(19) DANMARK

(10) **DK/EP 3282008 T3**

Patent- og
Varemærkestyrelsen

(12) Oversættelse af
europæisk patentskrift

---

(51) Int.Cl.: ***C 12 N 1/20 (2006.01)*** ***C 12 R 1/07 (2006.01)*** ***C 12 R 1/085 (2006.01)*** ***C 12 R 1/09 (2006.01)*** ***C 12 R 1/10 (2006.01)*** ***C 12 R 1/11 (2006.01)*** ***C 12 R 1/12 (2006.01)*** ***C 12 R 1/125 (2006.01)***

(45) Oversættelsen bekendtgjort den: **2020-03-16**

(80) Dato for Den Europæiske Patentmyndigheds
bekendtgørelse om meddelelse af patentet: **2019-12-25**

(86) Europæisk ansøgning nr.: **16776700.3**

(86) Europæisk indleveringsdag: **2016-04-08**

(87) Den europæiske ansøgnings publiceringsdag: **2018-02-14**

(86) International ansøgning nr.: **JP2016061604**

(87) Internationalt publikationsnr.: **WO2016163534**

(30) Prioritet: **2015-04-09 JP 2015079951**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Idemitsu Kosan Co., Ltd., 1-1 Marunouchi 3-chome, Chiyoda-ku, Tokyo 100-8321, Japan**

(72) Opfinder: **EGUCHI, Takanori, 1-1 Marunouchi 3-chome, Chiyoda-ku, Tokyo 100-8321, Japan**
MORISHITA, Yasuyuki, 1-1 Marunouchi 3-chome, Chiyoda-ku, Tokyo 100-8321, Japan
TSUKAGOSHI, Yuki, 1-1 Marunouchi 3-chome, Chiyoda-ku, Tokyo 100-8321, Japan

(74) Fuldmægtig i Danmark: **Budde Schou A/S, Hausergade 3, 1128 København K, Danmark**

(54) Benævnelse: **Sporulationsfremgangsmåde for Bacillus bacterium**

(56) Fremdragne publikationer:
JP-A- H0 479 879
JP-A- S4 875 720
JP-A- 2008 199 980
ZHEN-MIN CHEN ET AL: "Greater enhancement of Bacillus subtilis spore yields in submerged cultures by optimization of medium composition through statistical experimental designs", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 85, no. 5, 21 August 2009 (2009-08-21), pages 1353-1360, XP019778516, ISSN: 1432-0614
SANDRA M. MONTEIRO ET AL: "A Procedure for High-Yield Spore Production by Bacillus s ubtilis", BIOTECHNOLOGY PROGRESS., vol. 21, no. 4, 1 January 2005 (2005-01-01), pages 1026-1031, XP055226281, US ISSN: 8756-7938, DOI: 10.1021/bp050062z
ASWATHI SONI ET AL: "Bacillus Spores in the Food Industry: A Review on Resistance and Response to Novel Inactivation Technologies : Bacillus spores in response to technologies...", COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY, vol. 15, no. 6, 1 November 2016 (2016-11-01), pages 1139-1148, XP055490381, US ISSN: 1541-4337, DOI: 10.1111/1541-4337.12231
FARRERA RR ET AL.: 'Carbon:nitrogen ratio interacts with initial concentration of total solids on insecticidal

Fortsættes ...

crystal protein and spore production in Bacillus thuringiensis HD-73' APPL. MICROBIOL. BIOTECHNOL. vol. 49, no. 6, 1998, ISSN 1432-0614 pages 758 - 765, XP055321839
WAKISAKA Y ET AL.: 'Formation of Crystalline delta- Endotoxin or Poly-beta-Hydroxybutyric Acid Granules by Asporogenous Mutants of Bacillus thuringiensis' APPL. ENVIRON. MICROBIOL. vol. 43, no. 6, June 1982, ISSN 1098-5336 pages 1473 - 1480, XP055321843

DESCRIPTION

TECHNICAL FIELD

[0001] The present invention relates to a method of efficiently producing *Bacillus* spores.

BACKGROUND ART

[0002] *Bacillus* bacteria are used in various fields such as production of enzymes and useful substances, production of fermented foods, decomposition of organic substances, microbial pesticides and microbial fertilizers. In situations where such microbial pesticides, microbial fertilizers and the like are used, spores of *Bacillus* bacteria are commonly utilized. However, even strains that exert excellent performance for such applications have been difficult to commercialize without efficient sporulation ability.

[0003] Media frequently used for liquid culture of *Bacillus* bacteria include Nutrient Broth (DIFCO), Luria Bertani broth and Trypticase Soy Broth (Beckton Dickinson), but in these media, sufficient proliferation was not obtained and spore formation was hardly observed in some cases.

[0004] Patent Document 1 discloses a method of allowing for sporulation by carrying out culturing including a step of decreasing the dissolved oxygen concentration after proliferation. In certain *Bacillus* bacteria, however, it is difficult to efficiently allow for sporulation even by using the same technique. In addition, in this method, it is necessary to adjust stirring and ventilation conditions in the culturing step, which makes the manufacturing process complicated.

[0005] Patent Document 2 discloses a method of allowing for sporulation by continuing cultivation for a long period of time after the carbon source has been exhausted. However, this method is not suitable for actual production because the culturing cost will be high due to prolonged cultivation. In addition, in certain *Bacillus* bacteria, it is difficult to allow for sporulation even by using the same technique.

[0006] Patent Document 3 discloses a method of producing spores by defining the range of the phosphate concentration in the culture medium and the range of the oxygen supply and stirring rate as culture conditions. In certain *Bacillus* bacteria, however, it is difficult to efficiently form spores even by using the same technique. In addition, it is necessary to use a culture facility capable of achieving prescribed culture conditions in practice.

PRIOR ART REFERENCES

Patent Documents

[0007]

Patent Document 1: Japanese Laid-open Patent Application (Kokai) No. 2007-236286

Patent Document 2: Japanese Laid-open Patent Application (Kokai) No. 2000-217567

Patent Document 3: Japanese Laid-open Patent Application (Kokai) No. 2007-195542

SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0008] An object of the present invention is to provide a culturing method capable of efficiently producing spores of a *Bacillus* bacterium which hardly form spores in general liquid medium for bacteria.

MEANS FOR SOLVING THE PROBLEMS

[0009] The present inventors intensively studied in order to solve the above problems, and consequently has found liquid medium compositions suitable for efficient proliferation and sporulation of *Bacillus* bacteria whose spore production efficiency is not sufficient in cultivation using conventional liquid media for bacteria, thereby completed the present invention.

[0010] The present invention is as follows:

1. [1] A method of producing *Bacillus* spores comprising a step of culturing the *Bacillus* bacterium using a liquid medium having a C/N ratio (weight ratio of carbon content to nitrogen content) of greater than 4.0 and less than 9.5, wherein the Bacillus bacterium is *Bacillus siamensis, Bacillus simplex* or *Bacillus megaterium* and wherein the potassium content in the liquid medium is less than 2.0 g/L.
2. [2] The method of producing *Bacillus* spores described in [1], wherein the C/N ratio in the liquid medium used for culturing is 4.5 or more and less than 9.5.
3. [3] The method of producing *Bacillus* spores described in [1], wherein the C/N ratio in the liquid medium used for culturing is 4.5 or more and 7.5 or less.
4. [4] The method of producing *Bacillus* spores described in [1], wherein the C/N ratio in the liquid medium used for culturing is 6.0 or more and 7.5 or less.
5. [5] The method of producing *Bacillus* spores described in any one of [1] to [4], wherein the carbon content in the liquid medium is 50 g/L or less.
6. [6] The method of producing *Bacillus* spores described in any one of [1] to [4], wherein

the carbon content in the liquid medium is 25 g/L or less.

7. [7] The method of producing *Bacillus* spores described in any one of [1] to [6], wherein the potassium content in the liquid medium is 1.9 g/L or less.
8. [8] The method of producing *Bacillus* spores described in any one of [1] to [7], wherein the carbon and nitrogen sources contained in the liquid medium are carbon and nitrogen sources which can be utilized by the *Bacillus* bacterium.
9. [9] The method of producing *Bacillus* spores described in [8], wherein the carbon source which can be utilized by the *Bacillus* bacterium is one or more carbon sources selected from the group consisting of starch, glucose, lactose, glycerol, arabinose, ribose, xylose, galactose, fructose, mannose, inositol, mannitol, sorbitol, glucosamine, N-acetylglucosamine, cellobiose, maltose, sucrose, trehalose, xylitol, alcohols, organic acids, organic salts, and alkanes; and the nitrogen source which can be utilized by the *Bacillus* bacterium is one or more nitrogen sources selected from the group consisting of soybean-derived components, yeast-derived components, corn-derived components, animal and plant proteins and hydrolysates thereof, and ammonium salts such as ammonium nitrate, ammonium sulfate, ammonium chloride, ammonium acetate, ammonia, sodium nitrate, potassium nitrate, sodium glutamate, urea and the like.

EFFECT OF THE INVENTION

[0011] The present invention can enable stable proliferation and sporulation of *Bacillus* bacteria, and further proliferation to higher concentration and sporulation at a higher rate.

EMBODIMENTS FOR CARRYING OUT THE INVENTION

[0012] In the present disclosure, *Bacillus* bacteria are not particularly limited as long as they are bacteria classified as *Bacillus,* and include *Bacillus simplex*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Bacillus popilliae*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus firmus*, *Bacillus velezensis*, *Bacillus stearothermophilus*, *Bacillus pichinotyi*, *Bacillus acidocaldarius*, *Bacillus alcalophilus, Bacillus alkalicola, Bacillus coagulans, Bacillus azotoformans*, *Bacillus anthracis*, *Bacillus siamensis*, *Bacillus badius*, *Bacillus bataviensis*, *Bacillus brevis*, *Bacillus cycloheptanicus*, *Bacillus circulans*, *Bacillus aneurinilyticus*, *Bacillus migulanus*, *Bacillus abyssalis*, *Bacillus aestuarii*, *Bacillus polymyxa*, or *Bacillus* sp..

[0013] Among these, *Bacillus simplex, Bacillus siamensis* and *Bacillus megaterium* are preferred.

[0014] In the present invention, a liquid medium having a C/N ratio (weight ratio of carbon content to nitrogen content) of greater than 4.0 and less than 9.5 is used for culturing. The C/N ratio is preferably 4.5 or more and less than 9.5, more preferably 4.5 or more and 7.5 or less, still more preferably 6.0 or more and 7.5 or less. The C/N ratio is calculated as follows:

C/N ratio = total carbon content in each media component /total nitrogen content in each media component.

[0015] The carbon content in the liquid medium used in the present invention is preferably 50 g/L or less, more preferably 25 g/L or less. On the other hand, the carbon content is preferably 3 g/L or more.

[0016] For carbon and nitrogen sources in the liquid medium used for culturing, those which can be utilized by *Bacillus* bacteria can be used. Examples of the carbon source capable of being utilized include sugars which can be utilized by *Bacillus* bacteria (such as starch, glucose, lactose, glycerol, arabinose, ribose, xylose, galactose, fructose, mannose, inositol, mannitol, sorbitol, glucosamine, N-acetylglucosamine, cellobiose, maltose, sucrose, trehalose, xylitol), alcohols, organic acids, organic salts, alkanes or other common carbon sources. Examples of the nitrogen source capable of being utilized include soybean-derived components, yeast-derived components, corn-derived components, animal and plant proteins and hydrolysates thereof, and ammonium salts such as ammonium nitrate, ammonium sulfate , ammonium chloride and ammonium acetate, ammonia, sodium nitrate, potassium nitrate, sodium glutamate, urea.

[0017] In the liquid medium used in the present invention, the potassium content is preferably less than 2 g/L, more preferably 1.9 g/L or less, in order to achieve a higher sporulation rate. The potassium content is preferably 0.2 g/L or more. As potassium sources, for example, at least one of soybean-derived component, yeast-derived component, corn-derived component, animal and plant proteins and hydrolysates thereof, KH_2PO_4, K_2HPO_4, and KCl are selected for culturing.

[0018] Other medium compositions, such as trace metal salts commonly used for culturing *Bacillus* bacteria, may be added as long as they do not adversely affect sporulation, and if necessary, for example, amino acids or vitamins may be added.

[0019] Culture conditions may be those generally used for liquid culture of *Bacillus* bacteria, including culture conditions at 20 to 40°C under aerobic conditions (e.g., 15 to 50% oxygen concentration) with stirring for 10 to 100 hours. The pH of the medium is preferably 6.5 to 8.5, more preferably 7.0 to 8.0. Preculture may be performed before culturing in the liquid medium having the above-described C/N ratios.

[0020] In this way, *Bacillus* bacterial cells having a high sporulation rate (e.g., 50% or more, preferably 80% or more) can be obtained. Such *Bacillus* bacterial cells having a high sporulation rate can be used for a desired purpose after being subjected to proper operations such as concentration or removal of medium and drying.

EXAMPLES

[0021] The present invention will be described in detail below with reference to Examples, but is not limited to the following Examples.

Example 1

Evaluation of Bacillus simplex NBRC15720 strain

[0022] Using a 500 ml Erlenmeyer flask, each 100 ml of media containing glucose (Wako Pure Chemicals), defatted soy flour (Ajinomoto Healthy Supply), yeast extract (Difco), CSL (Corn Steep Liquor: ROQUETTE), peptone (Difco), and KH_2PO_4 (Wako Pure Chemicals) so that the final concentrations listed in Table 1 were achieved and further containing 100 ppm of $MnCl_2$ (Wako Pure Chemicals), 400 ppm of NaCl (Wako Pure Chemicals), 250 ppm of $MgCl_2$ (Wako Pure Chemicals), 75 ppm of $CaCl_2$ (Wako Pure Chemicals), and 0.3 ppm of $FeSO_4$ (Wako Pure Chemicals) were prepared, and autoclave sterilization was carried out with a SILICOSEN (glucose was separately sterilized and aseptically mixed in order to avoid Maillard reaction).

[0023] First, one loopful of *Bacillus simplex* NBRC15720 strain was taken from a colony grown on a nutrient agar plate, aseptically inoculated into the medium described in the medium condition 1 in Table 1 and cultured overnight with shaking at 37°C and 150 rpm to obtain a preculture medium.

[0024] Three milliliters from the obtained preculture medium was aseptically inoculated into various media described in Table 1 and cultured overnight with shaking at 37°C and 150 rpm for 40 hours to 72 hours to obtain a culture medium.

[0025] After cultivation, the bacterial cell concentration in the culture medium and the sporulation rate of the bacterial cells were measured using an optical microscope and a bacterial cell counter.

[0026] Methods for measuring bacterial cell concentration, spore concentration and sporulation rate are as follows. The *Bacillus simplex* strain grown in the culture medium was diluted with, for example, sterile water containing 0.01% Tween 20, and then the bacterial cell concentration (vegetative cells and spores) and the spore concentration were counted with a bacterial cell counter. The sporulation rate was calculated by: spore concentration / bacterial cell concentration.

[0027] The C/N ratio was calculated from the weight ratio of the carbon content to the nitrogen content in each medium component. C/N ratio = total carbon content in each media component / total nitrogen content in each media component.

[0028] The carbon content in each medium component was calculated by determining the reducing sugar concentration by Somogyi method after hydrolysis in acid and subsequently multiplying the total sugar amount by 0.4.

[0029] The nitrogen content in each medium component was determined by the Kjeldahl method.

[0030] The potassium content in each medium component was determined by atomic absorption spectrophotometry (measurement wavelength: 766.5 nm).

Table 1 Medium composition

Medium condition	g/L					
	Glucose	Delatted soy flour	Yeast extract	CSL	Peptone	KH_2PO_4
1	4	4.0	1.6	0.8	1.6	2.0
2	10.0	4.0	1.6	0.8	1.6	2.0
3	9.0	4.0	1.6	0.8	1.6	2.0
4	8.0	4.0	1.6	0.8	1.6	2.0
5	6.0	4.0	1.6	0.8	1.6	2.0
6	5.5	4.0	1.6	0.8	1.6	2.0
7	1.5	4.0	1.6	0.8	1.6	2.0
8	0.0	4.0	1.6	0.8	1.6	2.0
9	4.0	4.0	1.6	0.8	1.6	5.5
10	4.0	4.0	1.6	0.8	1.6	1
11	4.0	4.0	1.6	0.8	1.6	0
12	10.0	10.0	4.0	2.0	4.0	5.0
13	10.0	10.0	4.0	2.0	4.0	1.0
14	0.5	4.0	1.6	0.8	1.6	6.4
15	10	4.0	1.6	0.8	1.6	6.4
16	10	4.0	1.6	0.8	1.6	0
17	4	2.5	3.0	2.0	0	1.0
18	4	6.0	0	0	0	1.0

[0031] The results are shown in Table 2. At the C/N ratio of 9.5 or more, although growth of the bacterial cells was observed, a decrease in the sporulation rate was detected. On the other hand, at the C/N ratio of 4.0 or less, although growth of the bacterial cells was observed, a decrease in the sporulation rate was detected. The preferred potassium content was found to be in the range of 0.2 to 1.9 g/L.

Table 2 Culture results for NBRC15720 strain

Medium condition	Content of each component (g/L) and C/N ratio in medium				Culture result		
	Potassium content	C/N ratio	Carbon content	Nitrogen content	Bacterial cell concentration cell/ml	Sporulation rate %	Spore concentration spore/ml
1	0.8	6.0	4.1	0.7	2.4E+09	75%	1.8E+09

1	0.8	6.0	4.1	0.7	2.4E+09	75%	1.8E+09
2	0.8	9.5	6.5	0.7	1.6E+09	8%	1.3E+08
3	0.8	9.0	6.1	0.7	1.3E+09	89%	1.2E+09
4	0.8	8.4	5.7	0.7	1.6E+09	89%	1.5E+09
5	0.8	7.2	4.9	0.7	1.6E+09	67%	1.1E+09
6	0.8	6.9	4.7	0.7	1.8E+09	76%	1.4E+09
7	0.8	4.5	3.1	0.7	1.8E+09	70%	1.3E+09
8	0.8	3.7	2.5	0.7	1.1E+09	50%	5.4E+08
9	1.8	6.0	4.1	0.7	1.4E+09	97%	1.4E+09
10	0.5	6.0	4.1	0.7	2.0E+09	90%	1.8E+09
11	0.2	6.0	4.1	0.7	1.2E+09	94%	1.2E+09
12	1.9	6.0	10.2	1.7	2.2E+09	86%	1.9E+09
13	0.8	6.0	10.2	1.7	2.7E+09	82%	2.2E+09
14	2.0	4.0	2.7	0.7	1.3E+07	7%	9.4E+05
15	2.0	9.5	6.5	0.7	1.3E+07	0%	0.0E+00
16	0.2	9.5	6.5	0.7	1.2E+09	25%	3.1E+08
17	0.5	6.5	3.5	0.4	4.0E+09	68%	2.8E+09
18	0.4	7.5	3.5	0.5	1.5E+09	77%	1.2E+09

Example 2

Evaluation of Bacillus simplex NBRC104473 strain

[0032] Using a 500 ml Erlenmeyer flask, each 100 ml of media containing glucose (Wako Pure Chemicals), defatted soy flour (Ajinomoto Healthy Supply), yeast extract (Difco), CSL (ROQUETTE), peptone (Difco), and KH_2PO_4 (Wako Pure Chemicals) so that the final concentrations of medium conditions 1 to 3 listed in Table 3 were achieved and each further containing 100 ppm of $MnCl_2$ (Wako Pure Chemicals), 400 ppm of NaCl (Wako Pure Chemicals), 250 ppm of $MgCl_2$ (Wako Pure Chemicals), 75 ppm of $CaCl_2$ (Wako Pure Chemicals), and 0.3 ppm of $FeSO_4$ (Wako Pure Chemicals) were prepared, and autoclave sterilization was carried out with a SILICOSEN (glucose was separately sterilized and aseptically mixed in order to avoid Maillard reaction).

[0033] One loopful of *Bacillus simplex* NBRC104473 strain was taken from a colony grown on a nutrient agar plate, aseptically inoculated into the medium described in the medium condition 1 listed in Table 3 and cultured overnight with shaking at 37°C and 150 rpm to obtain a preculture medium. Each 3 ml from the obtained preculture medium used for culturing the *Bacillus simplex* NBRC104473 strain was aseptically inoculated into each medium described in Table 3 and cultured overnight with shaking at 37°C and 150 rpm for 40 hours to 72 hours to obtain a culture medium. After cultivation, the bacterial cell concentration in the culture medium and the sporulation rate of the bacterial cells were measured using an optical microscope and a bacterial cell counter.

Table 3 Medium composition

Medium condition	g/L					
	Glucose	Defatted soy flour	Yeast extract	CSL	Peptone	KH_2PO_4
1	4.0	4.0	1.6	0.8	1.6	2.0
2	0.5	4.0	1.6	0.8	1.6	6.4
3	10	4.0	1.6	0.8	1.6	6.4

[0034] The results are shown in Table 4. NBRC104473 strain also showed the same tendency as in Example 1.

Table 4 Culture results for NBRC 104473 strain

Medium condition	Content of each component (g/L) and C/N ration in medium				Culture result		
	Potassium content	C/N ratio	Carbon content	Nitrogen content	Bacterial cell concentration cell/ml	Sporulation rate %	Spore concentration spore/ml
1	0.77	6.01	4.09	0.68	1.2E+09	100%	1.2E+09
2	2.03	3.95	2.69	0.68	4.4E+07	0%	0.0E+00
3	2.03	9.54	6.49	0.68	3.2E+07	0%	0.0E+00

Example 3

Evaluation in Jar Fermentation System

[0035] Using a 5 L culture tank, each 2,000 ml of media containing glucose (Wako Pure Chemicals), defatted soy flour (Ajinomoto Healthy Supply), yeast extract (Difco), CSL (ROQUETTE), peptone (Difco), and KH_2PO_4 (Wako Pure Chemicals) so that the final concentrations of medium conditions 1 to 3 listed in Table 5 were achieved and each further containing 100 ppm of $MnCl_2$ (Wako Pure Chemicals), 400 ppm of NaCl (Wako Pure Chemicals), 250 ppm of $MgCl_2$ (Wako Pure Chemicals), 75 ppm of $CaCl_2$ (Wako Pure Chemicals), and 0.3 ppm of $FeSO_4$ (Wako Pure Chemicals) were prepared, and autoclave sterilization was carried out (glucose was separately sterilized and aseptically mixed in order to avoid Maillard reaction).

[0036] One loopful of *Bacillus simplex* NBRC15720 was taken from a colony grown on a nutrient agar plate, aseptically inoculated into the medium of medium condition 1 described in Example 1 (Table 1) prepared in a 500 ml Erlenmeyer flask and cultured overnight with shaking at 37°C and 150 rpm to obtain a preculture medium. Each 60 ml from the obtained preculture medium used for culturing the *Bacillus simplex* NBRC15720 strain was aseptically inoculated into each medium described in Table 5 and cultured overnight with aeration and

agitation at 37°C and 400 rpm for 40 hours to obtain a culture medium. After cultivation, the bacterial cell concentration in the culture medium and the sporulation rate of the bacterial cells were measured using an optical microscope and a bacterial cell counter.

Table 5 Medium composition

Medium condition	g/L					
	Glucose	Defatted soy flour	Yeast extract	CSL	Peptone	KH_2PO_4
1	10.0	10.0	12.0	8.0	0	1.0
2	20.0	16.0	12.0	8.0	0	1.0
3	35.0	20.0	12.0	8.0	0	0

[0037] The results are shown in Table 6. As long as C/N is within a certain range, 50% or more of sporulation rate of the *Bacillus simplex* NBRC15720 strain was obtained even in a jar fermentation system.

Table 6 Culture results for NBRC15720 strain (jar fermentation system)

Medium condition	Content of each component (g/L) and C/N ratio in medium				Culture result		
	Potassium content	C/N ratio	Carbon content	Nitrogen content	Bacterial cell concentration cell/ml	Sporulation rate %	Spore concentration spore/ml
1	1.24	5.41	11.67	2.16	9.0E+09	60%	5.4E+09
2	1.36	6.68	17.53	2.62	1.5E+10	53%	7.9E+09
3	1.15	8.45	24.76	2.93	1.8E+10	61%	1.1E+10

Example 4

Evaluation of Bacillus siamensis in Jar Fermentation System

[0038] Using a 5 L culture tank, each 2,000 ml of media containing glucose (Wako Pure Chemicals), defatted soy flour (Ajinomoto Healthy Supply), yeast extract (Difco), CSL (ROQUETTE), peptone (Difco), and KH_2PO_4 (Wako Pure Chemicals) so that the final concentrations of medium conditions 1 to 3 listed in Table 7 were achieved and each further containing 100 ppm of $MnCl_2$ (Wako Pure Chemicals), 400 ppm of NaCl (Wako Pure Chemicals), 250 ppm of $MgCl_2$ (Wako Pure Chemicals), 75 ppm of $CaCl_2$ (Wako Pure Chemicals), and 0.3 ppm of $FeSO_4$ (Wako Pure Chemicals) were prepared, and autoclave sterilization was carried out (glucose was separately sterilized and aseptically mixed in order to avoid Maillard reaction).

[0039] One loopful of *Bacillus siamensis* was taken from a colony grown on a nutrient agar plate, aseptically inoculated into the medium of medium condition 1 described in Table 7 prepared in a 500 ml Erlenmeyer flask and cultured overnight with shaking at 37°C and 150 rpm to obtain a preculture medium. Each 60 ml from the obtained preculture medium used for

culturing the *Bacillus siamensis* was aseptically inoculated into various media described in Table 7 and cultured overnight with aeration and agitation at 37°C and 400 rpm for 40 hours to obtain a culture medium. After cultivation, the bacterial cell concentration in the culture medium and the sporulation rate of the bacterial cells were measured using an optical microscope and a bacterial cell counter.

Table 7 Medium composition

Medium condition	g/L					
	Glucose	Defatted soy flour	Yeast extract	CSL	Peptone	KH_2PO_4
1	8.0	5.0	6.0	4.0	0	0.5
2	24.0	15.0	18.0	12.0	0	0.8
3	36.0	15.0	18.0	12.0	0	0.8

[0040] The results are shown in Table 8. As long as C/N is within a certain range, 88% or more of sporulation rate of the *Bacillus siamensis* was obtained even in a jar fermentation system.

Table 8 Culture results for *Bacillus siamensis* (jar fermentation system)

Medium condition	Content of each component (g/L) and C/N ratio in medium				Culture result		
	Potassium content	C/N ratio	Carbon content	Nitrogen content	Bacterial cell concentration cell/ml	Sporulation rate %	Spore concentration spore/ml
1	0.62	6.52	7.04	1.08	3.1E+09	93%	2.9E+09
2	1.64	6.52	21.11	3.24	6.8E+09	88%	6.0E+09
3	1.64	8.00	25.91	3.24	8.0E+10	95%	7.6E+10

Example 5

Culture results for *Bacillus megaterium* (jar fermentation system)

[0041] Using a 5 L culture tank, each 2,000 ml of media containing glucose (Wako Pure Chemicals), defatted soy flour (Ajinomoto Healthy Supply), yeast extract (Difco), CSL (ROQUETTE), peptone (Difco), and KH_2PO_4 (Wako Pure Chemicals) so that the final concentrations of medium conditions 1 to 3 listed in Table 9 were achieved and each further containing 100 ppm of $MnCl_2$ (Wako Pure Chemicals), 400 ppm of NaCl (Wako Pure Chemicals), 250 ppm of $MgCl_2$ (Wako Pure Chemicals), 75 ppm of $CaCl_2$ (Wako Pure Chemicals), and 0.3 ppm of $FeSO_4$ (Wako Pure Chemicals) were prepared, and autoclave sterilization was carried out (glucose was separately sterilized and aseptically mixed in order to avoid Maillard reaction).

[0042] One loopful of *Bacillus megaterium* was taken from a colony grown on a nutrient agar plate, aseptically inoculated into the medium of medium condition 1 described in Table 9 prepared in a 500 ml Erlenmeyer flask and cultured overnight with shaking at 37°C and 150

rpm to obtain a preculture medium. Each 60 ml from the obtained preculture medium used for culturing the *Bacillus megaterium* was aseptically inoculated into various media described in Table 9 and cultured overnight with aeration and agitation at 37°C and 400 rpm for 40 hours to obtain a culture medium. After cultivation, the bacterial cell concentration in the culture medium and the sporulation rate of the bacterial cells were measured using an optical microscope and a bacterial cell counter.

Table 9 Medium composition

Medium condition	g/L					
	Glucose	Defatted soy flour	Yeast extract	CSL	Peptone	KH_2PO_4
1	8.0	5.0	6.0	4.0	0	0.5
2	24.0	15.0	18.0	12.0	0	0.8
3	36.0	15.0	18.0	12.0	0	0.8

[0043] The results are shown in Table 10. As long as C/N is within a certain range, 78% or more of sporulation rate of the *Bacillus megaterium* was obtained even in a bulk culture system.

Table 10 Culture results for *Bacillus megaterium* (jar fermentation system)

Medium condition	Content of each component (g/L) and C/N ratio in medium				Culture result		
	Potassium content	C/N ratio	Carbon content	Nitrogen content	Bacterial cell concentration cell/ml	Sporulation rate %	Spore concentration spore/ml
1	0.62	6.52	7.04	1.08	2.7E+09	78%	2.1E+09
2	1.64	6.52	21.11	3.24	8.4E+09	95%	8.0E+09
3	1.64	8.00	25.91	3.24	9.2E+10	93%	8.6E+10

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- JP2007236286A [0007]
- JP2000217567A [0007]
- JP2007195542A [0007]

PATENTKRAV

1. Fremgangsmåde til fremstilling af *Bacillus* sporer omfattende et trin med dyrkning af *Bacillus* bacterium under anvendelse af et flydende medie med et C/N forhold (vægtforhold imellem carbonindhold og nitrogenindhold) på større end 4,0 og mindre end 9,5, hvor Bacillus bacterium er *Bacillus siamensis, Bacillus simplex* eller *Bacillus megaterium*, og hvor kaliumindholdet i det flydende medie er mindre end 2,0 g/l.

2. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge krav 1, hvor C/N forholdet i det flydende medie, som anvendes til dyrkning, er 4,5 eller mere og mindre end 9,5.

3. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge krav 1, hvor C/N forholdet i det flydende medie, som anvendes til dyrkning er 4,5 eller mere og 7,5 eller mindre.

4. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge krav 1, hvor C/N forholdet i det flydende medie, som anvendes til dyrkning er 6,0 eller mere og 7,5 eller mindre.

5. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge ethvert af kravene 1 til 4, hvor carbonindholdet i det flydende medie er 50 g/l eller mindre.

6. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge ethvert af kravene 1 til 4, hvor carbonindholdet i det flydende medie er 35 g/l eller mindre.

7. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge ethvert af kravene 1 til 6, hvor kaliumindholdet i det flydende medie er 1,9 g/l eller mindre.

8. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge ethvert af kravene 1 til 7, hvor carbon og nitrogenkilder indeholdt i det flydende medie er carbon og nitrogenkilder, som kan udnyttes af *Bacillus* bacterium.

9. Fremgangsmåde til fremstilling af *Bacillus* sporer ifølge krav 8, hvor carbonkilden, som kan udnyttes af *Bacillus* bacterium er én eller flere carbonkilder valgt fra gruppen bestående af stivelse, glucose, lactose, glycerol, arabinose, ribose, xylose, galactose, fructose, mannose, inositol, mannitol, sorbitol, glucosamin, N-acetylglucosamin, cellobiose, maltose, sucrose, trehalose, xylitol, alkoholer, organiske syrer, organiske salte og alkaner; og nitrogenkilden, som kan udnyttes af *Bacillus* bacterium er én eller flere

nitrogenkilder valgt fra gruppen bestående af sojabønne-afledte komponenter, gær-afledte komponenter, majs-afledte komponenter, animalske- og planteproteiner og hydrolysater deraf og ammoniumsalte såsom ammoniumnitrat, ammoniumsulfat, ammoniumchlorid, ammoniumacetat, ammoniak, natriumnitrat, kaliumnitrat, natriumglutamat, urea og lignende.