DESCRIPTION

STRAIN CAPABLE OF PRODUCING LARGE AMOUNT OF ε-POLY-L-LYSINE AND PROCESS FOR PRODUCING ε-POLY-L-LYSINE USING THE SAME

Technical Field

The present invention relates to a strain capable of producing a large amount of ε-poly-L-lysine (hereinafter, referred to as "εPL") and a process for producing εPL by the fermentation method using said strain.

Since εPL is a polymer of L-lysine which is an essential amino acids, it has high safety and, owing to a high cation content, it has unique physical properties. Accordingly, it is expected to find its utility in toiletries, cosmetics, feed additives, medicaments, pesticides, food additives, electronic materials and the like. Particularly in the field of food additives, it has drawn attentions as a naturally-occurring additive.

Background Art

As a production process for εPL in accordance with the fermentation method, a process is known (an examined Japanese patent publication No. 59-20359), which comprises incubating the strain of *Streptomyces albulus subsp. lysinopolymerus* No. 346-D (FERM P-3834), which is an εPL

production strain separated from the natural and belongs to the genus *Streptomyces*, on a medium, and separating and purifying εPL from the resulting cultured medium. Also known is a process (an examined Japanese patent publication No. 3-42070 or an examined Japanese patent publication No. 3-78998) which comprises subjecting the above-described strain of *Streptomyces albulus subsp. lysinopolymerus* No. 346-D to mutagenesis treatment, thereby transforming it into its mutant having resistance to S-(2-aminoethyl)-L-cysteine which is an analogous substance of L-lysine, incubating the resulting mutant, that is, the strain of *Streptomyces albulus lysinopolymerus* 11011A-1 (FERM BP-1109) on a medium, and separating and purifying εPL from the resulting cultured medium.

For the production of inexpensive εPL which can meet various applications, even the use of the above-described strain 11011A-1 has not yet been sufficient from the viewpoints of the production amount of εPL and glucose conversion efficiency. There has accordingly been a demand for the development of a strain improved in the ability to produce εPL and an industrially inexpensive and efficient process for the production of εPL by using said improved strain.

The present inventors have repeated extensive investigations with a view to obtaining a strain which is more improved in the ability to produce εPL compared with the conventional εPL-producing strain or mutant thereof. As a result, it has been found that a mutant having resistance to S-(2-aminoethyl)-L-cysteine (which will hereinafter be abbreviated as "AEC") at a concentration as high as 10 mg/ml or more can be a strain capable of producing εPL in a large amount and that a large amount of εPL can be produced by aerobically incubating said strain on a medium. Based on these findings, the present investigation has been completed.

As is apparent from the above description, an object of the present invention is to provide a strain which has a higher ability to produce εPL compared with the conventional εPL producing strain or improved strain thereof and can therefore improve the productivity of εPL; and also to provide a process for producing εPL at a low cost and in a large amount by using said strain.

Disclosure of the Invention

The present invention is constituted from the following technical means:

(1) A strain capable of producing a large amount of ε-poly-L-lysine which is obtained by subjecting a microorganism, which belongs to the species *Streptomyces albulus* and has an ability to produce ε-poly-L-lysine, to mutagenesis treatment, and has resistance to S-(2-aminoethyl)-L-cysteine at a concentration as high as 10 mg/ml or more.

(2) A strain B21021 (accession number: FERM BP-5926) capable of producing a large amount of ε-poly-L-lysine, which strain is obtained by subjecting the strain of *Streptomyces albulus subsp. lysinopolymerus* 11011A-1 (FERM BP-1109) to mutagenesis treatment and has resistance to S-(2-aminoethyl)-L-cysteine at a concentration as high as 10 mg/ml or more.

(3) A process for producing ε-poly-L-lysine, which comprises aerobically incubating a microorganism belonging to the genus *Streptomyces*, has a resistance to S-(2-aminoethyl)-L-cysteine at a concentration as high as 10 mg/ml or more and has a producing capacity of ε-poly-L-lysine, on a medium and collecting ε-poly-L-lysine formed and accumulated in the culture.

(4) A process for producing ε-poly-L-lysine, which comprises aerobically incubating the strain B21021

described in (2) above on a medium and collecting the ε-poly-L-lysine formed and accumulated in the culture.

The AEC to be used in the present invention is a structurally analogous substance (analog) to L-lysine and it is a compound having a structure different from L-lysine only in the sulfur atom replaced for the carbon atom at the 4-position. The addition of the AEC to a medium causes growth inhibition of microorganisms and growth inhibition becomes more apparent and stronger when L-threonine is used in combination with it.

Although the strain 11011A-1 disclosed in an examined Japanese patent publication No. 3-42070 is an εPL producing strain having resistance to AEC which is an analogous substance of L-lysine, it was screened in the presence of AEC at a concentration of 2 mg/ml and it shows growth only when the AEC concentration is 5 mg/ml or less and does not show growth at the AEC concentration of 10 mg/ml or more.

The improved strain according to the present invention is obtained by screening in the presence of AEC at a concentration of 10 mg/ml or more and it is a microorganism which belongs to the genus *Streptomyces* and has resistance permitting growth even at an AEC concentration of 40 mg/ml. In addition, the improved strain is recognized to have a

partial difference in mycological characteristics from the strain 1101A-1 which is a parent strain.

As a parent strain to be used upon obtaining such an improved strain, microorganisms belonging to the genus *Streptomyces* and capable of producing εPL can be used. Among them, the strain of *Streptomyces·albulus·lysinopolymerus* 11011A-1 (FERM BP-1109) is preferred.

The term "mutagenesis treatment" as used herein means a treatment for inducing mutation of a microorganism (strain) which belongs to the genus *Streptomyces* and is capable of producing εPL, thereby obtaining an improved strain resistant to AEC at a concentration as high as 10 mg/ml or more. Examples of the method for such a mutagenesis treatment include a method of adding N-methyl-N'-nitro-N-nitrosoguanidine (hereinafter, referred to as "NTG") to give a concentration of 0.1 mg to 3 mg per ml of a buffer and bringing the strain in contact with the NTG for 10 minutes to 2 hours; a method of exposing the strain to ultraviolet rays at an amount of irradiation of 100 to 1000 J/cm^2, a method of treating the strain with a chemical such as 5-bromouracil; and other ordinarily employed chemical or physical mutagenesis treatment methods.

Examples of the screening method of the strain which has been subjected to mutagenesis treatment include a

method of inoculating the strain on the minimum agar medium to which AEC has been added to give a concentration of 10 mg or more, L-threonine has been added to give a concentration of 1 mg, each per ml of the medium, and then collecting the strain growing on the medium.

The present invention will hereinafter be described in detail.

Illustrative example for obtaining the improved strain of the present invention, which is a strain resistant to high-concentration AEC is as follows. Spores of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* 11011A-1, which is an εPL-producing strain, are suspended in a tris-maleate buffer (pH 6.0). To the resulting suspension, NTG is added in an amount to give a concentration of 1.5 mg per ml of said buffer and the spores and NTG are brought into contact at 37°C for 60 minutes. The resulting spores are then collected by centrifugal separation, washed with a phosphate buffer (0.05 M, pH 7.0) and incubated on a liquid nutrient medium (glucose: 5%, ammonium sulfate: 1%, yeast extract: 0.5%, K_2HPO_4: 0.08%, KH_2PO_4: 0.136%, $MgSO_4 \cdot 7H_2O$: 0.05%, $ZnSO_4 \cdot 7H_2O$: 0.004%, $FeSO_4 \cdot 7H_2O$: 0.003%, pH: 6.8, wherein % means g/dl%) overnight. After incubation, the resulting cells are collected by centrifugal separation, washed with a phosphate buffer (0.05M, pH 7.0), applied to the minimum agar medium (glucose: 5%, ammonium sulfate: 1%,

K_2HPO_4: 0.08%, KH_2PO_4: 0.136%, $MgSO_4 \cdot 7H_2O$: 0.05%, $ZnSO_4 \cdot 7H_2O$: 0.004%, $FeSO_4 \cdot 7H_2O$: 0.003%, pH: 6.8, agar: 1.5%, wherein % means g/dl%) containing 20 mg of AEC and 1 ml of L-threonine, each based on 1 ml of the medium, and incubated at 30°C for 3 to 4 days. Colonies thus developed are then collected. Concerning the thus-obtained strain resistant to high-concentration AEC, productivity of εPL is evaluated. As a result, the strain exhibiting the highest productivity is the strain of *Streptomyces albulus subsp lysinopolymerus* B21021 [National Institute of Bioscience and Human Technology, Ministry of International Trade and Industry (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305, Japan), International Accession Number: FERM BP-5926, transferred from the original deposit (FERM P-15193, date of original deposit: September 20, 1995) to an international deposit under the Budapest Treaty on April 18, 1997], followed by the strains B22107 and B22201.

The resistance of the high-concentration-AEC-resistant strain, which is an improved strain, against AEC is measured as follows. That is, resistance is compared by applying each of the resistant strains to the minimum agar medium (described above) to which AEC has been added at a concentration as described below in Table 1 and L-threonine

has been added in an amount of 1 ml per mg of the medium, incubating at 30°C for 2 to 7 days and then visually observing the growth. The results are shown in Table 1. It is recognized that the strain 11011A-1, that is, a parent strain exhibits growth at the AEC concentration of 5 mg/ml, but not at all at the concentration of 10 mg/ml, while the strain B21021, that is, a high-concentration-resistant strain, exhibits growth even at the AEC concentration of 40 mg/ml. The improved strain according to the present invention has resistance to high-concentration AEC and in this respect, it can be distinguished clearly from the parent strain.

Table 1

Test strain	AEC concentration (mg/ml)	Incubated days (day) 2	3	4	5	6	7
B21021	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	20	+	+	+	+	+	+
	40	-	-	±	+	+	+
B22107	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	20	+	+	+	+	+	+
	40	-	-	±	+	+	+
B22201	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	20	+	+	+	+	+	+
	40	-	±	+	+	+	+
11011A-1 (parent strain)	2	-	-	±	±	+	+
	5	-	-	±	±	+	+
	10	-	-	-	-	-	-
	20	-	-	-	-	-	-

Note) +: growth was observed ±: slight growth was observed
-: no growth was observed

The mycological characteristics of the strain B21021 will next be described below.

(1) Morphological characteristics

(i) Branching manner and form of a sporulation hypha

simple branching, closed spiral

(ii) The number of spores: tens of spores

(iii) Surface structure and size of spores

The spores each has a spherical or oval shape of about 1.2 to 1.5 μm in size and has a spiny surface structure.

(vi) Existence or absence of a flagellum, sclerotium and sporangium: not recognized

(v) adhesion position of the tip of a spore: on an aerial hypya

(2) Cultural characteristics on each medium

Characteristics in each of the mediums as shown in Table 2 are the results of the observation after incubation at 30°C for 10 to 14 days.

Table 2

Medium	Substrate hypha	Aerial hypha	Soluble pigment
Sucrose·nitrate agar medium	Not formed	Not formed	None
Glucose·asparagine agar medium	White to pale yellow	Grayish brown	None
Glycerol·asparagine agar medium	Pale yellow	White, powdery	None
Tyrosine agar medium	Pale brown	White	Brown
Nutrient agar medium	Yellow	White Slightly formed	None
Yeast·malt agar medium	Yellowish brown	Grayish brown, powdery	None
Oat meal agar medium	Yellowish brown	White, Abundant	None

(3) Physiological characteristics

(i) Growth temperature range

About 15 to 40°C, optimal growth temperature: around 30°C.

(ii) Liquefaction of gelatin, hydrolysis of starch and peptonization of skim milk

Each, positive.

(iii) Coagulation of skim milk: Negative.

(iv) Formation of melanin-like pigment

A brown pigment is formed on a tyrosine agar medium.

(v) Composition of a cell wall

The type of diaminopimelic acid in the components of the composition of the cell wall was found to be of L,L

type as a result of analysis in accordance with the method of Becker, et al., [*Applied Microbiology*, Vol. 13, p. 236 (1965)].

(4) Assimilation of carbon sources (Pridham-Gottlieb agar medium)

L-Arabinose	-
D-Xylose	-
D-Glucose	+
D-Fructose	+
L-Rhamnose	-
D-Galactose	+
Sucrose	-
Raffinose	-
D-Mannitol	+
Inositol	+
Salicin	+

+: assimilable, -: not assimilable

As can be seen from the above-description, the strain B21021 which is one of the improved strains according to the present invention is different in some mycological characteristics from the strain 11011A-1 which is a parent strain. For example, concerning the assimilation of carbon sources, the strain 11011A-1 cannot assimilate salicin, while the strain B21021 can assimilate it. Concerning cultural characteristics, the grayish brown aerial hyphae

are formed on the sucrose · nitrate agar medium to which the strain 11011A-1 has been applied but no formation is found in the case of the strain B21021. White aerial hyphae are formed abundantly on the nutrient agar medium to which the strain 11011A-1 has been applied, while aerial hyphae are only slightly formed in the case of the strain B21021. The soluble pigment is observed on both the glucose · asparagine agar medium and glycerol · asparagine agar medium, while it is observed on neither of the mediums in the case of the strain B21021. Thus, the strain B21021 can also be distinguished clearly from the parent strain, that is, strain 11011A-1 in the mycological characteristics.

For producing εPL from the improved strain, the improved strain is inoculated and incubated on a medium and then, εPL thus formed and accumulated is separated and purified from the culture. As a medium, any medium can be employed insofar as it contains a carbon source, nitrogen source, inorganic substance and other nutrients in proper amounts. As the carbon source, those assimilable by the improved strain such as glucose, fructose, glycerin and starch can be used without any limitation. The amount to be added is preferably 1 to 5% (% means g/dl%). As a nitrogen source, any one of peptone, casein hydrolyzate,

amino acid, inorganic ammonium salt and the like can be used, of which ammonium sulfate is preferred. The nitrogen source is preferably added in an amount of 0.2 to 2% (% means d/l%). During incubation, a carbon source and nitrogen source can be added successively. Examples of the inorganic substance include phosphate ion, potassium ion, sodium ion, magnesium ion, zinc ion, iron ion, manganese ion, nickel ion and sulfuric acid ion. The incorporation of a yeast extract in an amount of 0.1 to 0.5% (% means d/l%) improves the growth of microorganisms and brings about good effects in the production of εPL.

Incubation is carried out under aerobic conditions by shaking culture, agitation culture or the like. The incubation temperature of 25 to 35°C is preferred. Although the pH of the medium is preferably near neutral (pH 6 to 8), pH lowers with the growth of the microorganisms after the incubation is started. When the pH is dropped to 4, an alkali is added to maintain the pH at 4. Aqueous ammonia is preferred as the alkali to be added, but sodium hydroxide, potassium hydroxide or the like can also be employed. εPL is accumulated in the culture in usually 1 to 7 days.

After the removal of the cells from the culture by centrifugal separation or a filter, the residual solution

was purified, decolored and concentrated. From the concentrate, εPL can be obtained by crystallizing it in an organic solvent such as acetone or ethanol.

Best Modes for Carrying Out the Invention

The present invention will hereinafter be described more specifically.

The production amount of εPL in the culture was measured in accordance with the method as described in Itzhaki, et al., "*Analytical Biochemistry*, 50, 569 (1972)". That is, the amount of εPL in the culture is determined by subjecting the culture to centrifugal separation to remove the cells, mixing 2 ml of the supernatant (εPL: 0 to 200 μg) with 2 ml of a 1 mM aqueous solution of methyl orange, allowing the resulting mixture to stand at room temperature for 30 minutes, removing the resulting εPL-methyl orange complex by centrifugal separation, and measuring the absorbance of the supernatant at 465 nm.

It should be noted that "%" in examples and comparative examples means weight(g)/volume(dl) % unless otherwise indicated specifically.

[Example 1]

In a jar having a volume of 3 liter, 2 liter of a medium composed of 5% of glucose, 0.5% of yeast extract, 1%

of ammonium sulfate, 0.08% of K_2HPO_4, 0.136% of KH_2PO_4, 0.05% of $MgSO_4 \cdot 7H_2O$, 0.04% of $ZnSO_4 \cdot 7H_2O$, 0.003% of $FeSO_4 \cdot 7H_2O$ and having a pH of 6.8 was charged. To the medium, 100 ml of a pre-culture of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021, that is, the improved strain obtained according to the present invention, was inoculated, followed by aerobic incubation at 30°C and 700 rpm for 72 hours at an air flow rate of 3 liter/min. The pH was maintained at 4 with 10% aqueous ammonia. When the remaining concentrations of glucose and ammonium sulfate lowered, they were added successively.

The production amount of εPL and glucose conversion efficiency after incubation for 72 hours are shown in Table 3. The term "glucose conversion efficiency (%)" as used herein means a value expressed by (εPL production amount/glucose consumption amount) x 100.

[Example 2]

Incubation was carried out in the same manner as in Example 1 except for the use of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B22107 instead of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021 and the amount of εPL in the culture was measured. The production amount of εPL and glucose conversion

efficiency after incubation for 72 hours are shown in Table 3.

[Example 3]

Incubation was carried out in the same manner as in Example 1 except for the use of the strain *Streptomyces·albulus·subsp·lysinopolymerus* B22201 instead of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021 and the amount of εPL in the culture was measured. The production amount of εPL and glucose conversion efficiency after incubation for 72 hours are shown in Table 3.

[Comparative Example 1]

Incubation was carried out in the same manner as in Example 1 except for the use of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* 11011A-1, that is, a parent strain instead of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021. The production amount of εPL and glucose conversion efficiency after incubation for 72 hours are shown in Table 3.

Table 3

Strain	Production amount of εPL (g/l)	Glucose conversion efficiency (%)
B21021 (Example 1)	16.2	12.1
B22107 (Example 2)	15.6	11.1
B22201 (Example 3)	14.3	13.0
11011A-1 (Comparative Example 1)	9.2	7.7

[Example 4]

In a jar having a volume of 3 liter, 2 liter of a medium composed of 5% of glucose, 0.5% of yeast extract, 1% of ammonium sulfate, 0.08% of K_2HPO_4, 0.136% of KH_2PO_4, 0.05% of $MgSO_4 \cdot 7H_2O$, 0.04% of $ZnSO_4 \cdot 7H_2O$, 0.003% of $FeSO_4 \cdot 7H_2O$ and having a pH of 6.8 was charged. To the medium, 100 ml of a pre-culture of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021, that is, the improved strain obtained according to the present invention, was inoculated, followed by aerobic incubation at 30°C and 700 rpm for 168 hours at an air flow rate of 3 liter/min. The pH was maintained at 4 with 10% aqueous ammonia. When the remaining concentrations of glucose and ammonium sulfate lowered, they were added successively.

The production amount of εPL and glucose conversion efficiency after incubation for 168 hours are shown in Table 4.

[Example 5]

Incubation was carried out in the same manner as in Example 4 except for the use of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B22107 instead of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021 and the amount of εPL in the culture was measured. The production amount of εPL and glucose conversion efficiency after incubation for 168 hours are shown in Table 4.

[Example 6]

Incubation was carried out in the same manner as in Example 4 except for the use of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B22201 instead of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021 and the amount of εPL in the culture was measured. The production amount of εPL and glucose conversion efficiency after incubation for 168 hours are shown in Table 4.

[Comparative Example 2]

Incubation was carried out in the same manner as in Example 4 except for the use of the strain of

Streptomyces·albulus·subsp·lysinopolymerus 11011A-1, that is, a parent strain instead of the strain of *Streptomyces·albulus·subsp·lysinopolymerus* B21021. The production amount of εPL and glucose conversion efficiency after incubation for 168 hours are shown in Table 4.

Table 4

Strain	Production amount of εPL (g/l)	Glucose conversion efficiency (%)
B21021 (Example 4)	31.0	12.4
B22107 (Example 5)	28.6	12.6
B22201 (Example 6)	25.0	11.4
11011A-1 (Comparative Example 2)	19.1	7.8

[Example 7]

The culture obtained in Example 4 by incubation for 168 hours was subjected to centrifugal separation to remove the cells, followed by the adjustment of pH to 7.5. Then the residue was separated and purified using each of IRC-50 (cation exchange resin), IRA-402 (anion exchange resin) and XT-1006 (cation exchange resin) and then concentrated through a reverse osmosis membrane (RO), whereby εPL was obtained. The yield of εPL is shown in Table 5.

[Comparative Example 3]

The culture obtained in Comparative Example 2 by incubation for 168 hours was subjected to centrifugal separation to remove the cells as in Example 7, followed by the adjustment of pH to 7.5. Then the residue was separated and purified using each of IRC-50 (cation exchange resin), IRA-402 (anion exchange resin) and XT-1006 (cation exchange resin) and then concentrated through a reverse osmosis membrane (RO), whereby εPL was obtained. The yield of εPL is shown in Table 5.

Table 5

Strain	Yield of εPL (g)
B21021 (Example 7)	39
11011A-1 (Comparative Example 3)	24

As is apparent from Tables 3, 4 and 5, it has been found that both the production amount of εPL and glucose conversion efficiency showed a marked increase compared with those of the strain 11011A-1.

Industrial Applicability

The improved strain of the present invention resistant to high-concentration-AEC has a capacity of producing εPL

in a large amount in a high yield. It is possible to produce εPL at a high productivity and in a high yield by using the εPL-producing strain.

CLAIMS

1. A strain for producing a large amount of ε-poly-L-lysine, which strain is obtained by subjecting a microorganism belonging to the species *Streptomyces·albulus* and having an ability to produce ε-poly-L-lysine to mutagenesis treatment and has resistance to S-(2-aminoethyl)-L-cysteine at a concentration as high as 10 mg/ml or more

2. A strain B21021 (Accession number FERM BP-5926) for producing a large amount of ε-poly-L-lysine, which strain is obtained by subjecting a strain *Streptomyces·albulus·subsp·lysinopolymerus* 11011A-1 (FERM BP-1109) to mutagenesis treatment and has resistance to S-(2-aminoethyl)-L-cysteine at a concentration as high as 10 mg/ml or more.

3. A process for producing ε-poly-L-lysine, which comprises aerobically incubating a microorganism belonging to the genus *Streptomyces*, which microorganism has resistance to S-(2-aminoethyl)-L-cysteine at a concentration as high as 10 mg/ml or more and has an ability to produce ε-poly-L-lysine, on a medium and collecting ε-poly-L-lysine formed and accumulated in the culture.

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4. A process for producing ε-poly-L-lysine, which comprises aerobically incubating a strain B21021 as claimed in claim 2 on a medium and collecting ε-poly-L-lysine formed and accumulated in the cultured solution.

