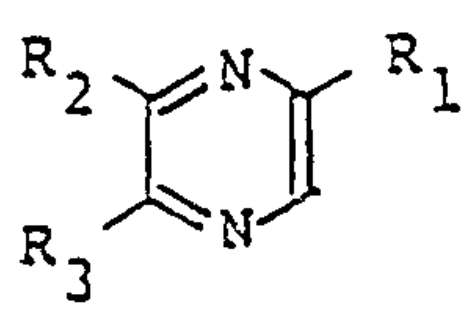
N-acetylglucusamine (NAG)	+
D-glycosaminic acid (GAT)	-
D-turanose (TUR)	-
2-hydroxyvalerate (o2V)	+
L-alanine (ALA)	+
L-proline (PRO)	-
tyramine (TYR)	-
4-aminobutyrate (o4B)	+
2-desoxythymidine-s-pup-phosphate (CDP)	+
galactose (GAL)	-
L-rhamnose (RHA)	-
aralite (ARA)	+
2-oxo-glutarate (o2G)	-
4-aminobutyrate (a4B)	-
L-serine (SER)	+
acetamide (ATA)	+

quinate (QUI)	+
D-glucarate (GCT)	-
D-ribose (RiB)	+
inositol (INO)	+
pimelase (PIM)	+
L-aspartate (ASP)	-
L-valine (VAL)	+
benzoate (BEN)	-
pNP-beta-D-xyloside (CXY)	+
gluconate (GDT)	+
sucrose (D-saccharose; SUC)	+
citrate (CIT)	+
succinate (SAT)	+
L-leucine (LEU)	+
putrescine (PUT)	+
3-hydroxybenzoate (o3B)	-
pNP-phosphoryl-choline (CCH)	+

Color code for colonies of coryne-shaped organisms:

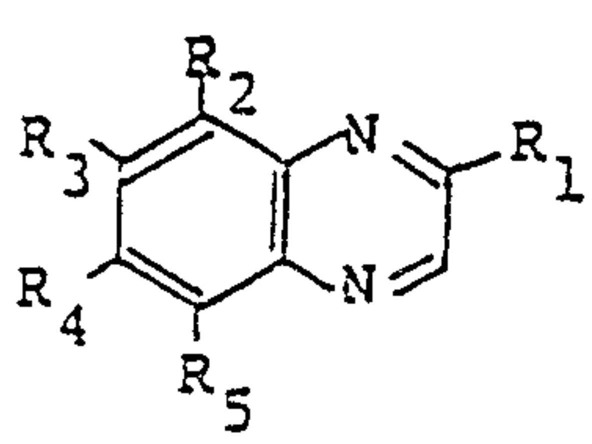
Type No. 60 according to Seiler (1983)

The invention further provides a process for the production of hydroxylated pyrazines of quinoxalines, wherein a pyrazine of general formula I as substrate:



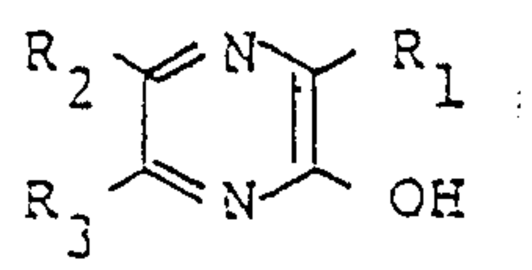
(I)

or a quinoxaline of general formula II as substrate:



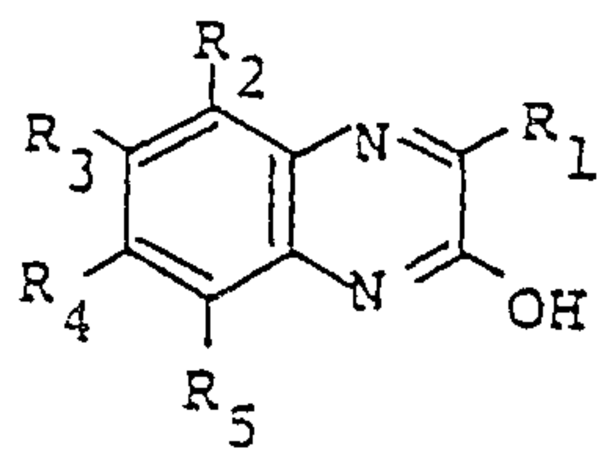
(II)

wherein R_1 is a C_1-C_4 alkyl group or a halogen atom and R_2, R_3, R_4 and R_5 are the same or different and each is a hydrogen atom, a C_1-C_4 alkyl group or a halogen atom is converted with the microorganisms set out above to form a hydroxylated pyrazine of general formula:



(III)

or a hydroxylated quinoxaline of general formula:



(IV)

respectively, wherein R_1, R_2, R_3, R_4 and R_5 have the above-stated meanings, and the concentrated product is isolated.

Compounds of general formula I or II are suitable as pyazines or quinoxalines to be hydroxylated.

In the preferred pyrazine derivatives or quinoxaline derivatives of general formula I or II, R_1 is a methyl group, ethyl group, or a chlorine atom and R_2 and R_3 are the same or different and each is a hydrogen atom, a methyl group or a chlorine atom.

Usually, the microorganisms are cultivated before the actual process (substrate reaction) in a medium containing a growth substrate. Growth substrate 2,5-dimethylpyrazine is suitably used in an amount of 0.001 to 10 percent (w/v), relative to the culture medium, preferably in an amount of 0.001 to 5 percent (w/v), relative to the culture medium.

The enzymes of the microorganism responsible for hydroxylation are suitably induced by 2,5-dimethylpyrazine. The compound used for induction can either be present during the reaction of the heterocyclic substrate or the feed of the induction compound can be stopped during the reaction. Preferably, the feed of the compound used for induction is stopped during the reaction of the heterocyclic substrate either by stopping the feed or by, for example, centrifuging the cells.

Before adding the substrate, the cells are suitably drawn in up to an optical density of 100 at 650 nm, preferably up to an optical density of 10 to 60 at 650 nm.

The substrate for the microorganisms, both for the cultivation and for the actual process, may be selected from those normally used among persons skilled in the art. The medium having the composition indicated in Table 1 below, is preferably used.

The actual process (substrate reaction) then takes place usually with resting cells.

The pyrazine or quinoxaline of general formula I or II can be fed once or continuously to the cell suspension, preferably so that the substrate concentration in the culture medium does not exceed 20 percent (w/v). In particular, the substrate concentration should not exceed 5 percent (w/v) in the culture medium.

The reaction is generally performed in a pH range of 4 to 10, preferably 6 to 8. Usually, the reaction is performed at a temperature of 0° to 50°C, preferably at 20° to 40°C.

After the reaction, the hydroxylated heterocycles can be isolated in a known way, e.g., by extraction with chlorinated hydrocarbons.

The following Examples illustrate the invention.

Example 1

Isolation of 2,5-dimethylpyrazine-metabolizing microorganisms

Aerobic 2,5-dimethylpyrazine-metabolizing microorganisms were concentrated in the A+N medium (Table 1) with 0.1 percent (w/v) of 2,5-dimethylpyrazine being added as sole carbon, nitrogen and energy source. The general techniques for isolating microorganisms are described, for example, in G. Drews, Mikrobiologisches Praktikum [Microbiological Workshop] 4th Ed. Springer Verlag, (1983). As an inoculum, samples from the earth, sewage treatment plants, compost and anthills were used. The concentrations were drawn into shaking flasks at 30°C. After over-inoculating three times in fresh medium, the concentrations of the same medium were streaked by adding 16 g of agar per liter and incubated at 30°C. After repeated streaking on agar medium, pure cultures were able to be isolated.

Table 1: A+N medium

Composition	Concentration (mg/l)
$(NH_4)_2SO_4$	2000
Na_2HPO_4	2000
KH_2PO_4	1000
NaCl	3000
$MgCl_2 \cdot 6H_2O$	400
$CaCl_2 \cdot 2H_2O$	14.5
$FeCl_3 \cdot 6H_2O$	0.8
pyridoxal hydrochloride	$10 \cdot 10^{-3}$
riboflavin	$5 \cdot 10^{-3}$
nicotinic acid amide	$5 \cdot 10^{-3}$
thiamine hydrochloride	$2 \cdot 10^{-3}$
biotin	$2 \cdot 10^{-3}$
pantothenic acid	$5 \cdot 10^{-3}$
p-aminobenzoate	$5 \cdot 10^{-3}$
folic acid	$2 \cdot 10^{-3}$
vitamin B12	$5 \cdot 10^{-3}$
$ZnSO_4 \cdot 7H_2O$	$100 \cdot 10^{-3}$
$MnCl_2 \cdot 4H_2O$	$90 \cdot 10^{-3}$
H_3BO_3	$300 \cdot 10^{-3}$
$CoCl_2 \cdot 6H_2O$	$10 \cdot 10^{-3}$
$NiCl_2 \cdot 6H_2O$	$20 \cdot 10^{-3}$
$Na_2MoO_4 \cdot 2H_2O$	$30 \cdot 10^{-3}$
$EDTANa_2 \cdot 2H_2O$	$5 \cdot 10^{-3}$
$FeSO_4 \cdot 7H_2O$	$2 \cdot 10^{-3}$

(pH of the solution was adjusted to 7.0)

Example 2

Conversion of 2,5-dimethylpyrazine to 2,5-dimethyl-3-hydroxypyrazine

Rhodococcus erythropolis (DSM No. 6138) was drawn in the A+N medium with 0.1 percent (w/v) of 2,5-dimethylpyrazine in a fermenter at pH 7 and a temperature of 25°C. Then, the cells were centrifuged and resuspended in the A+N medium and adjusted to an optical density of 10 at 650 nm. This cell suspension was added to a shaking flask and mixed with 92 mmol of 2,5-dimethylpyrazine per liter. After an incubation of 4 hours at 25° in a shaking machine, 83 mmol of 2,5-dimethyl-3-hydroxypyrazine per liter was detected corresponding to a yield of 90 percent.

Examples 3 to 9

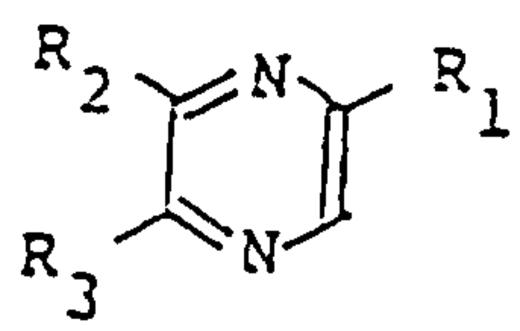
Examples 3 to 9 were performed according to the procedure outlined in Example 2 and are summarized in Table 2.

Table 2

Example	Substrate	Concentration of the heterocycle in % (w/v) in the medium	Reaction time in hours	End Product	Yield in %
3	2-methylpyrazine	0.2	1	3-hydroxy-2-methyl-pyrazine	90
4	2-chloropyrazine	0.2	24	3-hydroxy-2-chloro-pyrazine	60
5	2-ethylpyrazine	0.2	24	3-hydroxy-2-ethyl-pyrazine	80
6	2,6-dimethylpyrazine	0.2	1	3-hydroxy-2,6-dimethyl-pyrazine	90
7	2,5,6-trimethyl-pyrazine	0.2	6	3-hydroxy-2,5,6-tri-methyl-pyrazine	90
8	6-chloro-2,5-dimethylpyrazine	0.2	1	3-hydroxy-6-chloro-2,5-dimethyl-pyrazine	90
9	2-methyl-quinoxaline	0.4	5	3-hydroxy-2-methylquin-oxaline	50

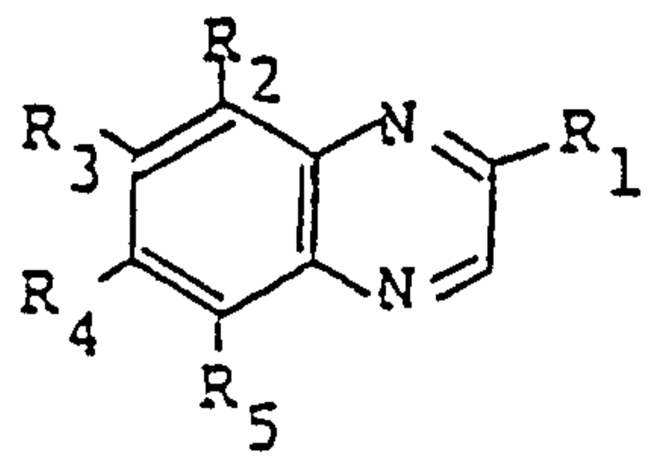
THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A microorganism capable of growing with 2,5-dimethylpyrazine as a sole carbon, nitrogen and energy source, the microorganisms being Rhodococcus erythropolis, DSM No. 6138, or arthrobacter sp., DSM No. 6137, or a descendent or mutant thereof and capable of converting a pyrazine of general formula I as substrate:



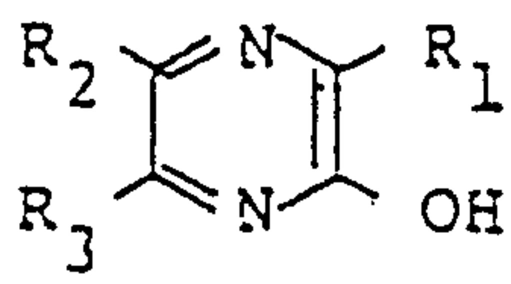
(I)

or a quinoxaline of general formula II as substrate:



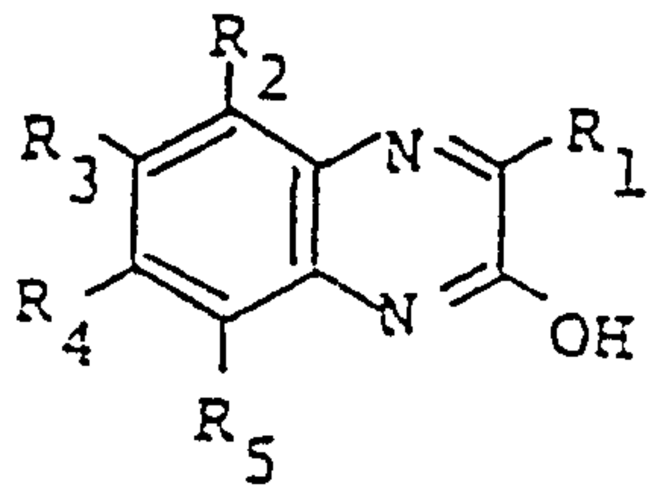
(II)

wherein R_1 is a C_1-C_4 alkyl group or a halogen atom and R_2, R_3, R_4 and R_5 are the same or different and each is a hydrogen atom, a C_1-C_4 alkyl group or a halogen atom to a hydroxylated pyrazine of general formula:



(III)

or to a hydroxylated quinoxaline of general formula:



(IV)

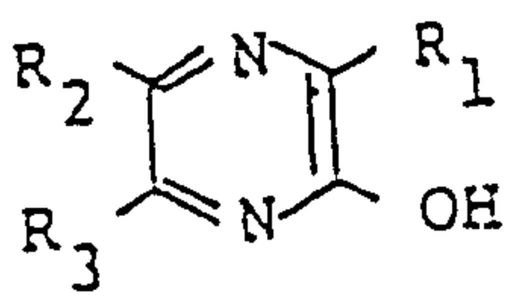
respectively, wherein R_1, R_2, R_3, R_4 and R_5 have the above-stated meanings, and achieving accumulation of the hydroxylated pyrazine of general formula III or hydroxylated quinoxaline of general formula IV in the growth medium.

2. A microorganism according to claim 1, with the designation Rhodococcus erythropolis, deposited with the DSM under the number 6138, a descendant thereof or a mutant thereof.

3. A microorganism according to claim 1, with the designation Arthrobacter sp., deposited with the DSM under the number 6137, a descendant thereof or a mutant thereof.

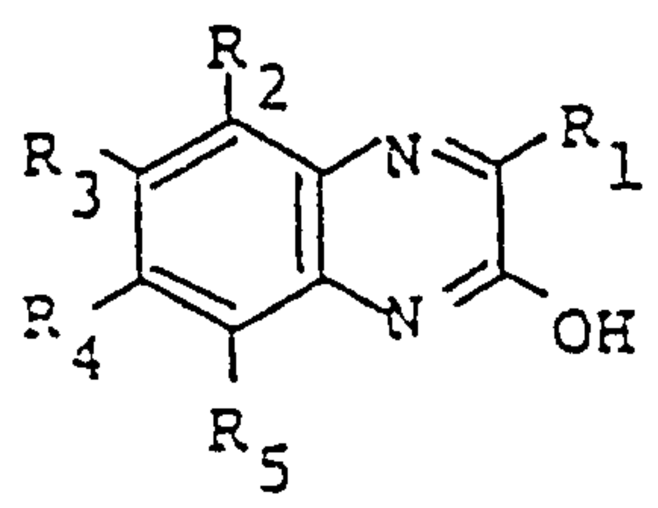
4. A biologically pure culture of the microorganism of claim 1, 2 or 3.

5. A process for the production of a hydroxylated pyrazine of the formula:



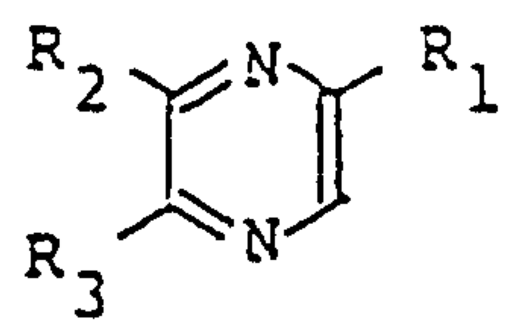
(III)

or a hydroxylated quinoxaline of general formula:



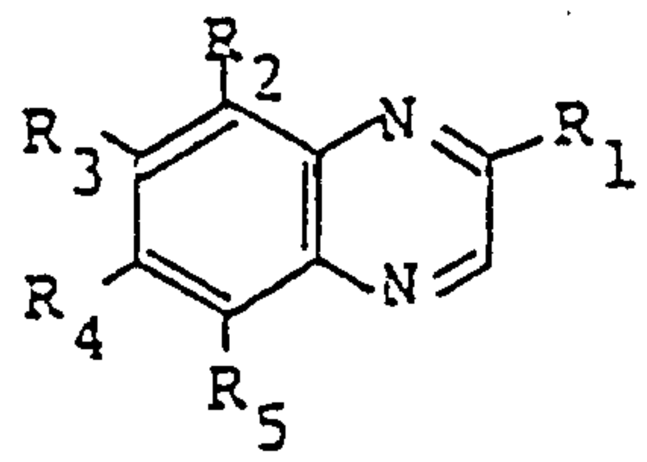
(IV)

wherein R_1 is a C_1-C_4 alkyl group or a halogen atom, and R_2, R_3, R_4 and R_5 are the same or different and each is a hydrogen atom, a C_1-C_4 alkyl group or a halogen atom, which comprises converting a pyrazine of general formula I as substrate:



(I)

or a quinoxaline of general formula II as substrate:



(II)

respectively, wherein R_1, R_2, R_3, R_4 and R_5 have the above-stated meanings, with a microorganism as defined in claim 1, 2 or 3, and isolating the concentrated hydroxylated pyrazine of general formula III or IV.

6. A process according to claim 5, wherein a biologically pure culture of the microorganism is used.

7. A process according to claim 5 or 6, wherein effective enzymes of the microorganism are induced with 2,5-dimethylpyrazine.

8. A process according to claim 5 or 6, wherein the reaction is performed with one-time or continuous addition of substrate so that the substrate concentration in the culture medium does not exceed about 20 percent (w/v).

9. A process according to claim 5 or 6, wherein the reaction is performed at a pH of from 4 to 10.

10. A process according to claim 5 or 6, wherein the reaction is performed at a temperature of from 0° to 55°C.