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Research Section

Safety evaluation of a xylanase expressed in *Bacillus subtilis*

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Abstract

A programme of studies was conducted to establish the safety of a xylanase expressed in a self-cloned strain of *Bacillus subtilis* to be used as a processing aid in the baking industry. To assess acute and subchronic oral toxicity, rat feeding studies were conducted. In addition, the potential of the enzyme to cause mutagenicity and chromosomal aberrations was assessed in microbial and tissue culture in vitro studies. Acute and subchronic oral toxicity was not detected at the highest dose recommended by OECD guidelines. There was no evidence of mutagenic potential or chromosomal aberrations. Furthermore, the organism used for production of the xylanase is already accepted as safe by several major national regulatory agencies.

Keywords: Xylanase; Hemicellulase; *Bacillus subtilis*; Safety; Toxicity

1. Introduction

Xylanases are xylan-hydrolysing enzymes that occur naturally and are widespread in plants and microorganisms. The systematic name of the xylanase that is the subject of this article is 1,4-β-D-Xylan xylanohydrolase. It has the CAS No. 9025-57-4 (xylanase, endo-1,4) and the EC classification EC 3.2.1.8. In addition to these classifications, xylanases are further classified as glycosyl hydrolases family 10 (F) and family 11 (G) xylanases (Davies and Henrissat, 1995). The family 10 xylanases differ from the family 11 xylanases primarily with regard to size, and the family 10 xylanases have relatively higher molecular weight than the family 11 xylanases. Xylanases used in food production generally belong to family 11, to which the subject of this article also belongs.

The xylanase discussed herein is intended for use as a processing aid in the milling and baking industry. In these industries xylanases often are referred to as hemicellulases or pentosanases. The term hemicellulase refers to the ability of the enzyme to hydrolyse insoluble non-starch compounds found in flour, whereas the term pentosanase indicates that the substrate for the enzyme, xylan, is composed of pentose monomers.

Kulp (1968) described the beneficial effect of fungal xylanases in baking. Since then the mechanism of action of xylanases has been discussed in several papers (e.g. Gruppen et al., 1993; Rouau, 1993; Rouau and Moreau, 1993; Rouau et al., 1994; Courtin et al., 1999; Lorenzen, 1999) and the use of these enzymes has become well-established within the milling and baking industries. In these industries, xylanases are used as dough strengtheners since they provide excellent tolerance to the dough towards variations in processing parameters and in flour quality. They also significantly increase volume of the baked bread. Until recently only xylanases of fungal origin have been used in baking. In some cases conventional xylanases may cause increased dough stickiness and provoke dough handling problems in industrial bread production. Recently, a new bacterial xylanase which reduces dough stickiness at high dosages was developed. This article presents the results of the safety evaluation of this enzyme.

2. Materials and methods

The gene encoding this xylanase was isolated from *Bacillus subtilis*. The host in which the gene was inserted is also a *B. subtilis*. The host organism is obtained by traditional strain development techniques and origins

Abbreviations: NOEL, no-observed-effect level; TXU, total xylanase units

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from the well-characterised *B. subtilis* 168. As both the donor of the xylanase gene and the host organism belong to the *B. subtilis* species, the recombinant organism is characterised as a self-cloned microorganism or as a microorganism developed by homologous recombination.

The safety evaluation of the xylanase was initiated by Danisco Cultor but the single safety studies were carried out at an independent and experienced contractor.

The test batches of *B. subtilis* xylanase expressed in *B. subtilis* used for all safety studies were produced in the same manner as production scale. The batch designated test material '150699-1' was used in the subchronic oral toxicity study. The batch designated test material '150699-2' was used in the two mutagenicity studies. The batch designated test material '150699-3' was used in the acute oral toxicity test. In the references cited, the xylanase appears under the name *Bacillus xylanase*. Key characteristics of the test materials are given in Table 1.

2.1. Toxicity studies

2.1.1. Acute oral toxicity in rats (Kaaber, 1999)

An acute oral toxicity study was performed in accordance with OECD guidelines (OECD, 1992).

The experiment was performed with Wistar rats of the stock Mol:WIST. The test article (150699-3) suspended in sterile water was administered orally by gavage to rats that had been fasted overnight. After dosing the feed was withheld for a further 3 h. The study was initiated with a sighting study. One female rat was given 2 g enzyme preparation/kg body weight. Only slight signs of toxicity were observed in this rat.

Based on the results of the sighting study, the main study was carried out with one group consisting of five male and five female rats given a dose of 2 g enzyme preparation/kg body weight. The dose volume administered was 10 ml/kg body weight both in the sighting and the main study. On the day of dosing the rats in the main study were 6–7 weeks old and weighed from 139 to 154 g.

Each rat was observed daily for a period of 14 consecutive days. Body weights were recorded on days 1, 2, 3, 4, 8 and 15. All animals both in the pilot study and main study were killed on day 15 and subjected to a gross necropsy examination.

The rats showed no clinical signs, and all survived treatment and the 14-day observation period. The median lethal dose (LD_{50}) was therefore greater than 2 g enzyme preparation/kg body weight.

Table 1
Characteristics of the xylanase test batches

	Test batch	
	150699-1, 150699-2	150699-3
Enzyme activity[a] (TXU/ml)	38,900	100,000
Dry matter content (%)	7.8	

[a] The activity of the xylanase is defined in TXU (total xylanase units). This unit is not defined in exact terms, but relies on a specific assay and a standard enzyme. The assay is based on solubilisation of Xylazyme (azurine-crosslinked wheat arabinoxylan) tablets (Megazyme, Australia) and the subsequent determination of solubilised substrate at 590 nm. The specific activity of the xylanase is 25,000 TXU/mg enzyme protein using this assay.

2.1.2. Subchronic oral toxicity study in rats (Glerup, 1999)

A repeated dose 90-day oral toxicity study was conducted in accordance with OECD guidelines (OECD, 1998). The experiment was performed in 80 SPF Wistar rats (40 males and 40 females) of the stock Mol:WIST. At the start of the acclimatisation period, the rats were 4–5 weeks old and the body weights were in the range of 69–91 g.

The test article (150699-1), suspended in sterile water, was administered orally by gavage at dose levels of 0 (control), 8000, 20,000 and 80,000 TXU (total xylanase units)/kg body weight/day (see Table 1). The dose was given according to the most recent body weight data. The dose volume used was 10 ml/kg body weight. Treatment was performed daily for 92–94 days, depending on the day of necropsy.

All visible signs of ill-health and any behavioural changes were recorded daily. Beginning prior to the start of treatment, detailed clinical observations were performed outside the home cage once per week at similar times. During week 12, all animals were examined once with respect to sensory reactivity to different types of stimuli, grip strength and motor activity.

All animals were weighed on arrival, on the first day of treatment (day 1) and weekly thereafter. Also the weight at necropsy was recorded. The consumption of food was recorded weekly for the two animals in each cage. Terminal haematology and clinical chemistry investigations were performed, and on completion of the treatment period, all animals were subjected to macroscopic examination and organ weight analyses. Microscopic examination of a comprehensive list of tissues was also undertaken.

No clinical signs related to the treatment were seen during the study. No treatment-related changes were seen with respect to stimuli-induced sensory reactivity and open field behaviour. No treatment-related changes in mean body weight and body weight gain were seen. In weeks 1 and 3–6, increased food consumption was seen for the males in group 3. In addition, food consumption was lower than that of controls in week 11 for the males in group 2 and 4. All the findings were statistically significant ($P<0.01$ or $P<0.05$). The body weights and food consumption of the male rats are presented in Table 2. Development in body weight is also illustrated in Fig. 1. As no difference in food consumption was seen among females throughout the study and

as no difference in body weight was seen between the groups for both males and females, these changes were considered incidental.

Clinical chemistry investigations did not reveal any changes. Statistically significant changes in the relative number of lymphocytes for the males were seen during the study. As no difference in the relative number of lymphocytes was seen among females throughout the study, as no difference in the absolute number of lymphocytes was seen, and as no dose dependency was seen, these changes were considered incidental.

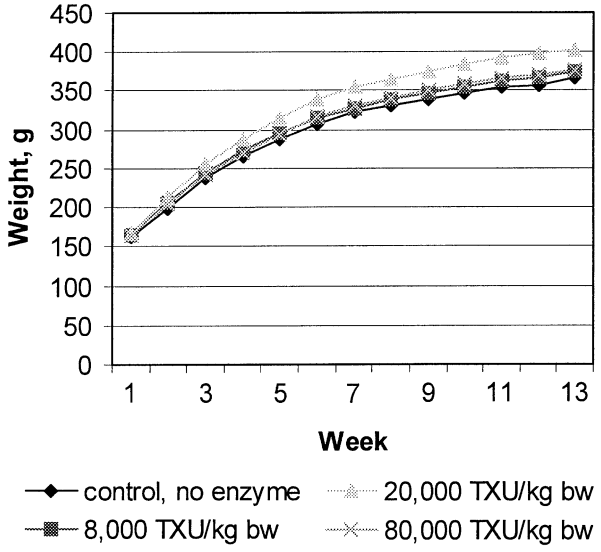

Fig. 1. 90-day oral toxicity study in rats, development in body weight, males.

Macroscopic and microscopic examinations and organ weight analysis gave no indication of adverse responses to treatment.

2.2. Mutagenicity

2.2.1. Bacterial gene mutation (Edwards, 1999a)

An Ames test was conducted in accordance with OECD guidelines (OECD, 1997a). The mutagenic effect of the test enzyme was assessed by exposing five strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535 and TA1537).

The tester strains carry a mutation in the histidine operon. As the test enzyme preparation was predicted to contain amounts of bioavailable histidine which would interfere with an Ames test conducted in the normal way, the test was performed using the 'treat and plate' method to avoid this potential problem.

Some chemicals do not exert a mutagenic effect in this system unless they are activated by mammalian enzymes. The metabolic activation is accomplished by incubating the bacteria together with the test compound and S-9 mix, consisting of rat liver postmitochondrial fraction (liver enzymes) supplemented with salts and co-factors.

Two independent tests were performed. In each test all five strains were used with and without metabolic activation, three plates per dose level. Positive and negative controls with and without metabolic activation were included in both tests. The negative control used was sterile distilled water. The positive control agents used without S-9 mix were sodium azide for TA100 and

Table 2
90-day oral toxicity study in rats, body weight and food consumption, males

Group[a]	Week 1		Week 2		Week 3		Week 4		Week 5	
	BW[b]	FC[b]	BW	FC	BW	FC	BW	FC	BW	FC
1	162.6	146.4	199.2	163.1	237.8	179.2	266.0	182.9	286.7	187.5
2	164.9	151.0	206.3	168.6	244.4	179.4	273.1	182.1	295.2	188.5
3	166.9	161.2**	211.6	178.9	255.1	192.3*	288.0	199.5*	315.1	206.5*
4	164.4	152.7	204.5	167.6	242.0	182.1	270.4	183.9	294.3	186.2
	Week 6		Week 7		Week 8		Week 9		Week 10	
	BW	FC	BW	FC	BW	FC	BW	FC	BW	FC
1	306.5	177.9	323.2	186.3	332.0	176.6	339.5	172.7	347.5	167.7
2	315.2	181.6	328.0	183.9	339.5	177.6	347.2	171.5	354.2	165.8
3	338.8	197.2*	354.6	203.3	364.2	184.5	375.1	186.7	384.5	181.4
4	316.2	178.7	331.7	183.7	341.8	172.9	350.3	174.8	359.3	165.9
	Week 11		Week 12		Week 13		Total 13 weeks			
	BW	FC	BW	FC	BW	FC	BW gain	FC		
1	354.9	200.5	357.4	162.2	367.3	162.7	240.8	2265.7		
2	362.7	167.1**	367.4	162.6	375.0	163.4	248.6	2243.1		
3	393.2	182.0	398.2	181.3	403.3	178.0	276.9	2452.0		
4	366.8	171.6**	370.1	164.3	377.1	166.3	250.6	2250.7		

[a] Group 1: 0 TXU/kg body weight/day, control; group 2: 8000 TXU/kg body weight /day; group 3: 20,000 TXU/kg body weight /day; group 4: 80,000 TXU/kg body weight /day.

[b] BW: body weight (g), mean values of groups of 10 animals; FC: food consumption (g), mean values of groups of five cages with two animals in each cage. *$P<0.05$, vs control group. **$P<0.01$, vs control group.

TA1535, 2-nitrofluorene for TA98 and TA1537, and cumene hydroperoxide for TA102. The positive control agent used with S-9 mix was 2-aminoanthracene.

The bacteria were treated at a range of doses (50–5000 μg/plate). All doses are expressed in terms of dry matter content of the test enzyme preparation. Test-tubes were set up with phosphate buffer or S-9 mix, concentrated bacterial suspension and test solution. After incubation at 37 °C for 30 min under gentle shaking, nutrient broth was added to each test tube and the incubation was continued for a further 3 h.

After this incubation period the bacteria were centrifuged, the supernatant removed and the bacteria resuspended in buffer. After another sedimentation/removal of supernatant, top agar was added to each tube and after mixing the suspensions were spread on selective agar plates. After incubation for 48–72 h at 37°C, the number of colonies was counted. The negative and positive control values were acceptable.

A small, statistically significant increase in the numbers of revertant colonies was observed in the second test with strain TA100 at the highest dose level (5000 μg/plate) in the presence of S-9 mix. This increase is not considered to be biologically significant because it was small and a statistically significant increase was not observed at this test point in the first of the two independent tests (Table 3).

No biologically or statistically significant increases in the numbers of revertant colonies, compared to the negative control values, were observed with any other tester strain in either test. Small, statistically significant decreases in the number of revertant colonies were observed in the second test with strain TA100 at dose levels of 50 and 160 μg/plate in the absence of S-9 mix. These decreases are not considered to be biologically significant because they were not dose related and they were not observed at this test point in the first test (Table 3).

The test enzyme preparation, *B. xylanase*, was concluded to be non-mutagenic in the Ames test.

2.2.2. Chromosome aberrations (Edwards, 1999b)

An in vitro mammalian chromosome aberration test was performed in accordance with OECD guidelines (OECD, 1997b).

Cultures of growing human lymphocytes were exposed to the test substance in the absence and in the presence of metabolic activation (S-9 mix). The negative control cultures were untreated. The positive control cultures were treated with daunomycin in the absence of S-9 mix and cyclophosphamide in the presence of S-9 mix. Duplicate cultures were included for all dose levels and controls with and without S-9 mix. Three independent tests were performed.

The study was performed with primary human lymphocytes obtained from two healthy male donors. One donor provided the lymphocytes for the first test, and the other donor provided the lymphocytes for the second and third tests. Cultures were set up using whole blood.

In the first test, all cultures were treated for 3 h with or without S-9 mix and harvested 20 h after the start of treatment. After treatment the cells were sedimented by centrifugation, resuspended in fresh medium and cultured for a further 17 h until harvest. In the second test, cultures were treated for 20 h in the absence of S-9 mix and 3 h in the presence of S-9 mix; all cultures were harvested 20 h after the start of the treatment. As the test article caused marked toxicity at all concentrations tested in the absence of S-9 mix in the second test, a lower range of concentrations was tested in the third test. In the third test, cultures were treated for 20 h in the absence of S-9 mix only.

A measure of the clastogenic effect of the test substance was obtained by comparing the frequency of chromosomal aberrations observed in the test cultures and in the control cultures. The number of cells at metaphase was counted in 1000 cells from each culture. The mitotic index was calculated as the percentage of cells at metaphase. One hundred metaphases from each culture were examined for the presence of chromosomal aberrations.

Treatment with *B. xylanase* caused dose-related toxicity in each test. Reductions in mean mitotic index to 21–48% of the corresponding negative control value were observed at the highest test concentrations selected for metaphase analysis. Results for mitotic index and number of aberrant metaphases are shown in Table 4.

The frequency of metaphases with chromosome aberrations was within the normal range in all negative control cultures. The positive control materials produced statistically significant increases in the frequency of aberrant metaphases, demonstrating the sensitivity of the test and the efficacy of the S-9 mix.

Treatment with *B. xylanase* did not affect the frequency of polyploid or endo reduplicated metaphases. No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with *B. xylanase* in any test, either in the presence or absence of S-9 mix.

Thus, *B. xylanase* was found to be non-clastogenic in this in vitro mammalian cytogenetic test.

3. Discussion

Toxicity was seen in cell cultures treated at high dose levels for 20 h without S-9 mix in the chromosome aberration test. However, no toxicity was seen either in the acute oral toxicity study or in the subchronic oral toxicity study and no clastogenic effect was seen in the chromosome aberration test.

Table 3
Ames test

Dose	No. of revertant colonies/plate		Dose	No. of revertant colonies/plate	
	Mean[a]	S.D.[b]		Mean	S.D.
Strain TA102, without S-9 mix, test 1			*Strain TA102, with S-9 mix, test 1*		
Control	680.7	61.1	Control	857.7	6.7
5000 μg	635.3	70.4	5000 μg	725.3	54.6
1600 μg	676.0	54.0	1600 μg	698.3	74.5
500 μg	631.0	94.8	500 μg	676.0	123.7
100 μg	680.3	66.8	100 μg	739.0	41.2
50 μg	685.0	94.8	50 μg	766.0	56.2
Cumene, 100 μg	1355.3	241.7	2-Aminoanthracene, 4 μg	1313.7	85.2
Strain TA102, without S-9 mix, test 2			*Strain TA102, with S-9 mix, test 2*		
Control	618.3	23.2	Control	643.0	5.0
5000 μg	604.3	11.2	5000 μg	645.3	1.5
1600 μg	606.3	5.1	1600 μg	639.7	8.5
500 μg	608.7	4.5	500 μg	639.7	2.1
100 μg	603.3	4.2	100 μg	630.3	32.1
50 μg	616.3	27.8	50 μg	633.7	14.2
Cumene, 100 μg	2507.0	113,4	2-Aminoanthracene, 4 μg	1532.0	112.4
Strain TA100, without S-9 mix, test 1			*Strain TA100, with S-9 mix, test 1*		
Control	144.3	15.3	Control	182.7	15.0
5000 μg	171.0	11.4	5000 μg	205.7	31.1
1600 μg	156.7	11.6	1600 μg	175.3	12.7
500 μg	165.0	20.4	500 μg	176.7	15.1
100 μg	126.3	16.0	100 μg	162.0	27.7
50 μg	127.3	9.5	50 μg	156.0	9.8
Sodium azide 1 μg	1738.3	430.8	2-Aminoanthracene, 2 μg	1681.3	28.5
Strain TA100, without S-9 mix, test 2			*Strain TA100, with S-9 mix, test 2*		
Control	113.3	6.4	Control	103.0	7.8
5000 μg	116.0	5.2	5000 μg	117.7*	5.8
1600 μg	112.0	3.6	1600 μg	111.0	8.2
500 μg	108.7	8.7	500 μg	101.3	1.5
100 μg	97.3*	9.5	100 μg	105.7	3.1
50 μg	92.7**	2.1	50 μg	101.7	5.5
Sodium azide 1 μg	656.0	50.1	2-Aminoanthracene, 2 μg	549.0	41.2
Strain TA98, without S-9 mix, test 1			*Strain TA98, with S-9 mix, test 1*		
Control	66.6	6.0	Control	85.3	16.3
5000 μg	61.3	19.1	5000 μg	98.7	41.7
1600 μg	67.7	12.7	1600 μg	99.0	8.2
500 μg	72.0	12.8	500 μg	98.3	6.4
100 μg	62.7	5.1	100 μg	80.0	21.4
50 μg	56.7	7.6	50 μg	88.3	15.0
2-Nitrofluorene, 1 μg	901.3	137.7	2-Aminoanthracene, 2 μg	1135.3	89.3
Strain TA98, without S-9 mix, test 2			*Strain TA98, with S-9 mix, test 2*		
Control	63.3	2.3	Control	69.7	2.1
5000 μg	63.0	2.6	5000 μg	68.7	3.1
1600 μg	64.7	3.2	1600 μg	67.0	1.0
500 μg	63.7	3.8	500 μg	66.7	1.5
100 μg	65.7	5.7	100 μg	64.3	2.9
50 μg	65.0	2.6	50 μg	70.3	6.5
2-Nitrofluorene, 1 μg	1189.7	141.1	2-Aminoanthracene, 2 μg	1692.7	257.5
Strain TA1537, without S-9 mix, test 1			*Strain TA1537, with S-9 mix, test 1*		
Control	24.0	5.6	Control	26.7	5.9
5000 μg	19.3	2.1	5000 μg	26.7	7.0
1600 μg	17.7	2.5	1600 μg	25.0	5.0
500 μg	18.7	3.5	500 μg	30.7	7.2
100 μg	26.7	6.0	100 μg	29.3	4.0
50 μg	17.0	3.6	50 μg	22.7	5.7
2-Nitrofluorene, 1 μg	214.0	6.9	2-Aminoanthracene, 2 μg	266.3	7.5

(*continued on next page*)

Table 3 (*continued*)

Dose	No. of revertant colonies/plate		Dose	No. of revertant colonies/plate	
	Mean[a]	S.D.[b]		Mean	S.D.
Strain TA1537, without S-9 mix, test 2			*Strain TA1537, with S-9 mix, test 2*		
Control	13.0	4.0	Control	18.0	1.0
5000 μg	12.0	1.0	5000 μg	15.3	0.6
1600 μg	10.3	0.6	1600 μg	14.3	2.5
500 μg	15.7	3.2	500 μg	16.0	1.7
100 μg	15.0	3.5	100 μg	17.3	1.5
50 μg	13.0	1.7	50 μg	14.0	3.5
2-Nitrofluorene, 1 μg	581.3	28.6	2-Aminoanthracene, 2 μg	364.7	35.4
Strain TA1535, without S-9 mix, test 1			*Strain TA1535, with S-9 mix, test 1*		
Control	26.3	4.7	Control	21.0	5.2
5000 μg	24.0	8.7	5000 μg	29.7	0.6
1600 μg	21.3	6.0	1600 μg	23.7	1.5
500 μg	23.3	7.1	500 μg	29.3	9.1
100 μg	20.0	6.2	100 μg	25.7	10.0
50 μg	33.0	7.0	50 μg	26.3	8.5
Sodium azide, 1 μg	224.7	19.1	2-Aminoanthracene, 2 μg	239.7	27.3
Strain TA1535, without S-9 mix, test 2			*Strain TA1535, with S-9 mix, test 2*		
Control	12.0	1.7	Control	16.0	5.2
5000 μg	13.7	2.1	5000 μg	19.0	3.5
1600 μg	16.0	1.0	1600 μg	20.3	2.1
500 μg	13.0	2.6	500 μg	20.3	2.5
100 μg	14.3	1.5	100 μg	13.7	5.7
50 μg	16.0	3.6	50 μg	19.3	4.5
Sodium azide, 1 μg	371.7	51.8	2-Aminoanthracene, 2 μg	157.0	34.0

*Statistically significant at 5% level; **statistically significant at 1% level; otherwise, not statistically significant at 5% level (the positive controls were not included in the statistical analysis).

[a] Mean = mean of three plates.

[b] S.D. = standard deviation.

The adverse effect levels from toxicology studies can be used in the calculation of safety margins based on the enzyme's applications, the use levels of the enzyme in the application and human consumption data. A theoretical calculation of human exposure to the xylanase through consumption of bread can be made on the following assumptions:

- The consumption of bakery products is estimated to be 200 g/person/day (OECD, 1991; USDA, ARS, 1997; Euromonitor, 1999).
- Flour content in bakery products is estimated to be 66%.

For a body weight of 70 kg the intake of flour from bakery products is then: 2 g flour/kg body weight/day.

- The maximum dose of xylanase to be expected in bakery products is 40,000 TXU/kg flour.

The maximum estimated daily intake (EDI) of xylanase through bakery products is then 80 TXU/kg body weight/day.

- The maximum dose used in the 90-day study in rats was 80,000 TXU/kg body weight/day giving a no-observed-effect level (NOEL) = 80,000 TXU/kg/day.

Thus the safety margin is: NOEL/EDI = 1000.

Studies of this specific enzyme product have shown a safety margin of 1000 based on a very high expected dose of the xylanase in food. The safety of the xylanase is further supported by the active approval of both *B. subtilis* as a production organism and of xylanase for use in food and feed as described below.

The US FDA in 1999 affirmed carbohydrase and protease from *B. subtilis* as GRAS (generally recognised as safe) (FDA, 1999). In this case, carbohydrase refers to the enzymes amylase and glucanase. The GRAS status was affirmed based on evidence of a substantial history of safe consumption of the preparations in food by a significant number of consumers prior to 1958. The use of the *Bacillus*-derived enzyme preparations in food is GRAS with no limits other than those in current good manufacturing practices (cGMP). Even though the specific xylanase enzyme of this article is not included in the

Table 4
The chromosome aberration test

Test no.	Treatment	Relative mitotic index[a] (%)	No. of aberrant metaphases[b]	Statistical significance[c]
1	*3 h without S-9 mix*			
	Untreated	100	1, 1	
	B. xylanase 1250 μg/ml	94	0, 1	NS[d]
	B. xylanase 2500 μg/ml	90	0, 0	NS
	B. xylanase 5000 μg/ml	21	0, 0	NS
	Daunomycin 0.015 μg/ml	73	8, 9	**[e]
3	*20 h without S-9 mix*			
	Untreated	100	0, 0	
	B. xylanase 156 μg/ml	94	1, 1	NS
	B. xylanase 313 μg/ml	55	0, 0	NS
	B. xylanase 625 μg/ml	48	1, 0	NS
	Daunomycin 0.015 μg/ml	97	10, 7	**
1	*3 h with S-9 mix (2% final concentration of S-9 homogenate in cultures)*			
	Untreated	100	1, 0	
	B. xylanase 1250 μg/ml	99	0, 1	NS
	B. xylanase 2500 μg/ml	76	1, 1	NS
	B. xylanase 5000 μg/ml	26	0, 0	NS
	Cyclophosphamide 6 μg/ml	13	53, 57	**
2	*3 h with S-9 mix (4% final concentration of S-9 homogenate in cultures)*			
	Untreated	100	0, 1	
	B. xylanase 1250 μg/ml	86	1, 0	NS
	B. xylanase 2500 μg/ml	62	0, 0	NS
	B. xylanase 4000 μg/ml	37	1, 0	NS
	Cyclophosphamide 6 μg/ml	18	56, 68	**

[a] Relative mitotic index: mitotic index relative to the untreated control values.
[b] No. of aberrant metaphases: Number of metaphases with structural chromosomal aberrations (excluding gaps), values for two replicate cultures are given, 100 metaphases scored/culture.
[c] Statistical significance: Fisher's exact test of the incidence of aberrant metaphases.
[d] NS = not significant compared to untreated control value, $P > 0.05$.
[e] ** = highly significant compared to untreated control value, $P < 0.01$.

1999 GRAS affirmation, that regulation indicates that the organism *B. subtilis* is regarded to be safe for use in the production of enzymes. In this case the inserted genes are well-characterised and will not compromise the safety of the host organism.

In the UK it is voluntary to have enzymes cleared for use in food. However, two hemicellulase products produced by *B. subtilis* are cleared (MAFF, 1997, 1999).

In the Australia/New Zealand Food Authority (ANZFA) proposal P 188 for processing aids, there is no distinction between enzymes produced by traditional or recombinant strains. The principle in Proposal P 188 is that an enzyme is approved if it is produced by a microorganism approved for production of this specific enzyme and if this microorganism contains additional copies of the gene or genes for the enzyme isolated from other approved microorganisms. In Proposal P 188 *B. subtilis* is approved as both donor and host for the production of hemicellulase (ANZFA, 1999).

In Canada, hemicellulase from *B. subtilis* is approved for use in distillers' mash, liquid coffee concentrate and mash destined for vinegar manufacture according to GMP. The enzyme pentosanase, which is most often identical to hemicellulase and xylanase, is also approved for use in Canada. Pentosanase from *B. subtilis* is approved for use in ale, beer, light beer, malt liquor, porter, stout, corn for degermination, distillers' mash, mash destined for vinegar manufacture, unstandardised bakery products and bread, flour, whole wheat flour. In all these applications the use level of the enzyme is according to GMP (Canadian HPB, 1995–1997).

Hemicellulase produced by self-cloned *B. subtilis* is on the French positive list for enzymes and is approved for use in standard bread and speciality bread and in biscuits (Arrêté, 1998). Hemicellulase is another term for xylanase as previously described. Furthermore, this specific xylanase has been approved by the French Direction Generale de la Concurrence, de la Consommation et de la Repression de Fraude (personal communication, 2001a).

Also, in Denmark, an approval of this specific xylanase has been granted (personal communication, 2001b) based on the guidelines prescribed by the Scientific Committee for Food of the Commission of the European Communities (SCF, 1992).

Hemicellulase from *B. subtilis* is also approved for the use in animal feed in the USA according to the Association of American Feed Control Officials (AAFCO, 1999).

4. Conclusion

The test program prescribed by the Scientific Committee for Food of the Commission of the European Communities (SCF, 1992) has been carried out. The tests were performed at the highest dose levels required by the OECD guidelines for materials of low toxicity. On the basis of the results of the test program it can be concluded that the *B. subtilis* xylanase expressed in *B. subtilis* can be considered a safe processing aid for use in milling and baking.

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