Fermentation Process For The Preparation Of Inosine And/Or Guanosine

This invention relates to a method of producing inosine and/or guanosine.

Inosine and guanosine are important starting materials for the synthetic production of condiments, namely 5'-inosinic acid and 5'-guanylic acid, and it is of great commercial significance to produce them in large quantities. For the production of inosine and/or guanosine by way of fermentation, it has been considered advantageous from industrial views to employ a mutant strain in which one or more of such properties as adenine/adenosine resistance, GMP reductase deficiency, and nucleotide phosphorylase deficiency have been constructed.

The intensive research undertaken by the present inventors for the purpose of improving the conventional fermentative production of inosine and/or guanosine led to the finding that the accumulation of inosine and/or guanosine by an inosine and/or guanosine producing microorganism of the genus Bacillus requiring adenine for growth can be remarkably increased by causing the microorganism to acquire resistance to antifolates such as methotrexate, aminopterin, pyrimethamine, trimethoprim, etc.

This invention therefore provides a method of producing inosine and/or guanosine, which comprises culturing a mutant strain of the genus Bacillus, which requires adenine for growth and is resistant to an antifolate (as hereinafter defined) and capable of produci-

ng inosine and/or guanosine, in a medium to cause the same strain to elaborate and accumulate inosine and/or guanosine in a culture broth, and recovering the inosine and/or guanosine so accumulated from said broth.

The invention also provides a biologically pure culture of a microorganism belonging to the genus Bacillus and having characteristics identifiable with those of FERM BP-288, FERM BP-289, FERM BP-290, FERM BP-291 or FERM BP-292, said culture being capable of producing, in a culture medium containing assimilable carbon and digestible nitrogen sources, a recoverable amount of inosine and/or guanosine.

The microorganism employed in accordance with this invention is an inosine and/or guanosine producing strain of the genus Bacillus which requires adenine for growth and has resistance to antifolates.

The antifolates (folic acid antagonistics) used in this invention are the drugs which show antagonistically inhibitory action to dihydrofolate reductase or to one carbon transfer reactions in which folic acid participates.

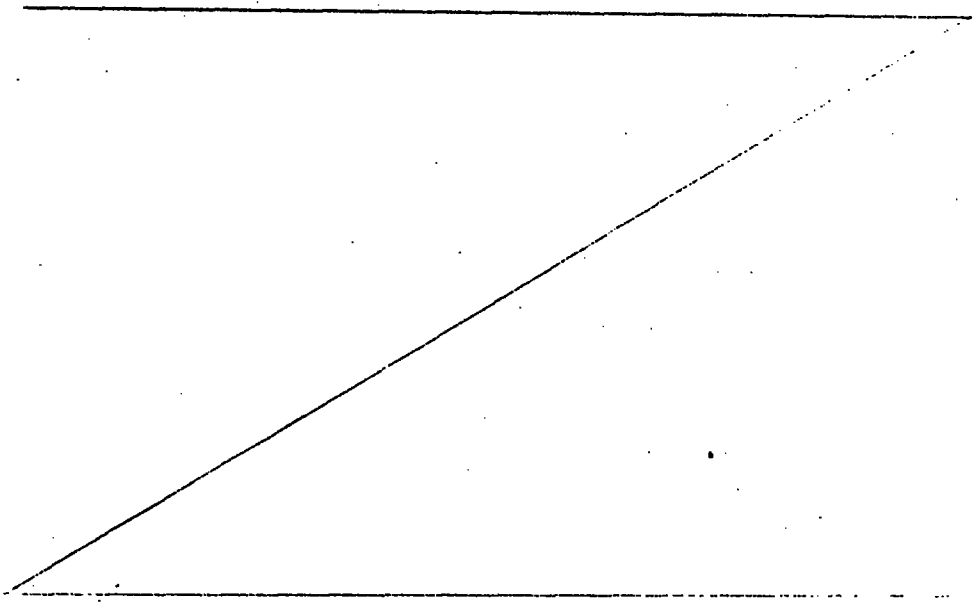
As such antifolates, there may be mentioned methotrexate, aminopterin, trimethoprim or pyrimethamine, etc.

The mutants used in the present invention can be derived from a broad range of parents including not only the wild strains of Bacillus pumilus, Bacillus subtilis, Bacillus licheniformis, etc. but also inosine and/or guanosine-producing strains of the genus Bacill-

us [e.g. Bacillus pumilus No. 158-A-11 (IFO 12476) described in Japanese Patent Publication No. 46839/1976/]

The derivation and isolation of the microorganism for use in accordance with this invention can be easily accomplished by the established procedures. Thus, the desired mutant strain can be obtained by subjecting a microorganism of the genus Bacillus, e.g. Bacillus pumilus, Bacillus subtilis, Bacillus licheniformis, to a conventional treatment, such as ultraviolet irradiation, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment, etc., then cultivating the treated microorganism on a plate medium containing an antifolate of concentration which does not permit growth of the parent strain, and selecting the resulting colony.

In the above plate medium, a concentration of antifolate varies with, among others, kinds of parent



microorganisms or medium composition. For example, in case of below-mentioned medium (A) containing agar is employed, methotrexate, aminopterin, trimethoprim or pyrimethamine is added thereto in a concentration as follows:

methotrexate: more than 10 μg/ml, preferably more than 50 μg/ml,

aminopterin: more than 20 μg/ml, preferably more than 100 μg/ml,

trimethoprim: more than 0.2 μg/ml, preferably more than 10 μg/ml,

pyrimethamine: more than 10 μg/ml, preferably more than 50 μg/ml.

The degree of resistance of derived mutant to antifolates can be evaluated by the following procedure. A test tube containing 5 ml of a medium (B) prepared by adding an antifolate in a predetermined concentration to the medium (A) shown in Table 1 is inoculated with about 10^6 cells/ml of the test strain previously grown on a nutrient agar (Difco (Trade Mark)) slant and shake culture is conducted at 37°C for 24 hours. The resulting broth is diluted 10-fold with water and the absorbance of the dilution at 590 nm was measured to find the degree of growth (B590). With the degree of growth (A590) of the same strain on the folic acid-free medium (A) being taken as 100, the relative growth on such an antifolate-containing medium can be expressed by (B590/A590) x 100.

Table 1

Composition of Medium	Concentration
Glucose	5.0%
Ammonium sulfate	0.3%
Sodium glutamate	1.0%
α-Alanine	0.5%
Magnesium sulfate	0.01%
Dipotassium hydrogen phosphate	0.1%

Composition of Medium	Concentration
Manganese sulfate	2 mg/ℓ
Zinc sulfate	2 mg/ℓ
Biotin	100 µg/ℓ
Thiamine	100 µg/ℓ
Adenosine	200 µg/ℓ
Histidine	200 µg/ℓ

The term "resistant to an antifolate" as used in this specification defines any mutant strain that gives such a relative degree of growth in excess of that of the parent strain in an antifolate-containing medium. As examples of the mutants that can be employed in the practice of this invention, there may be mentioned Bacillus pumilus NA-1101 (IFO 14184, FERM P-6639), Bacillus pumilus NA-1102 (IFO 14185, FERM P-6640), Bacillus pumilus NA-1103 (IFO 14186, FERM P-6641), Bacillus subtilis NA-6011 (IFO 14189, FERM P-6642) and Bacillus subtilis NA-6012 (IFO 14190, FERM P-6643).

The above IFO numbers are the deposit numbers assigned to the strains by the Institute for Fermentation, Osaka (IFO), 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka, Japan. Type cultures of the above-identified microorganisms have been deposited with IFO since July 13, 1982.

Sub-cultures of these microorganisms, which were deposited on July 22, 1982 at Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the accession numbers of FERM P as shown above, the deposit being converted to a deposit under the Budapest Treaty, have been stored at FRI under the accession numbers of FERM BP as follows:

Strain	The Accession Numbers under the Budapest Treaty
Bacillus pumilus NA-1101	FERM BP-288
Bacillus pumilus NA-1102	FERM BP-289
Bacillus pumilus NA-1103	FERM BP-290
Bacillus subtilis NA-6011	FERM BP-291
Bacillus subtilis NA-6012	FERM BP-292

The above-mentioned NA-1101, NA-1102 and NA-1103 strains were derived from Bacillus pumilus No. 158-A-11 strain (IFO 12476) as parent strain, and NA-6011 and NA-6012 strains from Bacillus subtilis No. 115 (IFO 14187) as the parent strain.

Microbiological characteristics of these mutant strains are similar to those of the respective parent strains.

Microbiological characteristics of the respective parent strains are as follows:

	Bacillus pumilus No. 158-A-11 (IFO 12476)	Bacillus subtilis No. 115 (IFO 14187)
a. Morphology		
1) Shape and size	Rods 0.6 × 3 μ	Rods 0.3 × 3 μ
2) Polymorphism	Singly or rarely in chains	Singly or rarely in pairs
3) Motility	Non-motile	Non-motile
4) Sporulation	Yes	Yes
Shape of spore	Ellipsoidal	Ellipsoidal
Swelling sporangium	No	No
Position of spore	Central or para-central	Central or para-central
5) Gram's stain	Positive	Positive
6) Acid fastness	Negative	Negative

	Bacillus pumilus No. 158-A-11 (IFO 12476)	Bacillus subtilis No. 115 (IFO 14187)
b. Cultural characteristics		
1) Bouillon agar plate	Round or undulated, convex, lenticular, opaque and white to pale yellow.	Amorphous and diffused, rough surface, flat, opaque and white to light brown.
2) Bouillon agar slant	Convex to raised, opaque and white to pale yellow.	Flat, opaque and white to light brown.
3) Bouillon liquid	Coherent pellicle, no turbidity, rarely no surface growth with some turbidity.	Coherent pallicle, no turbidity.
4) Litmus milk	Alkaline, peptonization, reduction	Alkaline, peptonization, reduction
c. Physiological characteristics		
1) Reduction of nitrates	Negative	Positive
2) V-P reaction	Positive	Positve
3) Hydrolysis of starch	Negative	Positive
4) Utilization of citrate	Positive	Positive
5) Utilization of propionate	Negative	Negative
6) Utilization of ammonium salts	Positive	Positive
7) Urease	Weakly positive	Weakly positive
8) Catalase	Positive	Positive

	Bacillus pumilus No. 158-A-11 (IFO 12476)	Bacillus subtilis No. 115 (IFO 14187)
9) Oxygen requirement	Aerobic, no growth under anaerobic conditions	Aerobic, no growth under anaerobic conditions
10) Growth in 7% sodium chloride	Positive	Positive
11) Growth in media, pH 5.7	Positive	Positive
12) Biotin requirement	Positive	Negative

Comparison of the above characteristics of IFO 12476 and IFO 14187 strains with the comparable descriptions in Bergey's Manual of Determinative Bacteriology, R. E. Buchanan and N. E. Gibbons (ed.), Edition 8, 1974 showed that they belong to Bacillus pumilus and Bacillus subtilis.

The medium used for cultivating such an inosine and/or guanosine-producing strain for the production of inosine and/or guanosine may be any of the media commonly employed for inosine and/or guanosine fermentation. Regarding the composition of the medium, the carbon sources may be sugars such as glucose, maltose, starch hydrolysate, etc., monohydric or polyhydric alcohols such as glycerin, ethanol, sorbitol, etc., and fatty acids such as acetic acid, propionic acid, stearic acid, oleic acid, etc., and these carbon sources are used either singly or in combination. The nitrogen sources include, among others, organic nitrogen sources such as peptone, soybean meal, corn steep liquor, yeast, meat extract, urea, etc., ammonium salts of sulfuric acid, nitric acid, hydrochloric acid, carbonic acid, etc., and inorganic nitrogen sources such as gaseous ammonia, aqueous ammonia, etc., and these nitrogen sources may be used singly or in combination. As other nutrient sources,

there may be employed various inorganic salts necessary for growth of the strain, such as the sulfates, hydrochlorides, carbonates, nitrates, phosphates, acetates, etc. of calcium, potassium, sodium, magnesium, manganese, iron, copper, zinc, etc. as well as the amino acids, vitamins, etc. necessary for growth of the organism. These nutrient sources may be used singly or in combination. As sources of adenine, there may be employed not only adenine, adenosine, adenylic acid, ribonucleic acid, etc. but also natural materials such as microbial cells containing them, extracts thereof, meat extract, etc. Moreover, defoaming agents and surfactants such as silicone oil, polyalkylene glycol ether, etc. may also be incorporated in the medium as necessary.

Cultivation is generally conducted under aerobic conditions, for example by shake culture or by aerated submerged culture. The medium is preferably maintained within the range of pH 4 to pH 9. If there is a fluctuation of pH in the course of fermentation, it may be corrected by adding an aqueous solution or suspension of alkali hydroxide, calcium carbonate, ammonia or the like or ammonia gas from time to time. The cultivation temperature is generally within the range of from 20° to 45°C and the optimum temperature for growth of the microorganism and for accumulation of inosine and/or guanosine is selected. Cultivation is continued until the accumulation of inosine and/or guanosine reaches a maximal level and it is generally sufficient to carry out fermentation for 24 to 144 hours.

Separation and recovery of inosine and/or guanosine from the fermentation broth can be effected by the conventional method, e.g. precipitation, ion exchange resin or activated carbon chromatography, etc.

According to the present invention, inosine and/or guanosine can be produced in much greater yields, compared with known methods.

The following examples are intended to illustrate this invention in further detail.

Example 1

Bacillus pumilus No. 158-A-11 (IFO 12476) [hereditary characteristics: adenine requiring, adenine/guanosine-resistant, guanylic acid reductase-deficient, and histidine-requiring] was treated with nitrosoguanidine (NTG) in the conventional manner and then streaked on a plate prepared by adding 50 µg/ml of methotrexate and 25 g/ℓ of agar to the medium A of Table 1. The inoculated medium was incubated at 37°C for 3 days and Bacillus pumilus NA-1101 (IFO 14184, FERM BP-288) was selected from among the colonies. The resistance to methotrexate (relative growth) of this strain as well as that of the parent strain was determined by the method hereinbefore described. The results are shown in Table 2.

Table 2

Strain	Relative growth Methotrexate added (µg/ml)					
	0	1	2	4	8	15
NA-1101	100	100	100	100	100	88
No. 158-A-11	100	80	65	43	40	18

Then, a 200 ml conical flask containing 20 ml of the seed culture medium of Table 3 was inoculated with a loopful of Bacillus pumilus NA-1101 grown on a nutrient agar (Difco) plate and shake culture was conducted at 37°C for 18 hours. A portion (1 ml) of the culture was transferred to a 200 ml creased flask containing 20 ml of the main fermentation medium indicated in Table 3, and cultivation was conducted on a rotary shaker at 37°C for 4 days. The yield of guanosine was 23.0 mg/ml.

Table 3

Seed culture		Main fermentation medium	
Composition of medium	Concentration	Composition of medium	Concentration
Sorbitol	2.0%	Glucose	15.0%
Dried yeast	1.5%	Ammonium sulfate	2.0%
Potassium dihydrogen phosphate	0.1%	Urea	1.0%
		Sodium glutamate	1.0%
Dipotassium hydrogen phosphate	0.3%	Corn steep liquor	2.0%
Histidine	0.01%	Crude nucleic acid	0.2%
		Calcium chloride	0.5%
		Magnesium sulfate	0.2%
		Potassium chloride	0.05%
		Calcium carbonate	3.0%
		Biotin	200 µg/ℓ
		Manganese sulfate	2.5 mg/ℓ

The above procedure was carried out for a total of about 50 flasks to obtain 1 liter of broth. This broth was adjusted to pH 11 with sodium hydroxide to dissolve the guanosine crystals and, then, centrifuged to remove the cells. The resulting supernatant was neutralized and cooled to give 21.0 g of crude crystals of guanosine. These crystals were dissolved in 900 ml of hot water, decolorized with activated carbon, and allowed to stand in the cold to precipitate the quanosine. 18.4 g of pure crystals of guanosine was obtained. Cultivation of the parent No.158-A-11 strain under the same conditions resulted in the accumulation of guanosine only in a yield of 4.5 mg/ml.

Example 2

Bacillus pumilus No. 158-A-11 was subjected to ultraviolet irradiation and NTG treatment in the conventional manner and, as described in Example 1, streak-cultured on the plate medium prepared by adding 200 µg/ml of amino-pterin and 25 g/l of agar to the medium A, followed by selection of a colony of Bacillus pumilus NA-1102 (IFO 14185, FERM BP-289). This strain was cultivated under the same conditions as Example 1. The yields of inosine and quanosine in the broth were 18 mg/ml and 6 mg/ml, respectively.

Example 3

Bacillus subtilis No. 115 (wild strain) (IFO 14187) was subjected to the conventional NTG treatment to obtain an adenine-requiring mutant NA-6001 (IFO 14188).

This strain was further treated with NTG three times to obtain Bacillus subtilis NA-6011 (IFO 14189, FERM BP-291), a strain which can grow on the plate medium prepared by adding 100 µg/ml of aminopterin and 25 g/l of agar to the medium A. The aminopterin-resistance and inosine/quanosine producing ability of each of these strains were determined by the procedures described in Example 1. The results are shown in Table 4 and Table 5, respectively.

Table 4

Strain	Relative growth					
	Aminopterin added (μg/ml)					
	0	4	8	15	30	60
NA-6011	100	100	97	95	89	52
NA-6001	100	85	75	55	22	13

Table 5

Strain	Accumulation of inosine	Accumulation of guanosine
NA-6011	18 mg/ml	5 mg/ml
NA-6001	1	-

Example 4

Bacillus pumilus No. 158-A-11 was subjected to the conventional NTG treatment to give Bacillus pumilus NA-1103 (IFO 14186, FERM BP-290), a mutant strain capable of growing on the plate medium prepared by adding 10 μg/ml of trimethoprim and 25 g/l of agar to the medium A. The trimethoprim resistance of this strain is shown in Table 6.

Table 6

Strain	Relative growth				
	Trimethoprim added (g/ml)				
	0	0.1	0.25	0.5	1.0
NA-1103	100	100	100	100	89
NA-158-A-11	100	36	17	10	7

When this strain was cultivated under the same conditions as Example 1, guanosine was accumulated at the

rate of 22 mg/ml.

Example 5

Bacillus subtilis NA-6001 (IFO 14188) was treated with NTG four times to obtain Bacillus subtilis NA 6012 (IFO 14190, FERM BP-292), a mutant strain capable of growing on the plate medium prepared by adding 50 µg/ml of pyrimethamine and 25 g/l of agar to the medium A. The pyrimethamine resistance of this mutant is shown in Table 7.

Table 7

Strain	Relative growth							
	Pyrimethamine (µg/ml)							
	0	0.5	1.5	3.0	6.0	12	25	50
NA-6012	100	100	86	76	66	52	49	26
NA-6001	100	85	56	26	6	2	2	2

When this mutant was cultivated under the conditions as Example 1, inosine and guanosine were accumulated at the rates of 9 mg/ml and 14 mg/ml, respectively.

CLAIMS:

1. A method of producing inosine and/or guanosine, which comprises culturing a mutant strain of the genus Bacillus, which requires adenine for growth and is resistant to an antifolate (as hereinbefore defined) and capable of producing inosine and/or guanosine, in a medium to cause the same strain to elaborate and accumulate inosine and/or guanosine in a culture broth, and recovering the inosine and/or guanosine so accummulated from said broth.

2. The method according to Claim 1, wherein the mutant is resistant to methotrexate.

3. The method according to Claim 1, wherein the mutant is resistant to aminopterin.

4. The method according to Claim 1, wherein the mutant is resistant to pyrimethamine.

5. The method according to Claim 1, wherein the mutant is resistant to trimethoprim.

6. The method according to Claim 1, wherein the mutant is of the species Bacillus pumilus.

7. The method according to Claim 1, wherein the mutant is of the species Bacillus subtilis.

8. The method according to Claim 1, wherein the mutant is Bacillus pumilus NA-1101 (FERM BP-288), Bacillus pumilus NA-1102 (FERM BP-289) or Bacillus pumilus NA-1103 (FERM BP-290).

9. The method according to Claim 1, wherein the mutant is Bacillus subtilis NA-6011 (FERM BP-291) or Bacillus

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subtilis NA-6012 (FERM BP-292).

10. A biologically pure culture of a microorganism belonging to the genus Bacillus and having characteristics identifiable with those of FERM BP-288, FERM BP-289, FERM BP-290, FERM BP-291 or FERM BP-292, said culture being capable of producing, in a culture medium containing assimilable carbon and digestible nitrogen sources, a recoverable amount of inosine and/or guanosine.

11. A microorganism of Bacillus pumilus NA-1101 (FERM BP-288), Bacillus pumilus NA-1102 (FERM BP-289), Bacillus pumilus NA-1103 (FERM BP-290), Bacillus subtilis NA-6011 (FERM BP-291) or Bacillus subtilis NA-6012 (FERM BP-292) which is resistant to an antifolate (as hereinbefore defined) and is capable of producing inosine and/or guanosine.

12. A method of producing inosine and/or guanosine, substantially as hereinbefore described with reference to any of the Examples.

13. Inosine or guanosine when produced by a method according to any of Claims 1 to 9 and 12.