Petition to Amend Standard 1.3.3 of the Australia New Zealand Food Standards Code to Include Glutaminase from *Bacillus amyloliquefaciens* as a Processing Aid

- Appendix to Section A -

Prepared for: Amano Enzyme Inc.
1-2-7, Nishiki, Naka-ku,
Nagoya 460-8630
Japan

Prepared by: Intertek Scientific and Regulatory Consultancy
2233 Argentia Road, Suite 308 Mississauga,
Ontario, Canada
L5N 2X7
www.intertek.com

December 22, 2014

Analytical Method for Detection

D 4 1 1 ASSAY METHOD OF GLUTAMINASE ACTIVITY

Principle

Hydrolysis of glutamine by glutaminase gives glutamic acid and ammonia. In this method, the resulting glutamic acid is determined enzymatically.

Definition of activity unit

One glutaminase unit (GTU) is defined as the amount of enzymes that forms 1 μ mol of glutamic acid per minute from glutamine at 37 ℃, pH 6.0.

Reagents and solutions

(1) 0.75 mol/L perchloric acid solution

Dilute 83 mL of 60 % perchloric acid to 1,000 mL with distilled water.

(2) 0.75 mol/L sodium hydroxide solution

Dissolve 30 g of sodium hydroxide in distilled water and bring to 1,000 mL. Store it in a polyethylene bottle.

(3) 1 mol/L acetic acid solution

Dilute 60 g of acetic acid to 1,000 mL with distilled water.

(4) 1 mol/L sodium acetate solution

Dissolve 136 g of sodium acetate trihydrate in distilled water and bring to 1,000 mL.

(5) 1 mol/L acetate buffer (pH 6.0)

Add 1 mol/L acetic acid solution to 1 mol/L sodium acetate solution to give a pH of 6.0.

(6) 10 % Triton X-100 solution

Dissolve 10 g of Triton X-100 in distilled water by heating and bring to 100 mL.

(7) Diluent

Dilute 10 mL of 1 mol/L acetate buffer (pH 6.0) and 0.5 mL of 10 % Triton X-100 solution to 1,000 mL with distilled water.

(8) YAMASA L-GLUTAMIC ACID DETERMINATION KIT (an article on the market)

a) colour reagent

Dissolve a bottle of the Enzyme Reagent (freeze-dried) in 100 mL of the Buffer Solution (pH 7.1). Store it in a light-resistant glass bottle at 2 ~ 10℃.(Do not freeze.) The solution is stable for 1 month.

b) L-glutamic acid standard solution (100 μg/mL)

(9) Substrate (2 % glutamine solution)

Dissolve 2.00 g of L-glutamine (special grade reagent) in about 70 mL of distilled water by stirring. Then add 10 mL of 1 mol/L acetate buffer (pH 6.0), and bring to 100 mL with distilled water. Prepare before use.

Preparation of sample solution

Using diluent, prepare the sample solution which contains 0.17 to 0.43 glutaminase units (GTU) per mL.

Procedure

(Test)

1. Pipet 1 mL of the sample solution into 15 mmϕ × 150 mm test tube, and preincubate it in a 37 ± 0.2 ℃ water bath for 5 minutes.
2. Add 1 mL of the substrate pre-warmed to 37 ± 0.2 ℃, mix, and incubate it in the water bath.
3. After 10 minutes, accurately timed from the addition of the sample solution, add 1 mL of 0.75 mol/L perchloric acid solution to stop the reaction,and mix by a vortex mixer. Then, immediately immerse it in the ice water, and allow to stand more than 1 minute.
4. Add 1 mL of 0.75 mol/L sodium hydroxide solution, and mix.
5. Determine the amount of L-glutamic acid in the mixture(reaction mixture) with YAMASA L-GLUTAMIC ACID DETERMINATION KIT.

(Blank)

1. Pipet 1 mL of the sample solution into 15 mmϕ × 150 mm test tube, add 1 mL of 0.75 mol/L perchloric acid solution, and mix by a vortex mixer.
2. After incubation in the water bath for 5 minutes, add 1 mL of the substrate to the test tube, and mix. Then, immediately immerse it in the ice water, and allow to stand more than 1 minute.
3. Add 1 mL of 0.75 mol/L sodium hydroxide solution, and mix.
4. Determine the amount of L-glutamic acid in the mixture(blank mixture) with YAMASA L-GLUTAMIC ACID DETERMINATION KIT.

Determination of L-glutamic acid

Pipet 3 mL of colour reagent into each of four separate test tubes, add 200μL of the reaction mixture, the blank mixture, L-glutamic acid standard and water separately, and mix. After allowing to stand at room temperature (15 ~ 25 ℃) for 10 minutes, determine the absorbance of each solution at 600 nm.

Note The time from addition of the test solution to colour reagent until determination of the absorbance shall not exceed 40 minutes.

Calculation

Calculate the activity of the enzyme preparation by the formula.

$$\text{GTU/g} = \frac{A_S - A_{SB}}{A_R - A_{RB}} \times 100 \times 4 \times \frac{1}{147} \times \frac{1}{10} \times \frac{1}{W}$$

A_S : absorbance of the reaction mixture
A_{SB} : absorbance of the blank mixture
A_R : absorbance of the L-glutamic acid standard
A_{RB} : absorbance of the standard blank (water)
100 : the weight, in μg, of the L-glutamic acid contained in 1 mL of L-glutamic acid standard solution
4 : final volume of the mixture, in mL
147 : molecular weight of L-glutamic acid
10 : time of the enzyme reaction, in min.
W : the weight, in g, of the enzyme preparation contained in 1 mL of the sample solution

Enzyme Characterisation

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN
PHONE : +81-748-75-1194
F A X : +81-748-75-0312

November 25, 2008

Comparison between GLUTAMINASE DAIWA C100S
and GLUTAMINASE DAIWA SD-C100S

Samples

Enzyme

GLUTAMINASE DAIWA C100S (alcohol precipitation preparation)	Lot. P8HC061
GLUTAMINASE DAIWA SD-C100S (spray dried preparation)	Lot. P8HC161

Results

There was no different between GLUTAMINASE C100S and GLUTAMINASE SD-100S.

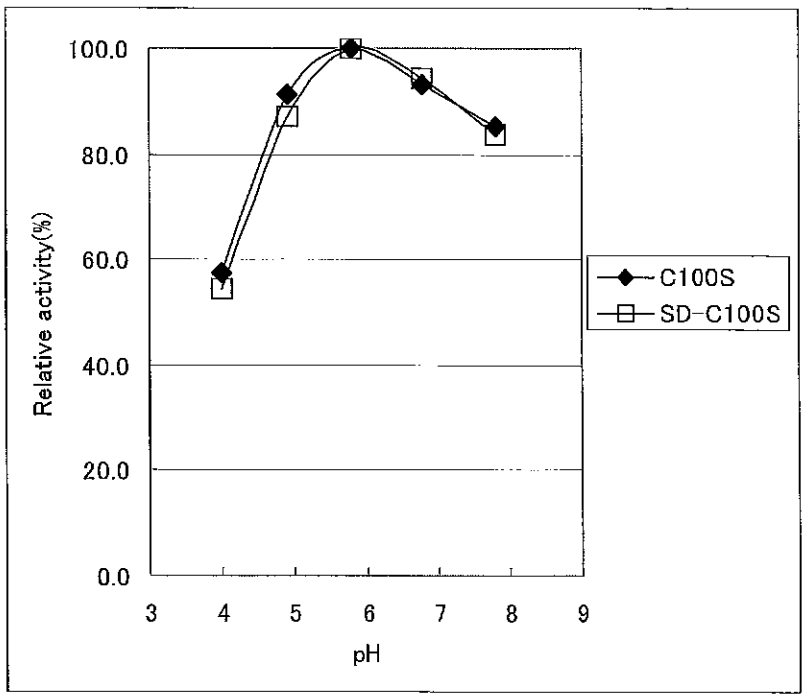


Figure 1. Effect of the pH on glutaminase activity

Experimental conditions:

37℃, 10min, 1.0% L-Glutamine, 50mmol/L Britton Robinson buffer

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN
PHONE : +81-748-75-1194
F A X : +81-748-75-0312

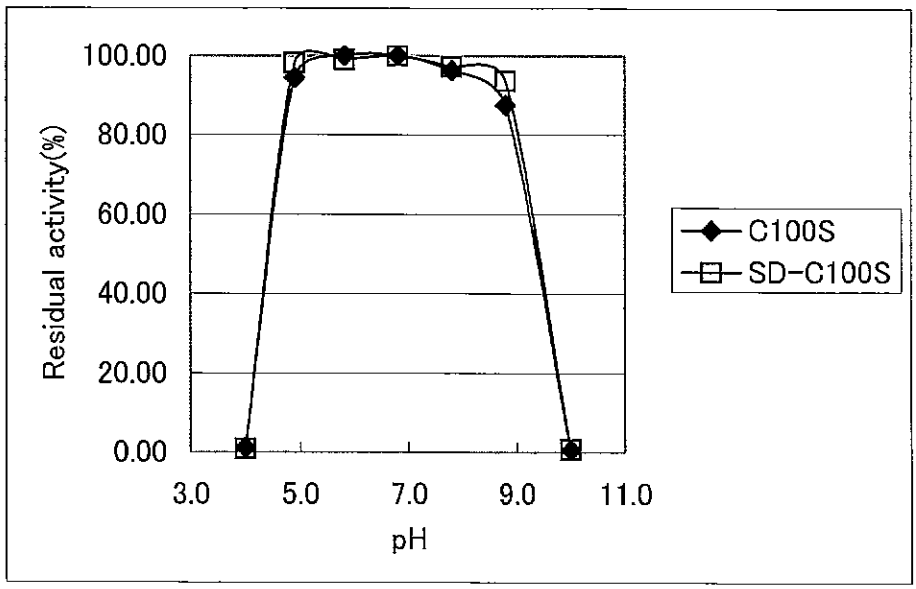


Figure 2. pH stability

Treatment conditions: 37℃, 16h, 50mmol/L Britton Robinson buffer

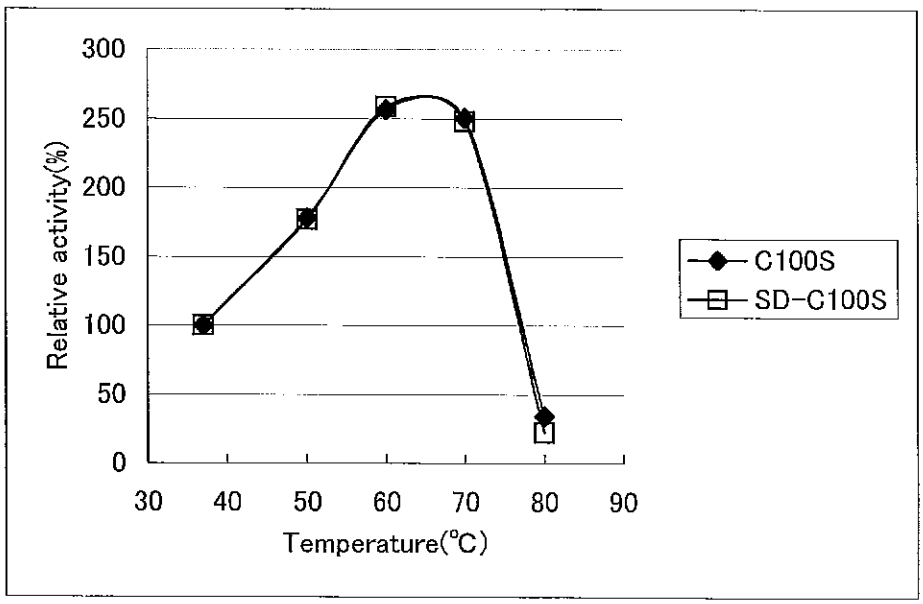


Figure 3. Effect of the temperature on glutaminase activity

Experimental conditions:

pH6.0, 5min, 1.0% L-Glutamine, 100mmol/L acetate buffer

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN
PHONE : +81-748-75-1194
F A X : +81-748-75-0312

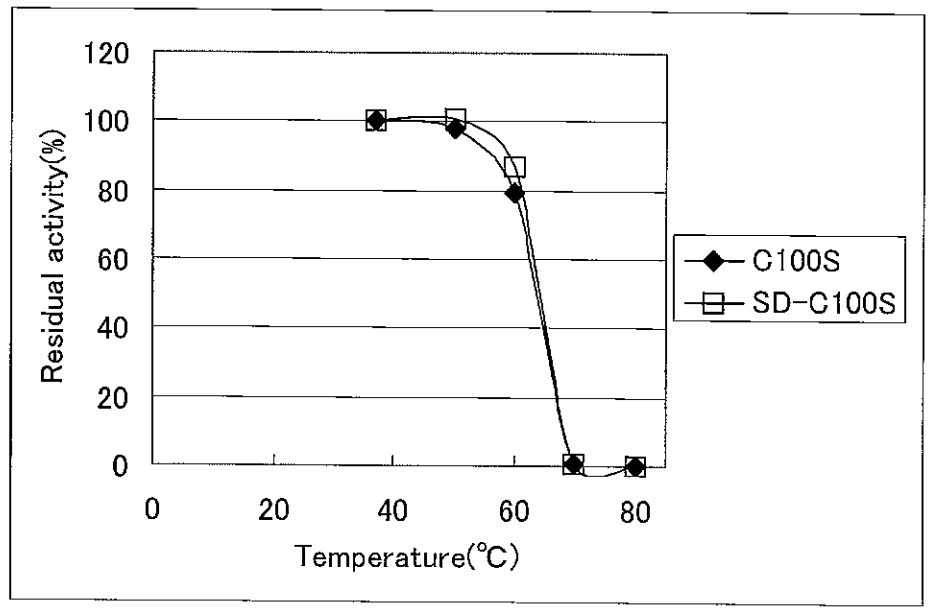


Figure 4. Thermal stability

Treatment condition: pH6.0, 60min, 50mmol/L acetate buffer

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN
PHONE : +81-748-75-1194
F A X : +81-748-75-0312

November 18. 2008

Stability Data of GLUTAMINASE DAIWA SD-C100S

Procedure

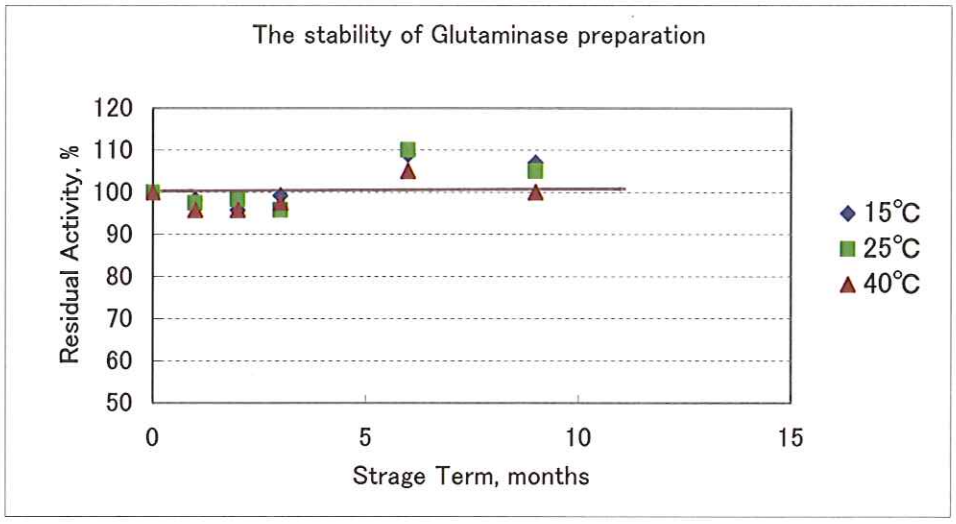
1. Place the sample in a GX film bag.
2. Keep the GX film bag at 15, 25 and 40℃.
3. Determine the enzyme activity after the storage of months mentioned below.
   Residual activity, (%) = b／a×100
   a: Initial enzyme activity
   b: Enzyme activity after storage

Method

Glutaminase activity: Daiwa Kasei method

Result

Months	Residual Activity, %						
Temp.	0	1	2	3	6	9	12
15℃	100	98.3	95.8	99.2	109	107	
25℃	100	97.5	98.3	95.8	110	105	
40℃	100	95.8	95.8	97.5	105	100	



Stability

When stored at 40℃ or below, the product will maintain around 100% activity for a minimum of 12 months. This product is an extremely stable enzyme preparation.

Secondary Activity Assessments

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN

PHONE : +81-748-75-1194
FAX : +81-748-75-0312

November 25, 2008

The enzymatic activities of glutaminase preparation

Samples

Glutaminase spray dried concentrate	Lot. GT0704132.00SP
Glutaminase alcohol precipitation Concentrate	Lot. GT0604272.00UP

Results

The enzymatic activity that existed in glutaminase preparation was gltaminase, protease and α-amylase. The peptidase activity was able hardly to confirm, and was below the detection limit. The protease and the amylase are the subsidiary enzymatic activities.

Table 1. Glutaminase activity and subsidiary enzymatic activity

	Glutaminase spray dried concentrate Lot. GT0704132.00SP	GLUTAMINASE DAIWA SD-C100S
Glutaminase (GTU/g)	1970	100-120 [1)]
Protease (PU/g)	145000	7360-8830 [1)]
α-Amylase (AU/g)	136000	6900-8280 [1)]
Peptidase (U/g)	< 0.25	< 0.001 [1)]

1):.calculation value based on the enzymatic activity of concentrate.

Table 2. Glutaminase activity and subsidiary enzymatic activity

	Glutaminase alcohol precipitation concentrate Lot. GT0704132.00SP	GLUTAMINASE DAIWA SD-C100S
Glutaminase (GTU/g)	2080	100-120 [1)]
Protease (PU/g)	158000	7600-9120 [1)]
α-Amylase (AU/g)	162000	7790-9350 [1)]
Peptidase (U/g)	< 0.25	< 0.001 [1)]

1): calculation value based on the enzymatic activity of concentrate.

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN

PHONE : +81-748-75-1194
F A X : +81-748-75-0312

November 25, 2008
DAIWA KASEI KK

Stability and fate of the enzyme preparation in the food ingredient

Samples

Extracts (Food ingredient)

Yeast extract: "Meast P1G" (ASAHI FOOD AND HEALTHCARE ,Ltd.)

Hydrolyzed vegetable protein (HVP): "ENZAP" (DAI-NIPPON MEIJI SUGAR Co., Ltd.)

Enzyme

GLUTAMINSE DAIWA SD-C100S (spray dried preparation) Lot. P7EB161

GLUTAMINSE DAIWA C100S (alcohol precipitation preparation) Lot. P8HC061

Results

The glutaminase activity and subsidiary enzymatic activities of "GLUTAMINASE DAIWA" was inactivated on incubation at 95℃ for 20 minutes in the presence of extract and hydrolyzed protein.

Example 1

A mixture containing 2 % yeast extract and 0.01% GLUTAMINSE DAIWA was incubated at 90 and 95℃ for 5, 10, 20 and 30 minutes. After that, the reaction mixture was cooled in ice water and assayed for residual activities of glutaminase, protease and amylase.

Table1. Inactivation of glutaminase, protease and amylase of "GLUTAMINASE SD-C100S" and "GLUTAMINASE C100S" in the presence of yeast extract.

Extract	Heat Treatment		GLUTAMINASE SD-C100S			GLUTAMINASE C100S		
	Temp (℃)	Time (min)	Glutaminase (GTU/mL)	Protease (U/mL)	Amylase (U/mL)	Glutaminase (GTU/mL)	Protease (U/mL)	Amylase (U/mL)
Yeast Extract	90℃	5	<0.000004	<0.0005	0.00031	0.000047	0.0005	0.00045
		10	<0.000004	<0.0005	0.00016	0.000019	0.0028	0.00140
		20	<0.000004	<0.0005	<0.00004	<0.000004	0.0022	<0.00004
		30	<0.000004	<0.0005	<0.00004	<0.000004	0.0031	<0.00004
	95℃	5	0.000010	<0.0005	0.00066	0.000078	0.0029	<0.00004
		10	0.000004	<0.0005	<0.00004	0.000039	<0.0005	0.00032
		20	<0.000004	<0.0005	<0.00004	<0.000004	<0.0005	<0.00004
Control		0	0.01	0.81	0.76	0.01	0.90	0.91

＊Detection limits: Glutaminase 0.000004GTU/mL
Protease 0.0005U/mL
Amylase 0.00004U/mL

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN
PHONE : +81-748-75-1194
F A X : +81-748-75-0312

Example 2

A mixture containing 10 % HVP and 0.1% GLUTAMINSE DAIWA was incubated at 90 and 95℃ for 5, 10, 20 and 30 minutes. After that, the reaction mixture was cooled in ice water and assayed for residual activities of glutaminase, protease and amylase.

Table2. Inactivation of glutaminase, protease and amylase of "GLUTAMINASE SD-C100S" and "GLUTAMINASE C100S" in the presence of HVP.

Extract	Heat Treatment		GLUTAMINASE SD-C100S			GLUTAMINASE C100S		
	Temp (℃)	Time (min)	Glutaminase (GTU/mL)	Protease (U/mL)	Amylase (U/mL)	Glutaminase (GTU/mL)	Protease (U/mL)	Amylase (U/mL)
HVP	90℃	5	0.000054	0.0077	0.01366	<0.000004	<0.0005	0.00775
		10	0.000018	0.0014	0.00605	<0.000004	<0.0005	0.00676
		20	0.000021	0.0018	0.00031	<0.000004	<0.0005	0.00164
		30	<0.000004	0.0014	<0.00004	<0.000004	<0.0005	<0.00004
	95℃	5	<0.000004	0.0023	0.00215	<0.000004	<0.0005	0.00219
		10	0.000013	<0.0005	<0.00004	<0.000004	<0.0005	<0.00004
		20	<0.000004	<0.0005	<0.00004	<0.000004	<0.0005	<0.00004
Control		0	0.10	8.14	7.59	0.10	8.97	9.07

＊Detection limits: Glutaminase 0.000004GTU/mL
Protease 0.0005U/mL
Amylase 0.00004U/mL

GMP Certificate

CERTIFICATE

This is to certify that the firm's quality system conforms to the Self-Imposed Standard of Good Manufacturing Practice for Food Additives (JAFA GMP) in the applicable scope as a result of audit based on the JAFA Quality Assessment and Registration Scheme for JAFA GMP.

Registered number : JAFAGMP – 011 – (3)
Registered date : February 10 , 2003
Registered firm : Amano Enzyme Inc.
Applicable scope :

* Applicable plant & its address
  Nagoya Plant
  27 Hanno, Kunotsubo, Kitanagoya-shi, Aichi-pref. 4818533 Japan
*Applicable production unit(s) in the plant
  Solid fermentation team (Fermentation・Purification)
  Products team (Preparation・Processing・Shipping)
*Applicable product(s) in the production unit(s)
  All of the food additives to be manufactured at the applicable production units in the applicable plant.

Renewal date : January 4 , 2012
Expiry date : February 9 , 2015
The date of issue : January 27 , 2012

Japan Food Additives Association (JAFA)

[redacted]

Shinji Sasaki, President

Japan Food Additives Association
3-9, Nihonbashi-Horidome-cho 1-chome, Chuo-ku, Tokyo 1030012, Japan

Quality Management System

CERTIFICATE OF CONFORMITY

Certificate No. :	JCQA-0043	Registration Date :	1995. 3. 27
Renewal Date :	2013. 3. 27	Expiry Date :	2016. 3. 26

Name of Registered Organization: **Amano Enzyme Inc. Head Office et al.**

Address of Registered Organization: **2-7, Nishiki 1-chome, Naka-ku, Nagoya-shi, Aichi, 460-8630, Japan et al.**

JAPAN CHEMICAL QUALITY ASSURANCE LTD. certifies that the Quality Management System of the above organization specified in the appendix, has been assessed and verified to be in accordance with the requirements of the quality management system standards as shown below:

Quality Management System Standards: **JIS Q 9001:2008**
ISO 9001:2008

The Date of Issue: **2013. 3. 11**

JAPAN CHEMICAL QUALITY ASSURANCE LTD. (JCQA)
Nittochi-Uchisaiwaicho BLDG. 7F, 1-2-1, Uchisaiwaicho,
Chiyoda-ku, Tokyo 100-0011 JAPAN

[redacted]

PRESIDENT REPRESENTATIVE DIRECTOR

Quality Management System

APPENDIX TO CERTIFICATE OF CONFORMITY

Certificate No. :	JCQA-0043	Registration Date :	1995. 3. 27
Renewal Date :	2013. 3. 27	Expiry Date :	2016. 3. 26
Extension Date :			

Name of Registered Organization: **Amano Enzyme Inc.**

Address of Registered Organization:

Head Office:
2-7, Nishiki 1-chome, Naka-ku, Nagoya-shi, Aichi, 460-8630, Japan

Gifu R&D Center:
6, Technoplaza 1-chome, Kakamigahara-shi, Gifu, 509-0109, Japan

Nagoya Plant:
27, Hanno, Kunotsubo, Kitanagoya-shi, Aichi, 481-8533, Japan

Yoro Plant:
3600-1, Makita, Kamiishizu-cho, Ogaki-shi, Gifu, 503-1602, Japan

Scope of Activity: **The Design/Development, Manufacture and Sales of Enzymes**

The Date of Issue: **2013. 3. 11**

JAPAN CHEMICAL QUALITY ASSURANCE LTD. (JCQA)
Nittochi-Uchisaiwaicho BLDG. 7F, 1-2-1, Uchisaiwaicho, Chiyoda-ku, Tokyo, 100-0011, JAPAN

[redacted]

PRESIDENT REPRESENTATIVE DIRECTOR

Quality Management System

CERTIFICATE OF CONFORMITY

Certificate No.:	JCQA-1357	Registration Date:	2003. 12. 24
Renewal Date:	2012. 12. 24	Expiry Date:	2015. 12. 23

Name of Registered Organization: **Amano Enzyme Inc.**
Shiga Plant

Address of Registered Organization: **4-19, Hie-cho, Konan-shi, Shiga, 520-3203, Japan**

JAPAN CHEMICAL QUALITY ASSURANCE LTD. certifies that the Quality Management System of the above organization specified in the appendix, has been assessed and verified to be in accordance with the requirements of the quality management system standards as shown below:

Quality Management System Standards: **JIS Q 9001:2008**
ISO 9001:2008

The Date of Issue: **2013. 4. 8**

JAPAN CHEMICAL QUALITY ASSURANCE LTD. (JCQA)
Nittochi-Uchisaiwaicho BLDG. 7F, 1-2-1, Uchisaiwaicho, Chiyoda-ku, Tokyo, 100-0011, JAPAN

[redacted]

PRESIDENT REPRESENTATIVE DIRECTOR

Quality Management System

APPENDIX TO CERTIFICATE OF CONFORMITY

Certificate No. :	JCQA-1357	Registration Date :	2003. 12. 24
Renewal Date :	2012. 12. 24	Expiry Date :	2015. 12. 23
Extension Date :			

Name of Registered Organization: **Amano Enzyme Inc.**
Shiga Plant

Address of Registered Organization: **4-19, Hie-cho, Konan-shi, Shiga, 520-3203, Japan**

Scope of Activity: **Development and Production of Enzyme Preparations for Food, Industrial and Laboratory Use, and Outsource Management, and Contract Production of Processed Food**

The Date of Issue: **2013. 4. 8**

JAPAN CHEMICAL QUALITY ASSURANCE LTD. (JCQA)
Nittochi-Uchisaiwaicho BLDG. 7F, 1-2-1, Uchisaiwaicho,
Chiyoda-ku, Tokyo, 100-0011, JAPAN

[redacted]
PRESIDENT REPRESENTATIVE DIRECTOR

SAFETY AND REGULATORY ASPECTS OF ENZYMES

The safety evaluation of fermentation enzymes should take three levels into account:

- the potential health hazard for the staff involved in the manufacturing and handling of the enzyme (occupational safety)
- the potential risk to the environment in which the micro-organism and/or its products may be released (environmental safety)
- the safety of use by the consumer (consumer safety)

For this last aspect, please refer to the food, feed and industrial uses.

1. Occupational safety

In an occupational setting, two issues have to be addressed:

- possible risks to employees associated with the production organism used
- possible risks to employees associated with the enzyme produced

1.1. Organisms

In the fermentation enzyme industry organisms are commonly used which have a long history of safe use or which have recently been established as being non-pathogenic (i.e. they have never been reported to be able to cause disease in healthy human beings) and non-toxicogenic (i.e. they have been proven not to produce toxic metabolites). In other words, the organisms used in the enzyme industry are not hazardous.

This is further underpinned by the fact that the so-called Biological Agent Directive (Directive 2000/54/EC, see References) does not require any additional measure for handling these organisms. This Directive essentially divides micro-organisms into four hazard categories, ranging from non-dangerous to very dangerous and it lays down occupational measures when using these micro-organisms in a manufacturing process. The dangerous micro-organisms (Group 2 to 4) are listed in Annex III of the Directive. None of the organisms used in the fermentation enzyme industry are listed.

Although the Directive defines the term "biological agent" as micro-organisms including those which have been genetically modified, no consideration has been given to genetically modified micro-organisms when drawing up the list. However, genetically modified micro-organisms are covered by another EU law (see below under Environmental Safety).

1.2. Enzymes

In the early days of the application of enzymes in the detergent industry it became apparent that enzymes could pose an occupational health risk. In hindsight this is not surprising since enzymes are essentially proteins and, like many proteins, inhalation of dust or aerosols may lead to respiratory allergy in susceptible individuals. The symptoms can be compared with those of allergy to pollen (hay-fever) and, like hay-fever, they will disappear when exposure is eliminated.

The risks can be minimised by minimising the exposure, either by engineering controls (ventilation), good handling practices and personal protection measures or by using enzyme preparations which are specifically designed to minimise inhalatory exposure (e.g. liquids, encapsulated or immobilised preparations). In this way the risks can be brought to acceptable levels (i.e. the enzyme preparation can be handled safely) as is proven by the safety record of the present detergent factories. More details on the safe handling of enzymes can be found in the **AMFEP Guide to the Safe Handling of Enzymes**.

Some enzymes, particularly proteases, may cause irritation when they come into contact with skin, eyes or mucous membranes. There is no evidence that enzymes may cause allergy by skin contact, a detailed summary of the relevant literature can be found in the **AISE/AMFEP document Enzymes: Lack of Skin Sensitisation Potential.**

Within the European Union this area is covered by quite a number of regulatory instruments. The most relevant for enzymes are the so-called Dangerous Substances Directive (Council Directive 67/548/EEC and its amendments, see References) and Dangerous Preparations Directive (Directive 1999/45/EC, see References). Both aim to protect man and the environment and one of the instruments to achieve this, is an obligatory provision of information on the hazards of products put on the market in the European Union. It should be noted that the classification, labelling and safety data sheet provisions apply to all enzyme preparations marketed by Amfep.

The system to be followed is:

- hazard identification of the product (also called classification) according to criteria laid down in the Directives
- if the product is classified as dangerous, it has to be labelled. This warning label consists of an indication of danger (symbol), risk phrases (R-phrases) describing the hazard in some detail and Safety phrases (S-phrases) giving a short advice on handling and/or first aid. All of these elements are standardised
- the label information has to be supplemented by a Safety Data Sheet, the contents of which have to comply with yet another Directive (Commission Directive 91/155/EEC and its amendments, see References).

The regulations require that manufacturers classify their products on the basis of the available data and label them accordingly. This label applies until the authorities have agreed on a classification/label and the product has been included onto a list, which is Annex 1 of an amendment to the Dangerous Substances Directive (Commission Directive 96/54/EC, see References).

16 enzymes are presently included in Annex I. All 16 enzymes are classified as R42 (May cause sensitisation by inhalation) and associated with a warning label symbol Xn (Harmful), risk phrase R 42 and the safety phrases S (2-)-22-24-36/37 .

Proteases other than subtilisin are additionally classified as Xi (irritant), R 36/37/38 (Irritating to eyes, respiratory system and skin) and are labelled Xn, R 36/37/38-42, S (2-)-22-24-26-36/37. Subtilisin is additionally classified as Xi (irritant), R 37/38-41 (Irritating to respiratory system and skin, risk of serious damage to eyes) and labelled Xn, R 37/38-41-42, S (2-)-22-24-26-36/37/39.

In case of classification of both Xn and Xi, only Xn is required on the label.

Amfep considers that the risk and safety phrases set by the EU Commission reflects a precautionary and reasonable approach to the risks and good handling practices associated with enzymes.

The above classification and labelling applies to the (pure) enzyme substances. No specific concentration limits have been entered in Annex 1 and in order to decide on the proper labelling of enzyme preparations according to the Dangerous Preparations Directive, the preparation can be tested for the exact amount of enzyme present. Normally, however, a default concentration limit for Xn, R 42 of 1% is used in the case of enzyme preparations.

As a result of the Dangerous Preparations Directive, enzyme containing products not classified Xn, R 42 but containing at least one allergenic component above 0.1%, must bear the inscription 'Contains (name of sensitising substance): May cause an allergic reaction' on the product label. The Dangerous Preparations Directive also introduces environmental classification of preparations but this will not in general affect enzyme preparations.

As enzymes derived from genetically modified organisms are proteins like all other enzymes, the above applies irrespective of the fact if genetic modification of the production organism has been involved.

2. Environmental safety

Also in the area of environmental safety the risks of the enzyme and of the production organism when released into the environment have to be assessed. Since enzymes are just proteins - albeit with a specific catalytic activity - and consequently readily biodegradable, their release into the environment does not raise concerns. Numerous studies have demonstrated that enzymes from all major classes (subtilisins, amylases, cellulase, lipase, etc) are biodegradable. This is expected considering their globular protein structure. Ready biodegradability tests with enzymes from genetically modified organisms, including protein-engineered variants, have not shown any different characteristics compared to naturally occurring or wild type enzymes. This would also be expected, since these techniques do not change the general globular protein structure.

The release of the production organism itself - whether it is genetically modified or not - is controlled by two categories of safety measures. The first one is called physical containment and consists of a fermenter system and recovery (downstreaming) equipment meeting high standards of hygiene, i.e. minimisation of micro-organisms entering or escaping from the equipment. The second, complimentary one, is called biological containment. Essentially, this is the inbred incapability of the organism to survive efficiently in the environment.

The production organisms used in the fermentation enzyme industry are specially bred - again either by traditional methods or by modern genetic techniques - to produce large amounts of one specific substance, the enzyme. Therefore, the production organisms are adapted to grow optimally only under the very specific fermenter conditions. The experience of our industry indicates that the growth in nature of these organisms, should they break the physical containment, is severely handicapped and they cannot compete with other micro-organisms which are present in the environment.

The EU Regulations which cover specifically genetically modified organisms are the so-called Contained Use Directive (Council Directive 90/219/EEC and its amendments, see References) and the Deliberate Release Directive (Directive 2001/18/EC, see References)). They deal exclusively with genetically modified organisms. The second directive is hardly relevant for the fermentation enzyme industry, since typically the enzyme products do not contain the production organism and consequently no genetically modified organisms are put on the market (i.e., they are not "deliberately released"). The Contained Use Directive lays down requirements to ensure a safe and controlled use of genetically

modified organisms, which are in summary:

- Assessment of the potential hazards of the organism
- Requirements for containment (physical and biological) of the organism, which become more stringent as the potential hazard of the organism increases
- A risk assessment of the use of the organism
- Notification of a competent authority before activities (R&D, manufacturing) start
- Depending on the risk category and the scale of the activities, authorisation from the competent authority is needed.

The organisms used in the production of enzymes invariably are classified in the least hazardous category for which no other containment measures are needed than Good Industrial Large Scale Practice (GILSP). These guidelines were formulated by the Organisation of Economic co-operation and Development (OECD) in 1986 and incorporated into the Contained Use Directive.

Batch Analysis

Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062

No.208010537 - 004 1/2
January 25, 2008

CERTIFICATE OF ANALYSIS

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi, Shiga 520-3203, Japan

Sample: GLUTAMINASE DAIWA SD-C100S LOT P7EB161

Received: January 11, 2008

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the client.

RESULT(S)

Item	Result	LD[1)]	Method
Loss on drying[2)]	0.5g/100g		1
Total nitrogen	0.53g/100g		2
Ether-soluble matter	Less than 0.1g/100g		3
Residue on ignition[3)]	94.0g/100g		4
Arsenic (as As_2O_3)	Not detected	0.1 ppm	5
Heavy metals (as Pb)	Not detected	1 ppm	6
Lead	Not detected	0.05 ppm	5
Cadmium	Not detected	0.01 ppm	5
Mercury	Not detected	0.01 ppm	7
Aerobic plate count	Not more than 300/g		8
Coliform bacteria (MPN)[4)]	Negative (not more than 3)/g		9
Escherichia coli	Negative/2.22 g		10
Coagulase positive Staphylococci[4)]	Negative/1g		10
Salmonella	Negative/25g		10
Clostridium perfringens	Negative/0.1g		10
Campylobacter jejuni/coli	Negative/10g		10
Listeria monocytogenes[5)]	Negative		
Clostridia[6)]	Negative/1g		11

1) LD: Minimum limit of determination.
2) Test conditions: Temperature; 105 ℃, Time; 5 hours.
3) Test conditions: Temperature; 550 ℃, Constant weight.
4) The test method was specified by the client.
5) FDA: Bacteriological Analytical Manual, 8th Ed., Chapter 10 (1995).
6) Heat-shocked conditions: at 70 ℃ for 20 minutes.

Method

1: Air oven method
2: Kjeldahl method
3: Method with acid hydrolysis
4: Ignition at 550 °
5: Atomic absorption spectrometry
6: Sodium sulfide colorimetric method
7: Cold vapor atomic absorption spectrometry
8: Standard Agar plating method
9: MPN method
10: Enrichment culture method
11: Clostridia Count Agar anaerobic plating method

JAPAN FOOD RESEARCH LABORATORIES

[redacted]
Principal Investigator
Japan Food Research Laboratories

Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062

No.208010537 · 005 1/2
January 25, 2008

CERTIFICATE OF ANALYSIS

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi, Shiga 520-3203, Japan

Sample: GLUTAMINASE DAIWA SD-C100S LOT P8AA791

Received: January 11, 2008

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the client.

RESULT(S)

Item	Result	LD[1)]	Method
Loss on drying[2)]	0.5g/100g		1
Total nitrogen	0.54g/100g		2
Ether-soluble matter	Less than 0.1g/100g		3
Residue on ignition[3)]	91.4g/100g		4
Arsenic (as As_2O_3)	Not detected	0.1 ppm	5
Heavy metals (as Pb)	Not detected	1 ppm	6
Lead	Not detected	0.05 ppm	5
Cadmium	Not detected	0.01 ppm	5
Mercury	Not detected	0.01 ppm	7
Aerobic plate count	Not more than 300/g		8
Coliform bacteria (MPN)[4)]	Negative (not more than 3)/g		9
Escherichia coli	Negative/2.22 g		10
Coagulase positive Staphylococci[4)]	Negative/1g		10
Salmonella	Negative/25g		10
Clostridium perfringens	Negative/0.1g		10
Campylobacter jejuni/coli	Negative/10g		10
Listeria monocytogenes[5)]	Negative		
Clostridia[6)]	Negative/1g		11

1) LD: Minimum limit of determination.
2) Test conditions: Temperature; 105 ℃, Time; 5 hours.
3) Test conditions: Temperature; 550 ℃, Constant weight.
4) The test method was specified by the client.
5) FDA: Bacteriological Analytical Manual, 8th Ed., Chapter 10 (1995).
6) Heat-shocked conditions: at 70 ℃ for 20 minutes.

Method

1: Air oven method
2: Kjeldahl method
3: Method with acid hydrolysis
4: Ignition at 550 °
5: Atomic absorption spectrometry
6: Sodium sulfide colorimetric method
7: Cold vapor atomic absorption spectrometry
8: Standard Agar plating method
9: MPN method
10: Enrichment culture method
11: Clostridia Count Agar anaerobic plating method

JAPAN FOOD RESEARCH LABORATORIES

[redacted]

Principal Investigator
Japan Food Research Laboratories

Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062

No.208010537 · 006 1/2
January 25, 2008

CERTIFICATE OF ANALYSIS

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi, Shiga 520-3203, Japan

Sample: GLUTAMINASE DAIWA SD-C100S LOT P8AA891

Received: January 11, 2008

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the client.

RESULT(S)

Item	Result	LD[1)]	Method
Loss on drying[2)]	0.4g/100g		1
Total nitrogen	0.52g/100g		2
Ether-soluble matter	Less than 0.1g/100g		3
Residue on ignition[3)]	92.8g/100g		4
Arsenic (as As_2O_3)	Not detected	0.1 ppm	5
Heavy metals (as Pb)	Not detected	1 ppm	6
Lead	Not detected	0.05 ppm	5
Cadmium	Not detected	0.01 ppm	5
Mercury	Not detected	0.01 ppm	7
Aerobic plate count	Not more than 300/g		8
Coliform bacteria (MPN)[4)]	Negative (not more than 3)/g		9
Escherichia coli	Negative/2.22 g		10
Coagulase positive Staphylococci[4)]	Negative/1g		10
Salmonella	Negative/25g		10
Clostridium perfringens	Negative/0.1g		10
Campylobacter jejuni/coli	Negative/10g		10
Listeria monocytogenes[5)]	Negative		
Clostridia[6)]	Negative/1g		11

1) LD: Minimum limit of determination.
2) Test conditions: Temperature; 105 ℃, Time; 5 hours.
3) Test conditions: Temperature; 550 ℃, Constant weight.
4) The test method was specified by the client.
5) FDA: Bacteriological Analytical Manual, 8th Ed., Chapter 10 (1995).
6) Heat-shocked conditions: at 70 ℃ for 20 minutes.

Method

1: Air oven method
2: Kjeldahl method
3: Method with acid hydrolysis
4: Ignition at 550 °
5: Atomic absorption spectrometry
6: Sodium sulfide colorimetric method
7: Cold vapor atomic absorption spectrometry
8: Standard Agar plating method
9: MPN method
10: Enrichment culture method
11: Clostridia Count Agar anaerobic plating method

JAPAN FOOD RESEARCH LABORATORIES

[redacted]
Principal Investigator
Japan Food Research Laboratories

Japan Food Research Laboratories
Authorized by the Japanese Government

Japan
Food
Research
Laboratories

HEAD OFFICE : 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
OSAKA BRANCH : 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
NAGOYA BRANCH : 5-13 Oosu 4-chome, Naka-ku, Nagoya 460-0011
KYUSHU BRANCH : 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka 812-0034
TAMA LABORATORY : 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
CHITOSE LABORATORY : 2-3 Bunkyo, Chitose-shi, Hokkaido 066-0052

REPORT

No. 205100812-002 1/2
November 4, 2005

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi
Shiga 520-3203
Japan

Received: October 12, 2005

Determination of Antibacterial Activity

1. Samples
   1) Glutaminase Concentrate GT812185.00UP
   2) Glutaminase Concentrate GT507105.00UP
   3) Glutaminase Concentrate GT0406045.00UP

2. Purpose
   The purpose of this test is to determine antibacterial activities of the samples.

3. Outline of methods
   The antibacterial activity was determined by the method based on the Joint FAO/WHO Expert Committee on Food Additives [Specifications for identity and purity of certain food additives (FAO FOOD AND NUTRITION PAPER 49)] ANNEX A, APPENDIX [DETERMINATION OF ANTIBIOTIC ACTIVITY].

4. Results

The test results are shown in Table 1.

Table 1. Inhibition zones with six bacterial strains

Strains	Diameter of inhibition zone (mm)		
	Sample 1)	Sample 2)	Sample 3)
Staphylococcus aureus ATCC 6538	–	–	–
Escherichia coli ATCC 11229	–	–	–
Bacillus cereus ATCC 2	–	–	–
Bacillus circulans ATCC 4516	–	–	–
Streptococcus pyogenes ATCC 12344	14.7	13.6	14.2
Serratia marcescens ATCC 14041	–	–	–

–: No zone formed.

It was concluded that the antibacterial activities of the samples were negative, because clear inhibition zones were observed on the plates of *Streptococcus pyogenes* only.

JAPAN FOOD RESEARCH LABORATORIES

[redacted]

Principal Investigator
Japan Food Research Laboratories

TNO Nutrition and Food Research

Nederlandse Organisatie voor toegepast-natuurwetenschappelijk onderzoek/Netherlands Organisation for Applied Scientific Research

Return address: P.O.Box 360, 3700 AJ, Zeist, The Netherlands

Dawai Kasei K.K.
4-19 Hie-cho, Kosei-cho, Koka-gun, Shiga
520-3203 JAPAN
attn. Mr. K. Hiratani

Risk Management & Microbiology
Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

www.tno.nl

T +31 30 694 41 44
F +31 30 695 72 24
infofood@voeding.tno.nl

[redacted]

Herewith we send you the results of the analysis of three samples of glutaminase concentrate, which we received on 29 January 2002.

The results of the analysis are summarized in Table 1 and Table 2 (see annex).

If you have any questions about the results of the analysis, please do not hesitate to contact us. The invoice of the costs of the analysis will be sent separately.

We hoped to have served you properly,

Yours faithfully,

[redacted]

Subject
Analysis of glutaminase

Date
14 October 2002

Our reference
ARMM 02-4216/REG-hos

E-mail
Vissers@voeding.tno.nl

Direct dialing
+31 30 694 47 71

Direct fax
+31 30 694 49 01

Project number
010.52024/01.26.01

Your reference
--

Enclosure(s)
2

Annex 2

Table 2
Results of the analysis in three samples of glutaminase concentrate, received 29-01-2002, from Dawai Kasei K.K., Japan

TNO code	Your code	Aflatoxin (µg/kg)				Fumonisin B1 (µg/kg)	Ochratoxin A (µg/kg)
		B1	B2	G1	G2		
3119/01/0874	Lot. No. GT00928501 UP	<1,0	<1,0	<1,0	<1,0	Not detectable[1)]	<1,0
3119/01/0875	Lot. No. GT00928502 UP	<1,0	<1,0	<1,0	<1,0	Not detectable	<1,0
3119/01/0876	Lot. No. GT10528500 UP	<1,0	<1,0	<1,0	<1,0	Not detectable	<1,0

1) not detectable with the method in use (too much interferences)

Japan Food Research Laboratories
Authorized by the Japanese Government

Japan
Food
Research
Laboratories

HEAD OFFICE : 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
OSAKA BRANCH : 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
NAGOYA BRANCH : 5-13 Oosu 4-chome, Naka-ku, Nagoya 460-0011
KYUSHU BRANCH : 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka 812-0034
TAMA LABORATORY : 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
CHITOSE LABORATORY : 2-3 Bunkyo, Chitose-shi, Hokkaido 066-0052

REPORT

No. 208102416-004 1/2
November 19, 2008

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi, Shiga 520-3203, Japan

Received: October 28, 2008

Determination of Antibacterial Activity

1. Samples
   1) GLUTAMINASE DAIWA SD-C100S Lot No. P8ID065
   2) GLUTAMINASE DAIWA SD-C100S Lot No. P8ID066
   3) GLUTAMINASE DAIWA SD-C100S Lot No. P8HC161

2. Purpose
   The purpose of this test is to determine antibacterial activities of the samples.

3. Outline of methods
   The antibacterial activities of the samples were determined by the method based on the Joint FAO/WHO Expert Committee on Food Additives [Specifications for identity and purity of certain food additives (FAO FOOD AND NUTRITION PAPER 49)] ANNEX A, APPENDIX [DETERMINATION OF ANTIBIOTIC ACTIVITY].

Japan Food Research Laboratories

4. Results

Table 1 shows the results of the test.

Table 1. Inhibition zones with six bacterial strains

Strains	Diameter of inhibition zone (mm)		
	Sample 1)	Sample 2)	Sample 3)
Staphylococcus aureus ATCC 6538	–	–	–
Escherichia coli ATCC 11229	–	–	–
Bacillus cereus ATCC 2	–	–	–
Bacillus circulans ATCC 4516	–	–	–
Streptococcus pyogenes ATCC 12344	18.5	18.7	18.5
Serratia marcescens ATCC 14041	–	–	–

–: No zone formed.

It was concluded that the antibacterial activities of the samples were negative, because clear inhibition zones were observed on only the plates of *Streptococcus pyogenes*.

JAPAN FOOD RESEARCH LABORATORIES

[redacted]

Principal Investigator
Japan Food Research Laboratories

MYCOTOXIN RESEARCH ASSOCIATION
(M. R. A.)

15, Daikoku Pier, Tsurumi-ku, Yokohama
230-0054 JAPAN.
T E L (045) 506-1151
F A X (045) 506-1153

Myco No.20-61997
Date:November 5, 2008

C E R T I F I C A T E

Article Examined	:	GLUTAMINASE DAIWA SD-C100S Lot No.P8ID065
Name and Adress of Applicant	:	DAIWA KASEI K.K. 4-19, Hie-Cho, Konan, SHIGA, 520-3203 JAPAN
Kind of Examination	:	Aflatoxin B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin, Zearalenone, T-2 Toxin
Method of Analysis	:	J.Assoc.Off.Anal.Chem.62,1265-1267(1979)
Result of Examination	:	Aflatoxin (B_1, B_2, G_1, G_2) not detected (detection limit:0.5ppb) Ochratoxin A not detected (detection limit:0.5ppb) Sterigmatocystin not detected (detection limit:20ppb) Zearalenone not detected (detection limit:50ppb) T-2 Toxin not detected (detection limit:200ppb)
Receiving No. and Date	:	No.20-61997 (October 28, 2008)

I hereby certify that the result of examination was as stated above.

Signed [redacted]

Chairman, Board of Directors
Mycotoxin Research Association
Registered by the Minister of
Health, Labour & Welfare

MYCOTOXIN RESEARCH ASSOCIATION
(M. R. A.)

15, Daikoku Pier, Tsurumi-ku, Yokohama
230-0054 JAPAN.
T E L (045) 506-1151
F A X (045) 506-1153

Myco No.20-61998
Date:November 5, 2008

C E R T I F I C A T E

Article Examined	:	GLUTAMINASE DAIWA SD-C100S Lot No.P81D066
Name and Adress of Applicant	:	DAIWA KASEI K.K. 4-19, Hie-Cho, Konan, SHIGA, 520-3203 JAPAN
Kind of Examination	:	Aflatoxin B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin, Zearalenone, T-2 Toxin
Method of Analysis	:	J.Assoc.Off.Anal.Chem.62,1265-1267(1979)

Result of Examination :

Aflatoxin (B_1, B_2, G_1, G_2)	not detected (detection limit:0.5ppb)
Ochratoxin A	not detected (detection limit:0.5ppb)
Sterigmatocystin	not detected (detection limit:20ppb)
Zearalenone	not detected (detection limit:50ppb)
T-2 Toxin	not detected (detection limit:200ppb)

Receiving No. and Date : No.20-61998 (October 28, 2008)

I hereby certify that the result of examination was as stated above.

Signed [redacted]

Chairman, Board of Directors
Mycotoxin Research Association
Registered by the Minister of
Health, Labour & Welfare

MYCOTOXIN RESEARCH ASSOCIATION
(M. R. A.)

15, Daikoku Pier, Tsurumi-ku, Yokohama
230-0054 JAPAN.
T E L (045) 506−1151
F A X (045) 506−1153

Myco No.20-61999
Date:November 5, 2008

C E R T I F I C A T E

Article Examined	:	GLUTAMINASE DAIWA SD-C100S Lot No.P8HC161
Name and Adress of Applicant	:	DAIWA KASEI K.K. 4-19, Hie-Cho, Konan, SHIGA, 520-3203 JAPAN
Kind of Examination	:	Aflatoxin B_1, B_2, G_1, G_2, Ochratoxin A, Sterigmatocystin, Zearalenone, T-2 Toxin
Method of Analysis	:	J.Assoc.Off.Anal.Chem.62,1265-1267(1979)

Result of Examination :

Aflatoxin (B_1, B_2, G_1, G_2)	not detected (detection limit:0.5ppb)
Ochratoxin A	not detected (detection limit:0.5ppb)
Sterigmatocystin	not detected (detection limit:20ppb)
Zearalenone	not detected (detection limit:50ppb)
T-2 Toxin	not detected (detection limit:200ppb)

Receiving No. and Date : No.20-61999 (October 28, 2008)

I hereby certify that the result of examination was as stated above.

Signed

Chairman, Board of Directors
Mycotoxin Research Association
Registered by the Minister of
Health, Labour & Welfare

Petition to Amend Standard 1.3.3 of the Australia New Zealand Food Standards Code to Include Glutaminase from *Bacillus amyloliquefaciens* as a Processing Aid

- Appendix to Section C -

Prepared for: Amano Enzyme Inc.
1-2-7, Nishiki, Naka-ku,
Nagoya 460-8630
Japan

Prepared by: Intertek Scientific and Regulatory Consultancy
2233 Argentia Road, Suite 308 Mississauga,
Ontario, Canada
L5N 2X7
www.intertek.com

December 22, 2014

Allergen Database for Food Safety (ADFS) Search Results

Evaluation of allergenicity

Comparison with known allergens for homology in primary sequence

Allergenicity survey of glutaminase from *Bacillus amyloliquefaciens* was performed using the following allergen databases. Amino acid sequence of the glutaminase is shown in Figure 1. The survey was performed by the sequence searches for the match for 8-consecutive amino acid sequence and for 80 amino acid sliding window (search for 35% or higher identities in 80 amino acid stretch).

Allergen Database for Food Safety (ADFS)
(http://allergen.nihs.go.jp/ADFS/index.jsp?pagen=top)
Database Specification
Last Updated: Feb. 18, 2014
Number of Registered allergen (isoallergen) sequences: 1698

AllergenOnline (http://www.allergenonline.org/index.shtml)
Database Specification
Released On: Jan. 20, 2014
Peer Reviewed Sequences: 1706

There was no hit in the search for 8-consecutive amino acid in these databases. In addition, there was no hit in the search for the 80 amino acid sliding window (Table 1).

The above results indicate that glutaminase from *B. amyloliquefaciens* is of low concern with regard to allergenicity.

Figure 1: Amino acid sequence of Glutaminase from *B. amyloliquefaciens*

MKKKKFMNLCFIVLLSALLTAGSIPYHAQAKKHPFSYDDYKQVDVGKDGMVATAHPLASQIGADVL
KKGGNAIDAAVAIQFALNVTEPMMSGIGGGGFMMVYDAKTKDTTIIDSRERAPAGATPDMFLDENG
KAIPFSERVTKGTAVGVPGTLKGLEKALDKWGTRSMKQLITPSIALASKGFPIDSVLADAISDYKDKL
SHTAAKDVFLPNGEPLKEGDTLVQKDLAKTFTAIKYKGTKAFYDGAFTKKLAETVQEFGGSMTEQD
IKNFNVTIDEPIWGDYQGYHIATAPPPSSGGVFLLQMLNLLDDFKLSQYDIRSWQKYQLLAETMHLA
YADRAAFAGDPEFVNVPLKGLLNPDYINARRQLIDINKVNKKPKAGDPWAYQEGSANYKQVEQPT
DKQEGQTTHFTVADRFGNVVSYTTTIEQLFGSGIMVPGYGVVLNNELTDFDAVPGGANEVQPNKR
PLSSMTPTILFKNNEPVLTVGSPGGATIISSVLQTILNKVEYGMDLKAAVEEPRIYTNSMTSYRYEEG
VPEEARTKLNEMGHKFGSKPVDIGNVQSILIDRENGTFTGVADSSRNGAAIGVNLKKCEK*

Table 1: Results of allergen database survey

Date of search	Name of Database	Search results	
		8mer Exact Match	Sliding 80mer Window
2014/4/7	Allergen database for Food Safety (ADFS)	No hit	No hit
2014/4/7	AllergenOnline	No hit	No hit

Petition to Amend Standard 1.3.3 of the Australia New Zealand Food Standards Code to Include Glutaminase from *Bacillus amyloliquefaciens* as a Processing Aid

- Appendix to Section D -

Prepared for: Amano Enzyme Inc.
1-2-7, Nishiki, Naka-ku,
Nagoya 460-8630
Japan

Prepared by: Intertek Scientific and Regulatory Consultancy
2233 Argentia Road, Suite 308 Mississauga,
Ontario, Canada
L5N 2X7
www.intertek.com

December 22, 2014

TNO Nutrition and Food Research Institute Characterization of *Bacillus amyloliquefaciens*

TNO report
V99.423

Characterisation of strains ATCC 13933 and production strain NP

TNO Nutrition and Food Research Institute

Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

Phone +31 30 694 41 44
Fax +31 30 695 72 24

Date
12 April 1999

Author(s)
[redacted]

Project number:
70096/0101

Approved by:
[redacted]

Number of specimen:
1
Number of copies:
7
Number of pages:
15
Number of appendices:
3

Contents

1. Introduction

1.1 General introduction

Microorganisms used for the production of enzymes, that be used in human food, need to be characterised. The production strain needs to be characterised to enable unique identification which will allow verification of the production process. Both genotypic and phenotypic characterisation need to be carried out to ensure differentiation between strains.

1.2 Scope

One *Bacillus* production strain is characterised using both genotypic and phenotypic methods. The results are compared to culture collection strain *Bacillus subtilis* ATCC strain 13933.

2. Materials and Methods

2.1 Microbiological strains

The test strain is the glutaminase production strain NP. This strain is provided by Daiwa Kasei K.K. The culture collection *B. subtilis* strain included in the assays is ATCC 13933 (TNO code B0275). Characteristics of the ATCC strain are presented in Appendix 1.

2.2 Methods

Experiments are carried out according to laboratory procedures as described in the protocol 848613, version 1.

2.2.1 Microbiology and biochemical identification

- Strains are subcultured on TSBA and incubated for 3 days at 30°C.
- One colony is transferred to two BM slants and incubated for 3 days at 30°C. Microscopy, gram staining and catalase test are carried out.
- Biochemical characterisation is carried out using the API 50 CHB system. Species are described both on biochemical profile and species identification.
- Colony material is stored at -80°C in two separate freezers.

2.2.2 DNA identification

Cells are grown according to standard protocols. Ribotyping is carried out using the Qualicon® riboprinter. The resulting riboprint patterns are stored in the database or future comparison. GTG5 fingerprinting is carried out according to standard laboratory procedures.

2.2.3 Pyrolysis-Mass spectrometry

Strains are inoculated on PCA medium supplemented with a standard salt solution and incubated at 30°C for 3 days. Pyrolysis-Mass spectrometry is carried out according to standard procedures. The results will be presented as mass spectra.

3. Results and Discussion

3.1 Microbiology and biochemical identification

The results of the microscopic characterisation are presented in Table 1 (Appendix B). The results of the biochemical tests (API 50 CHB) are presented in Tables 2 and 3 (Appendix B). The production strain NP and the *B. subtilis* ATCC 13933 strain are not identical, both microscopic and according to biochemical testing

3.2 DNA Analysis

3.2.1 Ribotyping

Riboprints are presented in figure 1 (Appendix C). The strains are represented by an individual pattern.

3.2.2 GTG5 fingerprinting

GTG5 patterns are presented in figure 2 (Appendix C). Both the strains are represented by an individual GTG5 pattern.

3.3 Pyrolysis-Mass spectrometry

Pyrograms of the production strain NP and *B. subtilis* ATCC 13933 are presented in figure 3 (Appendix C). The pyrograms are different for the strains.

4. Concluding remarks

Production strain NP could not be satisfactory identified using the biochemical API 50 CHB system. The system suggests that the strain represents either the *Bacillus* species *amyloliquefaciens, licheniformis* or *subtilis*.
The production strain NP and the *B. subtilis* strain ATCC 13933 could be differentiated based on their microbial, biochemical and DNA characteristics.

5. Signatures

[redacted]

Date 99 04 12

[redacted]

Date 99 04 12

[redacted]

Date 99 04 14

Appendix A Characteristics of *Bacillus subtilis* ATCC 13933

ATCC Number:	13933
Organism:	*Bacillus subtilis* (Ehrenberg) Cohn
Classification:	-
Designations:	NRRL B-1471
Depositors:	NRRL
History:	WRRL PT281
Isolation:	soil, Leicester, England
Descriptions:	Produces aterrimin and feed supplements
Propagation:	ATCC medium: 3
	Nutrient agar (Difco 0001) or nutrient broth (Difco 0003)
Temperature:	30°C
Biosafety Level:	1

Appendix B Tables 1, 2 and 3

Table 1: Microscopy

ID number	Med[1]	Tmp[2]	Cat.[3]		Gram		Microscopy									
							With			Length				Motility		
	TSBA	30	+	-	+	-	Slim	Normal	Large	Short	Normal	Long	Very long	Not	Motile	Very motile	Other
ATCC 13933	x	x	x		x			x			x			x			
Production strain NP	x	x	x		x			x				x		x			

[1] = Growth medium
[2] = Growth temperature
[3] = Catalase test

Table 2: Species identification according to API 50 CHB system

Number	API 50 CHB identification						
	1st identification		2nd identification		3rd identification		
ATCC 13933	*Bacillus subtilis*	95.2 %	-	-	-	-	Good identification
Production strain NP	*B. amyloliquefaciens*	37.1 %	*B. licheniformis*	36.5 %	*B. subtilis*	22.3 %	Good identification to the genus

Table 3: API 50 CHB identification

ID number	Substrate									
	Temoin/Control	Glycerol	Erythritol	D-Arabinose	L-Arabinose	Ribose	D-Xylose	L-Xylose	Adonitol	β Metyl-xyloside
	CTR	GLY	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX
	0	1	2	3	4	5	6	7	8	9
ATCC 13933	*	x#			x	x	x			
Production strain NP		x			x	x	x			

ID number	Substrate									
	Galactose	D-Glucose	D-Fructose	D-Mannose	L-Sorbose	Rhamnose	Dulcitol	Inositol	Manitol	Sorbitol
	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR
	10	11	12	13	14	15	16	17	18	19
ATCC 13933		x	x	x				x	x	x
Production strain NP		x	x	x				x	x	

ID number	Substrate									
	α Methyl-D-mannoside	α Methyl-D-glucoside	N Acetyl glucosamine	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Lactose
	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC
	20	21	22	23	24	25	26	27	28	29
ATCC 13933		x		x	x	x	x	x	x	
Production strain NP		x	x	x	x	x	x	x	x	x

ID number	Substrate									
	Melbiose	Saccarose/sucrose	Trehalose	Inulin	Melezitose	Rafinose	Amidon/Starch	Glycogen	Xylitol	β Gentiobiose
	MEL	SAC	TRE	INU	MLZ	RAF	AMO	GLYG	XLT	GEN
	30	31	32	33	34	35	36	37	38	39
ATCC 13933	x	x	x	x		x	x	x		
Production strain NP		x	x			x	x	x		x

ID number	Substrate									
	D-Turanose	D-Lyxose	D-Tagatose	D-Fucaose	L-Fucose	D-Arabitol	L-Arabitol	Gluconate	2-Ketogluconate	5-Ketogluconate
	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG
	40	41	42	43	44	45	46	47	48	49
ATCC 13933										
Production strain NP										

* blanc = negative reaction.
# x = positive reaction.

Appendix C Figures 1, 2 and 3

Figure 1: Riboprints

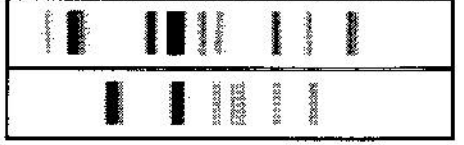
Sample Number	Original ID	RiboGroup	Sim to RiboGroup	RiboPrint Pattern
137-441-1	**ATCC 13933**	RIBO1 137-441-S-1	1.00	
137-441-3	**Production strain NP**	RIBO1 137-441-S-2	0.99	

Figure 2: GTG5 patterns

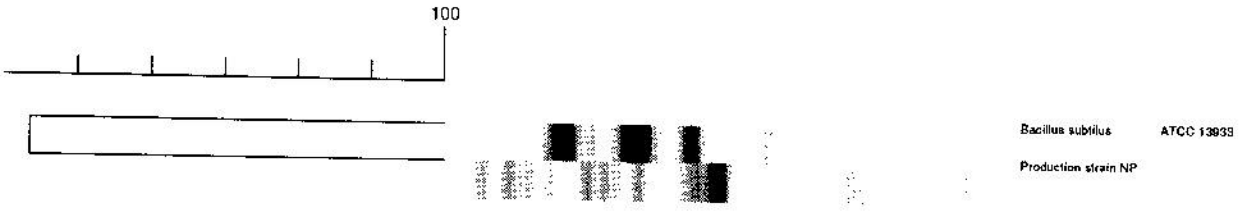
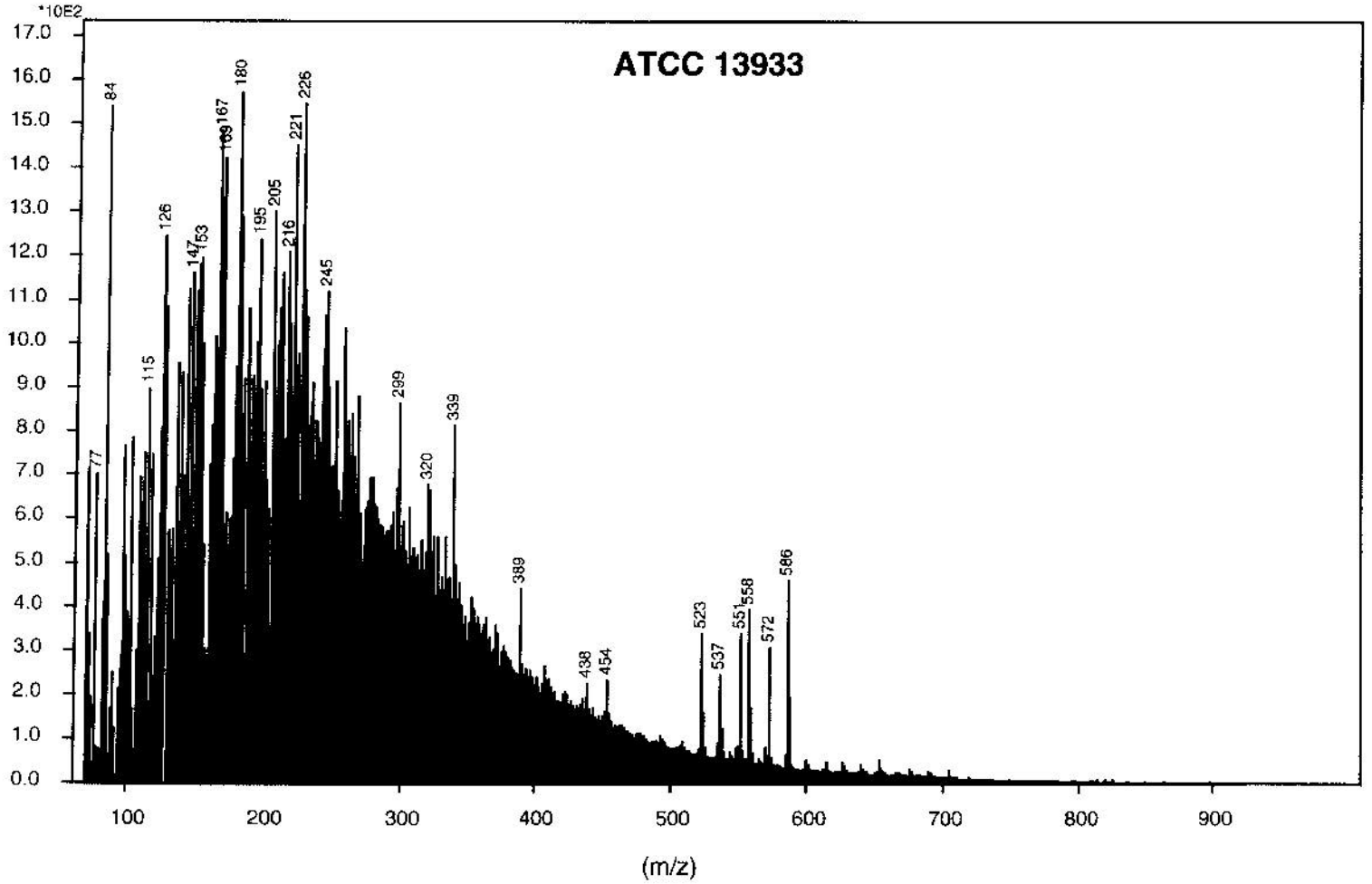
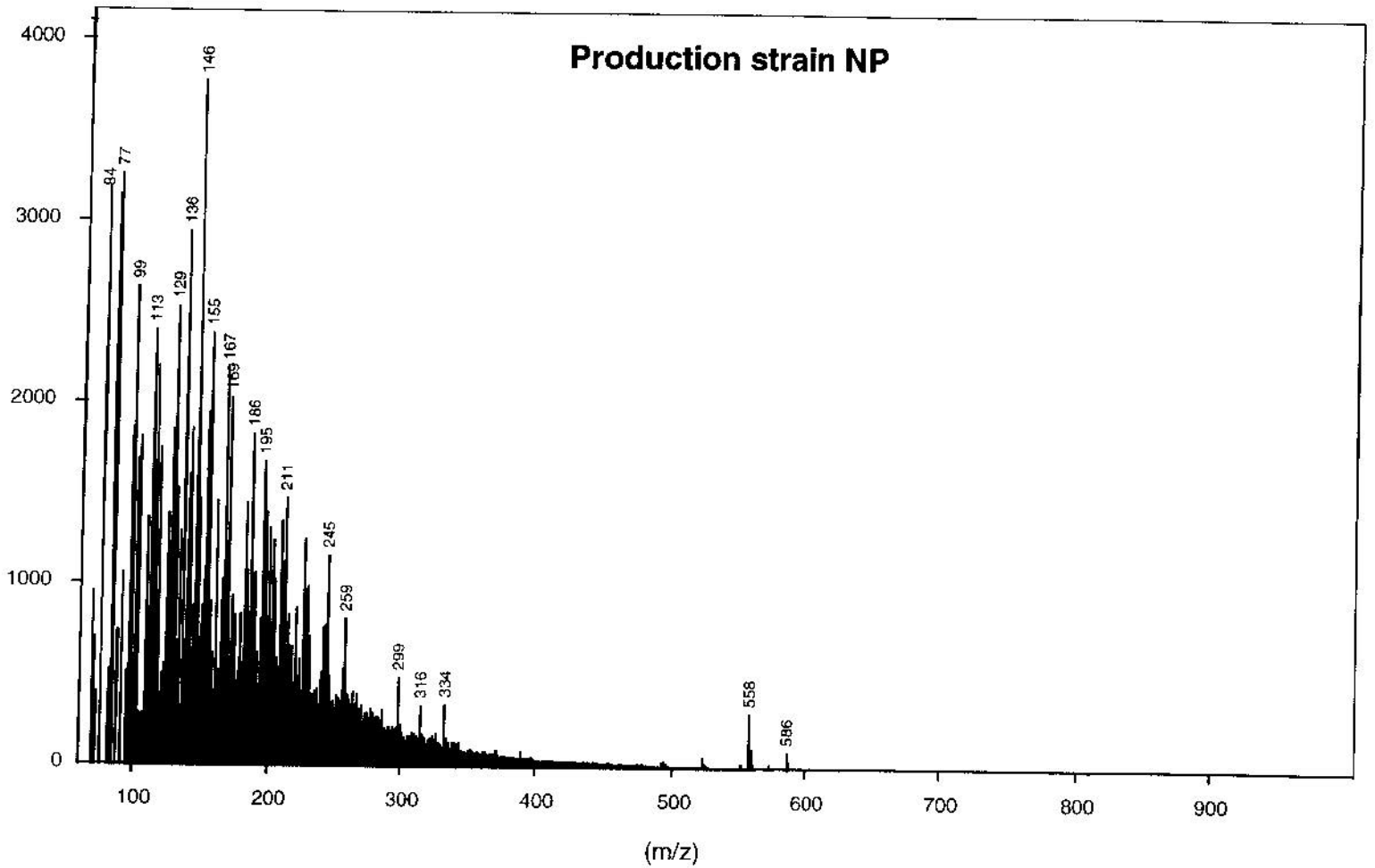


Figure 3: Pyrograms of ATCC 13933 and production strain NP



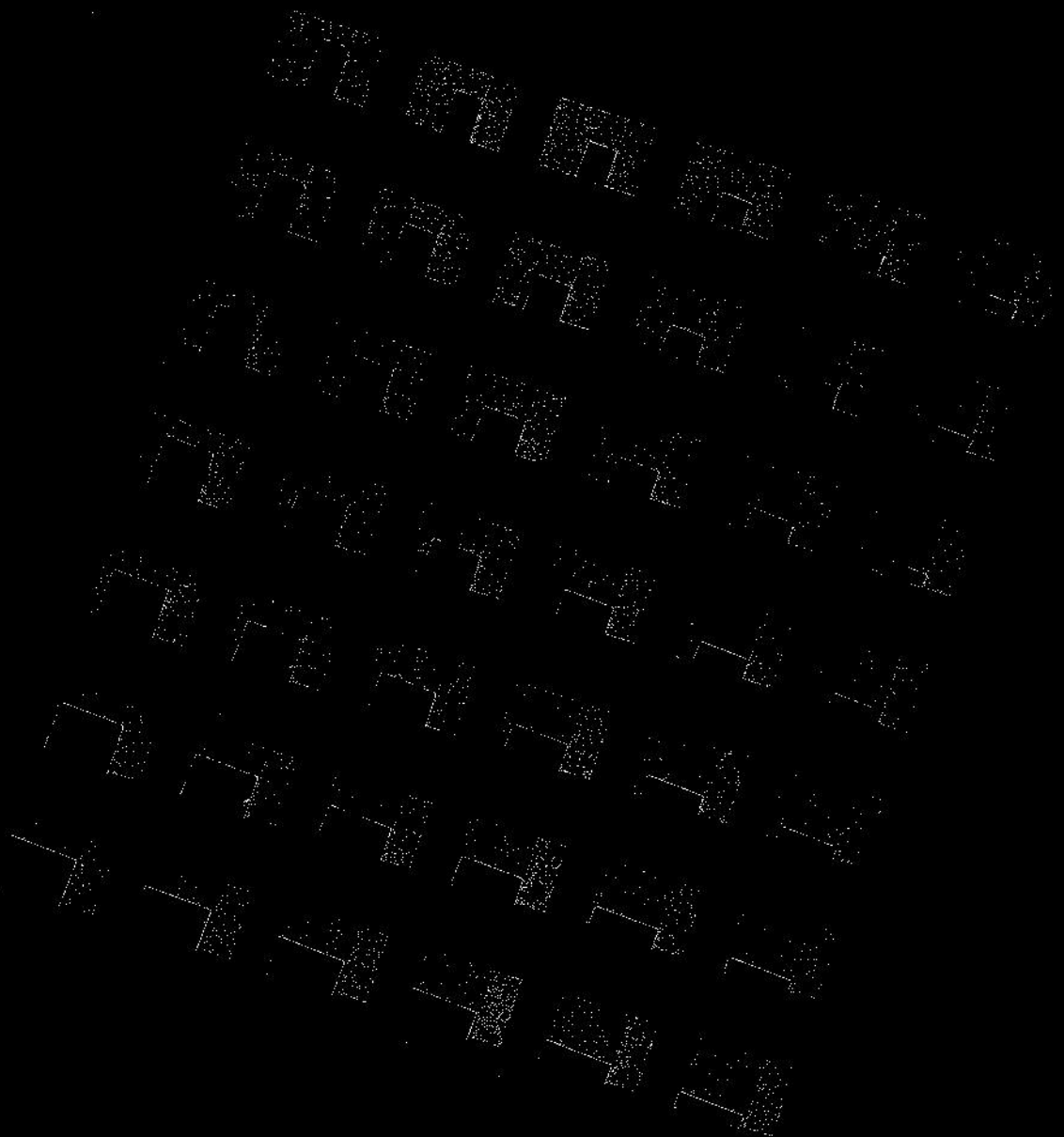

Production strain NP
4000
3000
2000
1000
0
146
84
77
136
99
129
113
155
167
169
186
195
211
245
259
299
316
334
558
586
100
200
300
400
500
600
700
800
900
(m/z)

Appendix 6-2:

TNO report
V99.424

Characterisation of strains ATCC 13933 and production strain GT2

TNO Nutrition and Food Research Institute



TNO report
V99.424

Characterisation of strains ATCC 13933 and production strain GT2

TNO Nutrition and Food Research Institute

Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

Phone +31 30 694 41 44
Fax +31 30 695 72 24

Date
12 April 1999

Author(s)
[redacted]

Project number:
70096/0101

Approved by:
[redacted]

Number of specimen:
1

Number of copies:
7

Number of pages:
15

Number of appendices:
3

Contents

1. Introduction

1.1 General introduction

Microorganisms used for the production of enzymes, that will be used in human food, need to be characterised. The production strain needs to be characterised to enable unique identification which will allow verification of the production process. Both genotypic and phenotypic characterisation need to be carried out to ensure differentiation between strains.

1.2 Scope

One *Bacillus* production strain is characterised using both genotypic and phenotypic methods. The results are compared to culture collection strain *Bacillus subtilis* ATCC strain 13933.

2. Materials and Methods

2.1 Microbiological strains

The test strain is the glutaminase production strain GT2. This strain is provided by Daiwa Kasei K.K. The culture collection *B. subtilis* strain included in the assays is ATCC 13933 (TNO code B0275). Characteristics of the ATCC strain are presented in Appendix 1.

2.2 Methods

Experiments are carried out according to laboratory procedures as described in the protocol 848613, version 1.

2.2.1 Microbiology and biochemical identification

- Strains are subcultured on TSBA and incubated for 3 days at 30°C.
- One colony is transferred to two BM slants and incubated for 3 days at 30°C. Microscopy, gram staining and catalase test are carried out.
- Biochemical characterisation is carried out using the API 50 CHB system. Species are described both on biochemical profile and species identification.
- Colony material is stored at -80°C in two separate freezers.

2.2.2 DNA identification

Cells are grown according to standard protocols. Ribotyping is carried out using the Qualicon® riboprinter. The resulting riboprint patterns are stored in the database or future comparison. GTG5 fingerprinting is carried out according to standard laboratory procedures.

2.2.3 Pyrolysis-Mass spectrometry

Strains are inoculated on PCA medium supplemented with a standard salt solution and incubated at 30°C for 3 days. Pyrolysis-Mass spectrometry is carried out according to standard procedures. The results will be presented as mass spectra.

3. Results and Discussion

3.1 Microbiology and biochemical identification

The results of the microscopic characterisation are presented in Table 1 (Appendix B). The results of the biochemical tests (API 50 CHB) are presented in Tables 2 and 3 (Appendix B). The production strain GT2 and the *B. subtilis* ATCC 13933 strain are not identical, both microscopic and according to biochemical testing.

3.2 DNA Analysis

3.2.1 Ribotyping

Riboprints are presented in figure 1 (Appendix C). The strains are represented by an individual pattern.

3.2.2 GTG5 fingerprinting

GTG5 patterns are presented in figure 2 (Appendix C). Both the strains are represented by an individual GTG5 pattern.

3.3 Pyrolysis-Mass spectrometry

Pyrograms of the production strain GT2 and *B. subtilis* ATCC 13933 are presented in figure 3 (Appendix C). The pyrograms are different for the strains.

4. Concluding remarks

Production strain GT2 could not be satisfactory identified using the biochemical API 50 CHB system. The system suggests that the strain represents the species *Bacillus subtilis* but indicates that the results are not valid.
The production strain GT2 and the *B. subtilis* strain ATCC 13933 could be differentiated based on their microbial, biochemical and DNA characteristics.

5. Signatures

[redacted]

Date 990412

[redacted]

Date 990412

[redacted]

Date 990414

Appendix A Characteristics of *Bacillus subtilis* ATCC 13933

ATCC Number:	13933
Organism:	*Bacillus subtilis* (Ehrenberg) Cohn
Classification:	-
Designations:	NRRL B-1471
Depositors:	NRRL
History:	WRRL PT281
Isolation:	soil, Leicester, England
Descriptions:	Produces aterrimin and feed supplements
Propagation:	ATCC medium: 3 Nutrient agar (Difco 0001) or nutrient broth (Difco 0003)
Temperature:	30°C
Biosafety Level:	1

Appendix B Tables 1, 2 and 3

Table 1: Microscopy

ID number	Med[1]	Tmp[2]	Cat.[3]		Gram		Microscopy									
							With			Length				Motility		
	TSBA	30	+	-	+	-	Slim	Normal	Large	Short	Normal	Long	Very long	Not	Motile	Very motile	Other
ATCC 13933	x	x	x		x			x			x			x			
Production strain GT2	x	x	x		x			x					x	x			

[1] = Growth medium
[2] = Growth temperature
[3] = Catalase test

Table 2: Species identification according to API 50 CHB system

Number	**API 50 CHB identification**		
ATCC 13933	*Bacillus subtilis*	95.2 %	Good identification
Production strain GT2	*B. subtilis*	73.7 %	Identification not valid

Table 3: API 50 CHB identification

ID number	Substrate												
	Temoin/Control	Glycerol	Erythritol	D-Arabinose	L-Arabinose	Ribose	D-Xylose	L-Xylose	Adonitol	β Metyl-xyloside	Galactose	D-Glucose	D-Fructose
	CTR	GLY	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU
	0	1	2	3	4	5	6	7	8	9	10	11	12
ATCC 13933	·	x[#]			x	x	x					x	x
Production strain GT2		x			x	x						x	x

ID number	Substrate												
	D-Mannose	L-Sorbose	Rhamnose	Dulcitol	Inositol	Manitol	Sorbitol	α Methyl-D-mannoside	α Methyl-D-glucoside	N Acetyl glucosamine	Amygdalin	Arbutin	Esculin
	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC
	13	14	15	16	17	18	19	20	21	22	23	24	25
ATCC 13933	x				x	x	x		x		x	x	x
Production strain GT2	x								x	x	x	x	x

ID number	Substrate												
	Salicin	Cellobiose	Maltose	Lactose	Melbiose	Saccarose/sucrose	Trehalose	Inulin	Melezitose	Rafinose	Amidon/Starch	Glycogen	Xylitol
	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMO	GLYG	XLT
	26	27	28	29	30	31	32	33	34	35	36	37	38
ATCC 13933	x	x	x		x	x	x	x		x	x	x	
Production strain GT2	x	x				x	x				x	x	

ID number	Substrate										
	β Gentiobiose	D-Turanose	D-Lyxose	D-Tagatose	D-Fucaose	L-Fucose	D-Arabitol	L-Arabitol	Gluconate	2-Ketogluconate	5-Ketogluconate
	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG
	39	40	41	42	43	44	45	46	47	48	49
ATCC 13933											
Production strain GT2											

· blanc = negative reaction.

[#] x = positive reaction.

Appendix C Figures 1, 2 and 3

Figure 1: Riboprints

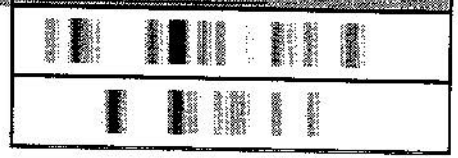
Sample Number	Original ID	RiboGroup	Sim to RiboGroup	RiboPrint Pattern
137-441-1	**ATCC 13933**	RIBO1 137-441-S-1	1.00	
137-441-2	**Production strain GT2**	RIBO1 137-441-S-2	0.99	

Figure 2: GTG5 patterns

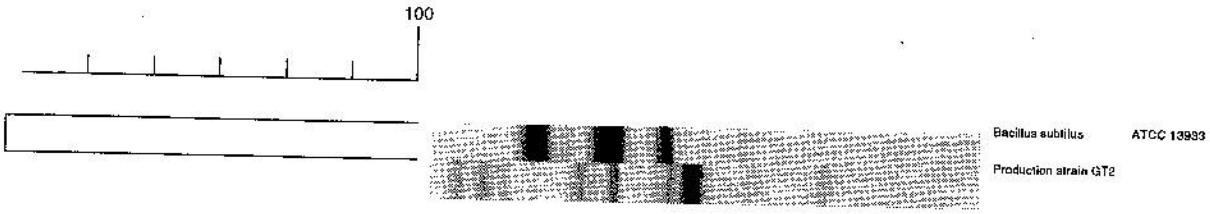
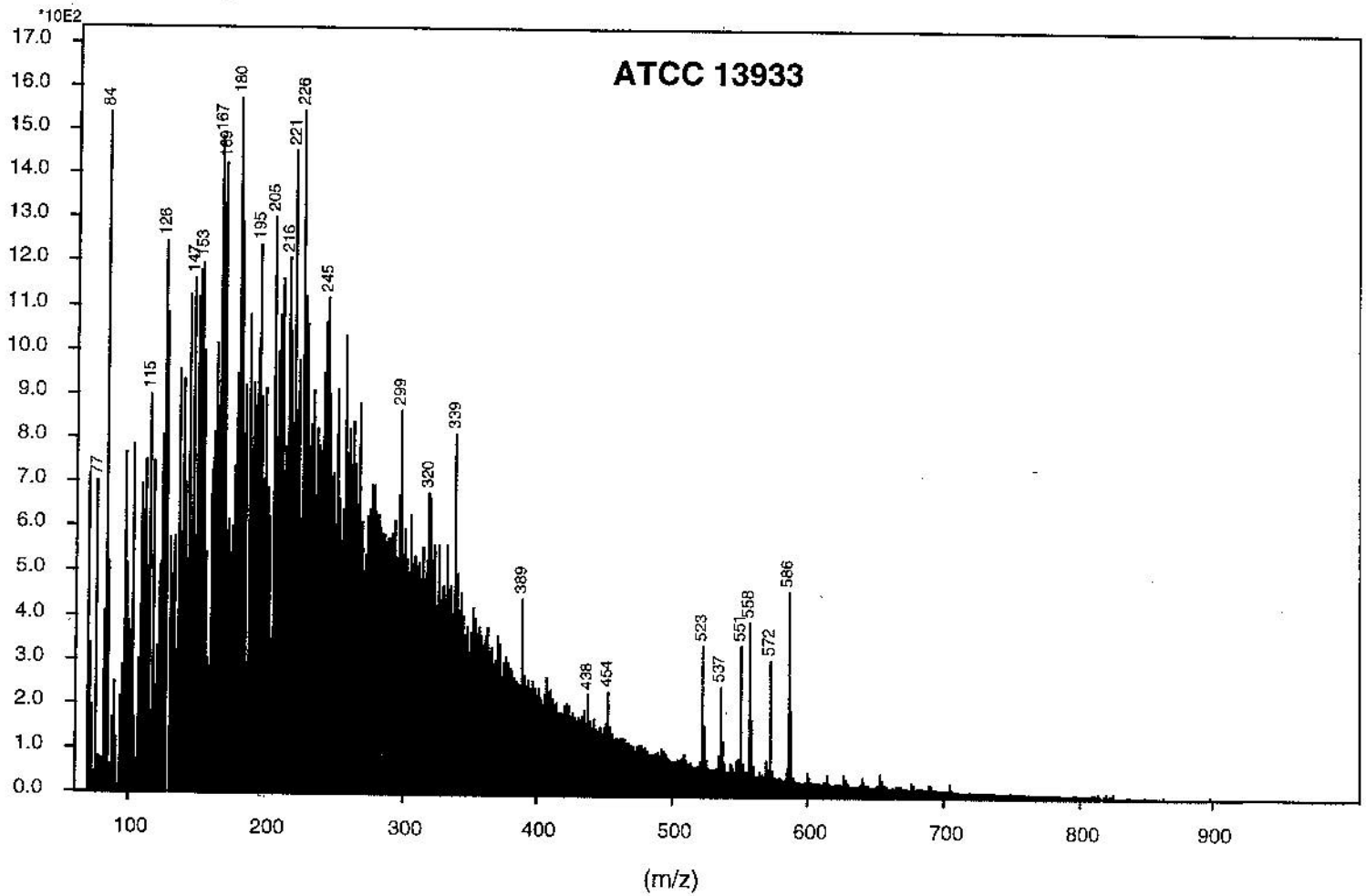
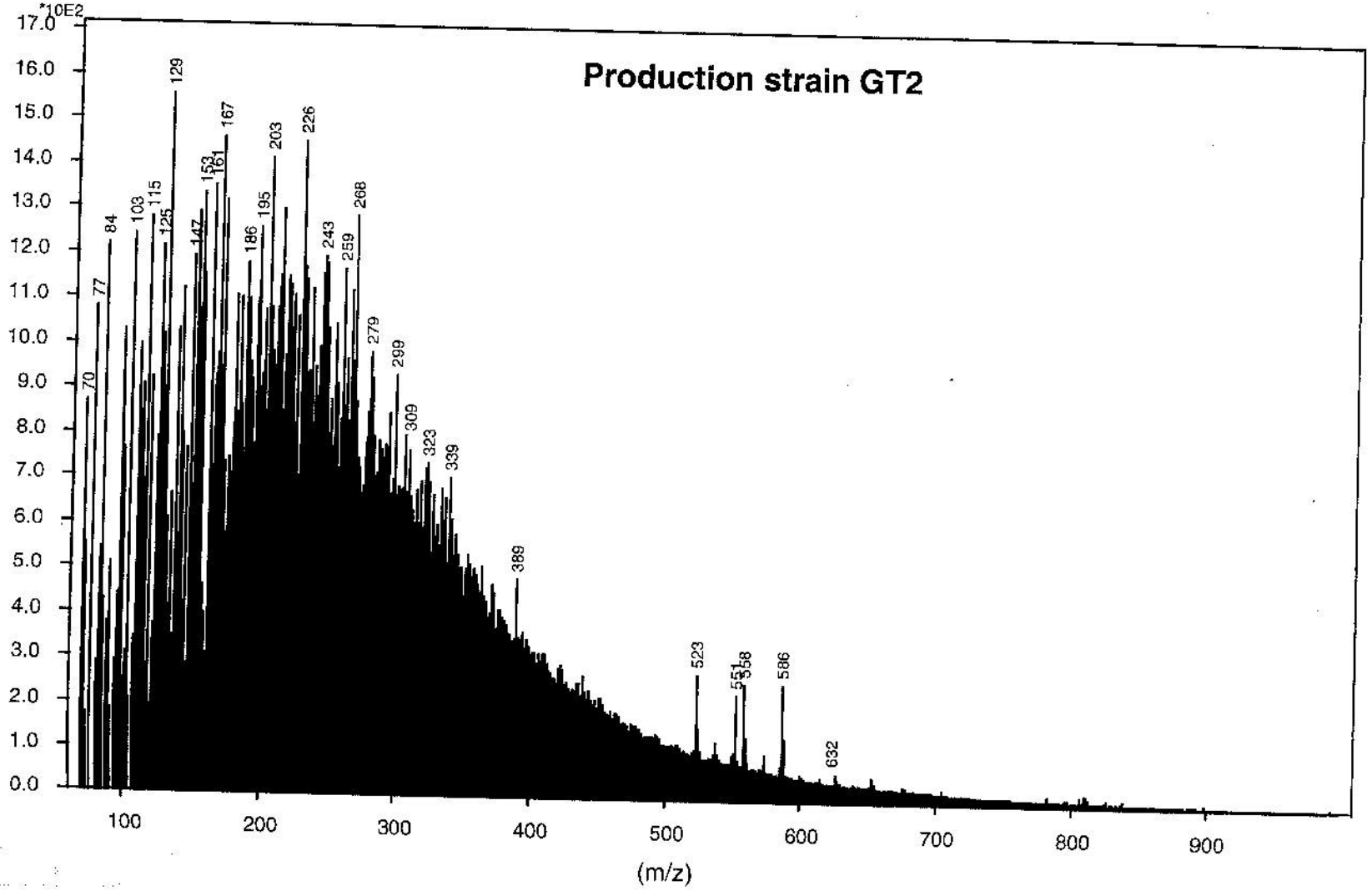


Figure 3: Pyrograms of ATCC 13933 and production strain GT2




Production strain GT2
*10E2
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
70
77
84
103
115
125
129
147
153
161
167
186
195
203
226
243
259
268
279
299
309
323
339
389
523
551
558
586
632
100
200
300
400
500
600
700
800
900
(m/z)

TNO report
V99.425

Characterisation of strains ATCC 13933 and isolated contamination strains

TNO Nutrition and Food Research Institute

Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

Phone +31 30 694 41 44
Fax +31 30 695 72 24

Date
12 April 1999

Author(s)
[redacted]

Project number:
70096/0101

Approved by:
[redacted]

Number of specimen:
1

Number of copies:
7

Number of pages:
15

Number of appendices:
3

Contents

1. Introduction

1.1 General introduction

Contamination flora from a commercial enzyme product, intended for human consumption, needs to be characterised. This will enable verification if the contamination is caused by the production strain or that after processing contamination has occurred. Both genotypic and phenotypic characterisation need to be carried out to ensure differentiation between strains.

1.2 Scope

Four spore forming bacteria, isolated from the enzyme product GT70916L12, are characterised using both genotypic and phenotypic methods. The results are compared to culture collection strain *Bacillus subtilis* ATCC strain 13933.

2. Materials and Methods

2.1 Microbiological strains

The test strains are isolated from the glutaminase enzyme product GT70916L12. This strains were isolated at TNO Nutrition and Food Research Institute as described in TNO report V98.920. The glutaminase enzyme product was provided by Daiwa Kasei K.K. The culture collection *B. subtilis* strain included in the assays is ATCC 13933 (TNO code B0275). Characteristics of the ATCC strain are presented in Appendix 1.

2.2 Methods

Experiments are carried out according to laboratory procedures as described in the protocol 848613, version 1.

2.2.1 Microbiology and biochemical identification

- Strains are subcultured on TSBA and incubated for 3 days at 30°C.
- One colony is transferred to two BM slants and incubated for 3 days at 30°C. Microscopy, gram staining and catalase test are carried out.
- Biochemical characterisation is carried out using the API 50 CHB system. Species are described both on biochemical profile and species identification.
- Colony material is stored at -80°C in two separate freezers.

2.2.2 DNA identification

Cells are grown according to standard protocols. Ribotyping is carried out using the Qualicon® riboprinter. The resulting riboprint patterns are stored in the database or future comparison. GTG5 fingerprinting is carried out according to standard laboratory procedures.

2.2.3 Pyrolysis-Mass spectrometry

Strains are inoculated on PCA medium supplemented with a standard salt solution and incubated at 30°C for 3 days. Pyrolysis-Mass spectrometry is carried out according to standard procedures. The results will be presented as mass spectra.

3. Results and Discussion

3.1 Microbiology and biochemical identification

The results of the microscopic characterisation are presented in Table 1 (Appendix B). The results of the biochemical tests (API 50 CHB) are presented in Tables 2 and 3 (Appendix B). Isolate A was identified as *B. subtilis*. Isolate B was identified as a species of *Brevibacillus*. The isolated strains A and B and the *B. subtilis* ATCC 13933 strain are not identical, both microscopic and according to biochemical testing. Isolate C did not give any sugar fermentation under the circumstances. Isolate D was not viable after storage.

3.2 DNA Analysis

3.2.1 Ribotyping

Riboprints are presented in figure 1 (Appendix C). The strains are represented by an individual pattern.

3.2.2 GTG5 fingerprinting

GTG5 patterns are presented in figure 2 (Appendix C). All the strains are represented by an individual GTG5 pattern.

3.3 Pyrolysis-Mass spectrometry

No pyrograms were obtained for isolates A and B since no growth was observed on the specific medium. Pyrograms of isolate C and *B. subtilis* ATCC 13933 are presented in figure 3 (Appendix C). The pyrograms are different for the strains.

4. Concluding remarks

Isolate A could be satisfactory identified as *Bacillus subtilis,* using the biochemical API 50 CHB system. Isolate B was identified, according to API 50 CHB as a species of the genus *Brevibacillus.* Isolate C could not be identified according to API 50 CHB.
The isolated strains A, B and C and the *B. subtilis* strain ATCC 13933 could be differentiated based on their microbial, biochemical and DNA characteristics.

5. Signatures

[redacted]

Date 990412

[redacted]

Date 990412

[redacted]

Date 99 04 14

Appendix A Characteristics of *Bacillus subtilis* ATCC 13933

ATCC Number:	13933
Organism:	*Bacillus subtilis* (Ehrenberg) Cohn
Classification:	-
Designations:	NRRL B-1471
Depositors:	NRRL
History:	WRRL PT281
Isolation:	soil, Leicester, England
Descriptions:	Produces aterrimin and feed supplements
Propagation:	ATCC medium: 3
	Nutrient agar (Difco 0001) or nutrient broth (Difco 0003)
Temperature:	30°C
Biosafety Level:	1

Appendix B Tables 1, 2 and 3

Table 1: Microscopy

ID number	Med[1]	Tmp[2]	Cat.[3]		Gram		Microscopy									
							With			Length				Motility		
	TSBA	30	+	-	+	-	Slim	Normal	Large	Short	Normal	Long	Very long	Not	Motile	Very motile	Other
ATCC 13933	x	x	x		x			x			x			x			
Isolate A	x	x	x		x			x				x		x			
Isolate B	x	x	x		x			x			x					x	
Isolate C	x	x	x		x			x			x				x		

[1] = Growth medium
[2] = Growth temperature
[3] = Catalase test

Table 2: Species identification according to API 50 CHB system

Number	API 50 CHB identification		
	1st identification		
ATCC 13933	*Bacillus subtilis*	95.2 %	Good identification
Isolate A	*B. subtilis*	82.5%	Acceptable identification
Isolate B	*Brevibacillus laterosporus*	96.5%	Good identification
Isolate C	No reactions		

Table 3: API 50 CHB identification

ID number	Temoin/Control	Glycerol	Erythritol	D-Arabinose	L-Arabinose	Ribose	D-Xylose	L-Xylose	Adonitol	β Metyl-xyloside
	CTR	GLY	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX
	0	1	2	3	4	5	6	7	8	9
ATCC 13933	•	x#			x	x	x			
Isolate A		x			x	x				
Isolate B										
Isolate C	No reactions at the given conditions									

ID number	Galactose	D-Glucose	D-Fructose	D-Mannose	L-Sorbose	Rhamnose	Dulcitol	Inositol	Manitol	Sorbitol
	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR
	10	11	12	13	14	15	16	17	18	19
ATCC 13933		x	x	x				x	x	x
Isolate A		x	x	x				x	x	x
Isolate B		x	x						x	
Isolate C										

ID number	α Methyl-D-mannoside	α Methyl-D-glucoside	N Acetyl glucosamine	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Lactose
	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC
	20	21	22	23	24	25	26	27	28	29
ATCC 13933		x		x	x	x	x	x	x	
Isolate A		x	x			x			x	
Isolate B			x		x	x	x	x	x	
Isolate C										

ID number	Melbiose	Saccarose/sucrose	Trehalose	Inulin	Melezitose	Rafinose	Amidon/Starch	Glycogen	Xylitol	β Gentiobiose
	MEL	SAC	TRE	INU	MLZ	RAF	AMO	GLYG	XLT	GEN
	30	31	32	33	34	35	36	37	38	39
ATCC 13933	x	x	x	x		x	x	x		
Isolate A		x	x	x			x			
Isolate B			x			x	x			x
Isolate C										

ID number	D-Turanose	D-Lyxose	D-Tagatose	D-Fucaose	L-Fucose	D-Arabitol	L-Arabitol	Gluconate	2-Ketogluconate	5-Ketogluconate
	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG
	40	41	42	43	44	45	46	47	48	49
ATCC 13933										
Isolate A										
Isolate B										
Isolate C										

• blanc = negative reaction.

\# x = positive reaction.

Appendix C Figures 1, 2 and 3

Figure 1: Riboprints

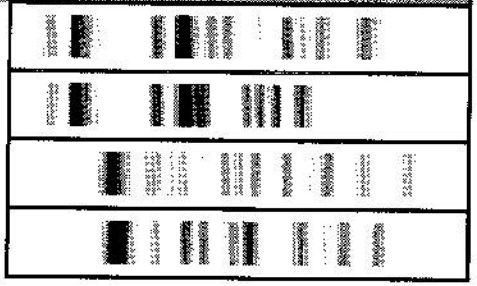
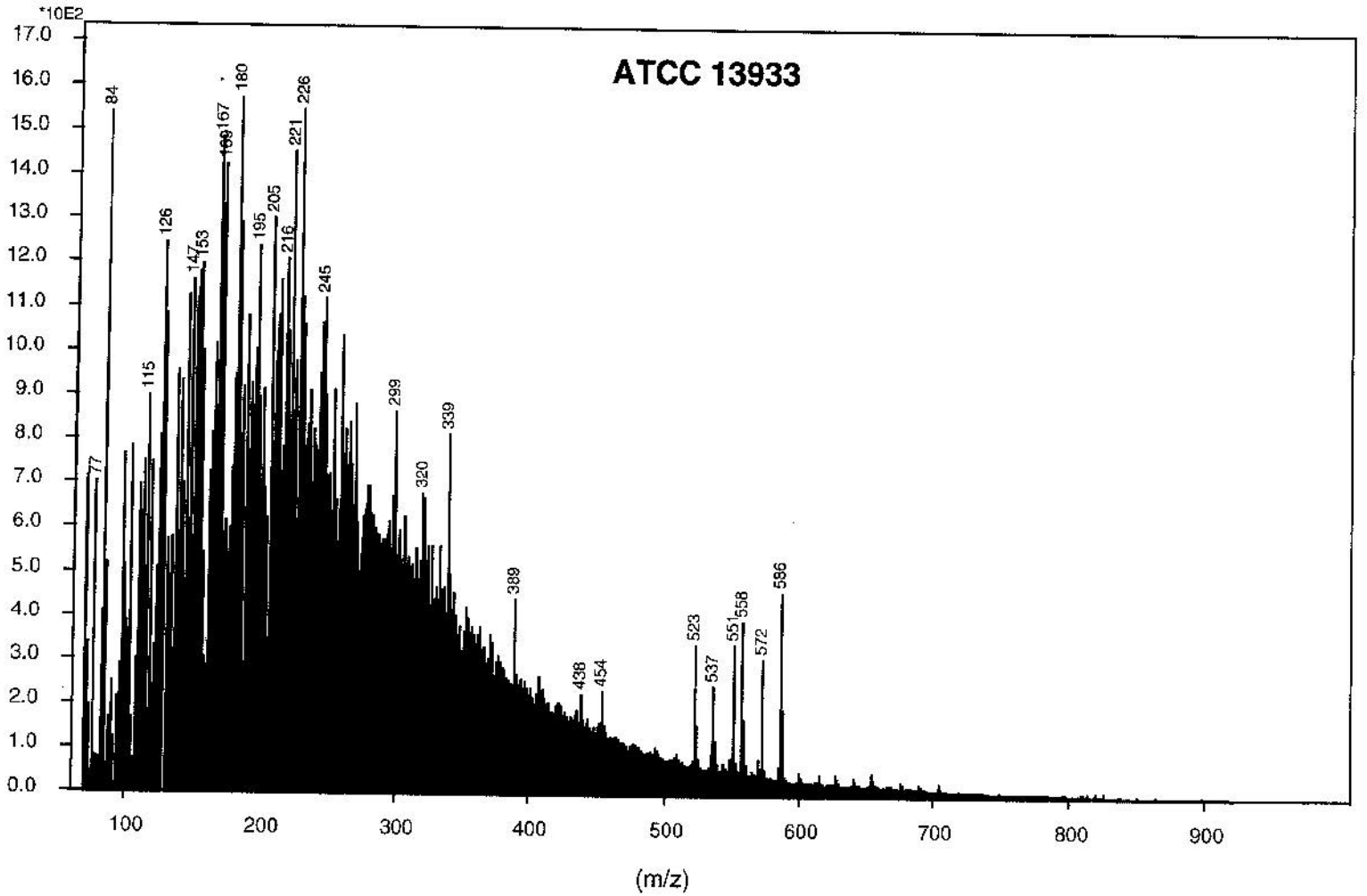
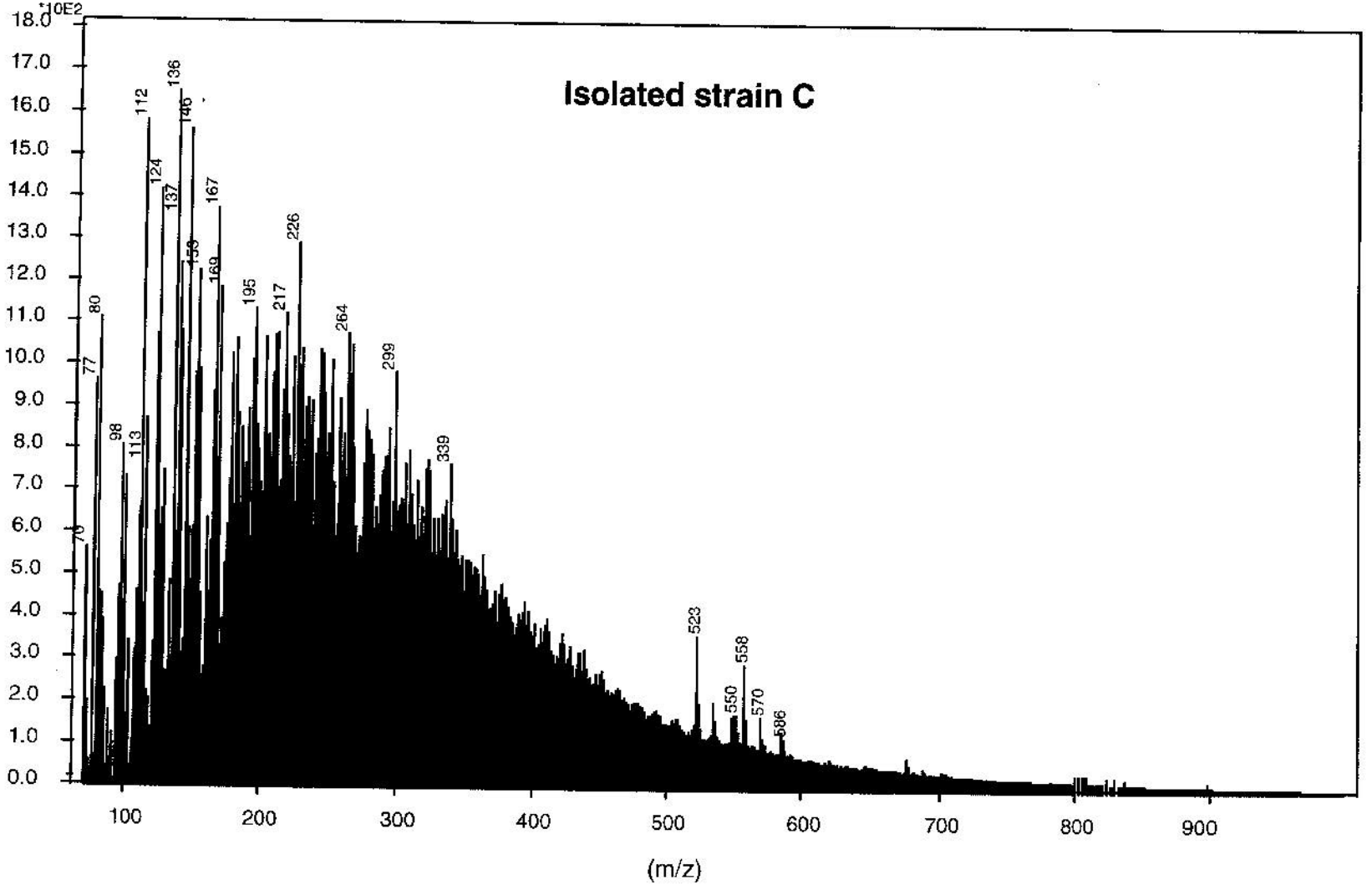
Sample Number	Original ID	RiboGroup	Sim to RiboGroup	RiboPrint Pattern
137-441-1	**ATCC 13933**	RIBO1 137-441-S-1	1.00	
137-441-4	**Isolate A**	RIBO1 137-441-S-4	1.00	
137-441-5	**Isolate B**	RIBO1 137-441-S-5	1.00	
137-441-6	**Isolate C**	RIBO1 137-441-S-6	1.00	

Figure 2: GTG5 patterns

Figure 3: *Pyrograms of ATCC 13933 and isolate C*




Isolated strain C
*10E2
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
70
77
80
98
113
112
124
136
137
146
155
167
169
195
217
226
264
299
339
523
550
558
570
586
100
200
300
400
500
600
700
800
900
(m/z)

PART 15

ENDOSPORE-FORMING RODS AND COCCI

FAMILY I. BACILLACEAE FISCHER 1895, 139

Ba.cil.la'ce.ae. M.L. n. *Bacillus* type genus of the family; *-aceae* ending to denote family; M.L. fem.pl.n. *Bacillaceae* the *Bacillus* family.

Cells rod-shaped, in one genus spherical. Mycelium not produced. Endospores formed. Endospores differ from vegetative cells in being more refractive and less susceptible to staining, in having greater resistance to heat and other destructive agents, and in containing dipicolinic acid (5–15% of dry weight). The spore contains a central cell (the core) which is enclosed by a cortex of peptidoglycan and an outer spore coat.

Majority Gram-positive. Motile by lateral or peritrichous flagella, or non-motile.

Aerobic, facultative or anaerobic.

Key to the genera of family **Bacillaceae**

I. Cells rod-shaped
  A. Aerobic or facultative, catalase usually produced.
    Genus I. *Bacillus*
  B. Microaerophilic, catalase not produced.
    Genus II. *Sporolactobacillus*, p. 550
  C. Anaerobic.
    1. Sulfate not reduced to sulfide.
      Genus III. *Clostridium*, p. 551
    2. Sulfate reduced to sulfide.
      Genus IV. *Desulfotomaculum*, p. 572

II. Cells spherical, in packets.
  Genus V. *Sporosarcina*, p. 573

Genus I. **Bacillus** *Cohn 1872, 174; nom.gen.cons.* Nomencl. Comm. Intern. Soc. Microbiol. 1937, 28; Opin. A. Jud. Comm. 1955, 39

T. Gibson† and Ruth E. Gordon

Ba.cil'lus. L. dim. n. *bacillus* a small rod; M.L. n. *Bacillus* a rodlet.

Cells rod-shaped, straight or nearly so, 0.3–2.2 by 1.2–7.0 μm. **Majority motile;** flagella typically lateral. **Heat-resistant endospores formed; not more than one in a sporangial cell.** Sporulation not repressed by exposure to air. Gram reaction: positive, or positive only in early stages of growth, or negative.

Chemoorganotrophs; metabolism strictly respiratory, strictly fermentative or both respiratory and fermentative, using various substrates. The terminal electron acceptor in respiratory metabolism is molecular oxygen, replaceable in some species by nitrate. Catalase formed by most species.

Strict aerobes or facultative anaerobes.

The G + C content of the DNA of those strains

† Deceased.

abundantly on nutrient agar and those that have attracted attention because of their pathogenicity or industrial importance. Other species have been examined less intensively. There are indications of further distinguishable groups which have not yet been defined and named.

Since endospore formation is the dominant feature in the characterization of *Bacillus*, it is essential that there is a boundary separating this genus from other genera in which endospores are produced. The genus *Clostridium* (see p. 551) is distinguished from *Bacillus* by inability to grow on the surface of agar media in air or, if growth does occur under these conditions, it is slight and does not lead to sporulation. There is also little or no catalase activity. *Sporosarcina* (see p. 573) is sharply separated from *Bacillus* by the coccal form of its vegetative cells. *Sporolactobacillus* (see p. 550) has the physiological properties of the genus *Lactobacillus* including the inability to form catalase.

Endospore-forming Bacteria of Uncertain Taxonomic Position

Various exceptionally large organisms which produce endospores occur in the alimentary tract of animals. They have been described solely by microscopical features; their response to oxygen and the physiology and the finer structure of their spores are still unknown. These large organisms have been placed in the following genera.

Genus *Bacillus*. Collin (1913), Hollande (1934) and Delaporte (1964) have described and named 15 species in the intestine of tadpoles. The spore in most of the species is larger and much more elongate than it is in other species of *Bacillus*. In seven of the species two spores may be formed in a single nonseptate cell, and Delaporte (1963) has shown that in *Bacillus camptospora* Collin 1913, 60 and *Bacillus enterothrix* Collin 1913, 60 one of the two spores may move rapidly through much of the interior of the long vegetative cell. The microscopical evidence thus suggests that the spores of these organisms are markedly different from typical endospores.

The genus *Fusosporus* Delaporte 1964, 857, in which two spores occur per cell, was separated from *Bacillus* because the vegetative cell tapers to pointed ends.

The genera *Arthromitus* Leidy 1850, 227, *Anisomitus* Grassé 1925, 343, *Entomitus* Grassé 1924, 30 and *Coleomitus* Duboscq and Grassé 1930, 28 comprise organisms which grow as septate filaments. In the first two genera the filaments occur attached by the basal end to a solid surface. The endospore in most of these organisms is morphologically not unlike the spore of cultivable species of *Bacillus*. In the 7th edition of THE MANUAL *Arthromitus* and *Coleomitus* were placed in the order *Caryophanales*.

The genera *Bacillospira* Hollande 1933, 1830 and *Sporospirillum* Delaporte 1964, 257, identified in batrachian gut contents, are distinguished from *Bacillus* by a rigid spiral form. Most produce two endospores in the vegetative cell. *Bacillospira* possesses an axial filament and was regarded as a spirochete by Hollande (1933, 1934).

Two specially large spore formers which occur in the alimentary tract of mammals appear to differ significantly from *Bacillus* in morphology. They are *Oscillospira* (see p. 574) and *Metabacterium* Chatton and Pérard 1913, 1234 which forms up to eight spores in a nonseptate cell 5 by 10–25 μm in size.

Interrelationships among these large intestinal organisms remain to be elucidated, as do their relationships to other genera and problems concerning the properties of their endospores.

Description of the species of genus **Bacillus**

The species in the following account are divided arbitrarily into two groups.

Group I comprises 22 species which are widely accepted as distinct entities. Their precise outlines may not be entirely clear, but at least some information is available on the internal variation in each and also on organisms which are distinguishable yet not markedly different.

Group II, which begins on page 545, contains 26 species which so far have received less widespread recognition.

Characteristics for primary differentiation of species in Group I are presented in Table 15.1. This table serves as a key and may be used as a lead to the appropriate description in the text or in Tables 15.2–15.7.

Group I

(1) **Bacillus subtilis** (Ehrenberg) Cohn 1872, 174; *Nom. cons.* Nomencl. Comm. Intern. Soc. Microbiol. 1937, 28; Opin. A. Jud. Comm. 1955, 39. (*Vibrio subtilis* Ehrenberg 1835, 279.)

sub'ti.lis. L. adj. *subtilis* slender.

See Tables 15.1 and 15.2.

Rods seldom in chains; stain uniformly. Flagella lateral. Endospores 0.8 by 1.5–1.8 μm; surface of free spore stains faintly. On germination, the spore coat breaks equatorially. After emergence of vegetative cell the lysis of spore coat is distinctly slow.

Colonies on agar media round or irregular; surface dull; become thick and opaque; may be

TABLE 15.1

Differential characteristics of the species of genus **Bacillus** *(Group I)*

The symbols used are: E, elliptical or cylindrical; S, spherical or nearly so; C, central; T, terminal or subterminal; CT, central to terminal, variation within or between strains; W, weakly positive.

	Spore			Products of action on glucose		
	Shape	Distends sporangium distinctly	Dominant position	Acid[a]	Gas[b]	Acetoin[c]
1. *B. subtilis*	E	–	C	+	–	+
2. *B. pumilus*	E	–	C	+	–	+
3. *B. licheniformis*	E	–	C	+	W or –	+
4. *B. cereus*	E	–	C	+	–	+
5. *B. anthracis*	E	–	C	+	–	+
6. *B. thuringiensis*	E	–	C	+	–	+
7. *B. megaterium*	E	–	C	+	–	–
8. *B. polymyxa*	E	+	CT	+	+	+
9. *B. macerans*	E	+	T	+	+	–
10. *B. circulans*	E	+	CT	+	–	–
11. *B. stearothermophilus*	E	+ or –	T	+	–	–
12. *B. coagulans*	E	+ or –	CT	+	–	+ or –
13. *B. alvei*	E	+	CT	+	–	+
14. *B. firmus*	E	–	C	W	–	–
15. *B. laterosporus*	E	+	C	+	–	–
16. *B. brevis*	E	+	CT	+ or –	–	–
17. *B. sphaericus*	S	+	T	–	–	–
18. *B. pasteurii*[d]	S	+	T	–	–	–
19. *B. fastidiosus*[d]	E	–	C	–	–	–
20. *B. larvae*[d]	E	+	CT	+	–	–
21. *B. popilliae*[d]	E	+	C	+	–	–
22. *B. lentimorbus*[d]	E	+	C	+	–	–

[a] Medium 1 (grams per liter): $(NH_4)_2HPO_4$, 1.0; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract, 0.2; agar, 15; bromcresol purple, 0.008; glucose (added after sterilization), 5; used as slants.

[b] Medium 2 (grams per liter): peptone, 5; yeast extract, 3; NaCl, 5; agar, 3; bromcresol purple, 0.008; glucose (added after sterilization), 10; pH 7.0; used for stab cultures; may show acid formation if growth fails on medium 1.

[c] Medium 3 (grams per liter): proteose peptone, 7; glucose, 5; NaCl, 5.

[d] Little or no growth in medium 1, 2 or 3; in media that support growth the results are as shown.

wrinkled and may become cream-colored or brown. Features of colonies vary greatly with composition of the medium. Active spreading occurs on agar with a moist surface. Cell material grown on agar does not disperse readily in liquids.

In 1% glucose nutrient agar stab surface growth becomes thick, often rugose and brown. A disc of reddish pigment may form below the growth. Deep growth starts but soon comes to a standstill. Weak acid formation (to bromcresol purple) occurs to the bottom and neutralization proceeds from the surface.

In broth dull, wrinkled, coherent pellicle; little or no turbidity.

Energy-yielding metabolism is predominantly respiratory, oxygen being the terminal electron acceptor. Anaerobically, in complex media containing glucose, growth and fermentation are weak; admission of oxygen permits abundant growth with the formation of 2,3-butanediol, acetoin and CO_2 as major products.

Pectin and polysaccharides of plant tissues are decomposed, and some strains produce a rot in live potato tubers.

Levan is formed extracellularly from sucrose and raffinose, the yield varying with the strain. The chief endocellular storage product is a glycogen-like carbohydrate.

Pigments, which in particular cases have been identified as pulcherrimin or melanins, may be produced in colonies or the adjacent medium. In many strains they are brown or red, in fewer orange or black. Occurrence of each pigment is dependent on composition of medium.

Nutrient gelatin (22 C) liquefied to 1 cm or more within 7 days.

TABLE 15.2

Differential characteristics of **Bacillus** *species 1–3*

Common characteristics: Gram-positive; motile; catalase positive; intracellular globules unstainable by fuchsin in cells grown on glucose agar not observed; acid produced from arabinose, xylose and mannitol in Medium 1 (Table 15.1) with glucose replaced; egg yolk reaction negative; no growth in 0.02% azide by strains growing at 55 C; growth in 7% NaCl and in Sabouraud dextrose broth and/or agar; variable growth in 0.001% lysozyme; alkali on citrate-salts agar; casein hydrolyzed; tyrosine not decomposed. Data are from Gordon *et al.* (1973).

	1. *B. subtilis*	2. *B. pumilus*	3. *B. licheniformis*
Rods			
Width, μm	0.7–0.8	0.6–0.7	0.6–0.8
Length, μm	2–3	2–3	1.5–3
Hydrolysis of			
Starch	+[a]	−	+
Hippurate	−	+	−
NO_3^- to NO_2^-	+	−	+
Growth in anaerobic agar	−	−	+
Temperature for growth, C			
Maximum	45–55	45–50	50–55
Minimum[b]	5–20	5–15	15

[a] + = positive for 90–100% of strains; − = negative for 90–100% of strains.

[b] Lowest temperature tested 3 C.

Litmus milk reduced; rapid digestion of casein without much clotting.

Arginine dihydrolase absent. Hemolysis variable. Lecithinase not produced.

Polypeptide antibiotics are produced; a single strain may form several. Enzymes are liberated which have a lytic action on live bacterial cells.

A minimal medium for vegetative growth has no vitamins and contains glucose, citrate and an ammonium salt as the sole sources of carbon and nitrogen.

Aerobic, excepting that glucose and, less effectively, nitrate permit a much restricted anaerobic growth in complex media. Growth active at pH 5.5–8.5; pH limits not recorded.

Endospores widespread; occur in many heat-treated materials. Vegetative growth, with participation in early stages of breakdown, in various materials of plant and animal origin. Growth in non-acid foods if oxygen available. Causative agent of slimy bread.

The G + C content of the DNA has been most frequently found to be 42–43 moles % (by analysis, T_m and buoyant density).

Neotype strain: Marburg strain; ATCC 6051; NCIB 3610; NCTC 3610.

Comments: The foregoing description applies to the neotype strain of *B. subtilis* and to other strains which appear to be essentially similar. Among freshly isolated organisms, derived more especially from severely heated materials (surgical dressings, canned foods, etc.), some may have properties that link them to *B. subtilis* yet they diverge from the typical form of that species in several of the following directions: cells larger (about 1 μm thick); surface of spore more stainable; diminished capacity to sporulate; colonies smooth, glossy, lacking pigments and easily dispersed in liquids; in some, colonies thin and translucent; turbidity without a pellicle in broth; little acetoin formed; no action on nitrate; more complex nutrient requirements. In laboratory work mutants showing most of these features have been obtained from typical strains of *B. subtilis*. This evidence, together with a gradation in the properties of fresh isolates, has led to the inclusion of the aberrant organisms in that species.

Subspecies most frequently recognized are:

a. *Bacillus subtilis* var. *aterrimus* Smith, Gordon and Clark 1946, 64 (*Bacillus aterrimus* Lehmann and Neumann 1896, 303), which produces a black pigment exclusively on carbohydrate media.

b. *Bacillus subtilis* var. *niger* Smith, Gordon and Clark 1946, 66 (*Bacillus niger* Migula 1900, 636), which forms a black pigment on media containing tyrosine. In neither is pigment formation fully stable.

Probable synonyms: Among the species of *Bacillus* that appear to lack a type culture, several as judged by description are indistinguishable from *B. subtilis*. Notable are *Bacillus mesentericus* Trevisan 1889, 19, *Bacillus vulgatus* Trevisan 1889, 19 and *Bacillus panis* Migula 1900, 576. Strains of *B. subtilis* that form a red pigment have sometimes been identified (possibly misidentified) as *Bacillus globigii* Migula 1900, 554. Original cultures of *Bacillus natto* Sawamura 1906, 109 were found to be identical with *B. subtilis* by Smith *et al.* (1946).

2. **Bacillus pumilus** Meyer and Gottheil in Gottheil 1901, 681.

pu'mi.lus. L. adj. *pumilus* little.

See Tables 15.1 and 15.2.

Differentiation from *B. subtilis* is the main problem in identification. Apart from the differences shown in Table 15.2, there are two significant distinguishing properties of *B. pumilus:* (1) the colony of most strains on nutrient agar is smooth and becomes slightly yellowish, and (2) there is a requirement for biotin with, in some strains, a

With a few exceptions, strains will sporulate readily when injected into the hemolymph or fed to susceptible insects (Pridham *et al.*, 1964). In laboratory media, only selected strains have been induced to sporulate (Haynes and Rhodes, 1966; Sharpe *et al.*, 1970). Spores formed *in vitro* caused milkiness in larvae infected by injection but not by feeding (Lüthy, 1968; Schwartz and Sharpe, 1970).

The parasporal body that distinguishes strains of *B. popilliae* from strains of *B. lentimorbus* was described by different authors as hemispherical or subconical, triangular, rhombohedral or indefinite in shape.

Krieg (1961, 244) recognized two subspecies of this species: (1) *Bacillus popilliae* subsp. *new zealand*, described by Dumbleton (1945) as the cause of a milky disease in *Odontria zealandica* White, and (2) *Bacillus popilliae* subsp. *fribourgensis*, first characterized as the cause of milky disease of *Melolontha melolontha* Linnaeus and named *Bacillus fribourgensis* by Wille 1956, 274.

22. **Bacillus lentimorbus** Dutky 1940, 57.

len.ti.mor'bus. L. adj. *lentus* slow; L. n. *morbus* disease; M.L. n. *lentimorbus* the slow disease.

See Tables 15.1 and 15.7.

Description based largely on Gordon *et al.*, 1973.

This species, which is more fastidious nutritionally and less widespread than *B. popilliae*, also infects the larvae of the Japanese beetle (*Popillia japonica* Newman) and the European chafer (*Amphimallon majalis* Razoumowsky).

Pure cultures can be isolated most readily from dry films of hemolymph of infected larvae. They can be maintained indefinitely by serial transfer in diphasic J-medium (Haynes and Rhodes, 1963). Spores are produced by injection of vegetative cells or spores into susceptible grubs (Haynes *et al.*, 1961). Sporulation by cultures *in vitro*, however, has been reported only by Steinkraus and Tashiro (1955); other methods producing sporulation *in vitro* by *B. popilliae* have been unsuccessful with *B. lentimorbus* (Rhodes *et al.*, 1965; Haynes and Rhodes, 1966; Sharpe *et al.*, 1970). Strains of *B. lentimorbus* have been separated from strains of *B. popilliae* by cross-agglutination reactions (Hrubant and Rhodes, 1968) and by the surface topography of their spores (Bulla *et al.*, 1969).

Beard (1956, 641) isolated strains in Australia that differed from strains of *B. lentimorbus* in having a somewhat different host range and being less infective for the Japanese beetle. He designated his strains as *Bacillus lentimorbus* subsp. *australis*.

Group II

The 26 species in this group are placed together because it might be considered that they need further investigation to show how they differ from other species or from closely similar but unnamed organisms. Several have the severe limitation that the original description was based on very few strains, while not more than a single culture may now be available for study.

Some differential properties of the species in Group II are given in Table 15.8.

Editorial Note: The status of the species in this section has been the subject of considerable correspondence between the authors and editors. In many genera, most, if not all of the species in this group would have been listed as *species incertae sedis*, and one author agrees. It is evident that more work is needed to establish their status as distinct species. *B. acidocaldarius* seems quite distinct and is placed at the end because its description appeared after the manuscript and tables had been written.

23. **Bacillus amyloliquefaciens** Fukumoto 1943, 488. (*Bacillus amyloliquifaciens* (*sic*) Fukumoto 1943, 488.)

am.yl.o.li.que.fac'i.ens. L. n. *amylum* starch; M.L. part.adj. *amyloliquefaciens* starch-digesting.

This species has been separated from *B. subtilis* by Welker and Campbell (1967) on the grounds that the G + C content of the DNA is 43.5–44.9 moles % (by analysis, T_m and buoyant density), DNA-DNA hybridization shows only about 15% homology and the α-amylase has different properties and is produced in larger amounts. Other differences were hitherto accepted among the variants of *B. subtilis*.

Neotype strain: ATCC 23350.

24. **Bacillus medusa** Delaporte 1969, 1131.

me.du'sa. L. n. *medusa* Gorgon with serpent hair.

Distinguished from *B. cereus* by forming a large spherical to elliptical parasporal body which consists mainly of protein and is refractile and deeply stainable.

Source: Cow dung.

Type strain not designated. Original strain: NCIB 10437.

Comment: It is not clear how this species differs from *Bacillus finitimus* Heimpel and Angus 1958, 539, more especially from the non-motile *Bacillus finitimus* var. *fowleri* Heimpel 1967, 292. See page 536.

25. **Bacillus maroccanus** Delaporte and Sasson 1967, 2346.

mar.oc.can'us. M.L. adj. *maroccanus* of Morocco.

Appendix 6-5:

TNO Nutrition and Food Research

Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands
Phone +31 30 694 41 44
Fax +31 30 695 72 24

Direct dialling [redacted]

Direct fax [redacted]

[redacted]
Kaiwa Kasei K.K.
4-19, Hie-cho, Kosei-cho, Koka-gun
Shigaken
520-3203 Japan

Date
14 November 2000

Our number
DAM 00-7635/VOJ

Your letter

Subject
DNA:DNA hybridisation

[redacted]

Please find enclosed the results of the DNA:DNA hybridisation performed with the strains belonging to the *Bacillus subtilis* group: TTC 00.0387 (your strain GT2), *Bacillus amyloliquefaciens* LMG 9814T, *Bacillus atrophaeus* LMG 16797T, *Bacillus majavensis* LMG17797T, *Bacillus subtilis* LMG 7135T, and *Bacillus vallismortis* LMG 18725T

From the study it can be concluded that strain TTC 00.0387 (your strain GT2) belongs to the species ***Bacillus amyloliquefaciens***. This is one of the species belonging to the *Bacillus subtilis* group.

Please feel free to contact me in case you have questions related to this project.

[redacted]
Product Manager Biomonitoring and Intestinal Ecology

Enclosure: 1

Our number
DAM 00-7635/VOJ

Page

Date

Analysis report

Methods

Genomic DNA was prepared from all strains and used for DNA:DNA hybridizations. Hybridisations were performed at 37°C according to a modification of the method described by Ezaki et al. (Ezaki, T., Y. Hashimoto, and E. Yabuuchi. 1989. Fluorimetric deoxyribonucleic acid-deoxyribonucleic acid hybridisation in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39:224-229).

Results

Stains	Homology
TTC 00.0387 (GT2)	100%
B. amyloliquefaciens LMG 9814T	95%
B. atrophaeus LMG 16797T	39%
B. mojavensis LMG 17797T	32%
B. subtilis LMG 7135T	27%
B. vallismortis LMG 18725T	25%

Strain TTC 00.0387 (GT2) shows 95% DNA homology with LMG9814, the type strain of *Bacillus amyloliquefaciens*. This is significant at the specieslevel (DNA homology is higher than 70%).
No species-significant DNA homology was observed between TTC 00.0387 (GT2) and any of the four other type strains currently constituting the *Bacillus subtilis* group.

Conclusion:

Identification of strain TTC 00.0387 (=GT2): ***Bacillus amyloliquefaciens***

Certificate of No Genetic Modification

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203, JAPAN

PHONE : +81-748-75-1194
F A X : +81-748-75-0312

June 15, 2007

CERTIFICATE OF GLUTAMINASE PRODUCTION STRAIN

We, hereby, certify that glutaminase production strain "GT2" was derived from the "NP" strain by the conventional mutation using N-methyl-N'-nitro-N-nitrosoguanidine.

[redacted]

General Manager, Quality Assurance

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203, JAPAN
PHONE : +81-748-75-1194
F A X : +81-748-75-0312

December 23, 2005

NO GENETIC MODIFICATION CERTIFICATE OF GLUTAMINASE PRODUCTION STRAIN

We, hereby, certify that no genetic modification strain is used in manufacturing the following product.

Product: GLUTAMINASE DAIWA C100S, GLUTAMINASE DAIWA C300S
Strain: *Bacillus subtilis*

[redacted]

General Manager, Quality Assurance
DAIWA KASEI K.K.

Petition to Amend Standard 1.3.3 of the Australia New Zealand Food Standards Code to Include Glutaminase from *Bacillus amyloliquefaciens* as a Processing Aid

- Appendix to Section F -

Prepared for: Amano Enzyme Inc.
1-2-7, Nishiki, Naka-ku,
Nagoya 460-8630
Japan

Prepared by: Intertek Scientific and Regulatory Consultancy
2233 Argentia Road, Suite 308 Mississauga,
Ontario, Canada
L5N 2X7
www.intertek.com

December 22, 2014

Analysis Employed for TOS Determination

Summary

The Analytical Sciences Division of TNO Nutrition and Food Research Institute was requested by Daiwa Kasei K.K. to carry out the analysis of the composition of five enzyme preparations, including the levels of some metal ions in the same enzyme preparations and to carry out a limit test on heavy metals.

This report gives the results obtained by TNO and specifies the test methods that have been used.

Contents

1. Introduction

Daiwa Kasei K.K. submitted five enzyme preparations to TNO Nutrition and Food Research Institute. TNO was requested to carry out a range of tests on these enzyme preparation.
The Analytical Sciences Division was requested to carry out the analysis of the chemical composition of the enzyme preparations as well as the determination of the levels of As, Pb, Cd and Hg and to carry out a limit test on total heavy metals.

2. Responsibilities and test facilities

2.1 Sponsor

DAIWA KASEI K.K.
7-12, Uehonmachi 5-chome
Tennouji-ku, Osaka 543
Japan

2.2 Test laboratory

TNO Nutrition and Food Sciences Institute
Analytical Sciences Division
PO Box 360
3700 AJ Zeist
The Netherlands
Phone : +31 30 6944144
Fax : +31 30 6957224

Project manager

[redacted]

Principal investigators
Sample management : [redacted]
Chemical testing :
Metal ions and heavy metals :

2.3 Responsibilities

[redacted] was overall responsible for the study. He was also responsible for the determination of the levels of moisture, protein, fat, ash and carbohydrate.
[redacted] was responsible for receipt, registration, management, distribution and storage of the test substances.
[redacted] was responsible for the analysis of the metal ions as well as for the limit test on heavy metals.

3. Test substances

The test substances had been submitted by Daiwa Kasei K.K. and were stored at TNO's Toxicology Division under the responsibility of their Test Substance Administration.

At receiving by TNO's Analytical Sciences Division the test substances had been identified with a code given by Daiwa Kasei K.K. (Glutaminase batch number) as well as with a separate code given by TNO's Toxicology Division (PSB reference number). For internal reasons the test laboratory identified the samples with a unique TNO sample number.

The test laboratory had obtained from the Test Substance Administration of TNO's Toxicology Division enough of each of the test substances to carry out all determinations.
The test substances were stored at 2 - 10 °C in the dark until needed for analysis. Any test substance remaining after completion of the analysis was returned to the Test Substance Administration of TNO's Toxicology Division.

4. Test methods

The chemical composition of the test substances was analysed. The levels of various heavy metals were determined.
The generic methods mentioned in Protocol DAS/PRT/638 were used, except for carbohydrate. As a deviation from the protocol the alternative method based on calculation by difference was used. The alternative method was preferred by the sponsor over the proposed generic method.

The principles of the methods which were used and the actual procedures are specified below.

- moisture:	loss of mass on heating at 103 °C; TNO standard method DAS/LNC/069 (qualified by STERLAB)
- protein:	calculated from Kjeldahl nitrogen (conversion factor 6.25); TNO standard methods DAS/LNC/101 and DAS/LNC/102 (qualified by STERLAB)
- fat:	extraction after acid hydrolysis (Weibull-Stoldt principle); TNO standard method DAS/LNC/066 (qualified by STERLAB)
- ash:	residue after ignition at 550 °C; TNO standard method DAS/LNC/006 (qualified by STERLAB)
- carbohydrate:	calculation by difference (100 % - (% fat + % protein + % moisture + % ash)) to obtain the level of total carbohydrate
- Arsenic:	hydride AAS; TNO standard method DAS/LSP/018 (qualified by STERLAB)
- Lead:	flameless AAS; TNO standard method DAS/LSP/023 (qualified by STERLAB)
- Cadmium:	flameless AAS; TNO standard method DAS/LSP/023 (qualified by STERLAB)
- Mercury:	cold vapour AAS; DANA/LSP/021 (qualified by STERLAB)
- total heavy metals:	limit test for metal ions coloured by sulfide ion after destruction of organic matter, specified as lead; TNO standard method AANA/LSP/038

5. Quality assurance

The testing was carried out in laboratory facilities which are GLP and STERLAB compliant.
The Quality Assurance Unit (QAU) of TNO Nutrition and Food Research Institute regularly inspects the laboratory facilities used for this study. No inspection was carried out during this study.
The QAU has not inspected the report of this study.

All determinations have been carried out in duplicate. The duplicate results fulfilled the requirements specified in the methods. Each time the mean of the two duplicate results is reported.

6. Results

The results of the analysis are given in Table 1 in Appendix A.

The results for total heavy metals were a reason for the sponsor to ask for a repeat of the determination. The repeated analyses were carried at a detection limit of 30 mg/kg. This level was preferred by the sponsor because it corresponds with the requirements for enzyme preparations given by the Food Chemical Codex[1].

The results of the repeated analysis are given in Table 2 in Appendix B.

[1]Food Chemicals Codex, fourth ed., 1996, Committee on Food Chemicals Codex, National Academy Press, Washington, D.C.

7. Discussion

The results show that protein is the main component in the enzyme preparations.

Fat was not detected in any of the samples above the level of 0.5 g/100g.

The individual metal ions (arsenic, cadmium, mercury and lead) were found below or slightly above the determination limits of the respective methods.

The test on total heavy metals detects a range of ions coloured by the sulfide ion, among them Ag, As, Bi, Cd, Cu, Hg, Pb, Sb and Sn. The colouring caused by the sample, after acid destruction, was first compared with a standard lead solution having a concentration corresponding with 50 ppm in the sample. Four samples showed less colouring than this standard lead solution. One sample (Glutaminase batch number GT70606L12, TNO sample number 200090055) showed equal colouring as the standard solution.
In the repeated analysis a larger portion of the test substance was used for the test. The colouring caused by the destructed sample was than compared with a standard lead solution having a concentration corresponding with 30 ppm in the sample. The results of the repeated test was the same for all samples: less colouring than the standard solution. This applies also to the Glutaminase batch number GT70606L12. The result of the repeated test is therefore less than 30 ppm heavy metals (as lead) in all samples.

The discrepancy between the results of the first and the repeated test for Glutaminase batch number GT70606L12 is probably caused by the inaccuracy of the test method. Because the repeated test used a larger test portion its result is seen as more reliable than the result of the first test. For this reason the results for heavy metals obtained in the first test should be neglected and the results of the repeated test should be accepted.

8. Approval

[redacted]

Dr. M.A. Bosma
Project manager

6 July 1998
Date

[redacted]

Head, Department Food and Non-Food Analysis

7 July 1998
Date

A Results of analysis

Table 1

TNO sample number	200090052	200090053	200090054	200090055	200090056
PSB reference number	980078 1A	980079 1A	980080 2A	980081 1A	980082 2A
Glutaminase batch number	GT70625L12	GT70613L12	GT70515L12	GT70606L12	GT70916L12
Moisture (g/100g)	11.2	8.7	7.8	8.2	9.2
Fat (g/100g)	<0.5	<0.5	<0.5	<0.5	<0.5
Protein (g/100g)	72.4	75.6	71.4	74.5	73.8
Ash (g/100g)	3.3	3.0	5.3	3.7	3.0
Carbohydrate (g/100g)	13.1	12.7	15.5	13.6	14.0
Arsenic (mg/kg)	0.03	0.01	0.02	0.02	0.03
Cadmium (mg/kg)	0.1	0.1	1.0	0.1	0.15
Mercury (mg/kg)	<0.005	<0.005	<0.005	<0.005	<0.005
Lead (mg/kg)	0.1	0.05	0.1	0.05	0.15
Total heavy metals as lead (mg/kg)	<50	<50	<50	50	<50

TNO report
V 98.707

Glutaminase Preparations, Determination of composition

TNO Nutrition and Food Research Institute

Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

Phone +31 30 694 41 44
Fax +31 30 695 72 24

Date
2 July 1998

Author(s)
[redacted]

Project number:
581136

Approved by:
[redacted]

Number of specimen:
1
Number of copies:
8
Number of pages:
10
Number of appendices:
2

B Results of repeated total heavy metals test

Table 2

TNO sample number	200093006	200093007	200093004	200093008	200093005
PSB reference number	980078 1A	980079 1A	980080 2A	980081 1A	980082 2A
Glutaminase batch number	GT70625L12	GT70613L12	GT70515L12	GT70606L12	GT70916L12
Total heavy metals as lead (mg/kg)	<30	<30	<30	<30	<30

Japan Food Research Laboratories
Authorized by the Japanese Government

HEAD OFFICE : 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
OSAKA BRANCH : 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
NAGOYA BRANCH : 5-13 Oosu 4-chome, Naka-ku, Nagoya 460-0011
KYUSHU BRANCH : 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka 812-0034
TAMA LABORATORY : 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
CHITOSE LABORATORY : 2-3 Bunkyo, Chitose-shi, Hokkaido 066-0052

ANALYSIS CERTIFICATE

No. 205051728-004 1/2
June 10, 2005

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi
Shiga 520-3203
Japan

Sample: Glutaminase concentrate
GT812185.00UP

Received: May 25, 2005

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the client.

RESULTS

Loss on drying [Air oven method]*1:	5.6 g/100 g
Total nitrogen [Kjeldahl method]:	9.31 g/100 g
Ether-soluble matter [Method with acid hydrolysis]:	Less than 0.1 g/100 g
Residue on ignition*2:	11.0 g/100 g
Arsenic (as As_2O_3)[Atomic absorption spectrometry]:	Not detected (LD*3 0.1 ppm)
Heavy metals (as Pb) [Sodium sulfide colorimetric method]:	7.4 ppm
Lead [Atomic absorption spectrometry]:	0.06 ppm
Cadmium [Atomic absorption spectrometry]:	0.19 ppm
Mercury [Cold vapor atomic absorption spectrometry]:	Not detected (LD 0.01 ppm)
Aerobic plate count [Standard Agar plating method]:	Not more than 300/g
Coliform bacteria (MPN)*4:	Negative (not more than 3)/g
Escherichia coli [Enrichment culture method]:	Negative/2.22 g
Coagulase positive *Staphylococci* [Enrichment culture method]*4:	Negative/1 g
Salmonella [Enrichment culture method]:	Negative/25 g
Clostridium perfringens [Enrichment culture method]:	Negative/0.1 g
Campylobacter jejuni/coli [Enrichment culture method]:	Negative/10 g
Listeria monocytogenes *5:	Negative
Clostridia [Clostridia Count Agar anaerobic plating method]*6:	Negative/1 g

*1 Test conditions: Temperature; 105 ℃, Time; 5 hours
*2 Test conditions: Temperature; 550 ℃, Constant weight
*3 LD: Minimum limit of determination
*4 By the method proposed by the client.
*5 By the method of FDA: Bacteriological Analytical Manual, 8th Ed., Chapter 10 (1995).
*6 Heat-shocked for 20 minutes at 70 ℃.

JAPAN FOOD RESEARCH LABORATORIES

[redacted]

Principal Investigator
Japan Food Research Laboratories

Japan Food Research Laboratories
Authorized by the Japanese Government

HEAD OFFICE : 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
OSAKA BRANCH : 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
NAGOYA BRANCH : 5-13 Oosu 4-chome, Naka-ku, Nagoya 460-0011
KYUSHU BRANCH : 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka 812-0034
TAMA LABORATORY : 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
CHITOSE LABORATORY : 2-3 Bunkyo, Chitose-shi, Hokkaido 066-0052

ANALYSIS CERTIFICATE

No. 205051728-005 1/2
June 10, 2005

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi
Shiga 520-3203
Japan

Sample: Glutaminase concentrate
GT507105.00UP

Received: May 25, 2005

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the client.

RESULTS

Loss on drying [Air oven method]*1:	5.7 g/100 g
Total nitrogen [Kjeldahl method]:	8.99 g/100 g
Ether-soluble matter [Method with acid hydrolysis]:	Less than 0.1 g/100 g
Residue on ignition*2:	10.5 g/100 g
Arsenic (as As_2O_3)[Atomic absorption spectrometry]:	Not detected (LD*3 0.1 ppm)
Heavy metals (as Pb) [Sodium sulfide colorimetric method]:	6.6 ppm
Lead [Atomic absorption spectrometry]:	0.12 ppm
Cadmium [Atomic absorption spectrometry]:	0.21 ppm
Mercury [Cold vapor atomic absorption spectrometry]:	Not detected (LD 0.01 ppm)
Aerobic plate count [Standard Agar plating method]:	Not more than 300/g
Coliform bacteria (MPN)*4:	Negative (not more than 3)/g
Escherichia coli [Enrichment culture method]:	Negative/2.22 g
Coagulase positive *Staphylococci* [Enrichment culture method]*4:	Negative/1 g
Salmonella [Enrichment culture method]:	Negative/25 g
Clostridium perfringens [Enrichment culture method]:	Negative/0.1 g
Campylobacter jejuni/coli [Enrichment culture method]:	Negative/10 g
Listeria monocytogenes *5:	Negative
Clostridia [Clostridia Count Agar anaerobic plating method]*6:	Negative/1 g

*1 Test conditions: Temperature; 105 ℃, Time; 5 hours
*2 Test conditions: Temperature; 550 ℃, Constant weight
*3 LD: Minimum limit of determination
*4 By the method proposed by the client.
*5 By the method of FDA: Bacteriological Analytical Manual, 8th Ed., Chapter 10 (1995).
*6 Heat-shocked for 20 minutes at 70 ℃.

JAPAN FOOD RESEARCH LABORATORIES

[redacted]

Principal Investigator
Japan Food Research Laboratories

Japan Food Research Laboratories
Authorized by the Japanese Government

HEAD OFFICE : 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
OSAKA BRANCH : 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
NAGOYA BRANCH : 5-13 Oosu 4-chome, Naka-ku, Nagoya 460-0011
KYUSHU BRANCH : 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka 812-0034
TAMA LABORATORY : 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
CHITOSE LABORATORY : 2-3 Bunkyo, Chitose-shi, Hokkaido 066-0052

ANALYSIS CERTIFICATE

No. 205051728-006 1/2
June 10, 2005

Requested by: DAIWA KASEI K.K.
4-19 Hie-cho, Konan-shi
Shiga 520-3203
Japan

Sample: Glutaminase concentrate
GT0406045.00UP

Received: May 25, 2005

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the client.

RESULTS

Loss on drying [Air oven method]*1:	5.5 g/100 g
Total nitrogen [Kjeldahl method]:	11.17 g/100 g
Ether-soluble matter [Method with acid hydrolysis]:	Less than 0.1 g/100 g
Residue on ignition*2:	7.3 g/100 g
Arsenic (as As_2O_3)[Atomic absorption spectrometry]:	Not detected (LD*3 0.1 ppm)
Heavy metals (as Pb) [Sodium sulfide colorimetric method]:	6.0 ppm
Lead [Atomic absorption spectrometry]:	0.11 ppm
Cadmium [Atomic absorption spectrometry]:	0.09 ppm
Mercury [Cold vapor atomic absorption spectrometry]:	Not detected (LD 0.01 ppm)
Aerobic plate count [Standard Agar plating method]:	Not more than 300/g
Coliform bacteria (MPN)*4:	Negative (not more than 3)/g
Escherichia coli [Enrichment culture method]:	Negative/2.22 g
Coagulase positive *Staphylococci* [Enrichment culture method]*4:	Negative/1 g
Salmonella [Enrichment culture method]:	Negative/25 g
Clostridium perfringens [Enrichment culture method]:	Negative/0.1 g
Campylobacter jejuni/coli [Enrichment culture method]:	Negative/10 g
Listeria monocytogenes *5:	Negative
Clostridia [Clostridia Count Agar anaerobic plating method]*6:	Negative/1 g