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Report number: **17.008**

Date: March 17, 2010

Chromosomal aberration test with enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity in cultured human lymphocytes
TNO V8402/03

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Department: TNO Quality of Life

Experimental work:

Keywords:
Toxicology
GLP
Chromosome aberration
In vitro
Mutagenicity
Meltamase
Amylomaltase
Bacillus amyloliquefaciens

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Signature authors: N. de Vogel Date manuscript: March 17, 2010	Signature responsible manager: Ms. M-J.S.T. Steenwinkel.

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TNO report

V8402/03 | Final |

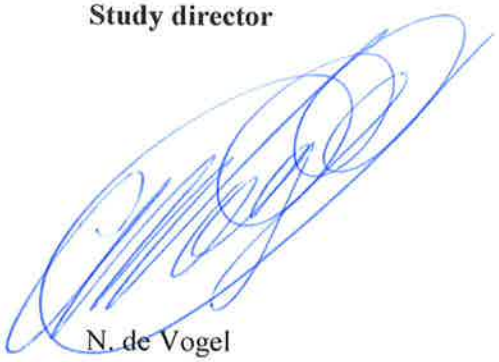
Chromosomal aberration test with Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity in cultured human lymphocytes

Date	17 March 2010
Author	N. de Vogel
At request of	DSM Food Specialties B.V. PO Box 1 2600 MA Delft The Netherlands
TNO Project number	031.20260/05.42
TNO Study number	8402/03
Sponsor Study code	-
Status report	Final
Previous version	Audited draft; 16 February 2010
Number of pages	35
Number of tables	8
Number of figures	-
Number of annexes	5
Number of appendices	-

Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results. All study activities performed by TNO Quality of Life were carried out in compliance with the current OECD Principles of Good Laboratory Practice[1]. The OECD principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan. Chemical analysis for the verification of Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity identity and properties was not performed in this study. "The test substance analysis of **stability** and TOS content are performed in TNO study 8791".

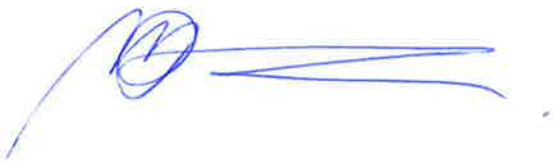
Study director



N. de Vogel

Date: 17 March 2010.

Approval by management



Ms. M-J.S.T. Steenwinkel, BSc

Date: 18 March 2010

[1] *The most recent endorsement of compliance of the test facility with these principles is attached to the report as Annex 5.*

Quality Assurance Statement

Report title : Chromosomal aberration test with Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity in cultured human lymphocytes
Report number : V8402/03
Report date : 17 March 2010

Phase	Start date of audit	Date of report
Authorized study plan	16 November 2009	16 November 2009
Test substance formulation	18 November 2009	18 November 2009
Test substance administration	18 November 2009	18 November 2009
Draft report (V8402/03) and study file	15 February 2010	15 February 2010
Final report	24 March 2010	24 March 2010

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all audits were reported to the study director and facility management on the dates indicated.

P.B. Davis B.A.
(Quality Assurance Auditor)

Date: 24 MARCH 2010

Contents

Summary

1. The test substance **Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity** was examined for its potential to induce structural chromosomal aberrations in cultured human lymphocytes, in both the absence and presence of a metabolic activation system (S9-mix). Culture medium was used as solvent. Two separate tests were conducted for which blood was obtained from two different donors. Dose levels, ranging from 10 to 5000 µg/ml (final concentrations in the culture medium), were tested. The purity of the test substance (5.5%) was taken into account while preparing the dosing solutions. Prior to preparation of the dose levels, the pH of each thawed sample was measured and adjusted to pH 7 with NaOH. In all instances, duplicate cultures were used. Cyclophosphamide, a clastogenic compound which requires metabolic activation, was used as positive control in the presence of S9-mix. Mitomycin C was used as positive control in the absence of S9-mix.

2. In the first test, in both the presence and absence of S9-mix, the treatment/harvesting times were 4/24 hours (pulse treatment). In both treatment groups, the cultures of three dose levels of the test substance (1250, 2500 and 5000 µg/ml), the cultures of the solvent control and the cultures of the positive controls were analysed for chromosomal aberrations. Only in the presence of S9-mix, the test substance was slightly toxic to the cells at all dose levels analysed. The test substance did not induce a statistically significant increase in the number of metaphases containing chromosomal aberrations.

3. In the second test, in the presence and absence of S9-mix, the treatment/harvesting times were 4/24 hours (pulse treatment) and 24/24 hours (continuous treatment), respectively. In both treatment groups, the cultures of three dose levels of the test substance (1000, 3000 and 5000 µg/ml), the cultures of the solvent control and the cultures of the positive controls were analysed for chromosomal aberrations. In the presence of S9-mix, the test substance was slightly toxic to the cells at the two highest dose levels analysed. In the absence of S9-mix, the test substance was slightly toxic at the intermediate dose level and clearly toxic at the highest dose level analysed. The test substance did not induce a statistically significant increase in the number of metaphases containing chromosomal aberrations.

4. Treatment with the positive controls Cyclophosphamide and Mitomycin C resulted in statistically significant increases in the numbers of metaphases containing chromosomal aberrations, when compared to the numbers observed in the cultures treated with the solvent control. This demonstrates the validity of the study.

5. From the results obtained in two chromosomal aberration tests it is concluded that, under the conditions used in this study, the test substance Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity was not clastogenic to cultured human lymphocytes.

1 General

1.1 Study sponsor and monitor

Sponsor:
DSM Food Specialties B.V.
PO Box 1, pp 530-0375
2600 MA, Delft
The Netherlands

1.2 Test facility

TNO Quality of Life
Business Unit Quality and Safety
Postal address: P.O. Box 360, 3700 AJ Zeist, the Netherlands
Location: Utrechtseweg 48, 3704 HE, Zeist, the Netherlands
Telephone: +31 30 694 4444
Telefax: +31 30 694 4777

1.3 Responsible personnel

Study director	: N. de Vogel
E-mail	: nico.devogel@tno.nl
Telephone	: +31 30 694 4494
Telefax	: +31 30 694 4986
Management	: Ms. M-J.S.T. Steenwinkel, BSc

1.4 Time schedule

The study was conducted between 16 November 2009 (start date of the first chromosomal aberration test) and 12 January 2010 (last day of slide analysis of the second test).

2 Introduction

2.1 Objective

The purpose of this study is to provide data on the ability of the test substance, **Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity** to induce structural chromosomal aberrations in cultured human lymphocytes in the absence and presence of a metabolic activation system (S9-mix). At predetermined intervals after treatment, the cells were arrested in the metaphase stage of their cell-cycle by the addition of the metaphase-arresting agent colcemid, harvested, fixed and dropped onto microscopic slides. After staining, the slides were analysed microscopically for the presence of metaphases containing chromosomal aberrations.

In human lymphocyte cultures, all cells are initially in the G_0 stage (resting stage) of the cell cycle. DNA synthesis begins approximately 24 h after stimulation by phytohaemagglutinin (PHA). Adequate numbers of cells in the first and second metaphases are present after 48 h and 72 h, respectively after initiation of the cultures.

2.2 Applicable guideline

The study was conducted in accordance with:

- OECD guideline 473, Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test, adopted 21 July 1997.

3 Study plan and deviations

3.1 Study plan

The study was conducted according to study plan P8402/03 entitled: "Chromosomal aberration test with Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity in cultured human lymphocytes". The study plan was approved by the study director and the sponsor on 13 November 2009.

3.2 Deviations

No deviations from the study plan occurred during the performance of the study.

4 Materials and methods

4.1 Test substance

Name[1]	: Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity
Chemical name[1]	: amylomaltase (IUPAC: 2.4.1.25)
Other name[1]	: Meltamase
Appearance[1]	: Brown liquid
Purity[1]	: 5.50%
Composition of carrier	: water
Batch number[1]	: MEG.GRZ.0905
Storage conditions[1]	: < -18°C
Date received	: 30 October 2009
Production date[1]	: January 2009
Expiry date[1]	: February 2010 (provisionally)
Supplier	: Sponsor
TNO dispense reference no.	: 090130

[1] Characteristics provided by the sponsor.

A Test Material Information Sheet (TMIS), a Material Safety Data Sheet (MSDS) and a Certificate of Analysis (CoA) of the used test substance batch, were provided by the sponsor. The Certificate of Analysis (CoA) is included as Annex 4 of this report.

4.2 Positive control substances

Indirect acting positive control (clastogen):

TNO dispense number	: 080025 (used in test 1)
Name	: Cyclophosphamide
Appearance	: white/grey powder
Batch number	: 6K035D
Purity	: 100%
Date received	: 28 January 2008
Expiry date	: 1 November 2009
Storage condition	: ambient temperature
Supplier	: Baxter B.V.

TNO dispense number	: 09015A (used in test 2)
Name	: Cyclophosphamide
Appearance	: white/grey powder
Batch number	: 9G039A
Purity	: 100%
Date received	: 4 December 2009
Expiry date	: 1 July 2012
Storage condition	: ambient temperature
Supplier	: Baxter B.V.

Direct acting positive control (clastogen):

Name	: Mitomycin C
Lot number	: 117K1684
CAS Reg. Number	: 50-07-7
Molecular formula	: $C_{15}H_{18}N_4O_5$
Molecular weight	: 334.3 g/mol
Date received	: 22 April 2009
Expiry date	: 1 April 2012
Storage conditions	: 2-10 °C
Supplier	: Sigma-Aldrich
TNO dispense number	: 090081

4.3 Tissue culture media and other chemicals

RPMI-1640 medium (with hepes and L-glutamine) and foetal calf serum were purchased from Cambrex Bioscience, Verviers, Belgium; penicillin-streptomycin and Dulbecco,s Phosphate Buffered Saline (DPBS) from Life Technologies (Gibco) B.V., Breda, The Netherlands; nicotinamide-adenine dinucleotide phosphate disodium salt (NADP) and D-glucose-6-phosphate disodium salt (G-6-P) from Roche Diagnostics, Almere, The Netherlands; Aroclor 1254 from Monsanto Chemical Company, St. Louis, USA; glacial acetic acid and Giemsa from Merck-Darmstadt, Darmstadt, Germany; methyl alcohol from Biosolve, B.V., Valkenswaard, the Netherlands; Mitomycin C and Demecolcine (colcemid) solution from Sigma-Aldrich Chemie GmbH, Germany; Cyclophosphamide from Baxter B.V. Utrecht, the Netherlands; Phytohaemagglutinin from Biochrom, AG, Berlin, Germany.

4.4 Blood samples

Blood samples were obtained by venapuncture from young (31-34 years old) healthy, non-smoking males with no known recent exposures to genotoxic chemicals or radiation. The blood was collected in sterile, heparinized vacutainer tubes and gently mixed before use to prevent clotting. A different donor was used for each chromosomal aberration test. The cultures were set up within 1 hour after withdrawal of the blood.

4.5 Culture medium for human lymphocytes

The medium for culturing the human peripheral blood lymphocytes consisted of RPMI-1640 medium (with Glutamax-I), supplemented with heat-inactivated (30 min, 56°C) foetal calf serum (20%), penicillin (100 IU/ml medium), streptomycin (100 µg/ml medium) and phytohaemagglutinin (2.4 µg/ml).

4.6 Metabolic activation system

The S9-mix consisted of a liver homogenate fraction (S9) and cofactors as described by Ames et al. (1975) and Maron and Ames (1983). The S9, used in this

study, was part of a batch prepared on 28 January 2009. The preparation and characterization of the batch is described in Annex 1. Immediately before use, a S9-mix was prepared by mixing the thawed S9 with a NADPH-generating system. The final concentrations of the various ingredients in the S9-mix were: $MgCl_2$ 8 mM; KCl 33 mM; G-6-P 5 mM; NADP 4 mM; sodium phosphate 100 mM (pH 7.4) and S9 40% (v/v). In all instances, the final concentration of the S9-mix in the culture medium was 4%.

4.7 Solubility, preparation of the test substance and measurements

To avoid multiple thawing before each study, the test substance sample was thawed once and 6 portions of ca. 10 ml were prepared and stored at < -18°C. Prior to each test, one portion was thawed and the pH was then measured and adjusted to pH 7 with NaOH.

Because the test substance is freely soluble in aqueous solvents (information provided by the sponsor), culture medium was chosen as solvent for the test substance. In the first and second test, prior to dosing, a stock concentration of 50 mg/ml, based on a purity of 5.5%, was prepared in culture medium. The stock concentration of 50 mg/ml was needed to yield the maximum final concentration in the culture medium of 5000 µg/ml. After pH adjustment and filter sterilization by passage through a 0.45 µm filter, serial stock dilutions were prepared in culture medium. The test solutions were used directly after preparation. The concentrations of the test substance were not determined analytically; the concentrations were therefore nominal concentrations.

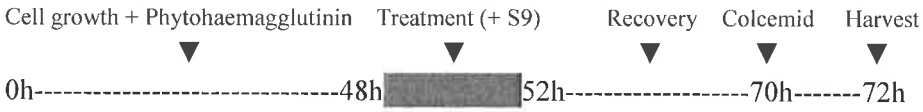
To avoid physiological effects as a result of treatment with the test substance, the osmolality of the maximum final concentration (5000 µg/ml) and two lower concentrations (2500 and 1250 µg/ml) were measured. The mean measured osmolality of the final concentrations was: 316, 307 and 304 mOsmol/kg, respectively. The measured values of the test substance concentrations were normal values, when compared to the values of culture medium (300 mOsmol/kg) and not expected to induce artefactual chromosome breakage.

4.8 The first chromosomal aberration test

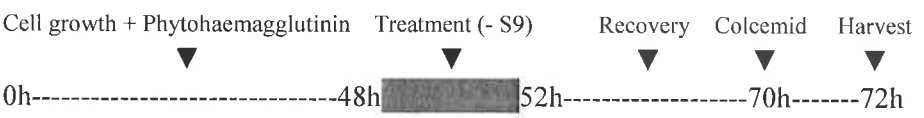
For the first chromosomal aberration test, aliquots of 0.5 ml of whole blood in 4.5 ml culture medium were incubated for 48 hours at ca. 37°C in humidified air containing 5% CO_2. The incubation was carried out in sterile screw-capped (loose) centrifuge tubes. After this incubation period, the cells were exposed to different concentrations of the test substance, in both the absence and presence of the S9-mix. Cyclophosphamide and Mitomycin C were used as positive control substances in the presence and absence of S9-mix, respectively. Culture medium was used as solvent/negative control for both positive control substances and the test substance. In all instances duplicate cultures were used. In both the absence and presence of S9-mix, the treatment times were 4 hours (pulse treatment) and the harvest time of the cells was 24 hours after onset of the treatment.

The test substance was dissolved in culture medium, at a concentration of 50 mg/ml, based on its purity. After pH adjustment and sterilization through 0.45 µm filter, the stock formulation appeared as a brown slightly turbid solution. Thereafter, serial dilutions of the stock formulation (25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2 and 0.1 mg/ml) were prepared in culture medium. The three highest serial stock formulations (25, 12.5 and 6.25 mg/ml) were slightly turbid brown to slightly brown solutions. The remaining serial stock formulations were clear solutions. The final concentrations of the test substance in the culture medium were: 5000, 2500, 1250, 625, 313, 156, 78, 39, 20 and 10 µg/ml.

Pulse treatment method (4 hours) in the presence of the S9-mix:
At time point 48 hours after initiation of the cultures, the cells were harvested by low speed centrifugation and resuspended in freshly prepared tissue culture medium without foetal calf serum and phytohaemagglutinin. Five-hundred µl of each of the stock dilutions of the test substance or negative control or 50 µl of the positive control substance Cyclophosphamide was added to the tissue culture medium in individual culture tubes. To all cultures, 0.5 ml of S9-mix (see paragraph 4.6) was added. The total volume in each culture was 5 ml. After the 4 hours treatment period, the culture medium with the test substances and S9-mix was removed. The cells were washed twice with phosphate-buffered saline (pH 7.4) and subsequently supplied with 5 ml freshly prepared culture medium enriched with foetal calf serum (20%) and phytohaemagglutinin. The cells were incubated for an additional 20 hours at 37°C in humidified air containing 5% CO_2 and harvested 72 hours after initiation of the cultures. The following schematic study design was followed.



Pulse treatment method (4 hours) in the absence of the S9-mix:
At time point 48 hours after initiation of the cultures, the cells were harvested by low speed centrifugation and resuspended in freshly prepared tissue culture medium without foetal calf serum and phytohaemagglutinin. Five-hundred µl of each of the stock dilutions of the test substance or negative control or 50 µl of the positive control substance Mitomycin C was added to the tissue culture medium in individual culture tubes. The total volume in each culture was 5 ml. After the 4 hours treatment period, the culture medium with the test substances was removed. The cells were washed twice with phosphate-buffered saline (pH 7.4) and subsequently supplied with 5 ml freshly prepared culture medium enriched with foetal calf serum (20%) and phytohaemagglutinin. The cells were incubated for an additional 20 hours at 37°C in humidified air containing 5% CO_2 and harvested 72 hours after initiation of the cultures. The following schematic study design was followed.



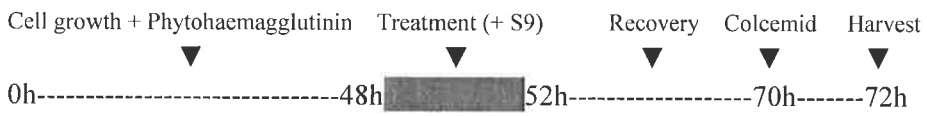
4.9 The second chromosomal aberration test

The second chromosomal aberration test consisted of two treatment groups. In the presence of S9-mix, cultures were pulse-treated for 4 hours and harvested 24 hours after onset of the treatment. In the absence of S9-mix, cultures were treated continuously with the test substance for 24 hours and harvested. Smaller intervals between the four highest dose levels were used.

The test substance was dissolved in culture medium, at a concentration of 50 mg/ml, based on its purity. After pH adjustment and sterilization through 0.45 µm filter, the stock formulation appeared as a brown slightly turbid solution. Thereafter, serial dilutions of the stock formulation (40, 30, 20, 10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 mg/ml) were prepared in culture medium. The four highest serial stock formulations (40, 30, 20 and 10 mg/ml) were slightly turbid brown to slightly brown solutions. The remaining serial stock formulations were clear solutions. The final concentrations of the test substance in the culture medium were: 5000, 4000, 3000, 2000, 1000, 500, 250, 125, 62.5, 31.3 and 15.6 µg/ml.

In the presence of S9-mix (pulse treatment group), the final concentrations of the test substance in the culture medium were: 5000, 4000, 3000, 2000, 1000, 500 and 250 µg/ml. In the absence of S9-mix (continuous treatment group), the final concentrations of the test substance in the culture medium were: 5000, 4000, 3000, 2000, 1000, 500, 250, 125, 62.5, 31.3 and 15.6 µg/ml.

Pulse treatment method (4 hours) in the presence of the S9-mix:
At time point 48 hours after initiation of the cultures, the cells were harvested by low speed centrifugation and resuspended in freshly prepared tissue culture medium without foetal calf serum and phytohaemagglutinin. Five-hundred µl of each of the stock dilutions of the test substance or negative control or 50 µl of the positive control substance Cyclophosphamide was added to the tissue culture medium in individual culture tubes. To all cultures, 0.5 ml of S9-mix (see paragraph 4.6) was added. The total volume in each culture was 5 ml. After the 4 hours treatment period, the culture medium with the test substances and S9-mix was removed. The cells were washed twice with phosphate-buffered saline (pH 7.4) and subsequently supplied with 5 ml freshly prepared culture medium enriched with foetal calf serum (20%) and phytohaemagglutinin. The cells were incubated for an additional 20 hours at 37°C in humidified air containing 5% CO_2 and harvested 72 hours after initiation of the cultures. The following schematic study design was followed.



Continuous treatment method (24 hours) in the absence of S9-mix:
At time point 48 hours after initiation of the cultures, the cells were harvested by low speed centrifugation and resuspended in freshly prepared tissue culture medium enriched with foetal calf serum and phytohaemagglutinin. Five-hundred µl

of each of the stock dilutions of the test substance or negative control or 50 µl of the positive control Mitomycin C was added to the tissue culture medium in individual culture tubes. The total volume in each culture was 5 ml. The cells were exposed to the test substance for 24 hours at 37°C in humidified air containing 5% CO_2 and harvested after the 24 hours treatment period. The following schematic study design was followed.

4.10 Cell fixation, slide preparation and analysis

Two hours before the end of the total incubation period (70 hours after initiation of the cultures), the cultured lymphocytes were arrested in the metaphase stage of their mitosis by the addition of colcemid (final concentration: 0.1 µg/ml). At the end of the total incubation period (72 hours after initiation of the cultures), the cells were harvested by low speed centrifugation, treated for 15 min at 37° C with a hypotonic solution (0.075 M KCl), fixed three times with a freshly prepared 3:1 (v/v) mixture of methanol and glacial acetic acid and processed for chromosome preparations. Two slides were prepared from each selected culture. The slides were stained in a 2% solution of Giemsa, rinsed in water, air-dried and mounted. The slides were coded by a qualified person not involved in scoring the slides, to enable "blind" scoring. 1000 stimulated lymphocytes (500 on each slide) were examined in each culture to determine the percentage of cells in mitosis (mitotic index). On the basis of the results of the mitotic index scoring and the observations with respect to the number and quality of the metaphases, three concentrations of the test substance together with the negative (vehicle) and positive controls were selected for chromosomal aberration analysis.

If possible, the highest concentration should reduce the mitotic index by at least 50 % (but not more than 70 %), when compared to the negative control value or exhibit some other clear indication of cytotoxicity. Subsequently, the cultures of the selected concentrations of the test substance, together with the negative and positive control cultures, were analysed for the induction of structural chromosomal aberrations.

For each treatment group, 200 well-spread metaphases per concentration (100 metaphases per culture and 50 metaphases per slide), each containing 46 ± 2 centromeres, were analysed by microscopic examination for chromatid-type aberrations (gaps, breaks, fragments, interchanges), chromosome-type aberrations (gaps, breaks, minutes, rings, dicentrics), according to the criteria recommended by Savage (1975). If heavily damaged cells, endoreduplicated cells or polyploid cells were observed, these cells were recorded but the cells were not counted and included in the 200 analysed cells. The Vernier readings of all aberrant metaphases were recorded. See also Annex 2 of this report for the definition of chromosomal aberrations.

4.11 Evaluation and interpretation of the test results

The number of metaphases containing one or more aberrations was compared with those of the concurrent negative controls using Fisher's exact test (one-sided). The results are considered statistically significant when the p-value of the Fisher's exact test is less than 0.05.

The study was considered valid because the positive controls gave a statistically significant increase in the number of aberrant cells and the negative controls were within the historical range.

There are several criteria for determining a positive response, such as a statistically significant concentration-related increase or a reproducible statistically significant increase in the number of metaphases containing one or more aberrations at one or more test concentrations.

A response was considered to be equivocal if the percentage of aberrant cells was statistically marginal higher than that of the negative control ($0.05<p<0.1$).

A test substance was considered to be negative if it produces neither a statistically significant concentration-related increase or reproducible statistically significant increase in the number of metaphases containing one or more aberrations, at any of the test concentrations analysed. A dose related but not significant increase in the number of aberrant cells (ie, a dose related response in the range of negative historical control values) was judged as negative.

The total number of metaphases containing one or more aberrations (excluding cells with only gaps) of the negative control was compared to the total number of metaphases containing one or more aberrations (excluding cells with only gaps) of the test substance treated groups.

Statistical methods were used as an aid in evaluating the test results but were not the only determining factor for a positive response. Both statistical methods and biological relevance of the results were considered together in the evaluation.

5 Results and discussion

The results of the first chromosomal aberration test are summarized in Table 1 and 2 (mitotic index scoring) and in Table 5 and 6 (chromosomal analysis of the selected cultures).

The results of the second chromosomal aberration test are summarized in Table 3 and 4 (mitotic index scoring) and in Table 7 and 8 (chromosomal analysis of the selected cultures).

Annex 2 gives a definition of different structural chromosomal aberrations. Annex 3 gives a presentation of the historical data of chromosomal aberration tests in cultured human lymphocytes, performed at the testing facility.

The study was considered valid because the positive controls cyclophosphamide and mitomycin C gave statistically significant increases in the number of aberrant cells and the negative controls were within the historical range.

In the first chromosomal aberration test, in the pulse treatment group with metabolic activation (S9-mix), the mitotic indices of the dose levels analysed (5000, 2500 and 1250 µg/ml), were slightly reduced to 76%, 78% and 88%, respectively, when compared to the mitotic index of the concurrent vehicle (culture medium) control. The test substance did not induce a statistically significant increase in the number of metaphases containing chromosomal aberrations at any of the concentrations analysed (see Table 1 and 5).

In the first chromosomal aberration test, in the pulse treatment group without metabolic activation (S9-mix), no reduction of the mitotic index could be demonstrated at all dose levels analysed (5000, 2500 and 1250 µg/ml), when compared to the mitotic index of the concurrent vehicle (culture medium) control. The test substance did not induce a statistically significant increase in the number of metaphases containing chromosomal aberrations at any of the concentrations analysed (see Table 2 and 6).

After the pulse treatment period (4 hours), prior to removal of the test substance, slightly turbid culture medium was observed at the two highest dose levels used (5000 and 2500 µg/ml). This observation did not influence the growth of the lymphocytes.

In the second chromosomal aberration test, in the pulse treatment group with metabolic activation (S9-mix), the mitotic indices of the two highest dose levels analysed (5000 and 3000 µg/ml), were slightly reduced to 80% and 92%, respectively, when compared to the mitotic index of the concurrent vehicle (culture medium) control. No reduction of the mitotic index could be demonstrated at the lowest dose level analysed (1000 µg/ml). The test substance did not induce a statistically significant increase in the number of metaphases containing chromosomal aberrations at any of the concentrations analysed (see Table 3 and 7).

At the end of the incubation period, after the first centrifugation step of cell harvesting, slightly turbid culture medium was observed at the two highest dose levels used (4000 and 5000 µg/ml). This observation did not influence the growth of the lymphocytes.

In the second chromosomal aberration test, in the continuous treatment group of 24 hours without metabolic activation (S9-mix), the mitotic indices of the two highest dose levels analysed (5000 and 3000 µg/ml), were reduced to 51% and 71%, respectively, when compared to the mitotic index of the concurrent vehicle (culture medium) control. No reduction of the mitotic index could be demonstrated at the lowest dose level analysed (1000 µg/ml). The test substance did not induce a statistically significant increase in the number of metaphases containing chromosomal aberrations at any of the concentrations analysed (see Table 4 and 8).

At the end of the continuous treatment period (24 hours), after the first centrifugation step of cell harvesting, slightly turbid culture medium was observed at the two highest dose levels used (4000 and 5000 µg/ml). This observation did not influence the growth of the lymphocytes.

6 Conclusion

From the results obtained in two chromosomal aberration tests it is concluded that, under the conditions used in this study, the test substance Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity was not clastogenic to cultured human lymphocytes.

7 Retention of records, samples and specimens

The following documents and materials will be retained for 5 years:

- Raw data or true copies of these
- Correspondence
- All other information related to the study

At the end of the retention period, the sponsor will be asked whether these documents should be discarded, retained for an additional period, or transferred to the archives of the sponsor.

Master copies of the approved study plan, any amendments thereof and the final report will be retained for at least 15 years.

Remaining test substance will be retained for at least one month and then returned to the sponsor. Remaining reference substance will be retained for at least one month and then discarded. Microscopic slides will be retained for at least 15 years and then removed from the archives.

Documents and materials will be retained in the archives of TNO Quality of Life, located in Zeist. The archiving period starts on the cover date of the final report.

8 References

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- OECD Principles of Good Laboratory Practice (as revised in 1997), Organisation for Economic Co-operation and Development (OECD), Paris; ENV/MC/CHEM(98)17.
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9 Tables of the results

Table 1: Mitotic index analysis of chromosomal aberration test 1

► *Pulse treatment method with metabolic activation (S9-mix)*					
treatment time: harvesting time:			4 h 24 h		
Treatment	dose (µg/ml) #	number of cells scored	Mitotic index		
			percentage of cells in mitosis	relative mitotic index (%)	selection for chromosomal aberration scoring
Culture medium	0	1000	9.4	100	+
		1000	9.3		+
Test substance	5000	1000	7.0	76	+
		1000	7.3		+
	2500	1000	7.8	78	+
		1000	6.8		+
	1250	1000	7.9	88	+
		1000	8.6		+
	625	1000	8.7	96	-
		1000	9.4		-
	313	1000	7.9	88	-
		1000	8.6		-
	156	1000	9.4	94	-
		1000	8.1		-
cyclophos-phamide	25	1000	4.4	54	+
		1000	5.8		+

The fixed cells of the lower dose levels (78, 39, 20 and 10 µg/ml) were stored without slide preparation

Table 2: Mitotic index analysis of chromosomal aberration test 1

► *Pulse treatment method without metabolic activation (S9-mix)*					
treatment time: 4 h harvesting time: 24 h					
Treatment	dose (µg/ml) #	number of cells scored	Mitotic index		
			percentage of cells in mitosis	relative mitotic index (%)	selection for chromosomal aberration scoring
Culture medium	0	1000	9.3	100	+
		1000	9.0		+
Test substance	5000	1000	9.4	97	+
		1000	8.4		+
	2500	1000	9.8	111	+
		1000	10.5		+
	1250	1000	9.6	107	+
		1000	10.0		+
	625	1000	10.1	104	-
		1000	8.9		-
	313	1000	9.5	100	-
		1000	8.9		-
	156	1000	10.1	116	-
		1000	11.1		-
Mitomycin C	0.4	1000	8.6	87	+
		1000	7.4		+

The fixed cells of the lower dose levels (78, 39, 20 and 10 µg/ml) were stored without slide preparation

Table 3: Mitotic index analysis of chromosomal aberration test 2

► *Pulse treatment method with metabolic activation (S9-mix)*					
treatment time: harvesting time:			4 h 24 h		
Treatment	Dose # (µg/ml)	number of cells scored	Mitotic index		
			percentage of cells in mitosis	Relative mitotic index (%)	selection for chromosomal aberration scoring
Culture medium	0	1000	13.2	100	+
		1000	12.3		+
Test substance	5000	1000	10.5	80	+
		1000	9.8		+
	4000	1000	9.8	79	-
		1000	10.3		-
	3000	1000	11.4	92	+
		1000	12.1		+
	2000	1000	13.9	111	-
		1000	14.3		-
	1000	1000	13.3	100	+
		1000	12.1		+
cyclophos-phamide	25	1000	8.6	78	+
		1000	7.9		+

The fixed cells of the lower dose levels (500 and 250 µg/ml) were stored without slide preparation

Table 4: Mitotic index analysis of chromosomal aberration test 2

►*Continuous treatment method without metabolic activation (S9-mix)*					
treatment time: harvesting time:			24 h 24 h		
Treatment	Dose # (µg/ml)	number of cells scored	mitotic index		
			percentage of cells in mitosis	relative mitotic index (%)	selection for chromosomal aberration scoring
Culture medium	0	1000	11.0	100	+
		1000	11.1		+
	5000	1000	5.3	51	+
		1000	5.9		+
	4000	1000	6.3	62	-
		1000	7.4		-
	3000	1000	8.3	71	+
		1000	7.3		+
	2000	1000	7.8	78	-
		1000	9.4		-
	1000	1000	11.6	105	+
		1000	11.5		+
	500	1000	10.2	97	-
		1000	11.2		-
	250	1000	11.9	101	-
		1000	10.5		-
Mitomycin C	0.2	1000	7.9	68	+
		1000	7.1		+

The fixed cells of the lower dose levels (125, 62.5, 31.3 and 15.6 µg/ml) were stored without slide preparation

Table 5: Chromosomal Aberration Analysis of Test 1

► *Pulse treatment method with metabolic activation (S9-mix)*

Treatment / harvest time (h)	Dose level (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)
		cells observed	chromatid break	chromatid exchange	chromo-some break	chromo-some exchange	others	Number of cells showing aberrations (%)	Statistics [2]		
4/24 (+ S9)	neg. control (medium)	100	0	0	0	0	0	0		0	100
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	1250	100	0	0	0	0	0	0	-	0	88
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	2500	100	0	0	0	0	0	0	-	0	78
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	5000	100	0	0	0	0	0	0	-	0	76
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	pos. control cyclophos-phamide (25.0)	100	14	4	5	0	0	22	***	0	54
		100	14	5	4	0	0	21		0	
		200	28	9	9	0	0	43 (21.5)		0	

[1] Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps

[2] Fisher's exact probability test (one-sided); *** $p \leq 0.001$

Table 6: Chromosomal Aberration Analysis of Test 1

► *Pulse treatment method without metabolic activation (S9-mix)*

Treatment / harvest time (h)	Dose level (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)
		cells observed	chromatid break	chromatid exchange	chromo-some break	chromo-some exchange	others	Number of cells showing aberrations (%)	Statistics [2]		
4/24 (- S9)	neg. control (medium)	100	0	0	0	0	0	0		1	100
		100	1	0	0	0	0	1		0	
		200	1	0	0	0	0	1 (0.5)		1	
	1250	100	0	0	0	0	0	0	-	0	107
		100	0	1	0	0	0	1		0	
		200	0	1	0	0	0	1 (0.5)		0	
	2500	100	0	0	0	0	0	0	-	0	111
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	5000	100	0	0	0	0	0	0	-	0	97
		100	1	0	0	0	0	1		1	
		200	1	0	0	0	0	1 (0.5)		1	
	pos.control mitomycin C (0.4)	100	12	20	1	0	0	32	***	1	87
		100	9	17	2	0	0	27		1	
		200	21	37	3	0	0	59 (29.5)		2	

[1] Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps

[2] Fisher's exact probability test (one-sided); *** $p \leq 0.001$

Table 7: Chromosomal Aberration Analysis of Test 2

► *Pulse treatment method with metabolic activation (S9-mix)*

Treatment / harvest time (h)	Dose level (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)
		cells observed	chromatid break	chromatid exchange	chromo-some break	chromo-some exchange	others	Number of cells showing aberrations (%)	Statistics [2]		
4/24 (+ S9)	neg. control (medium)	100	0	0	0	0	0	0		0	100
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	1000	100	0	0	0	0	0	0	-	0	100
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	3000	100	0	0	0	0	0	0	-	1	92
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		1	
	5000	100	1	0	0	0	0	1	-	0	80
		100	0	0	0	0	0	0		0	
		200	1	0	0	0	0	1 (0.5)		0	
	pos. control cyclophos-phamide (25.0)	100	15	7	3	1	0	24	***	1	78
		100	13	5	0	0	0	19		1	
		200	28	12	3	1	0	43 (21.5)		2	

[1] Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps

[2] Fisher's exact probability test (one-sided); *** $p \leq 0.001$

Table 8: Chromosomal Aberration Analysis of Test 2

►*Continuous treatment method without metabolic activation (S9-mix)*

Treatment / harvest time (h)	Dose level (µg/ml)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Relative Mitotic index (%)
		cells observed	chromatid break	chromatid exchange	chromo-some break	chromo-some exchange	others	Number of cells showing aberrations (%)	Statistics [2]		
24/24 (- S9)	neg. control (medium)	100	0	0	0	0	0	0		0	100
		100	0	0	0	0	0	0		0	
		200	0	0	0	0	0	0 (0.0)		0	
	1000	100	0	0	0	0	0	0		0	105
		100	0	0	0	0	0	0	-	0	
		200	0	0	0	0	0	0 (0.0)		0	
	3000	100	0	0	0	0	0	0		0	71
		100	0	0	0	0	0	0	-	1	
		200	0	0	0	0	0	0 (0.0)		1	
	5000	100	0	0	0	0	0	0		0	51
		100	0	0	0	0	0	0	-	0	
		200	0	0	0	0	0	0 (0.0)		0	
	pos.control mitomycin C (0.2)	100	6	12	3	0	0	20		0	68
		100	17	6	3	0	0	23	***	0	
		200	23	18	6	0	0	43 (21.5)		0	

[1] Gap(g) - total number of cells showing only (chromatid-type and chromosome-type) gaps

[2] Fisher's exact probability test (one-sided); *** p≤0.001

10 Annexes

Annex 1:

Preparation and characterization of Aroclor 1254-induced rat liver homogenate

The batch of S9 dated 28 January 2009 was prepared according to Ames et al. (1975) and Maron and Ames (1983) as follows.

Methods:
Male Wistar rats (n =12; obtained from Charles River Deutschland, Sulzfeld, Germany) were injected intraperitoneally with a single dose of Aroclor 1254 (nominal dose of 500 mg/kg body weight) in soy bean oil (20% w/v). The rats were provided with tap water and the Institute's stock diet ad libitum. Five days after the injection of Aroclor 1254 the rats were killed by CO_2 asphyxiation. The livers were removed aseptically and immediately put into a cold, sterile 0.15 M KCl solution. After washing in the KCl solution, the livers were weighed, cut into pieces and homogenized in 3 volumes of 0.15 M KCl solution in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for 10 minutes at 9,000 g. The supernatant, which is called S9, was collected and divided into small aliquots in sterile polypropylene vials. The vials were quickly frozen on dry ice and subsequently stored in a freezer at <-60 °C.

The S9 was checked for sterility. The protein and cytochrome P-430 content of the S9 fraction were determined according to the method published by Rutten et al. (1987).

Results:
The protein content of the batch was 21.8 g/litre.
The cytochrome P430 content of the batch was 22.6 µmol/litre.
The batch contained 1.04 µmol cytochrome P430 per gram protein.
The sterility check of the batch resulted in 0 colonies per 100 µl S9.

Conclusion:
- The batch of S9 of 28 January 2009 meets all of the in-house quality criteria.

References:
- Ames, B.N., J. McCann and E. Yamasaki (1975) "Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian microsome mutagenicity test." Mutation Res. 31: 347-365.
- Maron, D.M. and B.N. Ames (1983) "Revised methods for the Salmonella mutagenicity test." Mutation Res. 113: 173-215. + ERRATUM Mutation Res. 113: 533.
- Rutten, A.A.J.J.L., H.E. Falke, J.F. Catsburg, R. Topp, B.J. Blaauboer, I. van Holstein, L. Doorn and F.X.R. van Leeuwen (1987) "Interlaboratory comparison of total cytochrome P-430 and protein determinations in rat liver microsomes. Reinvestigation of assay conditions." Arch. Toxicol. 61: 27-33.

Annex 2: Definition of chromosomal aberrations

Chromatid gap:	An achromatic lesion smaller than the width of one chromatid, and with minimal misalignment of the chromatid.
Chromatid break:	A breakage of one chromatid larger than the width of one chromatid, or a clear misalignment of a chromatid.
Chromatid exchange:	A breakage and reunion between chromatids from different chromosomes (interchange) or within a chromosome (intrachange; including interstitial deletion).
Chromosome gap:	An achromatic lesion at an identical site in both chromatids smaller than the width of one chromatid, and with minimal misalignment of the chromatids.
Chromosome break:	A breakage at an identical site of both chromatids larger than the width of one chromatid, or a clear misalignment of the chromatids (misalignment of the chromatids can result in cases where only the acentric fragment but not the shortened monocentric chromosome can be identified).
Chromosome exchange:	A breakage of both chromatids with a reunion between chromatids from different chromosomes (dicentric) or within a chromosome (ring).
Multiple aberrations:	A cell containing more than 10 chromosomal aberrations.
Polyploidy:	A cell containing a multiple of the haploid chromosome number (n) other than the diploid number (i.e., 3n, etc.).
Endoreduplication:	A cell in which after an S (synthesis) period of DNA replication, the nucleus did not go into mitosis but started another S period. The result is chromosomes with 4, 8, 16 or more chromatids.

References:

- Savage, Annotation: Classification and relationships of induced chromosome structural change. *J. Med. Gen.* **13**, 103-122, 1975.
- Scott, D. Dean, B.J., Danford, N.D., and Kirkland, D.J. Metaphase chromosome aberration assays *in vitro*. In: Basic Mutagenicity Tests.
- UKEMS Recommended Procedures, editor D.J. Kirkland, Cambridge University Press, Report. Part 1 revised, pp. 62 - 86, 1990.

Annex 3:

Historical negative control data of Chromosomal Aberrations Tests performed with cultured human lymphocytes: Summarized data from 2000 (TNO report V99.1141) – 2010 (TNO report V8402/05)

		Percentage of metaphases with aberrations (excluding metaphases with only gaps)		
Treatment / harvest time	Vehicle	$\bar{X} \pm$ S.D.	Range	N
4 / 24 (– S9)	medium / saline DMSO gasmix*) ethanol	0.24 ± 0.4 0.14 ± 0.2 0.33 ± 0.6 0.5	0.0 – 1.5 0.0 – 0.5 0.0 – 1.0 -	17 14 3 1
4 / 24 (+ S9)	medium / saline DMSO gasmix*) ethanol	0.23 ± 0.3 0.16 ± 0.2 0.17 ± 0.3 0.25 ± 0.4	0.0 – 1.0 0.0 – 0.5 0.0 – 0.5 0.0 – 0.5	32 25 3 2
24 / 24 (– S9)	medium / saline DMSO ethanol	0.13 ± 0.3 0.07 ± 0.2 0.0	0.0 – 1.5 0.0 – 0.5 -	24 15 1
48 / 48 (– S9)	medium / saline DMSO	0.11 ± 0.2 0.0	0.0 – 0.5 -	9 1
4 / 48 (+ S9)	medium / saline DMSO gasmix*)	0.0 ± 0.0 0.0 0.0 ± 0.0	0.0 – 0.0 - 0.0 – 0.0	9 1 3
4 / 48 (– S9)	gasmix*)	0.17 ± 0.3	0.0 – 0.5	3

N = number of treatment groups performed
*) 19 % O_2, 5 % CO_2 and 76 % N_2

Annex 3 (continued):

Historical positive control (Cyclophosphamide) data of Chromosomal Aberrations Tests performed with cultured human lymphocytes: Summarized data from 2000 (TNO report V99.1141) – 2010 (TNO report V8402/05)

		Percentage of metaphases with aberrations (excluding metaphases with only gaps)		
Treatment / harvest time	Dose level (µg/ml)	$\bar{X} \pm$ S.D.	Range	N
4 / 24	15	21.5 ± 6.4	12.5 – 27.5	5
	17.5	27.5	-	1
	20	18.0 ± 0.7	17.5 – 18.5	2
	25	26.9 ± 6.5	14.0 – 44.5	47
	30	22.8 ± 4.1	18.5 – 28.0	4
4 / 48	15	9.5 ± 5.7	5.5 – 13.5	2
	20	11.5 ± 4.2	8.5 – 14.5	2
	25	13.2 ± 5.0	5.5 – 19.5	6
	30	13.7 ± 2.3	11.5 – 16.0	3

N = number of treatment groups performed

Annex 3 (continued):

Historical positive control (Mitomycin C) data of Chromosomal Aberrations Tests performed with cultured human lymphocytes: Summarized data from 2000 (TNO report V99.1141) – 2010 (TNO report V8402/05)

		Percentage of metaphases with aberrations (excluding metaphases with only gaps)		
Treatment / harvest time	Dose level (µg/ml)	$\bar{X}$ ± S.D.	Range	N
4 / 24	0.2	18.5 ± 1.4	17.0 – 19.0	2
	0.4	31.2 ± 4.9	22.0 – 39.5	31
24 / 24	0.05	12.8 ± 2.5	11.0 – 14.5	2
	0.2	26.7 ± 8.2	14.0 – 47.0	35
4 / 48	0.4	24.0 ± 3.1	21.5 – 27.5	3
48 / 48	0.05	14.0 ± 0.7	13.5 – 14.5	2
	0.1	23.8 ± 6.7	19.0 – 28.5	2
	0.2	45.3 ± 10.1	37.5 – 63.0	6

N = number of treatment groups performed

Annex 4:

Certificate of Analysis (provided by the sponsor) concerning batch MEG.GRZ.0905 of the test substance Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity

TNO Dispense reference
Orgi.1.32

DSM Food Specialties B.V.
R&D/REG

P02109

DSM

Page 1 of 1

CERTIFICATE OF ANALYSIS

Name of the product	Meltamase
Batch no.	MEG.GRZ.0905
Status	Batch for toxicity study
Date of manufacture	February 2009
Date of expiration	12 months (provisionally)
Active component	Meltamase
Date of issue	29 October 2009

Parameter	Method	Unit	Result
Characterization data			
Meltamase activity	B1903	ATU / g	2280
Dry Matter	60485	% (w/w)	6,65
Ash	60328	% (w/w)	1,15
TOS	Calculation	% (w/w)	5,50
Proteins by Kjeldahl Nitrogen x 6,25	61804	% (w/w)	2,20
Stability data; > 90% residual activity*			
Stability at 4°C, undiluted	B1903	Days	7
Stability at 4°C, 91 mg / ml	B1903	Days	7
Stability at 4°C, 273 mg / ml	B1903	Days	7
Stability at 4°C, 909 mg / ml	B1903	Days	7
Stability at RT, undiluted	B1903	Hours	4
Stability at RT, 91 mg / ml	B1903	Hours	4
Stability at RT, 273 mg / ml	B1903	Hours	4
Stability at RT, 909 mg / ml	B1903	Hours	4

Signature R&D QESH:

Remarks (if any):
CoA drafted for toxicity study.
*: analysis not performed under GLP

s552712

Annex 5: GLP compliance monitoring unit statement

voedsel en waren autoriteit

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 27-31 October 2008 at

TNO Quality of Life
Utrechtseweg 48, 3704 HE Zeist
P.O. Box 360, 3700 AJ Zeist

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, analytical and clinical chemistry, kinetics and metabolism, safety pharmacology, worker exposure and in-vitro studies.

Den Haag, 03 February 2009

Dr Th. Helder
Manager GLP Compliance Monitoring Program

Food and Consumer Product Safety Authority (VWA)
Prinses Beatrixlaan 2, 2595 AL Den Haag
Postbus 19506, 2500 CM Den Haag, The Netherlands

Annex 1 Certificate of Analysis of enzyme preparation

Certificate of Analysis of amylomaltase from *Bacillus amyloliquefaciens* updated with analytical data from GLP studies, TNO report V8791

DSM Food Specialties B.V.
R&D/RFG

DSM

CERTIFICATE OF ANALYSIS

Name of the product	**Meltamase**
Batch no	MEG GRZ 0905
Status	ocUF for toxicity study
Date of manufacture	February 2009
Date of expiration	12 months (provisionally) , extended 4 months
Active component	Amylomaltase or 4-α-glucanotransferase
Date of issue	29 October 2009, updated 17 August 2010

Parameter	Method	Unit	Result
	Characterization data		
Amylomaltase activity	B1903	ATU / g	2130
Dry Matter	60405	% (w/w)	6.70
Ash	60328	% (w/w)	1.26
TOS	Calculation	% (w/w)	5.50
Proteins by Kjeldahl Nitrogen x 6.25	61804	% (w/w)	2.20
	Stability data; > 90% residual activity		
Stability at 4°C, undiluted	B1903	Days	7
Stability at 4°C, 91 mg / ml	B1903	Days	7
Stability at 4°C, 273 mg / ml	B1903	Days	7
Stability at 4°C, 909 mg / ml	B1903	Days	7
Stability at RT, undiluted	B1903	Hours	4
Stability at RT, 91 mg / ml	B1903	Hours	4
Stability at RT, 273 mg / ml	B1903	Hours	4
Stability at RT, 909 mg / ml	B1903	Hours	4

Signature R&D QESH:	Remarks (if any): CoA drafted for toxicity study. Analyses performed under GLP