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Patents Act 1990

Patent Request: Standard Patent

We, the Applicant/Nominated Person specified below, request we be granted a patent for the invention disclosed in the accompanying standard complete specification.

[70,71] **Applicant/Nominated Person:**
Degussa Aktiengesellschaft, incorporated in Germany, of 9, Weissfrauenstrasse, D-6000 Frankfurt am Main, GERMANY

[54] **Invention Title:**
A process for the Fermentative Preparation of Amino Acids

[72] **Inventors:**
Walter Pfefferle, Hermann Lotter, Heinz Friedrich and Wolfgang Degener

[74] **Address for Service in Australia:**
Spruson & Ferguson, Patent Attorneys
Level 33 St Martins Tower
31 Market Street
Sydney New South Wales Australia [Code **SF**]

Basic Convention Application Details

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Basic Applicant: Degussa Aktiengesellschaft

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Registered Patent Attorney

AUSTRALIAN PATENT OFFICE IRN 216374

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NOTICE OF ENTITLEMENT

I, John David O'Connor, of 31 Market Street, Sydney, New South Wales, 2000, Australia, being authorised by the Applicant/Nominated Person in respect of an application entitled:

A Process for the Fermentative Preparation of Amino Acids

state the following:-

The Applicant/Nominated Person has entitlement from the actual inventors as follows:-

The Applicant/Nominated Person, by virtue of a Contract of Employment between the actual inventors as employees and the Applicant/Nominated Person as employer, is a person which would be entitled to have the patent assigned to it if a patent were granted on an application made by the actual inventors.

The Applicant/Nominated Person is the applicant of the basic application listed on the Patent Request.

The basic application listed on the Patent Request is the application first made in a Convention Country in respect of the invention.

Dated 27 July 1992

John David O'Connor

IRN: 216374

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A PROCESS FOR THE FERMENTATIVE PREPARATION OF AMINO ACIDS

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(71) Applicant(s)
DEGUSSA AKTIENGESELLSCHAFT

(72) Inventor(s)
WALTER PFEFFERLE; HERMANN LOTTER; HEINZ FRIEDRICH; WOLFGANG DEGENER

(74) Attorney or Agent
SPRUSON & FERGUSON , GPO Box 3898, SYDNEY NSW 2001

(56) Prior Art Documents
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AU 56005/90 C12P 013/08
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(57) Claim

1. A process for the fermentative preparation of amino acids, in which a strain of the genera *Brevibacterium* or *Corynebacterium* producing one or more amino acids is cultivated in a nutrient medium and the amino acid(s) is/are isolated from the culture fluid at the end of fermentation, characterised in that after the vigorous growth phase, the bacterial culture has less of an assimilable source of carbon available to it than it is capable of metabolising given the structure of the strain and the quantity of other necessary supplements provided in the nutrient medium.

2. A process according to claim 1, characterised in that the concentration of the source of carbon after the vigorous growth phase is from 0 to <3g/L.

4. A process according to any one of claims 1 to 3, characterised in that strains producing L-lysine and/or L-threonine are used.

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COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

Name and Address of Applicant: Degussa Aktiengesellschaft
9, Weissfrauenstrasse
D-6000 Frankfurt am Main
GERMANY

Actual Inventor(s): Walter Pfefferle, Hermann Lotter, Heinz Friedrich and Wolfgang Degener

Address for Service: Spruson & Ferguson, Patent Attorneys
Level 33 St Martins Tower, 31 Market Street
Sydney, New South Wales, 2000, Australia

Invention Title: A Process for the Fermentative Preparation of Amino Acids

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

A process for the fermentative preparation of amino acids

Description

This invention relates to an improved process for the fermentative preparation of amino acids such as L-lysine or L-threonine.
L-Lysine is an essential amino acid and is used in large quantities as animal feed supplement.

Numerous amino acids are generally produced biosynthetically and the method has long been known in the art. The bacterial strains for producing the amino acids are distinguished by the capacity of secreting these amino acids into the culture medium at high concentrations within a short time. Feed batch processes are generally carried out to avoid high initial concentrations of substrate. Due to the very high metabolic capacity of the production strains used, it is of decisive importance to carry out the fermentation process in such a manner that the maximum values of oxygen requirement and of evolution of heat will be of an economically acceptable order of magnitude.
Various strategies are therefore employed to regulate the metabolic activity of the organisms so as to ensure the supply of oxygen and removal of heat and at the same time balance the distribution of formation of biomass and of product.

A process entailing intermittent feeding is disclosed in CSFR-PS 212 558 in which the metabolic activity during the growth phase is adjusted by changes in pH and the total amount of biomass is adjusted by the α-aminonitrogen. SU-PS 157 059 describes a process entailing intermittent feeding, in which the threonine concentration serves as

criterion for the feeding and the proportion of the reducing compound is maintained at 3 to 5%. A very finely adjusted process is disclosed in FR-PS 8303487.

In this process, two feed solutions are continuously to be added: A leucine phosphate solution which is added at such a rate that both the intensity of metabolism and the formation of the biomass are limited by the rate of addition of supplement. The second feed solution, a sugar solution, is supplied at such a rate that the actual sugar concentration is maintained at 5 to 15g/L. This shows that due to a limitation by the leucine/phosphate supplements during the feed phase, the culture uses less sugar at any point in time than is available in the culture medium. This procedure is in line with the repeatedly documented view that both C-limitation and undue C-excess should be avoided (eg. DD-PS 269 167). Hadj Sassi *et al* in "Biotechn. Letters, Volume 10, No. 8, pages 583-586 (1988)" even propose from 90 to 140g/L of glucose for this purpose. The metabolic activity is therefore always regulated by a factor other than that of the source of C.

This invention provides a process for the fermentative preparation of amino acids which proceeds at a higher degree of conversion of the source of carbon used (sugar) and in which a higher concentration of amino acids is obtained in the dry mass free from biomass.

The invention relates to a process for the fermentative preparation of amino acids, in which a strain of the genera *Brevibacterium* or *Corynebacterium* producing one or more amino acids is cultivated in a nutrient medium and the amino acid(s) is/are isolated from the culture fluid at the end of fermentation, characterised in that after the vigorous growth phase (during the production phase), the bacterial culture has less of an assimilable source of carbon available to it than it is capable of metabolising given the structure of the strain and the quantity of other necessary supplements provided in the nutrient medium.

~~available to the bacterial culture than it could metabol-~~ ize on the basis of the structure of the ~~strain~~ and the quantity of other necessary ~~supplements~~ provided in the nutrient medium. ~~The~~ fermentation (nutrient) medium is in ~~other respects of conventional composition.~~

In addition to containing sources of carbon such as assimilable sugars, saccharose, glucose, molasses or starch hydrolysates and ammonium ions, in the case of autotrophic producers it contains complex components as source of organic supplements required due to one or more auxotrophies, such as protein hydrolysates as source of α-aminonitrogen, vitamins and inorganic salts. The vigorous growth at the beginning of fermentation is generally a logarithmic growth phase. This may be shortened if required by limiting the supplements and/or the source of carbon.

This phase is followed by cell growth but the extent of this growth is confined to a small fraction of the vigorous growth phase. Strains producing L-lysine and/or L-threonine are preferably used. The fermentation medium is chosen so that the temperature is from 25 to 40°C, preferably from 30 to 36°C, the pH from 6 to 8, preferably from 7 to 7.5, and the ammonium concentration from 0.5 to 8 g/l. The broth is stirred and amply supplied with oxygen. Metabolization of the sugar may be controlled by the quantity of amino acid added, especially in the case of amino acid-auxotrophic lysine secretors. The concentration of these supplements or of other necessary supplements after the growth phase is advantageously from 0 to 0.1 g/l each, in particular from 0 to 0.05 g/l each. Thus, for example, in a leucine-auxotrophic lysine secretor, the sugar/leucine ratio in a continuously added feed medium is chosen so that the formation of biomass is limited by the supply of leucine but at the same time the

amount of sugar provided is only a fraction of that which could be converted at the given leucine concentration.

The concentration of utilizable sugars after the vigorous growth phase is advantageously from 0 to <3 g/l, in particular from 0 to 1 g/l.

The concentration of 0 g/l in the fermentation broth does not mean, either with respect to any supplements required or with respect to the source of carbon, that these substances are not supplied continuously. It means that these compounds are supplied in a quantity which is immediately taken up by the bacterial cultures. This fermentation carried out by the process according to the invention has numerous very important advantages compared with the conventional processes mentioned above, namely:

1. The metabolic activity and hence the oxygen requirement and the evolution of heat of the culture can be influenced directly and without delay by the rate of supply of feed and adapted to the capacity of the fermenter.

2. The fermentation broths are distinguished by a higher product content of the dry mass as a whole and hence greater purity. Loss by the formation of by-products is prevented by the fact that over the whole period of feeding, the bacterial culture is offered less substrate than it would be capable of converting, so that the source of carbon constitutes the primary limitation.

3. The fermentations have a higher yield than fermentations in which limitation is primarily by way of supplements.

4. In the process of monitoring the product, fermentation can be stopped directly and without any time lag at an optimum or at a plateau and the gross yield is at all times equal to the net yield.

5. In a working up project which includes direct concentration of the fermentation broth by evaporation, the fermenter contents can be immediately used for working up in the event of technical breakdown without the quality of the product being impaired by a high residual sugar content.

The Examples which follow document possible embodiments of the process according to the invention.

Examples

Example 1 (Comparison Example)

5.1 kg of a sterile solution containing the following components were introduced into a fermentation container equipped with stirrer and ventilation system:

Water	4540 g
Molasses	26 g
Glucose	125 g
Corn gluten hydrolysate (hydrolysed with sulphuric acid)	35 g
Hydrolysate of the producer biomass (hydrolysed with sulphuric acid)	320 g
Ammonium sulphate	45 g
Phosphoric acid 85%	7 g
Magnesium sulphate	3 g

other mineral salts, traces and biotin and thiamine

and the solution was adjusted to pH 7.3 with ammonia solution. 0.6 l of an inoculum of a Corynebacterium Glutamicum DM 346-1 carrying the genetic markers leu$^-$, oxalysine resistance and aminoethyl resistance, were added to this solution at 33 to 35°C. The inoculum had been prepared by 15 hours' incubation at 33°C and pH 7 with stirring and ventilation in a medium containing 4.4 mass percent of molasses in addition to 2% of sucrose and 14% of soya bean meal hydrolysate (hydrolysed with sulphuric acid) with the addition of 3% of ammonium sulphate, 0.05% of phosphoric acid and 0.02% of magnesium sulphate and the vitamins, biotin and thiamine.

With vigorous stirring, ventilation and adjustment of the

pH to about 7.3 by means of aqueous ammonia solution, the following medium neutralised with aqueous ammonia solution was continuously added in the conventional manner within 32 hours after termination of the logarithmic growth phase in the main fermenter so that the measurable sugar concentration in the fermentation broth was from 5 to 35 g/l (enzymatic determination based on sucrose and glucose):

Water	1250 g
Molasses	94 g
Glucose	1465 g
Corn gluten hydrolysate (sulphuric acid)	39 g
Hydrolysate of the producer biomass (sulphuric acid)	265 g
Ammonium sulphate	31 g
Phosphoric acid 85%	4 g
Magnesium sulphate	2 g

other mineral salts, traces and biotin and thiamine.

At the end point of fermentation, when all the assimilable sugar in the fermentation medium had been used up, the degree of conversion of sugar into lysine was 35%, calculated as LysxHCl, and the lysine base content of the concentrated fermentation solution free from biomass was 45%.

Example 2

Preparation of the inoculum, the medium introduced into the main fermenter and the culture conditions are similar to those of Example 1.

Medium 2 also has the same composition with the exception of the following modification:

Water	1560 g
Molasses	75 g
Glucose	1170 g.

In this experiment, the feed medium was added at the same rate as in Example 1. Analyses of the progress based on assimilable sugar showed that, in accordance with the process according to the invention claimed here, the measurable concentration of assimilable sugars remained 3 g/l during the entire feed time and was almost always kept below 1 g/l. Analyses based on leucine in the fermentation broth, using amino acid analyser, showed that after the quantity of leucine provided in medium 1 had been used up, the leucine concentration during the feed time was at no point greater than 0.05 g/l.

After termination of the fermentation, the degree of conversion of sugar into lysine (calculated as LysxHCl) was 40% and the lysine base content of the concentrated fermentation broth free from biomass was 54%.

Example 3

3980 kg of a sterile medium having the following composition were introduced into a 10 m^3 reactor:

Saccharose	320 kg
Molasses	20 kg
Corn gluten hydrolysate	230 kg
25% Aqueous ammonium sulphate	150 kg
Citric acid . H_2O	2.3 kg
Phosphoric acid (89%)	6.6 kg
$MgSO_4.7H_2O$	2.8 kg
$CaCl_2.2H_2O$	75 g
$FeSO_4.H_2O$	113 g
Mn $SO_4.H_2O$	113 g
$ZnSO_4.7H_2O$	5.6 g
$CuSO_4.5H_2O$	0.6 g
Biotin	1.1 g
Thiamine.HCl	0.8 g
NH_4OH (2-3 %)	1010 kg
Water	2258 kg

pH: 7.0.

The contents of the reactor are stirred at 33°C and vigorously ventilated. After the transfer of 250 l of inoculum of the strain DM 282-2 carrying the genetic markers leucine auxotrophic and aminoethylcysteine resistant (after 16 hours' incubation in a medium containing 6% of molasses, 14% of soya bean meal hydrolysate, 1% of ammonium sulphate and 0.1% of phosphoric acid at pH 7 and 30°C) into a 10 mm^3 reactor, the pH is maintained at 7.0 by means of aqueous ammonia and the rate of ventilation is adjusted so that the dissolved oxygen content is always above 15% saturation.

After the culture had grown to an optical density (535 nm) of about 30, a production medium having the following composition was added at the rate of 30 l/h:

Saccharose	940	kg
Molasses	50	kg
Corn gluten hydrolysate	180	kg
25% Aqueous ammonium sulphate	80	kg
Citric acid . H_2O	1	kg
Phosphoric acid (89%)	2.8	kg
$MgSO_4.7H_2O$	1.2	kg
$FeSO_4.H_2O$	48	g
$MnSO_4.H_2O$	48	g
$ZnSO_4.7H_2O$	2.4	g
$CuSO_4.5H_2O$	0.3	g
Biotin	0.6	g
Thiamine.HCl	0.4	g
NH_4OH (25%)	80	kg
Water	740	kg

pH: 7.5

The pH was maintained at 7.3 during the production phase. In accordance with the invention claimed here, a concentration of assimilable sugar of 1 g/l was not exceeded during the feed phase after the sugar provided in the growth medium had been used up, and the measurable leucine concentration was below 0.05 g/l. At the end of fermentation, the degree of conversion of sugar into lysine (in the form of Lys.HCl) was 32.3% and the lysine base content of the concentrated fermentation broth free from biomass was 54.7%.

Example 4 (Comparison Example)

Preparation of the inoculum, the process parameters and

the media in the growth phase and in the production phase correspond to the conditions indicated in Example 3 although feeding was in this case carried out at a rate of about 100 l/h. As a result, the measurable concentrations of assimilable sugar during the feeding period after the sugar provided in the growth medium had been used up were always distinctly above 5 g/l but the concentration of leucine remained below 0.05 g/l. The degree of conversion of sugar into lysine (calculated as Lys.HCl) was 30.9% at the end of fermentation and the lysine base content of the fermentation broth free from biomass was 43.5%.

Example 5 (Comparison Example)

The media for culture, growth and production are similar in composition to the media of Example 1 except that the glucose was replaced by 25 g/l of saccharose in the growth medium and by 564 g/l of saccharose in the production medium. The incubation parameters including preparation of the inoculum are also identical. 0.82 Kg (0.8 l) of sterile growth medium were introduced into a small fermenter equipped with stirrer and ventilating means. To this solution were added 1.1 l of an inoculum of Corynebacterium glutamicum DSM 5717 at 33 to 35°C. When an optical density of about 30 (535 nm) had been reached, 533g (430 ml) of production medium were continuously added within 24h.

During the time of feeding, the measurable sugar content was always above 5 g/l in the fermentation medium and the leucine content after the quantity provided in the growth medium had been used up was always below 0.05 g/l. At the end of fermentation, 74 g of lysine were detected in the medium as Lys.HCl, which in the case of a total input of saccharose of 275 g corresponds to a degree of

conversion of 27%. The lysine content of the total dry biomass was 30.5%.

Example 6

In another experiment also using strain DSM 5715, in which all the parameters of media and incubation were identical to those of Experiment 5, the production medium was continuously fed in within 39 h. In accordance with the process claimed here, the actual saccharose concentration during the feed period after the source of C and leucine provided in the growth medium had been used up was below 1 g/l and the leucine concentration was below 0.05 g/l. At the end of fermentation, 89 g of lysine (in the form of lysine.HCl) were detected in the medium, and the degree of conversion was 32%. The lysine base content in the total dry mass was 36.3%.

The claims defining the invention are as follows:-

1. A process for the fermentative preparation of amino acids, in which a strain of the genera *Brevibacterium* or *Corynebacterium* producing one or more amino acids is cultivated in a nutrient medium and the amino acid(s) is/are isolated from the culture fluid at the end of fermentation, characterised in that after the vigorous growth phase, the bacterial culture has less of an assimilable source of carbon available to it than it is capable of metabolising given the structure of the strain and the quantity of other necessary supplements provided in the nutrient medium.

2. A process according to claim 1, characterised in that the concentration of the source of carbon after the vigorous growth phase is from 0 to <3g/L.

3. A process according to claim 1 or claim 2, characterised in that auxotrophic bacterial strains are used, and due to the occurrence in some cases of multiple strain auxotrophy, at least one organic compound having a concentration of from 0 to 0.1g/L is added after the vigorous growth phase.

4. A process according to any one of claims 1 to 3, characterised in that strains producing L-lysine and/or L-threonine are used.

5. A process according to claim 4, characterised in that leucine-auxotrophic strains of Corynebacterium glutamicum are used.

6. A process for the fermentative preparation of amino acids, substantially as hereinbefore described with reference to any one of the Examples but excluding the comparative examples.

7. Amino acids produced by the process of any one of the preceding claims.

Dated 13 September, 1994
Degussa Aktiengesellschaft

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

Abstract

The invention relates to a process for the fermentative preparation of amino acids, in which a strain of the genus Brevibacterium or Corynebacterium producing one or more amino acids is cultivated in a nutrient medium and the amino acid(s) is/are isolated from the culture fluid at the end of fermentation, characterised in that after the vigorous growth phase, the bacterial culture has at its disposal a smaller quantity of assimilable carbon source than it could metabolize on the basis of the structure of the strain and the quantity of other necessary supplements provided in the nutrient medium.