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(54) **METHODE MICROBIOLOGIQUE POUR LA PREPARATION DE L'ACIDE 6-HYDROXYPICOLINIQUE**

(54) **MICROBIOLOGICAL PROCESS FOR THE PRODUCTION OF 6-HYDROXYPICOLINIC ACID**

(57) A microbiological process is disclosed for the production of 6-hydroxypicolinic acid starting from 2-cyanopyridine. For this process new microorganisms are used, which are capable of growing with 2-cyanopyridine as the sole carbon, nitrogen and energy source and of converting it as the substrate into 6-hydroxypicolinic acid.

ABSTRACT OF THE DISCLOSURE

A microbiological process is disclosed for the production of 6-hydroxypicolinic acid starting from 2-cyanopyridine. For this process new microorganisms are used, which are capable of growing with 2-cyanopyridine as the sole carbon, nitrogen and energy source and of converting it as the substrate into 6-hydroxypicolinic acid.

This invention relates to a new microbiological process for the production of 6-hydroxypicolinic acid, starting from 2-cyanopyridine, as well as to new microorganisms suitable for use in this process.

It is known that microorganisms of the genus Bacillus hydroxylate picolinic acid to 6-hydroxypicolinic acid [O. Shukla and S.M. Kaul, Indian J. of Biochemistry and Biophysics, (1973), Vol. 10, pages 176 to 178; O. Shukla et al., Indian J. of Biochemistry and Biophysics, Vol. 14, (1977), pages 292 to 295]. A significant drawback of this process is that the further metabolization of the 6-hydroxypicolinic acid can be stopped only with the inhibitor sodium arsenite, and, thus, the growth of the microorganisms also is inhibited. Another drawback is that 6-hydroxypicolinic acid is not formed exclusively, but instead a mixture of 3,6-dihydroxypicolinic acid and 6-hydroxypicolinic acid results.

R.L. Tate and J.C. Ensign, Can. J. Microbiol., Vol. 20, (1974), pages 695 to 702, describes the hydroxylation of picolinic acid with microorganism of the genus Arthrobacter. Drawbacks of this process are that these microorganisms cannot use picolinic acid exclusively as a carbon, nitrogen and energy source, but in the hydroxylation, a yeast extract must be present, which can lead to undesirable impurities of the product. Another drawback lies in the fact that the 6-hydroxypicolinic acid is formed only in the case of low oxygen content, and the microorganisms are not present in the growth phase, and thus little product is formed.

The main object of the invention is to provide a simple, economical microbiological process for the production of 6-hydroxypicolinic acid starting from 2-cyanopyridine which reduces or eliminates the above drawbacks. A further object of the invention is to provide new microorganisms (and biologically pure cultures thereof) useful in the new microbiological process of the invention.

Thus, the invention provides a microbiological process for the production of 6-hydroxypicolinic acid, which includes biotransforming 2-cyanopyridine with certain microorganisms to form 6-hydroxypicolinic acid and accumulating the latter in the medium. The microorganisms are those that are capable of growing with 2-cyanopyridine as the sole carbon, nitrogen and energy source and of converting it as substrate to 6-hydroxypicolinic acid. Preferably the microorganism is Alcaligenes faecalis which has been deposited in the DSM with the deposit number 6335 (biologically pure cultures). The descendants and mutants thereof (biologically pure cultures) are also suitable. Preferably the effective enzymes of the microorganisms are induced with 2-cyanopyridine. The reaction preferably takes place under (with) substrate addition once or continuously so that the substrate concentration does not exceed about 20 percent by weight. The reaction is preferably performed at a pH of 4 to 10 and a temperature of 10° to 50°C.

The invention also provides microorganisms (biologically pure or substantially biologically pure cultures) that are capable of growing with 2-cyanopyridine as the sole carbon, nitrogen and energy source and of converting it as the substrate to 6-hydroxypicolinic acid. Preferably the microorganism is Alcaligenes faecalis DSM 6335 (biologically pure or substantially biologically pure cultures). Alcaligenes faecalis DSM 6335 is also termed Alcaligenes faecalis Kie 31. The descendants and mutants thereof (biologically pure or substantially biologically pure cultures) are also suitable.

6-Hydroxypicolinic acid is used, for example, for the production of 2-oxypyrimidine [Berichte der Deutschen Chemischen Gesellschaft, (Reports of the German Chemical Society), 45, (1912), pages 2456 to 2467], which in turn is an important intermediate product for the production of pharmaceutical agents.

According to the invention, all microorganisms are suitable that are capable of growing with 2-cyanopyridine as the sole carbon, nitrogen and energy source and of converting it as the substrate into 6-hydroxypicolinic acid. These microorganisms are a component of the invention and can be selected and isolated with the help of the usual microbiological techniques, for example, from sewage treatment plants, with 2-cyanopyridine as the growth substrate. The term "microorganisms which are capable of growing with 2-cyanopyridine as the sole carbon, nitrogen and energy source" comprises both mixtures of microorganisms and pure-isolates of the microorganisms, that can be used under sterile or nonsterile fermentation conditions.

Suitably, the microorganism Alcaligenes faecalis DSM 6335 or a descendant or mutant thereof is used. The microorganism Alcaligenes faecalis DSM 6335 was deposited with the Deutschen Sammlong fur Mikroorganismen und Zellkulturen GmbH [German Collection for Microorganisms and cell Cultures GmbH] (DSM), Mascheroderweg 1b, D-3300 Brunswick, Germany, on January 31, 1991 with the designation DSM No. 6335.

The scientific (taxonomic) description of Alcaligenes faecalis (DSM No. 6335) and properties of the strain are as follows:

cell shape	
width, micron	0.5 to 0.8
length, micron	1.0 to 2.0

mobility	+
flagella	peritrichous
gram reaction	-
lysis by 3 percent KOH	+
aminopeptidase (Cerny)	+
oxidase	+
catalase	+
growth	
anaerobic	-
37°/40°C	+/-
pH 5.6	+
MacConkey broth (agar)	+
pigments	-
nondiffusing	-
diffusing	-
fluorescent	-
pyocyanine	-
acid from (OF test)	
aerobic glucose	-
anaerobic glucose	-
aerobic xylose	-
gas from glucose	-
acid from ASA*	
glucose	-
fructose	-
xylose	-

ONPG	-
ADH	-
LDC	-
indole	-
VP	-
NO_2 from NO_3	-
denitrification	-
rods	
phenylalanine desaminase	-
levan from saccharose	-
lecithinase	-
urease	-
hydrolysis of starch	-
gelatin	-
casein	-
DNA	-
Tween®80	-
aesculin	-
tyrosine catabolism	-
use of substrate	
acetate	+
adipate	-
azelate	-
caprate	+
citrate	+
glycolate	+

laevulinate	-
malate	+
malonate	+
mesaconate	-
phenylacetate	+
pimelate	-
sebacinate	-
D-tartrate	-
L-arabinose	-
fructose	-
glucose	-
mannose	-
maltose	-
xylose	-
ribose	-
mannitol	-
gluconate	-
2-ketogloconate	-
N-acetylglucosamine	-
L-methionine	+
hydroxybenzoate	-

RESULT: Strain Kie 31 (DSM No. 6335) = Alcaligenes faecalis

*ASA = acetylsalicylic acid

The process for the production of 6-hydroxypicolinic acid is performed according to the invention in such a way that 2-cyanopyridine is biotransformed with one of the microorganisms of the

invention to form 6-hydroxypicolinic acid and the latter is accumulated in the medium.

Before the actual reaction, these microorganisms are usually cultivated (cultured) and the effective enzymes of the microorganisms are suitably induced with 2-cyanopyridine. Usually the cultivation (culture) and induction take place with 2-cyanopyridine in a concentration of 0.01 to 20 percent by weight, preferably in a concentration of 0.1 to 1 percent by weight. Then the microorganisms can be harvested either before the substrate addition (2-cyanopyridine) by the usual separation processes or the substrate (2-cyanopyridine) can be directly added to the microorganisms.

For the actual process, the cell suspension is then suitably adjusted to an optical density at 650 nm of 1 to 100, preferably to an optical density of 5 to 80. As the medium, those known to persons skilled in the art can be used, preferably one of the media whose compositions are given in Tables and 2 below. The substrate (2-cyanopyridine) for the production of 6-hydroxypicolinic acid can be added once or continuously. Suitably, the substrate addition takes place so that the substrate concentration in the medium does not exceed 20 percent by weight, preferably so that the substrate concentration does not exceed 10 percent by weight. Usually the conversion of 2-cyanopyridine to 6-hydroxypicolinic acid takes place with dormant cells. The pH of the reaction is suitably in the range of 4 to 10, preferably in the range of 5 to 9. Suitably the reaction is performed at a temperature of 10° to 50°C, preferably at a temperature of 20° to 40°C. After a usual reaction time of 1 to 100 hours, 6-hydroxypicolinic acid can be isolated, for example, by acidification of the cell-free fermentation solution.

The following Examples illustrate the invention.

Example 1

Isolation of 2-Cyanopyridine-Metabolizing Microorganisms

Aerobic 2-cyanopyridine-metabolizing microorganisms were concentrated in the A+N medium (see Table 1 below) with the addition of 0.1 percent (w/v) 2-cyanopyridine as the sole carbon and energy source. The general techniques for isolating microorganisms are described, for example, in G. Drews, Mikrobiologisches Praktikum (Microbiological Workshop), 4th edition, (1983), Springer Verlag. Samples from sewage treatment plants were used as an inoculum. The concentrations were cultivated in shaking flasks at 30°C. After inoculating three times in fresh medium, the concentrations were plated out on the same medium with the addition of 16 g of agar per liter and incubated at 30°C. After repeated plating-out 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_2	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	$5 \cdot 10^{-3}$
thiamin 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	$300 \cdot 10^{-3}$
$CoCl_2 \cdot 6H_2O$	$200 \cdot 10^{-3}$
$CuCl_2 \cdot 2H_2O$	$10 \cdot 10^{-3}$
$NiCl_2 \cdot H_2O$	$20 \cdot 10^{-3}$
$Na_2MoO_4 \cdot H_2O$	$30 \cdot 10^{-3}$
$EDTANa_2 \cdot H_2O$	$30 \cdot 10^{-3}$
$FeSO_4 \cdot H_2O$	$2 \cdot 10^{-3}$

(The pH of the solution was adjusted to 7.0)

Example 2

Conversion of 2-Cyanopyridine To 6-Hydroxypicolinic Acid

(a) Alcaligenes faecalis DSM No. 6335 (Kie 31) was cultivated in A+N medium (see Table 1 above) with the addition of 0.1 percent (w/v) 2-cyanopyridine in a fermenter at pH 7 and at a temperature of 30°C. Then the cells were centrifuged off, resuspended in A+N medium and adjusted to an optical density of 10 at 650 nm. This cell suspension was poured into a shaking flask and mixed with 0.1 mol/l (10.4 g/l) of 2-cyanopyridine. After an incubation of 16 hours at 30°C on a shaking machine, 0.04 mol/l (5.5 g/l) of 6-hydroxypicolinic acid was detected by analytical methods in the cell-free solution, which corresponded to a yield of 40 percent, relative to the 2-cyanopyridine used.

(b) Alcaligenes faecalis DSM No. 6335 was cultivated in a mineral salt medium (see Table 2 below) with addition of 0.1 percent (w/v) 2-cyanopyridine in a fermenter (working volume 5.5 liters) at pH 7 and a temperature of 30°C. 3 mol/l of sodium hydroxide and 8.5 percent (w/v) of phosphoric acid was used for the pH adjustment. During the growth, additional 2-cyanopyridine was added to the fermenter until, after 24 hours of growth, the optical density at 650 nm was 5.1. Altogether 35 g of 2-cyanopyridine was metabolized during the growth phase. The microorganism suspension was mixed with 2-cyanopyridine (220 g) for the production of 6-hydroxypicolinic acid. After another incubation of 18 hours, 108 g of 6-hydroxypicolinic acid was isolated from the cell-free solution, corresponding to a yield of 37 percent relative to the 2-cyanopyridine used.

Table 2

Composition Of The Mineral Salt Medium

$MgCl_2 \cdot 6H_2O$	0.8 g/l
$CaCl_2$	0.16 g/l
Na_2SO_4	0.25 g/l
KH_2PO_4	0.4 g/l
Na_2HPO_4	0.9 g/l
SLF	1 ml/l
FeEDTA	15 ml/l

Composition Of The Trace Elements (SLF) In The Mineral Salt Medium

KOH	15 g/l
$EDTANa_2 \cdot 2H_2O$	100 g/l
$ZnSO_4 \cdot 7H_2O$	9 g/l
$MnCl_2 \cdot 4H_2O$	4 g/l
H_3BO_3	2.7 g/l
$CoCl_2 \cdot 6H_2O$	1.8 g/l
$CuCl_2 \cdot 2H_2O$	1.5 g/l
$NiCl_2 \cdot 6H_2O$	0.18 g/l
$Na_2MoO_4 \cdot 2H_2O$	0.2 g/l

Composition of FeEDTA

$EDTANa_2 \cdot 2H_2O$	5 g/l
$FeSO_4 \cdot 7H_2O$	2 g/l

(The pH of the solution was adjusted to 7.0.)

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

1. An isolated microorganism, wherein the microorganism is Alcaligenes faecalis DSM 6335, or a descendant or a functional mutant thereof, which is capable of growing with 2-cyanopyridine as a sole carbon, nitrogen and energy source and capable of converting 2-cyanopyridine as a substrate into 6-hydroxypicolinic acid.

2. A biologically pure culture of the microorganism according to claim 1 which is capable of growing with 2-cyanopyridine as a sole carbon, nitrogen and energy source and of converting 2-cyanopyridine as the substrate into 6-hydroxypicolinic acid.

3. A microbiological process for the production of 6-hydroxypicolinic acid, which comprises biotransforming 2-cyanopyridine with a microorganism, wherein the microorganism is Alcaligenes faecalis DSM 6335, or a descendant or a functional mutant thereof, which is capable of growing with 2-cyanopyridine as the sole carbon, nitrogen and energy source, and of converting the 2-cyanopyridine as substrate into 6-hydroxypicolinic acid, and accumulating the 6-hydroxypicolinic acid in the medium.

4. A process according to claim 3, wherein effective enzymes of the microorganism is induced with 2-cyanopyridine.

5. A process according to claim 3 or 4, wherein the reaction takes place under substrate addition once or continuously so that the substrate concentration does not exceed about 20 percent by weight.

6. A process according to claim 3 or 4, wherein the reaction is performed at a pH of from 4 to 10 and a temperature of from 10° to 50°C.