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Report number: 16.326

Date: 14-10-2003

BACTERIAL REVERSE MUTATION TEST WITH ENZYME PREPARATION FROM *ASPERGILLUS NIGER* GEP 44

CRO report number V5005/10

Author(s): C.A.M. Krul

Department: TNO Nutrition and Food Research, Zeist

Experimental work:

Keywords:
GLP
Toxicology
Mutagenicity
Ames
Endopro
GEP44
Aspergillus niger

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Signature author: Date manuscript 14 October 2003 TNO Nutrition and Food Research, Zeist, The Netherlands	Signature department manager:

TNO Nutrition and Food Research

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

Location Zeist
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

TNO report

V 5005/10

Bacterial reverse mutation test with Enzyme preparation from Aspergillus niger GEP 44

Date	14 October 2003
Authors	Ms. Dr. C.A.M. Krul
At request of	DSM Food Specialties PP 001-0490 A.Fleminglaan 1 P.O. Box 1 2600 MA Delft The Netherlands
TNO Project number	010.45264/01.41
TNO Study number	5005/10
Sponsor Study code	-
Status report	Final
Previous version	-
Number of pages	24
Number of tables	1
Number of figures	-
Number of annexes	5
Number of appendices	-

Summary

1. The test substance Enzyme preparation from Aspergillus niger GEP 44 was examined for mutagenic activity in the bacterial reverse mutation test using the histidine-requiring *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, the tryptophan-requiring *Escherichia coli* strain WP2 *uvrA*, and a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix).

2. The test substance was diluted in milli-Q water. Two bacterial reverse mutation tests were performed with all strains in the first assay, and with TA 1535, TA 1537 and TA 98 in the second assay, in the absence and the presence of S9-mix with five different concentrations of the test substance, ranging from 62 - 5000 μg/plate in the first assay and from 312 – 5000 μg/plate in the second assay. Negative controls (milli-Q water) and positive controls were run simultaneously with the test substance.

3. Enzyme preparation from Aspergillus niger GEP 44 was not toxic as was evidenced by the absence of a dose-related decrease in the mean number of revertant colonies compared to the negative controls.

4. In the first assay, in strain TA 1535 and TA 1537 a more than two-fold increase in reverse mutations was observed, and in TA 98 an increase was observed, although this never reached a two-fold increase above the negative control. Additionally an increase in background lawn was observed on the plates, indicating the presence of histidine or proteinous compounds in the test substance solution. In order to exclude false positive results, for these strains a second assay according to the treat-and-plate method was chosen.
   In the second assay in both the absence and the presence of S9-mix, Enzyme preparation from Aspergillus niger GEP 44 did not cause a more than two-fold increase in the mean number of revertant colonies appearing in the test plates compared to the background spontaneous reversion rate observed with the negative control.

5. 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.

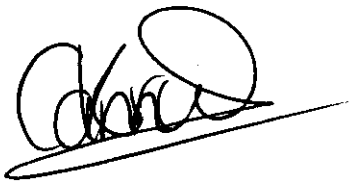
6. It is concluded that Enzyme preparation from Aspergillus niger GEP 44 was not mutagenic under the conditions employed in this study.

Contents

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 Nutrition and Food Research were carried out in compliance with the current OECD Principles of Good Laboratory Practice (Organisation for Economic Co-operation and Development, Paris, ENV/MC/CHEM (98) 17).

The OECD Principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan.

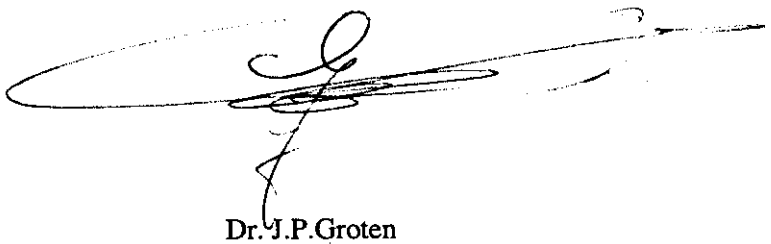


14 October 2003

Ms. Dr. C.A.M. Krul
Study Director

Date

Approved by:



14 October 2003

Dr. J.P.Groten
Head, Department of Biomolecular Sciences

Date

Quality Assurance Statement

On: Bacterial reverse mutation test with Enzyme preparation from Aspergillus niger GEP 44
Report Number: V5005/10
Date: 14 October 2003

The protocol was audited as follows:

Date of audit	Date of report
4 July 2003	4 July 2003

This type of short-term study is carried out frequently and the Quality Assurance Unit does not audit the experimental phase of each individual study; the processes involved are audited at regular intervals according to a predetermined schedule. The audits of experimental phases listed below were carried out of this type of study during the period relevant to this particular study.

Date of audit	Date of report
20 May 2003	20 May 2003
23 May 2003	23 May 2003

This report was audited as follows:

Date of audit	Date of report
2 October 2003	7 October 2003
20 October 2003	20 October 2003

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 the management on the dates indicated.

M.Th.A. Wolters, BSc.
Quality Assurance Auditor

Date: 20 October 2003

GLP compliance monitoring unit statement

[redacted]

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 88/320/EEC the conformity with the OECD Principles of GLP was assessed on 4-7 and 17-21 February and19-20 June 2003 at

TNO Nutrition and Food Research
Utrechtseweg 48, 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 studies, mutagenicity studies, studies on behaviour in water, soil and air, bio-accumulation, residue studies, and analytical and clinical chemistry.

The Hague, 01 July 2003

NEDERLAND STAATSTOEZICHT OP DE VOLKSGEZONDHEID

R. Jaspers, PhD
GLP Compliance Monitoring Department

Inspectorate for Health Protection and Veterinary Public Health
Ministry of Health, Welfare and Sport

Testing facility

TNO Nutrition and Food Research
Department of Biomolecular Sciences
P.O. Box 360, 3700 AJ ZEIST, the Netherlands
Telephone +31 30 69 44 144
Telefax +31 30 69 60 264
Visitors address: Utrechtseweg 48, Zeist, the Netherlands

Contributors

Study director:	Ms. Dr. C.A.M. Krul
Technician:	Ms. G.C.D.M. Bruijntjes-Rozier
	Ms. M.J.M. van den Wijngaard
Management:	Dr. J.P. Groten

Sponsor

Sponsor:	DSM Food Specialties A. Fleminglaan 1 PP001-490 P.O. Box 1 2600 MA Delft The Netherlands
Study monitor:	Ms. Dr. Ir. M.E.M. Kuilman-Wahls

1 Objective

The objective of this study was to provide data on the mutagenic activity of Enzyme preparation from Aspergillus niger GEP 44 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).

This study was conducted in accordance with the following guideline:

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

The study was conducted according to a protocol, entitled: 'Bacterial reverse mutation test with Enzyme preparation from Aspergillus niger GEP 44', which was approved by the study director on 27 June 2003.

2 Experimental

2.1 Test substance

Name	Enzyme preparation from Aspergillus niger GEP 44
Chemical name	enzyme protein
Other name	endoprotease, prolyl oligopeptidase
CAS Number	72162-84-6
Purity	25.9% dry matter
Lot number	JLL 03006 IDF
Appearance	brown liquid
Date of received	27 June 2003
Expiry date	March 2004
Storage conditions	< -18 °C
Supplier	DSM Food Specialties
TNO test substance no.	030087

A test substance information sheet, enzyme-safety data sheet and a certificate of analysis with information concerning physico-chemical properties and purity of the test substance were provided by the sponsor.
Characterization and verification of the test substance identity and properties are the responsibility of the sponsor.

2.2 Other chemicals

Nicotinamide adenine dinucleotide phosphate, disodium salt (NADP) and D-glucose-6-phosphate, disodium salt (G-6-P) were obtained from Boehringer Mannheim GmbH, Germany; 9-aminoacridine (9-AA), N-ethyl-N-nitrosourea (ENU), dimethylsulphoxide (DMSO) and benzo(a)pyrene (B[a]P) from Sigma Chemical Company, St. Louis, USA; and 2-nitrofluorene (2-NF), 2-amino-anthracene (2-AA), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and sodium azide (NaN_3) from Aldrich, Brussels, Belgium.

2.3 Origin of strains

The *Salmonella typhimurium* strains, has been originally obtained from Dr. B.N. Ames (University of California Berkeley, U.S.A.) and the strain *Escherichia coli* WP2 *uvrA*, has been 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 Annex 1).

2.4 Metabolic activation system (S9-mix)

The S9 liver homogenate used in study was prepared as described in Annex 2.

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), 46 mM NaCl, and S9 10 %. The S9-mix was kept on ice until use.

2.5 Time schedule

The study was conducted between 8 July 2003 and 8 August 2003.

2.6 Mutagenicity assay

Two mutagenicity assays were performed. For the first assay, 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 used. 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.
Since in the first assay a more dense background lawn was observed together with higher levels of revertants in strain TA 98 in the presence of S9-mix and in TA 1535 and 1537 in the absence and presence of S9-mix, a second assay was applied for those strains according to the "treat and plate" method.

MilliQ water was chosen as vehicle. Just before use, the test substance was diluted in the vehicle at 50 mg/ml, assuming a dry matter content of 25.9%. A light brown solution was obtained, which was sterilized by passage through a micropore filter (0.45 μm). Serial 3-fold dilutions in milliQ water were prepared, and in total five concentrations were tested, ranging from 62 to 5000 μg/plate. In the second assay, 2-fold dilutions in milli-Q water were prepared, and in total five concentrations were tested, ranging from 312 to 5000 μg/plate. The sterility of the test substance solutions were checked on minimal glucose agar plates.
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 to be 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

positive control substances for ' treat and plate' method		
Strain	**in the absence of the S9-mix**	**in the presence of the S9-mix**
TA 1535	1-methyl-3-nitro-1-nitrosoguanidine: 5.0 μg/plate	2-aminoanthracene: 2.0 μg/plate
TA 1537	9-aminoacridine: 10.0 μg/plate	2-aminoanthracene: 2.0 μg/plate
TA 98	2-nitrofluorene: 2.0 μg/plate	2-aminoanthracene: 2.0 μg/plate

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 for the *S. typhimurium* strains, and supplemented with 0.05 mM tryptophane for the *E. coli* WP2 *uvrA* strain), maintained at ca. 46 °C, were added subsequently: 0.1 ml of a fully grown culture of the appropriate strain, 0.1 ml of the appropriate test

substance solution, or of the negative or positive control substance solution, and 0.5 ml S9-mix for with metabolic activation or 0.5 ml sodium phosphate 100 mM (pH 7.4) for 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 °C for 48-72 hours. Subsequently, the his^+ and trp^+ revertants were counted. The background lawn of bacterial growth was examined microscopically to determine any growth-diminishing or growth-enhancing effects by the test substance, if a two-fold or greater increase in the mean number of his^+ or trp^+ revertants was observed. Cytotoxicity is defined as a reduction in the number of revertant colonies and/or a clearing of the background lawn of bacterial growth.

For TA 1535, TA 1537 and TA 98 in the second assay, the treat and plate procedure was used. Briefly, the procedure was as follows: to 2 ml nutrient broth was added 0.5 ml of the bacterial suspension, 0.5 ml of the appropriate dilution of the test material or of the negative control (vehicle) or of the positive control solution, and 2 ml of the S-9 mix for with metabolic activation or 2 ml sodium phosphate 100 mM (pH 7.4) for without metabolic activation. The mixtures were incubated for 3 hours at ca. 37 °C while shaking. Thereafter, the mixtures were centrifuged for 10 min at 3000 rpm, the bacterial pellets were resuspended in 5 ml saline and centrifuged again. Finally, the pellets were resuspended in 0.5 ml saline. Of the resulting bacterial suspensions, 0.1 ml was added to 2 ml molten top agar, and the mixture was poured onto minimal glucose agar plates. All determinations were made in triplicate. The plates were incubated for 48-72 hours at ca. 37 °C.

2.7 Evaluation of test results

The mutagenicity study is considered valid if the mean colony counts of the control values of the strains are within acceptable ranges, if the results of the positive controls meet the criteria for a positive response (all as recorded in Annex 1), and if no more than 5 % of the plates are lost through contamination or other unforeseen events.

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 is concentration-related increased or if a reproducible two-fold or more increase is observed compared to that on the negative control plates.

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.

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

Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base 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.

No statistical analysis was performed.

Both numerical significance and biological relevance are considered together in the evaluation.

Historical data on the bacterial reverse mutation tests, including data on positive and negative controls, are presented in Annex 4.

2.8 Retention of records, samples and specimens

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study, will be retained in the archives of the TNO Nutrition and Food Research for a period of at least 15 years after submission of the final report.

2.9 Deviations from the protocol

No deviations occurred while conducting the study.

3 Results and discussion

The results of the main bacterial reverse mutation test are shown in Table 1 and 2.

The test substance was diluted in milli-Q Water. The sterility of the test substance was checked and the test substance was sterile. Two bacterial reverse mutation tests were performed with all strains in the first assay, and with TA 1535, TA 1537 and TA 98 in the second assay, in the absence and the presence of S9-mix with five different concentrations of the test substance, ranging from 62 - 5000 μg/plate in the first assay and from 312 – 5000 μg/plate in the second assay. Negative controls (milli-Q water) and positive controls were run simultaneously with the test substance.

Enzyme preparation from Aspergillus niger GEP 44 was not toxic, as was evidenced by the absence of a dose-related decrease in the mean number of revertant colonies compared to the negative controls.

In the first assay, in strain TA 1535 both in the absence and presence of S9-mix a dose related increase in reverse mutations was observed. This increase was more than two-fold at 5000 μg/plate in the presence of S9-mix. In strain TA 1537 a dose related increase in reverse mutations was observed in the absence of S9-mix and this increase was more than two-fold at 1667 μg/plate. In the presence of S9-mix a more than two fold increase was observed at 556 μg/plate. In strain TA 98 a dose related increase was observed in the presence of S9-mix, although this never reached a two-fold increase above the negative control. Additionally an increase in background lawn was observed at 556-5000 μg/plate on the plates of all strains, except for strain TA100 this increase was observed only at the highest dose level, indicating the presence of histidine or proteinous compounds in the test substance solution. In order to exclude false positive results, for these three strains a second assay according to the treat-and-plate method was performed.
In the second assay in both the absence and the presence of S9-mix, Enzyme preparation from Aspergillus niger GEP 44 did not cause a more than two-fold increase in the mean number of revertant colonies appearing in the test plates compared to the background spontaneous reversion rate observed with the negative control.

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.

4 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 from Aspergillus niger GEP 44 was not mutagenic under the conditions employed in this study.

5 References

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Tables

Table 1: Bacterial reverse mutation test with Enzyme preparation from Aspergillus niger GEP 44 (first assay)

		TA 1535		TA 1537		TA 98		TA 100		E.coli	
Dose		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0 µg/plate		25	11	13	18	37	49	162	191	48	40
		23	18	18	16	41	65	159	178	31	42
		32	16	18	14	38	73	150	160	32	41
	Mean	27	15	16	16	39	62	157	176	37	41
	StDev	5	4	3	2	2	12	6	16	10	1
62 µg/plate		25	12	20	34	34	83	186	178	38	35
		14	20	13	26	49	71	169	171	48	43
		20	19	16	29	44	74	163	40	47	38
	Mean	20	17	16	**30**	42	76	173	130	44	39
	StDev	6	4	4	4	8	6	12	78	6	4
185 µg/plate		20	14	12	31	49	62	161	138	53	47
		26	25	22	25	44	88	147	173	49	34
		24	19	8	20	61	97	172	198	35	52
	Mean	23	19	14	**25**	**51***	**82**	160	170	46	44
	StDev	3	6	7	6	9	18	13	30	9	9
556 µg/plate		40	28	31	47	55	96	166	210	56	65
		35	***	18	24	35	64	157	171	**47**	53
		32	30	41	24	53	80	165	202	48	73
	Mean	**36**	**29**	**30**	**32**	**48***	**80**	163	194	**50**	**64**
	StDev	4	1	12	13	11	16	5	21	5	10
1667 µg/plate		37	32	24	32	64	96	180	186	44	42
		36	35	37	26	38	83	159	174	34	58
		37	17	43	19	56	112	161	171	36	52
	Mean	**37**	**28**	**35**	**26**	**53***	**97**	167	177	**38**	**51**
	StDev	1	10	10	7	13	15	12	8	5	8
5000 µg/plate		50	34	20	22	53	103	202	241	43	47
		60	44	11	26	50	115	204	222	46	41
		46	38	18	31	52	131	190	219	58	50
	Mean	**52**	**39**	**16***	**26**	**52***	*116*	**199**	**227**	**49**	*46*
	StDev	7	5	5	5	2	14	8	12	8	5
Pos. Control		528	928	5245	325	1607	2043	673	3508	203	1613
		571	926	5042	411	1518	2250	705	3173	304	1599
		532	871	4702	360	1459	2296	729	3520	324	1344
	Mean	544	908	4996	365	1528	2196	702	3400	277	1519
	StDev	24	32	274	43	75	135	28	197	65	151

Mean	Average number of revertants per plate
StDev	Standard deviation
S9	Liver homogenate from rats treated with aroclor
***	not counted due to contamination
bold	slightly more dense background lawn of the bacterial growth
*	slightly more dense background lawn of the bacterial growth partly on the agar plates
italic	more dense background lawn of the bacterial growth
Pos. Control	Positive control; for actual concentrations of reference mutagens see text

Table 2: Bacterial reverse mutation test with Enzyme preparation from Aspergillus niger GEP 44 (second assay)

Dose		TA 1535 -S9	TA 1535 +S9	TA 1537 -S9	TA 1537 +S9	TA 98 -S9	TA 98 +S9
0 µg/plate		16	23	17	17	41	26
		28	18	13	7	24	30
		20	16	10	20	23	30
	Mean	21	19	13	15	29	29
	StDev	6	4	4	7	10	2
312 µg/plate		10	23	10	16	26	43
		24	20	6	5	32	48
		23	22	10	12	22	42
	Mean	19	22	9	11	27	44
	StDev	8	2	2	6	5	3
625 µg/plate		22	29	11	13	35	43
		20	11	16	7	34	46
		24	34	8	17	20	38
	Mean	22	25	12	12	30	42
	StDev	2	12	4	5	8	4
1250 µg/plate		19	26	17	13	20	38
		22	29	8	12	23	38
		19	25	12	13	32	30
	Mean	20	27	12	13	25	35
	StDev	2	2	5	1	6	5
2500 µg/plate		26	20	7	12	20	40
		29	23	13	22	30	46
		26	26	17	14	30	28
	Mean	27	23	12	16	27	38
	StDev	2	3	5	5	6	9
5000 µg/plate		30	22	12	11	36	28
		24	26	16	13	31	40
		18	31	11	11	22	35
	Mean	24	26	13	12	30	34
	StDev	6	5	3	1	7	6
pos.control		6023	226	649	120	933	2379
		6265	240	645	138	850	2396
		6435	219	625	169	878	2145
	Mean	6241	228	640	142	887	2307
	StDev	207	11	13	25	42	140

Mean — Average number of revertants per plate
StDev — Standard deviation
S9 — Liver homogenate from rats treated with aroclor
pos. control — Positive control; for actual concentrations of reference mutagens see text

Annexes

Annex 1

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	16 May 2002	+	+	+	+	NT
TA 1537	10 July 2001	+	+	+	+	NT
TA 98	10 July 2001	+	+	-	+	NT
TA 100	13 May 2002	+	+	-	+	NT
WP2 *uvrA*	16 May 2002	±	+	+	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 (1975) 347-365.
- Maron, D.M. and B.N. Ames (1983) "Revised methods for the *Salmonella* mutagenicity test." Mutation Res. 113 (1983) 173-215. + ERRATUM, Mutation Res. 113 (1983) 533.

Annex 2
Preparation and characterization of Aroclor 1254-induced rat liver homogenate

The batch of S9 dated 25 September 2002 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-450 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 34.9 g/litre.
The cytochrome P450 content of the batch was 35.7 µmol/litre.
The batch contained 1.02 µmol cytochrome P450 per gram protein.
The sterility check of the batch resulted in 2 colonies per 100 µl S9.

Conclusion
- The batch of S9 of 25 September 2002 meets all of the in-house quality criteria.

References
- Ames, B.N., J. McCann and E. Yamasaki "Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian microsome mutagenicity test." Mutation Res. 31 (1975) 347-365.
- Maron, D.M. and B.N. Ames "Revised methods for the Salmonella mutagenicity test." Mutation Res. 113 (1983) 173-215.
- 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 "Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes. Reinvestigation of assay conditions." Arch. Toxicol. 61 (1987) 27-33.

Annex 3 Acceptable ranges for negative and positive control data

Strain	revertant colonies per plate (with and without S9-mix): negative controle, 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.

Annex 4 Historical data of bacterial reverse mutation tests

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)

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

Strain	Mean ± SD number of revertants (number of assays)			
	methanol	saline	water	DMSO
without S9-mix				
TA 1535	26 ± 1 (2)	33 ± 12 (6)	21 ± 4 (7)	23 ± 3 (6)
TA 1537	17 ± 4 (2)	15 ± 2 (6)	17 ± 4 (7)	21 ± 5 (6)
TA 98	39 ± 11 (2)	37 ± 5 (6)	38 ± 9 (7)	41 ± 3 (14)
TA 100	173 ± 8 (2)	174 ± 13 (6)	184 ± 20 (7)	176 ± 26 (9)
WP2 *uvrA*	35 ± 5 (2)	40 ± 2 (6)	34 ± 7 (7)	39 ± 7 (6)
with S9-mix				
TA 1535	32 ± 2 (2)	23 ± 5 (6)	22 ± 6 (7)	19 ± 4 (6)
TA 1537	20 ± 4 (2)	20 ± 4 (6)	19 ± 5 (7)	23 ± 5 (6)
TA 98	61 ± 3 (2)	58 ± 5 (6)	64 ± 8 (7)	58 ± 8 (14)
TA 100	189 ± 30 (2)	185 ± 16 (6)	187 ± 19 (7)	180 ± 24 (9)
WP2 *uvrA*	44 ± 11 (2)	45 ± 5 (6)	43 ± 8 (7)	44 ± 6 (6)

Historical negative control (vehicle) data from studies started in 1995 to May 2002, all vehicles together.

Strain	Number of revertants per plate mean ± standard deviation; range; (number of assays)					
	without S9-mix			with S9-mix		
TA 1535	23 ± 9	11-71	(287)	18 ± 4	9-44	(287)
TA 1537	12 ± 4	4-27	(289)	15 ± 5	6-30	(292)
TA 98	32 ± 7	20-66	(295)	49 ± 9	25-81	(295)
TA 100	147 ± 19	100-197	(293)	150 ± 17	112-206	(294)
WP2 *uvrA*	27 ± 5	16-46	(183)	29 ± 5	19-44	(182)

Historical positive controls

Overview historical positive control data from studies between 1995 and May 2002.

Strain	Compound[@]	Mutation Ratio[#] mean ± standard deviation; range (number of assays)		
without S9-mix				
TA 1535	Sodium azide, 1 µg/plate	26 ± 10	6-82	(267)
	ENNG, 5 µg/plate	7 ± 2	2-11	(20)
TA 1537	9-AA, 80 µg/plate	110 ± 45	38-318	(289)
TA 98	2-NF, 2 µg/plate	34 ± 13	10-88	(295)
TA 100	Sodium azide, 1 µg/plate	4 ± 1	3-11	(274)
	ENNG, 3 µg/plate	3 ± 0.4	2-3	(19)
WP2 *uvrA*	ENNG, 2 µg/plate	13 ± 5	4-23	(49)
	MNNG, 5 µg/plate	14 ± 5	7-24	(23)
	ENU, 100 µg/plate	10 ± 6	4-42	(111)
with S9-mix				
TA 1535	2-AA, 2 µg/plate	29 ± 11	8-65	(287)
TA 1537	2-AA, 80 µg/plate	20 ± 9	7-43	(61)
	BP, 4 µg/plate	17 ± 6	5-37	(231)
TA 98	2-AA, 2 µg/plate	21 ± 7	6-64	(295)
TA 100	2-AA, 2 µg/plate	10 ± 2	2-16	(294)
WP2 *uvrA*	2-AA, 80 µg/plate	32 ± 11	8-65	(182)

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

[@] ENNG = N-ethyl-N'-nitro-N-nitrosoguanidine
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
ENU = N-nitroso N-ethylurea
MMS = methyl methanesulphonate
2-AA = 2-aminoanthtacene
9-AA = 9-aminoacridine
BP = benzo(a)pyrene
2-NF = 2-nitrofluorene

Annex 5 Certificate of analysis (provided by the sponsor)

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

DSM

F-12202 version 1
Page 1 of 1

CERTIFICATE OF ANALYSIS

Name of the product	Enzyme preparation from *Aspergillus niger* GEP44
Batch no.	JLL 03 006 IDF
Study no.	ANA/03/D68
GLP-archive no.	GLP-0302
Status	ISO 9002
Date of manufacture	March 2003
Date of expiration	March 2004 (provisional)
Active component	Endoprotease
Date of issue	17 June 2003

Analysis type	Method number	Dimension	Result
Endoprotease activity	62186	PPU/g	11.0
Dry matter	60485	% (w/w)	25.9
Ash	60328	% (w/w)	0.7
Total organic solids (TOS)	W-10850NLv2	% (w/w)	25.2
Proteins by Kjeldahl Nitrogen x 6.25	62186	% (w/w)	13.9
Stability in water 21°C, 100 mg/ml	62186	hours	48
Stability in water 21°C, 350 mg/ml	62186	hours	48
Stability in water 21°C, undiluted	62186	hours	48
Stability in water 4°C, 100 mg/ml	62186	days	7
Stability in water 4°C, 350 mg/ml	62186	days	7
Stability in water 4°C, undiluted	62186	days	7

Signature Study Director: P.P.J.M. Snuverink

Date: 17-06-2003 18-06-03

Remarks (if any):