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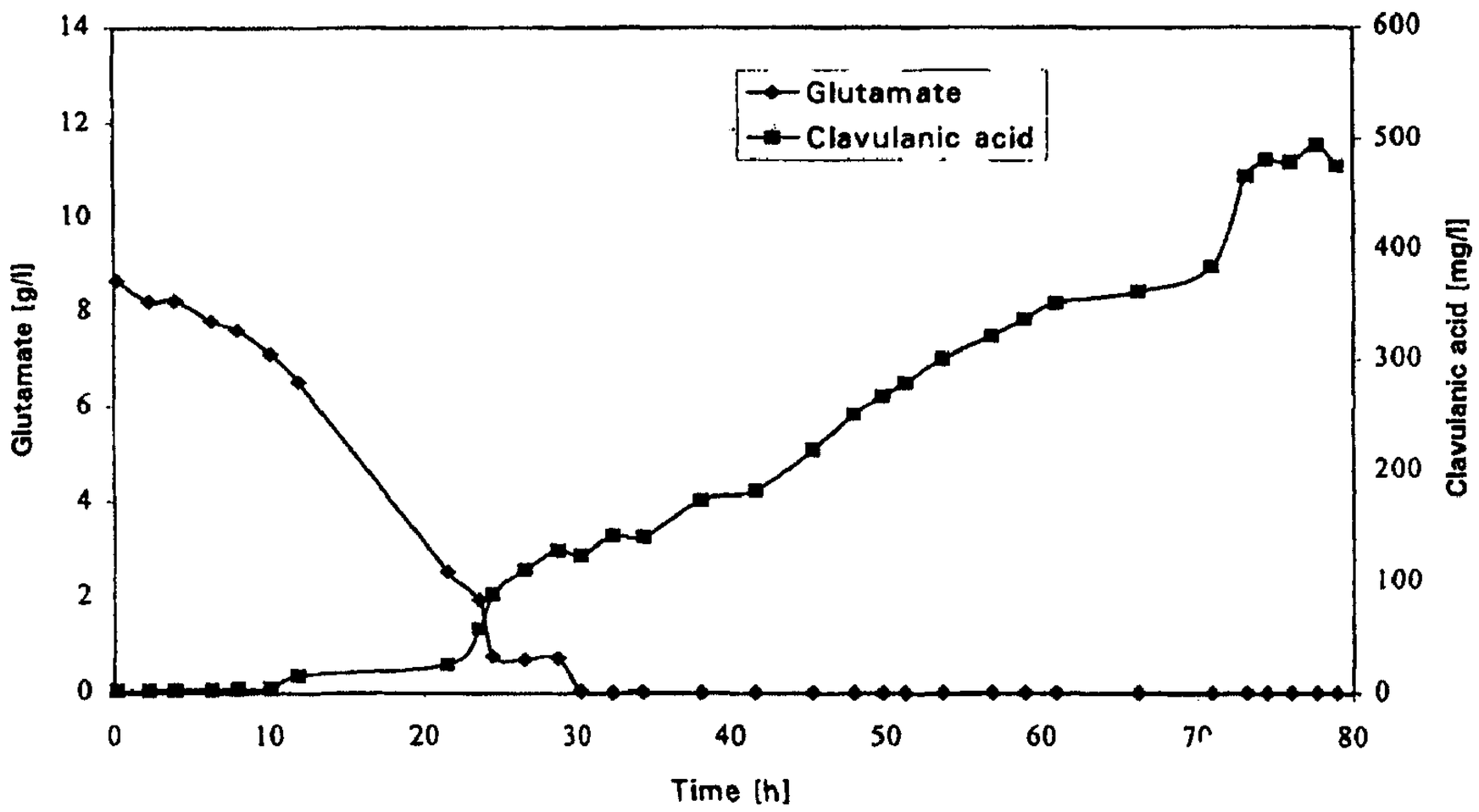
(54) PROCESSUS DE FERMENTATION VISANT LA PRODUCTION D'ACIDE CLAVULANIQUE A FAIBLE CONCENTRATION D'ACIDES AMINES LIBRES

(54) FERMENTATION PROCESS TO PRODUCE CLAVULANIC ACID AT A LOW CONCENTRATION OF FREE AMINO ACIDS



(57) L'invention concerne un procédé amélioré visant la production de métabolites secondaires par la fermentation d'une souche de Streptomyces. Si la concentration d'acides aminés libres reste faible, on obtient un rendement étonnamment élevé.

(57) An improved method for the production of secondary metabolites by the fermentation of a Streptomyces strain has been disclosed. If the concentration of free amino acids is kept low, a surprisingly high yield is obtained.

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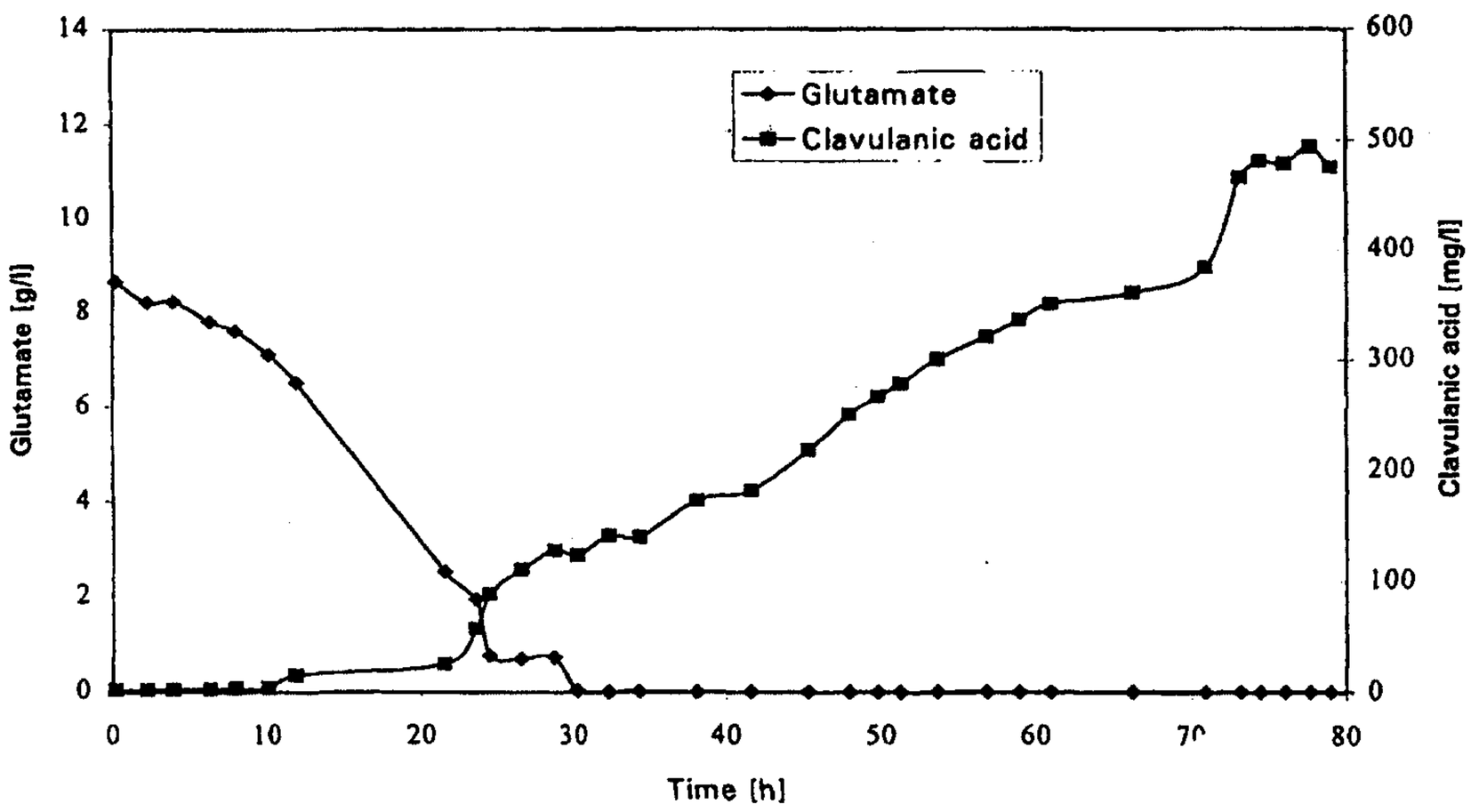

(57) Abstract

An improved method for the production of secondary metabolites by the fermentation of a Streptomyces strain has been disclosed. If the concentration of free amino acids is kept low, a surprisingly high yield is obtained.

FERMENTATION PROCESS TO PRODUCE CLAVULANIC ACID AT A LOW CONCENTRATION OF FREE AMINO ACIDS

Field of the invention

The present invention relates to the field of the fermentative production of secondary metabolites from a Streptomyces strain on a suitable medium comprising carbon and nitrogen sources.

Background of the invention

A large variety of different secondary metabolites is produced fermentatively from Streptomyces microorganisms, as for instance β-lactams, polyketides and macrolides, such as clavulanic acid, pimaricine and erythromycine. Clavulanic acid is an important inhibitor of β-lactamases and is produced by various microbial strains belonging to the genus of *Streptomycetes* such as *S. clavuligerus* ATCC 27064, *S. jumonjinensis* (GB patent 1563103), *S. katsurahamanus* IFO 13716 FERM 3944 (JP patent 83009679B) and *Streptomyces* sp. P6621 FERM 2804 (JP patent application 55162993A).

Clavulanic acid can be produced in substantial amounts and production conditions are being optimized continuously in order to increase the yield and the purity of the end product. The production of clavulanic acid has been optimized with respect to continuous fermentation (GB 1508977), feeding of carbon to a batch process (EP-182522), maintaining ammonia at low concentrations (WO 96/18743 and Romero J., Liras P. and Martin J.F., Appl. Microbiol. Biotechnol. (1984), Vol. 20, 318-325), reduction of phosphate concentration in fermentation media during growth and production (Romero (1984), v.s., Lebrihi A., Germain P. and Lefebre G., Applied Microbiol. Biotechnology. (1987), Vol.

26, 130-135 and International patent applications WO 97/19187 and WO 97/39137). With respect to carbon sources it was mentioned that triglycerides are the preferred carbon sources compared to glycerol (Butterworth (1984); Biotechnology of Industrial antibiotics, E.J. Vandamme, 1st edition, Marcel Dekker Inc. pages 225-235 and the patent application WO 97/19187).

With respect to the nitrogen source a lot of research has been done by Brana A.F., Paiva N. and Demain A.L., J. of General Microbiology (1986), Vol 132, 1305-1317. It was found that growth on media containing an amino acid is faster than growth on ammonia as sole nitrogen source.

Furthermore, on a medium containing ammonia as the sole nitrogen source, the maximum specific growth rate is $< 0.05\ h^{-1}$, Brana (1986) v.s., and Aharonowitz Y. and Demain A.L., Can J. Microbiol. (1979), Vol. 25, 61-67, while the maximum specific growth rate is $> 0.05\ h^{-1}$ in media containing at least one amino acid like asparagine, aspartate, glutamate, glutamine, alanine, histidine, proline, threonine or arginine. Therefore, one would prefer the use of a medium containing one or more amino acids. In such media, the production of biomass takes less time compared to inorganic media containing only ammonia which is of course an advantage over a slower production process.

Asparagine is described in the non-prepublished International application WO 98/37179 as fermentation ingredient for the production of clavulanic acid and also for cephalosporin, e.g. cephamycin C production from Streptomyces clavuligerus (Aharonowitz Y. and Demain A.L., v.s.). Besides this, glutamate was shown to repress clavulanic acid formation in a batch process (Romero (1984), v.s.), one would not be directed to use glutamate or glutamine.

Furthermore it is even more common to use proteins as source of amino acids as this is much cheaper than the individual ones. The disadvantage is obviously the heterogeneity of proteins and the

irreproducible quality generally associated with these complex nutrients. For clavulanic acid production typical complex nitrogen sources applied are: fishmeal, soybean meal, peanut meal for *S. clavuligerus* (EP 0 182 522 B1 and WO 97/19187), soybean meal for *S. jumonjinensis* (UK patent 1563103), soybean meal and corn steep liquor for *Streptomyces* sp. P6621 FERM 2804 (WO 97/39137 + JP patent application 55162993), and soybean flour and cotton seed flour for *S. katsurahamanus* (JP patent 83009679B). In an overview article it was reported that soybean protein was the most important protein for clavulanic acid production (Butterworth (1984), v.s.).

Here we describe the surprisingly advantageous application of hydrolysed proteins preferably rich in glutamate and proline in the fermentation broth of a secondary metabolite producing Streptomyces and the particular advantage of gluten hydrolysate and casein hydrolysate in this respect. Moreover we describe the unexpected advantageous application of a source of amino acids, in particular glutamate as a feeding nutrient in a fed batch process for the production of these compounds.

Description of the Figures

Figure 1: titre of clavulanic acid during a batch fermentation at low levels of free glutamate.

Figure 2: titre of clavulanic acid during a fed batch fermentation at low levels of free glutamate.

Summary of the invention

The present invention provides a method for the production of secondary metabolites by the fermentation of such a secondary metabolite producing Streptomyces on a suitable medium by keeping the concentration of free amino acids lower than 5 g/l fermentation broth, preferably lower than 2.5 g/l and more preferably lower than 0.5 g/l,

provided that a concentration of 4 g/l free asparagine supplied to a fermentation broth of Streptomyces clavuligerus has been excluded. This process is especially favourable for the production of -lactams, polyketides and macrolides, preferably clavulanic acid, pimaricine, erythromycine, nystatine or amphotericine. According to one aspect of the invention to maintain such low concentrations in the fermentation broth, one or more of the amino acids, preferably glutamate or proline is applied in a fed-batch or continuous mode. According to another aspect of the invention, besides amino acids itselves, also protein hydrolysate, more preferably glutenhydrolysate or caseinhydrolysate and most preferably wheat glutenhydrolysate is applied in a batch, fed-batch, semi-continuous or continuous mode.

Description of the invention

The present invention describes the use of media poor in free amino acids, by the application of said amino acids in a fed batch or continuous mode or by the application of protein hydrolysate in any mode.

For the present patent application a protein is defined as a polymer of amino acids with a size expressed by a Molecular Weight >20,000 Dalton which has not been processed by any means to degrade the protein to smaller fragments. A protein hydrolysate is defined to be a polymer of amino acids with an average size between 300 Dalton and 20,000 Dalton and a free amino acid content of less than 30% of the total amino acids. These protein hydrolysates can be produced either by enzymatic or by chemical hydrolysis of the corresponding proteins. Alternatively the advantage of using hydrolyzed proteins is achieved by using strains improved for protease activity in a process using non-hydrolyzed proteins as raw materials. Furthermore, glutamate stands in the present application for glutam like compounds as glutamate and glutamine. In the present application a protein extract is defined to be a

protein hydrolysate with a molecular weight lower than 300 Dalton and wherein > 30% of the amino acids are present as free amino acids.

When a protein or the hydrolysate thereof is defined as rich in glutamate it is meant that >15% of the amino acid content consists of glutamate and glutamine. Proteins described as rich in glutamate are casein (21%) and wheat gluten (35%).

According to one aspect of the present invention it was surprisingly found that clavulanic acid production was especially high when a protein hydrolysate was included in the medium, especially when it was derived from wheat gluten or casein. As the production levels were reduced dramatically when protein extracts were used of other protein sources like yeast and corn, especially at high hydrolysis degree (>35%) compared to the peptides (<30% free amino acids), it is shown that protein hydrolysates should preferably contain less than 30% free amino acids, even more preferably less than 5% and most preferably less than 1% free amino acids. A protein hydrolysate preferably rich in glutamate can be used as well supplied in all forms of feeding, viz. batch, fed batch, continuous or semi-continuous. By semi-continuous feeding is meant the continuous addition of nutrients to the fermentation broth while intermittently a small volume of the broth is removed.

The fermentation medium may either be a defined medium comprising $(NH_4)_2SO_4$, free amino acid, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, 3-(N-morpholino), propanesulfonic acid, glycerol, sodium succinate and a solution of trace elements, with a low concentration of amino acid, or with a protein hydrolysate which results in a complex medium. Also the application of a complex medium as for instance flours from nuts, vegetables, seeds, cereals, grasses such as those useful in fermentation industry, soybean flour, lineseed flour, peanut flour, potato flour, sunflower, pea- or beanflour, cotton seed flour, wheat gluten, whole wheat, rice meal to which protein hydrolysate is added, wherein the concentration of free amino acid is low, is part of the invention. The

medium may also contain mixtures of mentioned flours with mixtures of protein hydrolysates of various sources and of various peptide sizes as desired to achieve the optimal result.

Besides the wild type strains, also microbial strains which are capable of being fermented in a chemically defined medium and/or improved by subjecting a parent strain of interest to a classical mutagenic treatment using physical means, such as UV irradiation, or a suitable chemical mutagen, such as N-methyl-N'-nitro-N-nitrosoguanidine or ethylmethane sulfonate may be used for the process of the present invention. The same does apply to a parent strain of interest to recombinant DNA technology, whereby the parent strain is transformed with a one or more functional genes of interest.

Any assimilable carbon source may be added to the above said mixture, like sugars such as glucose, fructose, sucrose, maltose, lactose, or polysaccarides like starch, maltodextrines and inuline or other fructose polymers, triglycerides such as soybean oil, sunflour oil, olive oil, tri-oleate etc., (poly-) alcohols such as ethanol, propanol, glycerol, mannitol, or organic acids or a salt thereof such as acetate, propionate, succinate, adipate, malonate, citrate, lactate, gluconate etc.

An inorganic nitrogen source may be added to the medium such as ammonia and/or nitrate or any of its salts. Ureum may be used as well. Furthermore also vitamins, and various sorts of inorganic anions such as sulphates, phosphates, chlorides, borates, molybdate, iodate or their salts and the cations potassium, sodium, zinc, manganese, magnesium, iron, copper, cobalt, nickel etc. may be added to the medium.

A fermentation is started by inoculating from a preculture or inoculum fermentation at a volume of about 1 to 50% of the main fermentation medium, particularly from 5 to 20%. The process may last from about 24 to 400 hours and especially from 48 to 168 hours. The temperature will be kept between 20 and 40 °C, preferably between 25 and 35 °C, and even more preferably between 26 and 30 °C. The pH can

be maintained at pH 6 to 8 by means of titration with an alkaline substance such as ammonia, sodium hydroxide, potassium hydroxide, calcium hydroxide, or an organic base like lysine, arginine and histidine and an acid substance, such as the anorganic acids like sulphuric acid and hydrochloric acid. Alternatively, an organic acid may be used such as glutamate, citrate, gluconate or acetate.

The dissolved oxygen concentration may be controlled in the optimal range for the process by varying the oxygen concentration in the inlet gas, application of overpressure, modification of stirrerspeed and airflow. The range may vary between 0 and 100% of air saturation.

The process may be carried out by controlling various non-growth limiting nutrients in their optimal concentrations. Dependent on the growth limiting nutrient of choice, these growth-non-limiting nutrients may contain any relevant carbon, nitrogen, phosphor, sulphur source or oxygen.

Carbon dioxide should be kept at non-toxic concentrations by for instance increasing the airflow through the fermentor so that the carbon dioxide concentration in the outlet-gas is less than 5%, preferably less than 2.5%.

The fermentation can be carried out in a batch, fed batch, or (semi-) continuous fermentation process mode.

Of course, the recovery of the impure clavulanic acid solution as formed by the fermentative process of the present invention as well as the subsequent conversion thereof into a pharmaceutically acceptable salt by methods known in the art do form an aspect of the present invention. One of the most advantageous procedures is the conversion of the impure clavulanic acid into an amino salt thereof by adding the corresponding amino salt forming compound as for instance N,N,N',N'-tetramethylethylenediamine, 1,3-bis(di-methylamino)-2-propanol, t-butylamine, t-octylamine, benzhydrylamine and bis (2-(dimethyl-amino)ethyl)ether and reacting said amine clavulanate with a non-toxic

pharmaceutially acceptable salt as for instance potassium ethylhexanoate to form the corresponding purified salt, for instance potassium clavulanate.

Example 1

Batch fermentations of clavulanic acid comparing the use of proteins, protein hydrolysates and protein extracts.

Streptomyces clavuligerus ATCC27064 was improved for clavulanic acid production by means of several rounds of classic mutation (UV, nitroso guanidine (NTG)) and selection in shake flask cultures whereby clavulanic acid production was tested by imidazole methods as known in the art. The strain was conserved as vegetative mycelium grown for 48 hours in Tryptone Soytone Broth-medium (TSB-medium) at 28 °C in a shaker incubator shaken at 280 rpm and stored frozen at -80 °C.

1 ml of the frozen mycelium was inoculated to 100 ml of a sterilized (30 minutes, 121 °C) preculture medium containing 5-20 g/l maltose.1aq, 15-30 g/l bacto tryptone, 15-30 g/l bacto peptone, 1-10 g/l bacto soytone, mono potassium phosphate 1-5 g/l and 0.2 g/l synthetic antifoam.

After 72 hours of cultivation at 27 to 28 °C, 2.5% of this preculture is transferred to a sterile production medium containing 2.5 g/l from a complex nitrogen source such as a protein, a protein hydrolysate and/or or protein extract. The production medium further contains 50-100 g/l glycerol, 5-20 g/l soybean flour, 0.5-2 g/l mono potassium phosphate, a suitable trace element cocktail and 0.2 - 2 g/l synthetic antifoam. After 4 days of cultivation at 28 °C and adequate shaking, the cultures were harvested and assayed for clavulanic acid by means of standard HPLC-methods.

When different nitrogen sources were compared in a batch process, it was surprisingly found that the application of protein hydrolysates

increased the production of clavulanic acid with at least 10% compared to the use of proteins. The best protein hydrolysates were those derived from gluten, followed by casein- and soy-proteins respectively (see table 1).

Table 1. Clavulanic acid production with a mutant strain from *S. clavuligerus* ATCC 27064 using different complex nitrogen sources additional to soybean flour.

Nutrient name	Type	Supplier	Class	Free amino acid (g/l)	Yield percentage
Potato flour	Alburex	Roquette Freres	protein	<1	100
Yeast extract	Baker's YE (65% free amino acids)	Gist brocades	extract	13.0	7
	Brewers YE (50% free amino acids)	Gist brocades	extract	7.9	11
	Maxarome® (35% free amino acid)	Gist brocades	extract	5.1	20
Corn Steep Liquor	Phytase treated CSL	Roquette Frères	extract	5.7	4
	Corn Steep Powder	Roquette Frères	extract	5.4	5
Glutamate			amino acid	25	0
Caseine hydrolysate	Casein hydrolysee	Armor Proteines	hydrolysate	2.0	115
Wheat gluten hydrolysate	GPU	Marcor Inc.	hydrolysate	0.1	121
	GPN	Marcor Inc.	hydrolysate	0.3	136
	GPA	Marcor Inc.	hydrolysate	0.3	132
Soybean protein hydrolysate	HXP-90	PTI Inc.	hydrolysate		115

Example 2

Fermentation of clavulanic acid at low levels of free glutamate in the medium

Batch fermentation of clavulanic acid

Streptomyces clavuligerus ATCC 27064 was precultivated for 26 h at a temperature of 28°C and a start pH of 6.8 in a shaken incubator rotated at 220 RPM on a medium containing 5 to 30 g/l glycerol, 5 to 30 g/l soy peptone, 2 to 6 g/l sodium chloride, and 0.5 to 3 g/l calcium carbonate.

The preculture was inoculated at a volume of 10 % into 1 l of chemically defined medium containing 10-30 g/l glycerol, 0.5 to 3 g/l KH_2PO_4, 1 to 3 g/l $(NH_4)_2SO_4$, 15-25 g/l monosodium glutamate, 0.05 to 0.2 g/l $FeSO_4 \cdot 7\ H_2O$, 0.1 to 1 g/l $MgSO_4 \cdot 7\ H_2O$, 10-20 g/l 2-(N-morpholine) propane sulfonic acid (MOPS), 0.2-1 g/l basildon antifoam, and a suitable trace element solution. The pH was adjusted to 6.8 with 4N NaOH. The second preculture was cultivated for 20 h at a temperature of 28°C and a start pH of 6.8 in a shaken incubator rotated at 220 RPM.

The second preculture was inoculated at a volume of 3.3 % into 29 l of a chemically defined medium containing 10 to 30 g/l glycerol, 0.5 to 1 g/l KH_2PO_4, 0.5 to 3 g/l $(NH_4)_2SO_4$, 10-30 g/l monosodium glutamate, 0.05-0.15 g/l $FeSO_4 \cdot 7\ H_2O$, 0.1 to 1 g/l $MgSO_4 \cdot 7\ H_2O$, 0.1 to 1g/l basildon antifoam, and a suitable trace elements solution.

The fermentation was carried out at 30°C and the pH was controlled at 6.95 to 7.05 by titration with 4N NaOH and 4N H_2SO_4. The dissolved oxygen tension was maintained above 50 % of air saturation or regulated at 50 % of air saturation by the stirrer speed.

Fed-batch fermentation of clavulanic acid

Streptomyces clavuligerus ATCC 27064 was precultivated for 26 h at a temperature of 28°C and a start pH of 6.8 in a shaken incubator rotated at 220 RPM on a medium containing 5 to 30 g/l glycerol, 5 to 30

g/l soy peptone, 2 to 6 g/l sodium chloride, and 0.5 to 3 g/l calcium carbonate.

The preculture was inoculated at a volume of 10 % into 1 l of chemically defined medium containing 10-30 g/l glycerol, 0.5 to 3 g/l KH_2PO_4, 1 to 3 g/l $(NH_4)_2SO_4$, 15-25 g/l monosodium glutamate, 0.05 to 0.2 g/l $FeSO_4 \cdot 7\ H_2O$, 0.1 to 1 g/l $MgSO_4 \cdot 7\ H_2O$, 10-20 g/l 2-(N-morpholine) propane sulfonic acid (MOPS), 0.2-1 g/l basildon antifoam, and a suitable trace element solution. The pH was adjusted to 6.8 with 4N NaOH. The second preculture was cultivated for 20 h at a temperature of 28°C and a start pH of 6.8 in a shaken incubator rotated at 220 RPM.

The second preculture was inoculated at a volume of 4 % into 24 l of a chemically defined medium containing 5 to 15 g/l glycerol, 0.5-2 g/l K_2HPO_4, 0.5 to 3 g/l $(NH_4)_2SO_4$, 10 g/l monosodium glutamate, 0.05 to 0.15 g/l FeSO4.7H2O, 0.1 to 1 g/l $MgSO_4 \cdot 7H_2O$, 0.05 to 1 g/l basildon antifoam, and a suitable trace elements solution.

The fermentation was carried out at 30°C and the pH was controlled at 6.95 to 7.05 by titration with 4N NaOH and 4N H_2SO_4. The dissolved oxygen tension was maintained above 50 % of air saturation or regulated at 50 % of air saturation by the stirrer speed.

Five hours after the phosphate was exhausted from the batch medium a carbon feed containing 300 to 500 g glycerol/kg feed was added at a rate of 30-60 g/h and a nitrogen-phosphate-feed containing 50 to 100 g Na-glutamate/kg feed, 5 to 10 g $(NH_4)_2SO_4$/ kg feed, and 5 to 10 g K_2HPO_4/kg feed was added at a rate of 50 to 150 g/h.

Clavulanic acid and glutamate concentrations in the medium are demonstrated in figures 1 and 2 respectively for the batch process and the fed batch process. From figure 1 it can seen that clavulanic acid production starts when the glutamate concentration is dropping below 5 and even more preferably below 2.5 g/l and ends at 250-300 mg clavulanic acid /liter. Figure 2 shows that when glutamate is fed to the

fermentor keeping the concentration very low (<1 g/L) after 30 hours, clavulanic acid titers can increase to 500 mg/L in this experiment, giving a doubling compared to the batch experiment.

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FERMENTATION PROCESS TO PRODUCE A SECONDARY METABOLITE AT A LOW CONCENTRATION OF FREE AMINO ACIDS

NEW SET OF CLAIMS

1. A method for the production of a secondary metabolite by the fermentation of a Streptomyces strain on a suitable medium comprising carbon and nitrogen sources, wherein protein gluten hydrolysate is present as a source of free amino acids.

2. A method according to claim 1 wherein the concentration of free amino acid is lower than 5 g/l fermentation broth, more preferably lower than 2.5 g/l and most preferably lower than 0.5 g/l.

3. A method according to claim 1 or 2 wherein the secondary metabolite produced is a β-lactam, polyketide or macrolide.

4. A method according to claims 1 - 3 wherein the secondary metabolite produced is clavulanic acid, pimaricine, erythromicine, nystatine or amphotericine.

5. A method according to claims 1 - 4 characterized by the application of protein gluten hydrolysate in a batch, fed-batch, semi-continuous or continuous mode.

6. A method according to claim 5 wherein the protein gluten hydrolysate is formed by the addition of protein gluten to a strain with an increased protease activity compared to the corresponding wild type strain.

DSM N.V.

7. A method according to claim 5 or 6, characterized by the application of a protein gluten hydrolysate rich in anyone of glutamate or proline.

8. A method according to anyone of the claims 1 - 7, characterized by the application of wheat gluten hydrolysate.

9. A method for the production of a secondary metabolite by the fermentation of a Streptomyces characterized by the application of free amino acids in a fed-batch, semi- continuous or continuous mode with the proviso that a concentration of free asparagine of 4 g/l has been excluded.

10. A method according to claim 9, characterized by the application of glutamate or proline.

11. A method according to anyone of the claims 1 - 10, characterized by the production of clavulanic acid by the fermentation of Streptomyces clavuligerus.

12. A method for the further purification of clavulanic acid characterized by the purification of clavulanic acid obtained by the process of claim 11.

13. Process for the purification of clavulanic acid according to claim 12 and subsequent conversion to a salt thereof characterized by converting the impure clavulanic acid into an amine salt thereof by adding the corresponding amine salt forming compound and reacting said amine.

14. Process for the purification of clavulanic acid according to claim 13 and subsequent conversion to a salt thereof characterized by converting the impure clavulanic acid into an amine salt thereof by adding the corresponding amine salt forming compound and reacting said amine clavulanate with a non-toxic pharmaceutically acceptable salt to form the corresponding purified salt of clavulanic acid.

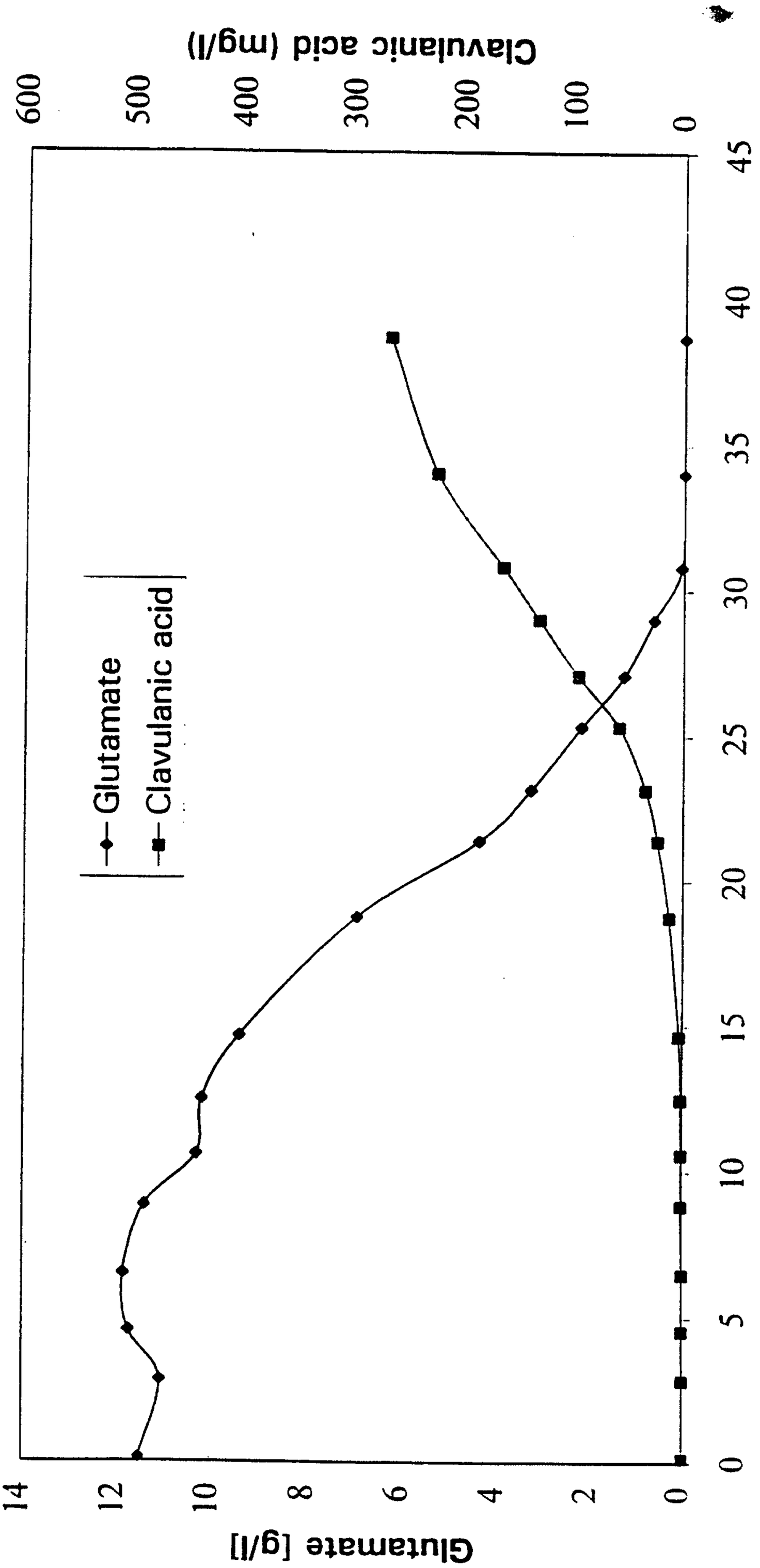

Clavulanic acid (mg/l)
600
500
400
300
200
100
0
45
40
35
30
25
20
15
10
5
0
Time [h]
Glutamate
Clavulanic acid
14
12
10
8
6
4
2
0
Glutamate [g/l]

Figure 1

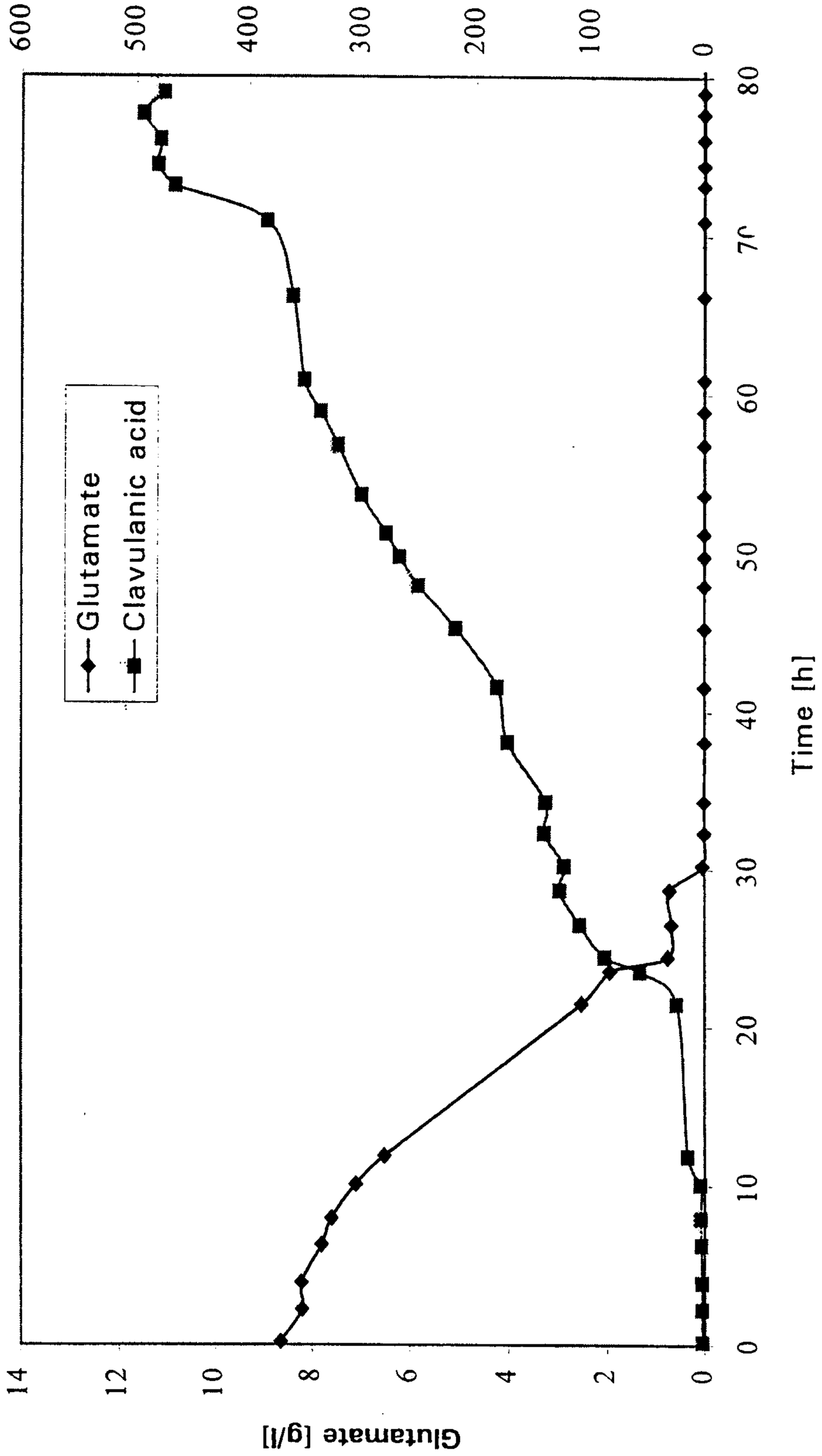
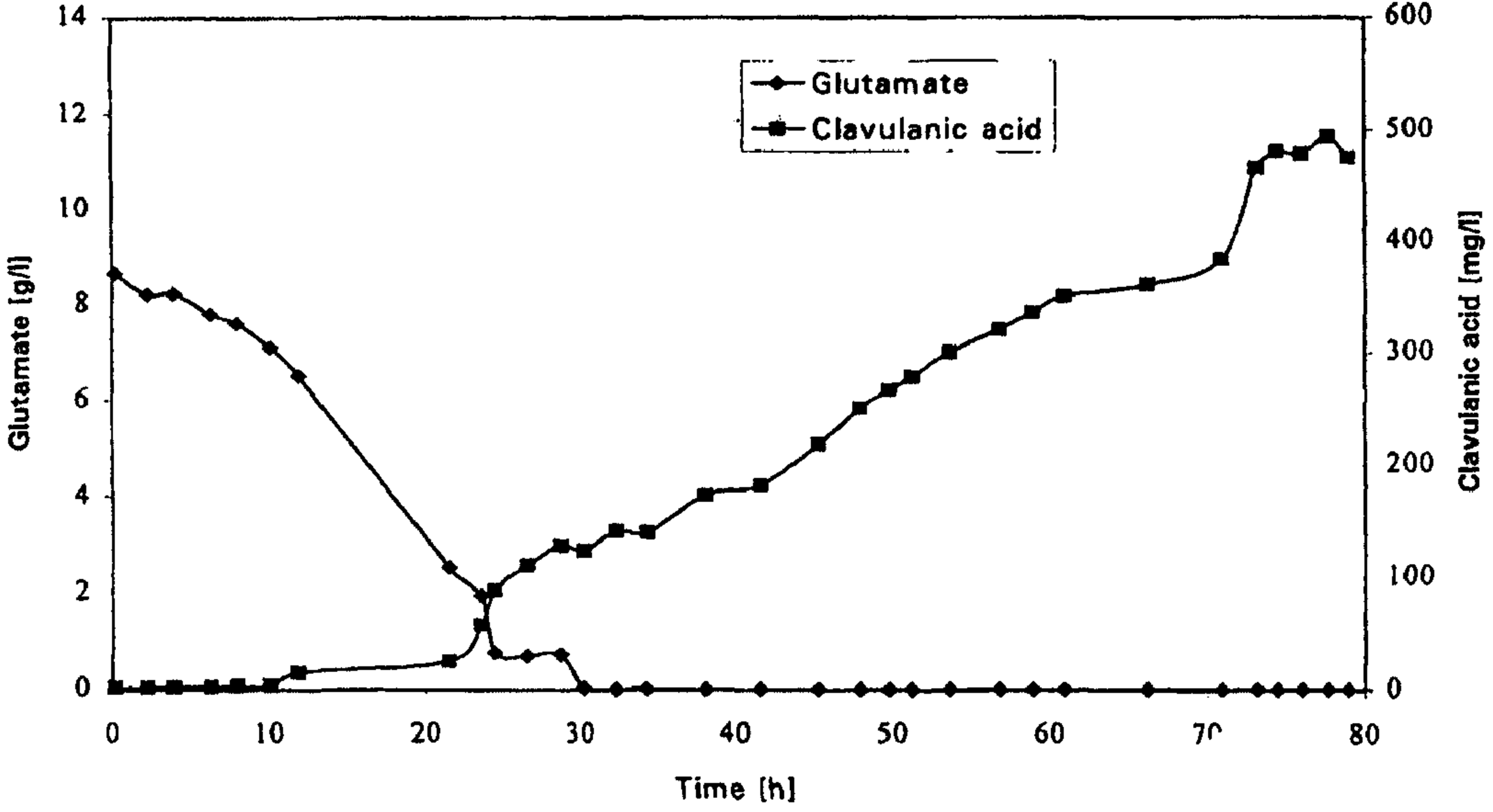

Clavulanic acid [mg/l]
600
500
400
300
200
100
0
Glutamate
Clavulanic acid
80
70
60
50
40
30
20
10
0
Time [h]
14
12
10
8
6
4
2
0
Glutamate [g/l]

Figure 2


Glutamate
Clavulanic acid
Glutamate [g/l]
Clavulanic acid [mg/l]
Time [h]
0
2
4
6
8
10
12
14
0
100
200
300
400
500
600
0
10
20
30
40
50
60
70
80