Title: FERMENTATION PROCESS FOR PREPARING L-LYSINE

Abstract: The present invention relates to a method of activating a Corynebacterium sp. strain by pre-seed culture and seed culture of the same in a medium supplemented with pyrrolidone carboxylic acid (PCA), and a fermentation process for preparing L-lysine by main culturing the same.
Description

FERMENTATION PROCESS FOR PREPARING L-LYSINE

Technical Field
[1] The present invention relates to a method of activating a Corynebacterium sp. strain by pre-seed culture and seed culture of the same in a medium supplemented with pyrrolidone carboxylic acid (PCA), and a fermentation process for preparing L-lysine by main culturing the same.

Background Art
[2] L-lysine is one of the essential amino acids, and it has been used as an animal feed additive, a food additive and a medicinal ingredient. In particular, the market for L-lysine as an animal feed additive grew to approximately 860,000 tons in 2005, which shows L-lysine is a huge factor for producing a fermented product.

[3] L-lysine is industrially produced by direct fermentation using a medium supplemented with a carbon source such as raw sugar, glucose and molasses (sugar cane, sugar beet), a nitrogen source such as corn steep liquor (CSL), soybean meal hydrolysate and ammonium sulfate, vitamins and minerals.

[4] The conventional methods for producing L-lysine are described in patent documents; for example, Japanese Laid-Open Patent Publication No. Hei 4-66558 describes a method in which cane molasses and beet molasses diluted in water are passed through a cation exchange resin and the eluent is loaded in a fermentation medium to increase the fermentation concentration and yield of L-lysine. Japanese Laid-Open Patent Publication No. 1982-026594 describes a method for producing L-lysine by fermentation of the reaction product of cane molasses and beet molasses diluted in water with invertase using Corynebacterium.

Disclosure of Invention

Technical Problem
[5] The inventors completed the present invention by establishing a novel process for the fermentation of L-lysine with excellent fermentation efficiency. This was achieved by activating a Corynebacterium sp. strain by pre-seed culture and seed culture of the same in a medium supplemented with pyrrolidone carboxylic acid (PCA).

Technical Solution
[6] It is an object of the present invention to provide a method of activating a Corynebacterium sp. strain to be used for the fermentation of L-lysine.

[7] It is another object of the present invention to provide a process for the fermentation of L-lysine using the activated Corynebacterium sp. strain.

[8] To achieve the above objects, the present invention provides a method of activating
a Corynebacterium sp. strain by pre-seed culture and seed culture of the same in a medium supplemented with pyrrolidone carboxylic acid (PCA).

In the present invention, any Corynebacterium sp. strain that is a member of the Corynebacterium genus and is able to produce L-lysine can be included. For example, a strain selected from a group consisting of Corynebacterium glutamicum, Corynebacterium thermoaminogenes, Corynebacterium flavum and Corynebacterium lactofermentum can be used.

The Corynebacterium sp. strain of the present invention is preferably Corynebacterium glutamicum (Accession No: KFCC 11043).

The present invention relates to a method of activating a Corynebacterium sp. strain to produce L-lysine by fermentation, in which pre-seed and seed culture media are supplemented with pyrrolidone carboxylic acid or a substance containing pyrrolidone carboxylic acid, and the Corynebacterium sp strain is cultured on the medium. It is not necessary to add pyrrolidone carboxylic acid to the medium during the main fermentation process following pre-seed or seed culture, so the post-seed culture addition of pyrrolidone carboxylic acid does not affect the fermentation efficiency.

In general, the fermentation procedure is composed of the three steps of pre-seed culture, seed culture and main fermentation (main culture).

Pre-seed culture is carried out to ensure the culture is pure before seed culture for efficient production and to maintain continuous activity of the bacterium.

Seed culture is an interim process between pre-seed culture and main fermentation to increase the cell density and activity of the strain. To increase the efficiency of the fermentation process, the volume of a culture solution has to be 5 ~ 30 % of the initial volume of main fermentation, so it is preferred to perform seed culture before the main fermentation process. Seed culture consists of culturing the pure culture strain obtained from pre-seed culture until the strain is fully activated and the numbers have increased enough for the main culture. The seed culture solution is used directly for the main fermentation process.

According to the batch fermentation process, a purely isolated strain is cultured in a small volume reactor. When the population of the strain reaches a certain level, they are transferred to a large volume reactor (in some cases, this process has to be repeated several times). After being through the above process, the required amount of cells for the main culture and a culture solution are created, wherein the activity is secured.

According to the present invention, pyrrolidone carboxylic acid, known as a natural moisturing factor, or a substance containing pyrrolidone carboxylic acid is added to a medium for pre-seed culture or/and seed culture of a Corynebacterium sp. strain before the main fermentation process, so as to activate the strain. As a result, the productivity of L-lysine produced from the main fermentation process is also increased.
In the present invention, pyrrolidone carboxylic acid is added independently, or a substance containing pyrrolidone carboxylic acid can be added.

In the present invention, the preferable content of pyrrolidone carboxylic acid in a pre-seed culture and seed culture medium is 0.1 to 5 g/l.

The substance containing pyrrolidone carboxylic acid is exemplified by animal skin, aloe, sugar beet, etc, and beet molasses is preferred. The preferable content of beet molasses in a pre-seed culture and seed culture medium is 5 to 100 g/l. The content of pyrrolidone carboxylic acid in beet molasses is approximately 2 to 3%.

In addition to pyrrolidone carboxylic acid, various components such as a carbon source, a nitrogen source and minerals can be added to a pre-seed culture medium, a seed culture medium and a main fermentation medium.

The carbon source is exemplified by carbohydrates such as glucose, sucrose, fructose, maltose and starch, and organic acids such as acetic acid and lactic acid. The carbon source can be added independently or combined with others. The nitrogen source is exemplified by organic nitrogen spruces such as peptone, yeast extract, gravy, malt extract, corn immersion solution and soybean molasses, and inorganic nitrogen sources such as urea, ammonium sulfate, ammonium chloride, ammonium phosphate and ammonium nitrate. The nitrogen source can be added independently or combined with others. The medium is additionally supplemented with potassium dihydrogen phosphate or dipotassium hydrogen phosphate and its corresponding sodium salt as a phosphate source. A metal salt such as magnesium sulfate or iron sulfate might be added to the medium. In addition, amino acids, vitamins and a proper precursor can also be added. Such a medium or precursor can be added to the culture solution by batch-type or continuous addition.

In order to regulate the pH of the culture medium, sodium hydroxide, potassium hydroxide or ammonia can be added during the culture. The generation of air bubbles during the culture is inhibited by using an antifoaming agent such as fatty acid polyglycol ester. To maintain aerobic conditions, oxygen or an oxygen-containing gas (for example: air) is added to the culture medium. The preferable temperature of the culture medium is 20 to 45°C and more preferably 25 to 40°C. The culture period is determined as the duration of L-lysine generation, which is preferably 10 to 160 hours.

The method for producing the above culture is selected from a group consisting of batch culture, continuous culture and fed culture. Fed culture includes fed-batch culture and repeated fed-batch culture, but is not always limited thereto.

The present invention relates to a fermentation process for producing L-lysine, in which the Corynebacterium sp. strain activated as described above is cultured.

The isolation of L-lysine from the culture medium in the fermentation process of the present invention is performed by any conventional method known to those in the
art, which is exemplified by centrifugation, membrane filtration, ion exchange chromatography and crystallization. For example, low-speed centrifugation can be performed to separate L-lysine from the culture medium or ion chromatography can be performed on the supernatant with the elimination of biomass using a membrane.

**Best Mode for Carrying Out the Invention**

[26] Practical and presently preferred embodiments of the present invention are illustrated as shown in the following examples.

[27] However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

[28]

[29] **Example 1**

[30] In this preferred embodiment of the present invention, the effect of pyrrolidine carboxylic acid (PCA) on the activity of the *Corynebacterium* sp. strain was investigated.

[31] *Corynebacterium glutamicum* (Accession No: KFCC 11043), separated from freeze-dried cells and tested for titer, was cultured in a LB (Luria Bertani) liquid medium for 20 hours, followed by washing three times with sterilized saline. The culture solution was inoculated in a minimum medium supplemented with PCA at the concentrations of 0, 0.1, 0.25, 0.5 and 1.0 g/l, followed by shaking-culture at 30°C, 220 rpm for 20 hours.

[32] **Composition of the minimum medium (pH 7.0, before sterilization):**

<table>
<thead>
<tr>
<th></th>
<th>glucose</th>
<th>(NH₄)₂SO₄</th>
<th>urea</th>
<th>KH₂PO₄</th>
<th>K₂HPO₄</th>
<th>MgSO₄·7H₂O</th>
<th>FeSO₄·7H₂O</th>
<th>MnSO₄</th>
<th>MoO₃</th>
<th>CaG₃H₂O₄</th>
<th>NaCl</th>
<th>MnCl₂</th>
<th>ZnSO₄·7H₂O</th>
<th>CuSO₄·5H₂O</th>
<th>FeCl₃·6H₂O</th>
<th>L-lysine</th>
<th>thiamine-HCl</th>
<th>riboflavin</th>
<th>biotin</th>
<th>CaG₃H₂O₄·2H₂O</th>
<th>nicotinic acid</th>
<th>in distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(g/l)</strong></td>
<td>1.0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The reaction solution was diluted and distributed on a LB solid medium, followed by further culture at 30°C to 32°C for 2 days, and the generated colonies were counted.

[34] To count the live bacteria, the culture solution was diluted with 0.85% sterilized saline and loaded on the LB medium (peptone 10g/l, yeast extract 5g/l, NaCl 5g/l, agarose 20g/l) and the colonies were counted. The equivalent was quantified by the Bertrand method.

[36] **Table 1**

<table>
<thead>
<tr>
<th>PCA conc. (g/l)</th>
<th>0</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number (cells/mL)</td>
<td>5×10⁶</td>
<td>6×10⁸</td>
<td>7×10⁸</td>
<td>3×10⁸</td>
<td>5×10⁸</td>
</tr>
</tbody>
</table>
As shown in Table 1, the activity of the *Corynebacterium glutamicum* KFCC 11043 was increased by the addition of PCA. From the above result, it was presumed that PCA physiologically affected the cell membrane to increase resistance against the external environment (pH in a medium, osmotic change, etc.), resulting in the increase of the population of the strain.

Example 2: Fermentation in a flask and a small fermentor

In this preferred embodiment of the present invention, *Corynebacterium glutamicum* KFCC-1043, an L-lysine producing strain, was fermented in a flask and a small fermentor.

(1) Fermentation in a flask

25 D of a pre-seed medium was added to a 250 D baffle flask, followed by shaking-culture. 50 D of fermentation medium was added to a 500 D baffle flask, followed by shaking-culture.

PCA was added to the pre-seed culture medium and the fermentation medium at different concentrations of 0, 0.5, 1.0 and 2.5 g/l. The composition of a medium excluding PCA is as follows.

Composition of a pre-seed medium (pH 7.0, before sterilization):

- Raw sugar 50 g/l, soybean meal hydrolysate 10 g/l, yeast extract 1 g/l, KH$_2$PO$_4$ 1.5 g/l, MgSO$_4$·7H$_2$O 0.5 g/l, urea 5 g/l, biotin 50 μg/l.

Composition of a fermentation medium (pH 7.0, after sterilization):

- Raw sugar or glucose 100 g, soybean meal hydrolysate 10 g, (NH$_4$)$_2$SO$_4$ 40 g, urea 4 g, KH$_2$PO$_4$ 2 g, NaCl 2.5 g, MgSO$_4$·7H$_2$O 0.5 g, FeSO$_4$·7H$_2$O 10D, MnSO$_4$·5H$_2$O 10D, biotin 100D, thiamine-HCl 200D, CaC$_2$O$_4$G (in 1 L of process water).

Each culture was performed at 32°C, 222 rpm for 72 hours.

Upon completion of the culture, the culture solution was analyzed by High Performance Liquid Chromatography (HPLC) to measure the concentration of L-lysine and OD. The remaining sugar was measured by the Bertrand method and the results are as follows:

Table 2

<table>
<thead>
<tr>
<th>PCA conc.</th>
<th>L-lysine HCl conc.</th>
<th>L-lysine HCl yield (based on sugar, %)</th>
<th>OD$^*$ (×100)</th>
<th>Remaining sugar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>52.1</td>
<td>52.1</td>
<td>0.35</td>
<td>0</td>
</tr>
<tr>
<td>0.5 g/l</td>
<td>54.8</td>
<td>54.8</td>
<td>0.29</td>
<td>0</td>
</tr>
<tr>
<td>1.0 g/l</td>
<td>56.3</td>
<td>56.3</td>
<td>0.32</td>
<td>0</td>
</tr>
<tr>
<td>2.5 g/l</td>
<td>56.0</td>
<td>56.0</td>
<td>0.33</td>
<td>0</td>
</tr>
</tbody>
</table>

$^*$0.1 N HCl was used as a dilution solution (x100) to eliminate the remaining CaCO$_4$, then OD$_{562}$ was measured.

(2) Fermentation in a small fermentor
After pre-seed culture in a shaking incubator, seed culture was performed in a 5 L fermentor, and the main fermentation process was performed in a 30 L fermentor by fed-batch fermentation.

Particularly, pre-seed culture was performed in a shaking incubator (25D medium/250D baffle flask) at 30°C and 220 rpm for 20 hours with the addition of PCA at the concentrations of 0 (non-added), 1.0 and 2.5 g/l in the pre-seed culture medium, or with the addition of beet molasses at the concentrations of 10 and 20 g/l.

**Composition of a pre-seed culture medium (pH 7.0 before sterilization):**

Raw sugar 50g/l, soybean hydrolysate 10g/l, yeast extract 1g/l, KH\(\text{PO}_4\) 1.5g/l, Mg\(\text{SO}_4\) -7H\(\text{O}\) 0.5g/l, urea 5g/l, biotin 50D/1.

After pre-seed culture, seed culture was performed at 30°C, pH 7.2 (adjusted with ammonia gas) and 600 rpm for 24 hours in a 5 L fermentor with the addition of PCA at the concentration of 0 (non-added), 1.0 and 2.5 g/l in the medium, or with the addition of beet molasses at the concentrations of 10 and 20 g/l, and aeration was 1 vvm.

**Composition of a seed culture medium:**

Raw sugar 70g/l, soybean hydrolysate 5g/l, \((\text{NH}_4)^2\)\(\text{SO}_4\) 10g/l, KH\(\text{PO}_4\) 1.0g/l, Mg\(\text{SO}_4\) -7H\(\text{O}\) 0.5g/l, biotin 300D/1, thiamine-HCl 1,000D/l, niacinamide 2,000D/l, calcium pentothenate 500D/1, antifoaming agent 3D/1.

After seed culture, main fermentation was performed at 32°C, pH 7.0 (adjusted with ammonia gas) and 600 rpm for 50 to 80 hours in a 30 L fermentor with the addition of PCA at the concentrations of 0 (non-added), 1.0 and 2.5 g/l in the main fermentation medium, and aeration was 1 vvm.

**Composition of a main fermentation medium:**

Raw sugar 360g/l, molasses (reducing sugar) 10g/l, soybean hydrolysate 30g/l (pH 2-3, adjusted with ammonia gas, added independently after sterilization), \((\text{NH}_4)^2\)\(\text{SO}_4\) 55g/l, KH\(\text{PO}_4\) 2.0g/l, Mg\(\text{SO}_4\) -7H\(\text{O}\) 1.5g/l, Fe\(\text{SO}_4\) -7H\(\text{O}\) 50g/l, Mn\(\text{SO}_4\) -5H\(\text{O}\) 50D/1, biotin 300D/1, thiamine-HCl 1,000D/l, niacinamide 2,000D/l, calcium pentothenate 500D/1, antifoaming agent 3D/1.

Upon completion of the culture, the culture solution was analyzed by High Performance Liquid Chromatography (HPLC) to measure L-lysine concentration and the results are shown in Tables 3 and 4.

**Table 3**

<table>
<thead>
<tr>
<th>Composition of pre-seed culture medium (pH 7.0 before sterilization):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw sugar 50g/l, soybean hydrolysate 10g/l, KH(\text{PO}_4) 1.5g/l, Mg(\text{SO}_4) -7H(\text{O}) 0.5g/l, urea 5g/l, biotin 50D/1.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Composition of seed culture medium:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw sugar 70g/l, soybean hydrolysate 5g/l, ((\text{NH}_4)^2)(\text{SO}_4) 10g/l, KH(\text{PO}_4) 1.0g/l, Mg(\text{SO}_4) -7H(\text{O}) 0.5g/l, biotin 300D/1, thiamine-HCl 1,000D/l, niacinamide 2,000D/l, calcium pentothenate 500D/1, antifoaming agent 3D/1.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Composition of main fermentation medium:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw sugar 360g/l, molasses (reducing sugar) 10g/l, soybean hydrolysate 30g/l (pH 2-3, adjusted with ammonia gas, added independently after sterilization), ((\text{NH}_4)^2)(\text{SO}_4) 55g/l, KH(\text{PO}_4) 2.0g/l, Mg(\text{SO}_4) -7H(\text{O}) 1.5g/l, Fe(\text{SO}_4) -7H(\text{O}) 50g/l, Mn(\text{SO}_4) -5H(\text{O}) 50D/1, biotin 300D/1, thiamine-HCl 1,000D/l, niacinamide 2,000D/l, calcium pentothenate 500D/1, antifoaming agent 3D/1.</td>
</tr>
</tbody>
</table>
Table 4

<table>
<thead>
<tr>
<th>PCA conc. (g/l)</th>
<th>L-lysine HCl conc. (g/l)</th>
<th>L-lysine HCl yield (based on sugar, %)</th>
<th>Productivity (g/L.hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-seed/seed culture</td>
<td>Main fermentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-/-</td>
<td>-</td>
<td>165</td>
<td>51.0</td>
</tr>
<tr>
<td>0.5/0.5</td>
<td>-</td>
<td>190</td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
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<td>52.0</td>
</tr>
<tr>
<td>1.0/1.0</td>
<td>-</td>
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<td>51.8</td>
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<td></td>
<td>1.0</td>
<td>186</td>
<td>51.6</td>
</tr>
<tr>
<td>2.5/2.5</td>
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<td>191</td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>187</td>
<td>51.6</td>
</tr>
</tbody>
</table>

As shown in Table 3, the addition of PCA to the pre-seed culture/seed culture medium at the concentrations of 0.5, 1.0 and 2.5 g/l increased fermentation productivity up to 15% and yield by 2%, compared with the conventional methods, regardless of the addition of PCA to the main fermentation medium.

Similar effects were confirmed when pyrrolidone carboxylic acid containing beet molasses was added (Table 4).

**Industrial Applicability**

As explained hereinbefore, the fermentation process for preparing L-lysine of the present invention provides excellent productivity and yield based on sugar.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the present invention as set forth in the appended claims.
Claims

[1] A method of activating a *Corynebacterium* sp. strain by pre-seed culture and seed culture of the same in a medium supplemented with pyrrolidone carboxylic acid.

[2] The method according to claim 1, wherein the concentration of the pyrrolidone carboxylic acid in the medium is 0.1 to 5 g/l.

[3] The method according to claim 1, wherein the pyrrolidone carboxylic acid is supplied to the medium as beet molasses.

[4] The method according to claim 3, wherein the concentration of the beet molasses in the medium is 5 to 100 g/l.

[5] The method according to claim 1, wherein the *Corynebacterium* sp. strain is *Corynebacterium glutamicum* (Accession No: KFCC 11043).

[6] A fermentation process for preparing L-lysine, which includes the step of main culturing one of the activated *Corynebacterium* sp. strains of claim 1 to claim 5.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

C12N 1/20(2006.01)1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 C12N 1/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975
Korean Utility models and applications for Utility models since 1975
Japanese Utility models and Utility models for Utility models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS (KIPO internal), 'PCA', 'Corynebacterium

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>A</td>
<td>JP 57-026594 A (Azinomoto Co ) 12 February 1982 See whole document</td>
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Further documents are listed in the continuation of Box C

Date of the actual completion of the international search

13 MARCH 2007 (13 03 2007)

Date of mailing of the international search report

14 MARCH 2007 (14.03.2007)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
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Facsimile No 82-42-472-7140

Authorized officer

LEE, CHUNG HO
Telephone No 82-42-481-8160

Form PCT/ISA/210 (second sheet) (April 2005)
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