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Bacterial reverse mutation test with enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity TNO V8405/15

Authors: Ms. M.J.M. van den Wijngaard

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TNO Report

V8405/15 | Final |

Bacterial reverse mutation test with Enzyme preparation of Bacillus amyloliquefaciens containing amylomaltase activity

Date	19 February 2010
Authors	Ms. M.J.M. van den Wijngaard, BSc.
Sponsor	DSM Food Specialties B.V. PO Box 1 2600 MA Delft the Netherlands
TNO project number	031.20260/05.41.01
TNO study code	8405/15
Sponsor's study code	-
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Number of appendices	7

Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete 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

Ms. M.J.M. van den Wijngaard, BSc.

19-02-2010
Date (dd-mm-yyyy)

[1] *The most recent endorsement of compliance of the test facility with these principles is attached to the report as Appendix 7.*

Quality Assurance Statement

Report title : Bacterial reverse mutation test with Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity
Report number : V8405/15
Report date : 19 February 2010

Phase	*	**Start date of Audit**	**Date of audit report**
Authorized study plan	Yes	17 November 2009	18 November 2009
Authorized amendment 1	Yes	19 November 2009	19 November 2009
Strain management	No	23 November 2009	23 November 2009
Test substance dissolution	No	18 December 2009	18 December 2009
Preparation of dosing solutions	No	18 December 2009	18 December 2009
Start of exposure	No	30 October 2009	30 October 2009
Counting revertants	Yes	7 December 2009	7 December 2009
Draft report and study file	Yes	15 January 2010	15 January 2010
Final report	Yes	19 February 2010	19 February 2010

* This type of short-term study is carried out frequently and the Quality Assurance does not audit the experimental phase of each individual study; the processes involved are audited at regular intervals according to a predetermined schedule. This column indicates whether or not the audit was of this particular study.

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.

19 February 2010

H. Visscher
Quality Assurance Auditor

Date (dd-mm-yyyy)

Contents

Summary

1. The test substance, Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity, was examined for mutagenic activity in the bacterial reverse mutation test using the histidine-requiring *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 and the tryptophan-requiring *Escherichia coli* strain WP2 *uvrA*, in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix).

2. The test substance was diluted in milli-Q water. One bacterial reverse mutation test was performed. All strains were used, in the absence and presence of S9-mix, with five concentrations of the test substance, ranging from 62 to 5000 µg/plate. Negative controls (milli-Q water) and positive controls were run simultaneously with the test substance.

3. The mean number of his^+ and trp^+ revertant colonies of the negative controls were within the acceptable range and the positive controls gave the expected increase in the mean number of revertant colonies. The test was considered valid.

4. The test substance was slightly toxic to strain TA 1537, in both the absence and presence of S9-mix, at and above 1667 µg/plate and at 5000 µg/plate, respectively. Toxicity was evidenced by a clearing of the background lawn of bacterial growth compared to the negative controls.

5. In both the absence and presence of S9-mix in all strains, Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity did not induce a minimal 2-fold and/or dose related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control.

6. It is concluded that the results obtained with the test substance in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, and in the *Escherichia coli* strain WP2 *uvrA*, in both the absence and the presence of the S9-mix, indicate that **Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity** is **not mutagenic** under the conditions employed in this study.

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 Testing Facility

TNO Quality of Life
Business Unit Quality and Safety
Postal address: P.O. Box 360, 3700 AJ Zeist, the Netherlands
Location: Utrechtseweg 48, Zeist, the Netherlands
Telephone +31 30 694 4144; Telefax +31 30 694 4777

1.3 Responsible personnel

Study director	: Ms. M.J.M. van den Wijngaard, BSc.
Management	: Ms. M-J.S.T. Steenwinkel, BSc.

1.4 Time schedule

The study was conducted between 4 December 2009 and 8 December 2009.

2 Introduction

2.1 Objective

The objective of this study was to provide data on the possible mutagenic activity of Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity, in four selected strains of *Salmonella typhimurium,* TA 1535, TA 1537, TA 98 and TA 100, and in the *Escherichia coli* mutant WP2 *uvrA*, in both the absence and presence of a metabolic activation system (S9-mix).

2.2 Guidelines

This study was conducted in accordance with the following guideline:

- OECD guideline no. 471, Genetic Toxicology: Bacterial Reverse Mutation Test, adopted 21 July 1997

3 Study plan and deviations

3.1 Study plan

The study was conducted according to the study plan and amendment 1 P8405/15, which was approved by the study director on 12 November 2009 and 16 November 2009, respectively.

3.2 Deviations

The expiry date of the test substance is 1 February 2010 instead of 1 January 2010 as stipulated in the study plan.

This deviation is considered not to have affected the validity of the study.

4 Materials and Methods

4.1 Characterization of the test substance

Name	: Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity
Chemical name	: amylomaltase (IUPAC: 2.4.1.25)
Other name	: Meltamase
Appearance	: Brown liquid
Purity	: 5.50%
Composition of carrier	: water
Batch number	: MEG.GRZ.0905
Storage conditions	: <-18ºC
Date of receipt	: 30 October 2009
Expiry date	: 01 February 2010
Supplier	: Sponsor
TNO dispense reference no.	: 090130

Analysis data provided by the sponsor are included in appendix 6.

4.2 Other chemicals

Nicotinamide adenine dinucleotide phosphate, disodium salt (NADP) was obtained from Roche Diagnostics, Woerden, The Netherlands; Biotine, L-histidine and L-Tryptophan from Merck KGaA, Darmstadt, Germany; D-glucose-6-phosphate, disodium salt (G-6-P), 9-aminoacridine (9-AA), N-ethyl-N-nitrosourea (ENU), dimethylsulphoxide (DMSO), Benzo(a)pyrene (B[a]P) from Sigma Chemical Company, St. Louis, USA; Aroclor 1254 from Monsanto Chemical Company, St. Louis, USA and 2-nitrofluorene (2-NF), 2-amino-anthracene (2-AA) and sodium azide (NaN_3) from Aldrich, Brussels, Belgium.

4.3 Characterization of the test system

The four *Salmonella typhimurium* strain (TA 1535, TA 1537, TA 98 and TA 100) are originally obtained from Dr. B.N. Ames (University of California Berkeley, U.S.A.), the strain *Escherichia coli* WP2 *uvrA* were provided by Dr. C. Voogd (National Institute of Public Health, Bilthoven, the Netherlands).

The genotype of the *Salmonella typhimurium* and *Escherichia coli* strains are given below:

		Additional mutations[1]		
Strain	Amino acid mutation	LPS	UV-repair	R-factor
TA 98	His D3052	rfa^-	$uvrB^-$	+R
TA 100	His G46	rfa^-	$uvrB^-$	+R
TA 1535	His G46	rfa^-	$uvrB^-$	-R
TA 1537	His C3076	rfa^-	$uvrB^-$	-R
WP 2 *uvrA*	Trp	rfa^+	$uvrA^-$	-R

[1] rfa: this mutation causes partial loss of the lipopolysaccharide (LPS) barrier that coats the surface of the bacteria; it increases the permeability to large molecules, e.g. crystal violet

uvrB/A: these mutations comprise deletions of a gene coding for the DNA excision repair system, which results in greatly increased sensitivity in detecting many mutagens including UV radiation

R-factor: the R-factor strains contain the plasmid pKM 101, which increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA-repair system normally present in *S. typhimurium*. It carries an ampicillin resistance gene

Frozen stocks of each strain were checked for histidine or tryptophan requirement and for sensitivity to ampicillin, crystal violet and UV radiation (the results for the stocks used in the present assay are presented in Appendix 2).

The S9 liver homogenate used in the study was prepared as described in Appendix 3. On the day of use, aliquots of S9 liver homogenate were thawed and mixed 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), NaCl 46 mM, and S9 10 %. The S9-mix was kept on ice until use.

4.4 Experimental procedures

4.4.1 Dose levels of the test substance

The plate-incorporation method with the histidine-requiring *S. typhimurium* mutants TA 1535, TA 1537, TA 98 and TA 100 and the tryptophan-requiring *Escherichia coli* mutant WP2 *uvrA* as indicator strains was applied. The assay has been described in detail by Ames et al. (1975) and by Maron and Ames (1983). A preliminary test to assess the toxicity of the test substance was not performed. Therefore the toxicity test was incorporated in the mutagenicity assay.

One bacterial reverse mutation test was performed. The pH of the thawed test substance was adjusted to pH 7.0 with 1M sodiumhydroxide. The test substance was diluted in milli-Q water to a concentration of 50 mg/ml based on a purity of 5.5%, resulting in a brown, turbid suspension. The stock solution was sterilized by passage through a 0.45µm filter (Corning Incorporated). A brown, slightly-turbid suspension was obtained. The sterility of the stock solution was tested. Serial dilutions in milli-Q water were made. Five concentrations were tested, ranging from 62 to 5000 µg/plate.
Negative controls (milli-Q water) and positive controls were run simultaneously with the test substance.
The actual concentrations of the test substance in the test solutions were not determined. Therefore, the concentrations quoted in this report are nominal concentrations.

The reference mutagens used as positive controls were:

positive control substances		
Strain	**in the absence of the S9-mix**	**in the presence of the S9-mix**
TA 1535	sodium azide: 1.0 µg/plate	2-aminoanthracene: 2.0 µg/plate
TA 1537	9-aminoacridine: 80.0 µg/plate	benzo(a)pyrene: 4.0 µg/plate
TA 98	2-nitrofluorene: 2.0 µg/plate	2-aminoanthracene: 2.0 µg/plate
TA 100	sodium azide: 1.0 µg/plate	2-aminoanthracene: 2.0 µg/plate
WP 2 *uvrA*	N-ethyl-N-nitrosourea: 100 µg/plate	2-aminoanthracene: 80 µg/plate

4.4.2 Mutation analysis

Fresh bacterial cultures were prepared by inoculation of nutrient broth with a thawed aliquot of the stock culture and subsequent incubation for approximately 10-16 h at 37°C while shaking. Briefly, the mutagenicity assay was carried out as follows. To 2 ml molten top agar (containing 0.6 % agar, 0.5 % NaCl and 0.05 mM L-histidine.HCl/0.05 mM biotin or 0.05 mM tryptophane for the *S. typhimurium* strains, and *E. coli* WP2 *uvrA* strain, respectively), maintained at ca. 46 ^{o}C, were added subsequently: 0.1 ml of a fully grown culture of the appropriate strain, 0.1 ml of the test substance or of the negative control or of the positive control substance solution, and 0.5 ml S9-mix for the experiments with metabolic activation or 0.5 ml sodium phosphate 100 mM (pH 7.4) for the experiments without metabolic activation. The ingredients were thoroughly mixed and the mix was immediately poured onto minimal glucose agar plates (1.5 % agar in Vogel and Bonner medium E with 2 % glucose). All determinations were made in triplicate. The plates were incubated at ca. 37 ^{o}C for approximately 48-72 hours. Subsequently, the his^{+} and trp^{+} revertants were counted.

4.5 Analysis of test results

The mutagenicity study is considered valid if the mean colony counts of the vehicle control values of the strains are within acceptable ranges (Appendix 4), if the results of the positive controls meet the criteria for a positive response (all as recorded in Appendix 4 and 5), and if no more than 5 % of the plates are lost through contamination or other unforeseen events.

Toxicity is defined as a reduction (by at least 50%) in the number of revertant colonies and/or a clearing of the background lawn of bacterial growth as compared to the negative (vehicle) control

A test substance is considered to be positive in the bacterial gene mutation test if the mean number of revertant colonies on the test plates shows a concentration-related increased or if a reproducible two-fold or more increase is observed compared the negative controls.

A test substance is considered to be negative in the bacterial gene mutation test if it produces neither a dose-related increase in the mean number of revertant colonies nor a reproducible positive response at any of the test points.

Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base pair substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested strains.

Omission of a second test under these conditions is acceptable as a single test does not, or hardly ever results in false negative conclusions (TNO historical data in Appendix 5 and Kirkland and Dean, 1994).

Both numerical significance and biological relevance are considered together in the evaluation. No statistical analysis was performed.

Historical data on the bacterial reverse mutation tests, including data on positive and negative controls, are presented in Appendix 5.

5 Results and discussion

The results of the bacterial reverse mutation test are shown in Table 1 (Appendix 1).

One bacterial reverse mutation test was performed. Just before use, the test substance was adjusted to pH 7.0, diluted to 50 mg/ml with milli-Q water and sterilized by passage through a 0.45 µm filter. A slightly-turbid, brown suspension was obtained. Sterility was tested and the test substance solution was sterile. Five concentrations were tested, ranging from 62 to 5000 µg per plate. Negative controls (milli-Q water) and positive controls were run simultaneously with the test substance.

The mean number of his^+ and trp^+ revertant colonies of the negative controls were within the acceptable range, and positive controls gave the expected increase in the mean number of revertant colonies as defined in Appendices 4 and 5.

The test substance was slightly toxic to strain TA 1537, in both the absence and presence of S9-mix, at and above 1667 µg/plate and at 5000 µg/plate, respectively. Toxicity was evidenced by a clearing of the background lawn of bacterial growth compared to the negative controls. Since only slight toxicity was observed, no decrease in the mean number of revertants was observed and at least 3 concentrations of the test substance were non-toxic, the test was regarded as valid.

In both the absence and presence of S9-mix in strains TA 1535, TA 1537, TA 98, TA 100 and WP2 *uvrA*, Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity did not induce a minimal 2-fold and/or dose related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control.

6 Conclusion

It is concluded that the results obtained with the test substance in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, and in the *Escherichia coli* strain WP2 *uvrA*, in both the absence and the presence of the S9-mix, indicate that **Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity** is **not mutagenic** under the conditions employed in this study.

7 Documentation and retention of records and test substance

The following documents 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.

The master copy of the approved study plan and the final report will be retained for at least 15 years.

Remaining test substance will be retained for at least one month.

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

8 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.
- Kirkland, D.J. and Dean, S.W. (1994) On the need for confirmation of negative genotoxicity results *in vitro* and on the usefulness of mammalian cell mutation tests in a core battery: experiences of a contract research laboratory. Mutagenesis 9: 491-501.
- Organisation for Economic Co-operation and Development. OECD Guideline for Testing of Chemicals no. 471, Genetic Toxicology: Bacterial Reverse Mutation Test, Paris, 21 July 1997.
- Organisation for Economic Co-operation and Development. OECD Principles of Good Laboratory Practice (as revised in 1997), Paris, ENV/MC/CHEM(98)17.

9 Appendices

Appendix 1 Table 1: Number of revertants counted in bacterial reverse mutation test with Enzyme preparation of *Bacillus amyloliquefaciens* containing amylomaltase activity

	TA 1535		TA 1537		TA 98		TA 100		E. Coli	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0 µg/plate	14	23	13	14	42	50	126	135	14	37
	30	12	11	17	37	52	123	148	42	38
	19	24	5	24	47	53	143	156	25	40
Mean	21	20	10	18	42	52	131	146	27	38
StDev	8	7	4	5	5	2	11	11	14	2
62 µg/plate	16	13	17	12	36	37	119	119	26	35
	16	18	16	14	22	53	121	141	34	31
	17	10	11	27	28	43	117	157	38	35
Mean	16	14	15	18	29	44	119	139	33	34
StDev	1	4	3	8	7	8	2	19	6	2
185 µg/plate	24	16	8	12	46	38	103	156	34	52
	22	17	1	19	34	54	127	147	42	28
	20	15	12	24	32	68	141	149	46	36
Mean	22	16	7	18	37	53	124	151	41	39
StDev	2	1	6	6	8	15	19	5	6	12
556 µg/plate	29	22	6	10	35	48	120	154	24	40
	25	11	5	18	28	49	126	132	29	42
	36	24	7	17	35	54	127	153	35	42
Mean	30	19	6	15	33	50	124	146	29	41
StDev	6	7	1	4	4	3	4	12	6	1
1667 µg/plate	17	19	10	20	29	73	142	153	31	55
	25	24	7	18	42	49	129	153	29	43
	28	14	17	22	28	80	153	173	18	41
			SLD							
Mean	23	19	11	20	33	67	141	160	26	46
StDev	6	5	5	2	8	16	12	12	7	8
5000 µg/plate	13	24	6	20	37	79	119	168	22	28
	19	25	12	18	23	68	98	167	34	41
	24	26	14	14	28	74	108	155	25	47
			SLD	*SLD*						
Mean	19	25	11	17	29	74	108	163	27	39
StDev	6	1	4	3	7	6	11	7	6	10
Pos. Control	492	500	3142	238	877	1704	667	2157	334	1217
	550	595	2353	276	910	2072	687	2220	334	1333
	537	552	2439	341	739	1996	694	2198	362	1589
Mean	526	549	2645	285	842	1924	683	2192	343	1380
StDev	30	48	433	52	91	194	14	32	16	190

Mean Average number of revertants per plate
StDev Standard deviation
S9 Liver homogenate from rats treated with aroclor
Pos. Control Positive control; see text for actual concentrations of reference mutagens
SLD Slightly less dense background lawn of bacterial growth

Appendix 2
Characteristics of *Salmonella typhimurium* and *Escherichia coli* strains

Frozen stocks of each strain are checked for histidine or tryptophan requirement and for sensitivity to ampicillin, crystal violet and UV radiation at the date of freezing. The results for the stocks used in the present assays are:

Strain	Stock date	Additional mutations[1]				
		rfa	uvr	R-factor	his	trp
TA 1535	26 October 2007	-	-	-	-	NT
TA 1537	15 August 2007	-	-	-	-	NT
TA 98	15 August 2007	-	-	+	-	NT
TA 100	15 August 2007	-	-	+	-	NT
WP2 *uvrA*	15 August 2007	±	-	-	NT	-

[1]rfa : - = sensitive to crystal violet; ± = weak sensitive to crystal violet
uvr : - = sensitive to UV radiation
R-factor : - = sensitive to ampicillin; + = resistant to ampicillin
His : - = requires histidine
Trp : - = requires tryptophan
NT : not tested

- 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.

Appendix 3
Preparation and characterization of Aroclor 1254-induced rat liver homogenate

The batch of S9 dated 28 January 2009 was used for the bacterial reverse mutation test and 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 ^{o}C.

The S9 was checked for sterility. The protein and cytochrome P-450 content of the S9 fraction were determined according to the method published by Rutten et al. (1987).

Results batch 28 January 2009
The protein content of the batch was 21.8 g/litre.
The cytochrome P450 content of the batch was 22.6 µmol/litre.
The batch contained 1.04 µmol cytochrome P450 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-450 and protein determinations in rat liver microsomes. Reinvestigation of assay conditions." Arch. Toxicol. 61: 27-33.

Appendix 4
Acceptable ranges for negative and positive control data

Strain	**revertant colonies per plate (with and without S9-mix): negative control, acceptable range**
TA 1535	10 – 75
TA 1537	4 – 40
TA 98	20 – 95
TA 100	100 – 230
WP2 *uvrA*	18 – 60

Strain	**in the absence of the S9-mix**	**Minimum Mutation Ratio**	**in the presence of the S9-mix**	**Minimum Mutation Ratio**
TA 1535	sodium azide: 1.0 µg/plate	5	2-aminoanthracene: 2.0 µg/plate	5
TA 1537	9-aminoacridine: 80.0 µg/plate	10	Benzo(a)pyrene: 4.0 µg/plate	3
TA 98	2-nitrofluorene: 2.0 µg/plate	5	2-aminoanthracene: 2.0 µg/plate	3
TA 100	sodium azide: 1.0 µg/plate	3	2-aminoanthracene: 2.0 µg/plate	3
WP 2 *uvrA*	N-ethyl-N-nitrosourea: 100 µg/plate	3	2-aminoanthracene: 80 µg/plate	5

Mutation Ratio: number of induced revertants/number of control revertants.

Appendix 5
Historical data of bacterial reverse mutation test

Bacterial viability
Number of viable bacterial cells at various time points after the start of the culture as determined by plating appropriate dilutions.

Strain	Viable cell count at several hours after start of culture[#]									
	2	3	4	5	6	7	8	9	10	11
Assay 1, November 11, 1994										
TA 1535	2.8	3.1	2.9	4.5	4.3	6.0	5.4	6.5	6.9	
TA 1537	1.2	2.6	3.5	4.3	4.5	5.8	6.3	5.8	6.7	
TA 98	0.9	2.3	3.4	4.1	5.2	4.9	7.0	6.0	7.2	
TA 100	1.9	2.5	2.5	2.8	3.4	4.4	5.7	5.9	5.5	
WP2*uvrA*	7.5	5.3	8.6	11	12	13	12	13	12	
Assay 2, November 24, 1995										
TA 1535								5.9	8.1	8.4
TA 1537								6.5	5.7	7.3
TA 98								4.1	6.1	6.6
TA 100								7.5	6.8	7.7

[#] number of viable cells per ml (x 10^8)

False negative responses
Reproducibility between first and second assay with respect to predicting overall absence of mutagenicity. Data from studies until 2008. Overview from January 2009.

Mutagenicity: overall judgement	-			+	+		-
Mutagenicity: judgement First / second assay	- / -	- / +	+ / -	- / +	+ / +	+/-	+/- (second assay according to 'treat and plate')
Number of studies	138	3	19	0	17	0	10

Vehicle controls
Demonstration of the absence of mutagenic effects for several commonly used vehicles.
Data from assays conducted between May 2002 - December 2008

	Mean ± SD number of revertants (number of assays)				
Strain	Methanol/ ethanol	saline	water	DMSO	PBS
without S9-mix					
TA 1535	28 ± 3 (9)	26 ± 9 (17)	26 ± 6 (36)	24 ± 5 (66)	22 ± 4 (9)
TA 1537	15 ± 4 (9)	14 ± 2 (17)	17 ± 4 (36)	16 ± 5 (63)	13 ± 3 (9)
TA 98	53 ± 27 (10)	34 ± 5 (17)	36 ± 6 (36)	35 ± 7 (74)	32 ± 6 (9)
TA 100	163 ± 18 (9)	157 ± 25 (17)	168 ± 36 (35)	148 ± 31 (69)	134 ± 10 (9)
WP2 *uvrA*	36 ± 5 (9)	36 ± 7 (17)	37 ± 8 (34)	35 ± 6 (65)	38 ± 5 (7)
with S9-mix					
TA 1535	32 ± 5 (9)	21 ± 4 (17)	21 ± 5 (36)	19 ± 4 (66)	20 ± 5 (8)
TA 1537	19 ± 2 (9)	19 ± 4 (17)	21 ± 5 (36)	21 ± 5 (63)	18 ± 6 (8)
TA 98	70 ± 11 (11)	53 ± 7 (17)	59 ± 11 (36)	54 ± 9 (74)	47 ± 4 (8)
TA 100	173 ± 21 (9)	166 ± 30 (17)	176 ± 31 (35)	148 ± 31 (69)	141 ± 21 (8)
WP2 *uvrA*	40 ± 6 (9)	40 ± 7 (17)	42 ± 7 (34)	38 ± 6 (65)	38 ± 10 (7)

Historical negative control (vehicle) data from studies started in May 2002 to December 2008, all vehicles together.

Strain	Number of revertants per plate mean ± standard deviation; range; (number of assays)					
	without S9-mix			with S9-mix		
TA 1535	25 ± 6	13-53	(138)	21 ± 5	13-42	(137)
TA 1537	16 ± 4	6-30	(135)	20 ± 5	9-34	(134)
TA 98	36 ± 10	20-127	(147)	56 ± 10	29-96	(147)
TA 100	155 ± 32	82-225	(140)	158 ± 32	90-226	(139)
WP2 *uvrA*	36 ± 7	19-56	(133)	40 ± 7	27-58	(133)

Historical positive controls

Overview historical positive control data from studies between May 2002 and December 2008.

Strain	Compound[@]	Mutation Ratio[#] mean ± standard deviation; range (number of assays)		
without S9-mix				
TA 1535	NaN_3, 1 µg/plate	30 ± 24	12-297	(138)
TA 1537	9-AA, 80 µg/plate	213 ± 87	43-494	(135)
TA 98	2-NF, 2 µg/plate	41 ± 16	16-98	(147)
TA 100	NaN_3, 1 µg/plate	6 ± 1	3-11	(140)
WP2 *uvrA*	ENU, 100 µg/plate	8 ± 6	2-53	(133)
with S9-mix				
TA 1535	2-AA, 2 µg/plate	28 ± 11	11-69	(137)
TA 1537	BP, 4 µg/plate	14 ± 5	5-35	(134)
TA 98	2-AA, 2 µg/plate	23 ± 10	6-80	(147)
TA 100	2-AA, 2 µg/plate	14 ± 4	5-27	(139)
WP2 *uvrA*	2-AA, 80 µg/plate	35 ± 9	10-63	(133)

[#] Mutation Ratio: number of induced revertants/number of control revertants

[@] NaN_3 = natrium azide
ENU = N-nitroso N-ethylurea
2-AA = 2-aminoanthtacene
9-AA = 9-aminoacridine
BP = benzo(a)pyrene
2-NF = 2-nitrofluorene

Appendix 6 Analysis data (provided by sponsor)

TNO Dispense reference
Oct 13.2

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

P021109

DSM

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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	60185	% (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

#55271.2

Appendix 7 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