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(54) Titre : PROCEDE MICROBIOLOGIQUE DE PRODUCTION D'ACIDE 5-HYDROXYPYRAZINECARBOXYLIQUE
(54) Title: MICROBIOLOGICAL PROCESS FOR THE PRODUCTION OF 5-HYDROXYPYRAZINECARBOXYLIC ACID

(57) Abrégé/Abstract:
A microbiological process for the production of 5-hydroxypyrazinecarboxylic acid and/or a salt thereof from 2-cyanopyrazine. Suitable microorganisms for the process of the present invention are capable of utilizing 3-cyanopyridine as the sole carbon, nitrogen and energy source and of catabolizing 3-cyanopyridine to the corresponding 6-hydroxypyridinecarboxylic acid derivative. Preferably, the reaction is performed with a microorganism of the species Agrobacterium sp. DSM 6336 or a descendant or mutant thereof.

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ABSTRACT OF THE DISCLOSURE

A microbiological process for the production of 5-hydroxypyrazinecarboxylic acid and/or a salt thereof from 2-cyanopyrazine. Suitable microorganisms for the process of the present invention are capable of utilizing 3-cyanopyridine as the sole carbon, nitrogen and energy source and of catabolizing 3-cyanopyridine to the corresponding 6-hydroxypyridinecarboxylic acid derivative. Preferably, the reaction is performed with a microorganism of the species Agrobacterium sp. DSM 6336 or a descendant or mutant thereof.

The present invention relates to a novel process for the production of 5-hydroxypyrazinecarboxylic acid and/or a salt thereof from 2-cyanopyrazine with microorganisms utilizing 3-cyanopyridine.

In the following, the term 5-hydroxypyrazinecarboxylic acid also represents salts thereof, for example, alkali and ammonium salts thereof.

In the metabolism of dogs and humans 5-hydroxyprazinecarboxylic acid is formed from pyrazinamide by pyrazinecarboxylic acid (J Pharmacol Exp Ther, 180:2:411-434; 1972).

5-Hydroxypyrazinecarboxylic acid can be used, for example, as an intermediate product for the production of pharmaceutical agents, such as, for the production of pyrazine-nucleoside analogs with cytostatic effect (M. Bobek, J Heterocyclic Chem, 28:1131; 1991).

European Published Patent Application Number 0519512 describes a microbiological process for the production of 5-hydroxypyrazinecarboxylic acid from pyrazinecarboxylic acid by microorganisms utilizing nicotinic acid. A drawback of this process is that the feedstock, pyrazinecarboxylic acid, is difficult to obtain.

An object of the present invention is to provide a simple, economical and ecological process for the production of 5-hydroxypyrazinecarboxylic acid.

The process involves converting a substrate, 2-cyanopyrazine, with a microorganism which utilizes 3-cyanopyridine as the sole carbon, nitrogen and energy source, into 5-hydroxypyrazinecarboxylic acid and/or a salt thereof. The product accumulates in the reaction medium.

Preferably the conversion reaction is performed with a microorganism of the species Agrobacterium sp. DSM 6336, or a descendant or mutant thereof. Preferably the effective enzymes of the microorganism are induced with 3-cyanopyridine. The substrate can be added all at once or continuously during the conversion reaction, preferably so

that the substrate concentration does not exceed about 20% by weight. Preferably the conversion reaction is performed at a pH in the range of from about 4 to 10 and at a temperature in the range of from about 10 to 60°C.

According to the present invention, there is provided a microbiological process for the production of 5-hydroxypyrazinecarboxylic acid and/or a salt thereof, comprising the steps of converting 2-cyanopyrazine in a reaction medium, with a microorganism capable of utilizing 3-cyanopyridine as the sole carbon, nitrogen and energy source, into 5-hydroxypyrazinecarboxylic acid and/or a salt thereof, and accumulating 5-hydroxypyrazinecarboxylic acid and/or a salt thereof in the reaction medium.

Examples of salts of 5-hydroxypyrazinecarboxylic acid are an alkali metal salt, for example, sodium, potassium and lithium salts, and an ammonium salt thereof.

All microorganisms which are capable of utilizing 3-cyanopyridine as the sole carbon, nitrogen and energy source, and which catabolize 3-cyanopyridine to the corresponding 6-hydroxypyridinecarboxylic acid derivative are suitable microorganisms for the process of the present invention.

Suitable microorganisms include both mixtures of microorganisms and pure isolates of microorganisms which can be used under asceptic or nonasceptic fermentation conditions. Suitably, the reaction is performed with microorganisms of the species Agrobacterium sp. DSM 6336, or a descendant or mutant thereof. Such mutants and descendants are those which have substantially the same biological capability as Agrobacterium sp. DSM 6336 to effect such conversion and are capable of utilizing 3-cyanopyridine as the sole carbon, nitrogen and energy source. The species Agrobacterium sp. DSM 6336 was deposited with the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH [German Collection for Microorganisms and Cell Cultures GmbH] (DSMZ), Mascheroder Weg 1b, D-38124

Braunschweig, Germany, on February 7, 1991, with the designation DSM 6336.

The following is a taxonomic description of Agrobacterium sp. DSM 6336.

Properties of the strain:

Cell shape	rods	ADH	-
width, micron	0.6-0.8		
length, micron	1.5-3.0	ADC	-
Mobility	+	ONPG	-
Gram reaction	-	VP	-
Lysis by 3% KOH	+		
Aminopeptidase (Cerni)	+	Indole	-
Spores	-	NO_2 from NO_3	+
Oxidase	+	Denitrification	+
Catalase	+	Phenylalanine Desaminase	k.W.
Growth		Lecithinase	-
anaerobic	-		
37°/41°C	+/-	Urease	+
pH 5.6	-		
MacConkey broth	+	Simmons citrate	-
SS agar	-		
Cetrimide agar	-	Malonate	-
2% NaCl	+	Ketolactose	-
Pigments	-		
nondiffusing	-	Hydrolysis of starch	-
diffusing	-		
fluorescent	-	gelatin	-
pyocyanine	-	casein	-
		DNA	-
		Tween 80	-
		aesculin	+

Acid from (OF test)	
aerobic glucose	-
anaerobic glucose	-
Gas from Glucose	-
Acid from (ASA)	
glucose	+
fructose	+
xylose	+
m-erythritol	+
melezitose	-
arabinose	+
saccharose	-
cellobiose	+
trehalose	-
rhamnose	+
dulcitol	-
sorbitol	+
glycerol	+
L-arabinose	+
fructose	+
glucose	+
mannose	+
maltose	+
xylose	+
saccharose	+
sorbose	-
mannitol	+
2-ketogluconate	-
N-acetylglucosamine	+
L-serine	-
hydroxybutyrate	-
L-lysine	+
L-ornithine	+
Alkalization of litmus milk	-
Growth substance requirement	-
Use of substrate	
acetate	+
adipate	-
caprate	-
citrate	-
glycolate	-
lactate	+
levulinate	-
malate	+
malonate	-
phenylacetate	-
suberate	-

The microorganisms are cultured in any suitable medium known to those skilled in the art before the actual conversion reaction and their effective enzymes are induced. Preferably, the culture and the induction reaction take place with 3-cyanopyridine as the sole carbon, nitrogen and energy source.

The microorganisms can then be harvested by any of the separating processes known to those skilled in the art and resuspended in fresh medium prior to addition of the substrate (2-cyanopyrazine). Alternatively, the substrate can be added directly to the culture broth.

The cell suspension suitably is then adjusted to an optical density (OD_{650}) in the range of from about 1 to 100, preferably in the range of from about 10 to 40.

The substrate (2-cyanopyrazine) can be added all at once or continuously. Suitably, the substrate addition takes place so that the substrate concentration does not exceed about 20% by weight, preferably about 8% by weight. Usually the reaction of 2-cyanopyrazine to 5-hydroxypyrazinecarboxylic acid takes place with dormant cells. Suitably the reaction takes place at a pH in the range of from about 4 to 10, preferably at a pH in the range of from about 6 to 8. The reaction temperature suitably is between 10 and 60°C, preferably between 15 and 45°C.

After a reaction time of from about 4 to 100 hours, the product can be precipitated by acidification of a cell-free supernatant and isolated by methods known to those skilled in the art.

The following Example illustrates the invention.

EXAMPLE

Agrobacterium sp. DSM 6336 was cultivated in a mineral salt medium (Table 1) and 0.1% (w/v) 3-cyanopyridine at a temperature of 30°C in a 300 ml Erlenmeyer flask. After 36 hours, the cells were centrifuged and washed with mineral salt medium without 3-

cyanopyridine. 25 ml of the cell suspension (OD_{650} = 10) was mixed with 297 mg (2.83 mmol) of 2-cyanopyrazine and incubated for 16 hours on a shaker. After this incubation period no substrate was detected in the UV spectrum. The cells were then centrifuged and the supernatant was concentrated five-fold with a rotary evaporator and acidified with concentrated HCl to pH 2.0. The precipitated 5-hydroxypyrazinecarboxylic acid was filtered and dried. 307 mg (2.21 mmol) of product was isolated, corresponding to a yield of 78%. No impurities were detected in the ^{1}H-NMR spectrum (D_2O).

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
$FeCl_2 \cdot 6H_2O$	0.8
Pyridoxal hydrochloride	10×10^{-3}
Riboflavin	5×10^{-3}
Nicotinic acid amide	5×10^{-3}
Thiamin hydrochloride	2×10^{-3}
Biotin	2×10^{-3}
Pantothenic acid	5×10^{-3}
p-Aminobenzoate	5×10^{-3}
Folic acid	2×10^{-3}
Vitamin B12	5×10^{-3}
$ZnSO_4 \cdot 7H_2O$	100×10^{-3}
$MnCl_2 \cdot 4H_2O$	90×10^{-3}
H_3BO_3	300×10^{-3}
$CoCl_2 \cdot 6H_2O$	200×10^{-3}
$CuCl_2 \cdot 2H_2O$	10×10^{-3}
$NiCl_2 \cdot 6H_2O$	20×10^{-3}
$Na_2MoO_4 \cdot 2H_2O$	30×10^{-3}
EDTA $Na_2 \cdot 2H_2O$	5.0
$FeSO_4 \cdot 7H_2O$	2.0
pH adjusted to 7.0	

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

1. A microbiological process for the production of 5-hydroxypyrazinecarboxylic acid and/or a salt thereof, comprising the steps of converting 2-cyanopyrazine in a reaction medium, with a microorganism of the species Agrobacterium sp. DSM 6336, or a descendant or functional mutant thereof, capable of utilizing 3-cyanopyridine as the sole carbon, nitrogen and energy source, into 5-hydroxypyrazinecarboxylic acid and/or a salt thereof, in the reaction medium.

2. A process according to claim 1, wherein the effective enzymes of the microorganisms are induced with 3-cyanopyridine.

3. A process according to claim 1, wherein the substrate is added all at once or continuously, so that the substrate concentration does not exceed about 20% by weight.

4. A process according to claim 2, wherein the substrate is added all at once or continuously, so that the substrate concentration does not exceed about 20% by weight.

5. A process according to claim 3, wherein the substrate concentration does not exceed about 8% by weight.

6. A process according to claim 4, wherein the substrate concentration does not exceed about 8% by weight.

7. A process according to claim 1, 4, 5 or 6, wherein the reaction is performed at a pH in the range of from about 4 to 10 and at a temperature in the range of 10 to 60°C.

8. A process according to claim 2, wherein the reaction is performed at a pH in the range of from about 4 to 10 and at a temperature in the range of 10 to 60°C.

9. A process according to claim 3, wherein the reaction is performed at a pH in the range of from about 4 to 10 and at a temperature in the range of 10 to 60°C.

10. A process according to claim 7, wherein the pH is in the range of from about 6 to 8 and the temperature is in the range of from about 15 to 45°C.

11. A process according to claim 1, 4, 5, 6, 8, 9 or 10, wherein the salt of 5-hydroxypyrazinecarboxylic acid is an alkali salt thereof.