DSM

Product liability dossier

(SCF format) of

xylanase from a

genetically modified strain of

Bacillus subtilis

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2007/XAS-PLI/REG#44319

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4 INTERNAL AUTHORISATION

The product liability dossier (SCF format) of xylanase from a genetically modified strain of *Bacillus subtilis* was authorised by the following persons:

Dossier for approval
of xylanase from B si

Dossier for approval
of xylanase from B si

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II TECHNICAL DATA

1 ACTIVE COMPONENTS

1.1 The principal enzyme activity

Nature provides micro-organisms such as *Bacillus subtilis* with a wide range of different enzyme activities capable of degrading the complex polysaccharides found in the cell walls of plants.

Partial degradation of cereal flour polysaccharides proved to have a positive influence on baking processes. Various commercial microbial enzyme preparations are used to exert this function.

Polysaccharides found in cereal flours are ß-glucans, arabinogalactans and arabinoxylans. Of these, arabinoxylans are the most predominant. Several enzymes are involved in the breakdown of this polysaccharide. The arabinoxylan-degrading enzyme which is considered to be the most important for increasing loaf volume and improvement of dough handling is Endo-1,4-ß-xylanase (further called 'xylanase'), which is the principal activity of the enzyme from *Bacillus subtilis* described in this dossier:

Recommended Name	:	Endo-1,4-ß-xylanase
Systematic Name	:	1,4-ß-D-Xylan xylanohydrolase
IUPAC/IUB Number	:	EC 3.2.1.8
CAS Number	:	9025-57-4

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1.2 The activity of the enzyme preparation

Endo-1,4-ß-xylanase catalyses the hydrolysis of the 1,4-ß-D-xylosidic linkages in the centre of the xylan chains (endo), liberating reducing sugars.

In Annex 1.2.1 the test for measuring xylanase activity as described in the JECFA Compendium of Food Additive Specifications is given. The well-defined and readily available substrate for this method is a water insoluble cross-linked and dyed birch-wood xylan [Xyla-zyme7 tablets, supplied by Megazyme (Aust.) Pty. Ltd].

DSM developed its own method to measure xylanase, which is given in Annex 1.2.2 (relative method) and 1.2.3 (reference method). This method is based upon the amount of xylose residues formed as result of the xylanase activity on the glycosidic bonds in wheat arabinoxylan. The enzyme activity is expressed as New Bakery Xylanase Units (NBXU).

One NBXU is defined as the amount of enzyme that can produce 0.5 mg of xylose residues in the incubation mixture under the assay conditions as given in Annex 1.2.2.

One NBXU corresponds to 1.4 units of the JECFA method.

The purified enzyme protein, when subjected to SDS gel electrophoresis, has a molecular weight of 22,000. This is conform the apparent molecular weight based on the amino acid composition (see Section 2.5). The enzyme has an optimum pH of 5.5 to 6.5, a temperature optimum of 50°C and an inactivation temperature of 60°C (see Annex 1.2.4). This is in agreement with the data found in literature (Bernier et al, 1983 and Murakami et al., 2005)

The specific activity of the pure enzyme is about 1330 NBXU per mg protein.

1.3 Subsidiary enzymatic activities

Apart from xylanase, the enzyme preparation will also contain non-standardised amounts of other enzymes, which are also produced by the micro-organism. These enzymes, such as xylan-1,4-ß-xylosidase and α-L-arabinofuranosidase, are present only in small amounts. They are not needed for the technological function of xylanase in the bakery application.

2 MICROBIAL SOURCE

2.1 Taxonomy of the recombinant production strain

The recombinant xylanase production strain XAS-1 (DS 47435) has been taxonomically identified by experts of the Deutsche Sammlung von Mikoorganismen und Zellkulturen GmbH (DSMZ), Braunschweig (Germany). DSMZ is an independent, internationally recognised laboratory. Presently, another single colony isolate from the same transformant (XASB154-10#18, see Section 2.5) is being used as xylanase production strain, designed XAS-3.

As can been seen in Annex 2.1.1, strain XAS-1 (DS 47435) was identified as *Bacillus subtilis.*

Bacillus subtilis is classified as follows:

Kingdom	:	Procaryotae
Division	:	Bacteria
Order	:	Bacillales (endospore-forming rods and cocci)
Family	:	Bacillaceae
Genus	:	Bacillus
Species	:	*Bacillus subtilis*

2.2 Genealogy of current production strain

The original ancestor of the current production strain is the characteristic ('type strain') *Bacillus subtilis* 168, also called the Marburg strain. This strain, which is autotrophic for tryptophane (genotype: *trp*C2), was originally isolated in 1901 from nature.

The genealogy of the recombinant *Bacillus subtilis* xylanase production strain is schematically shown in Annex 2.2.1.

A detailed description of the construction of the recombinant strain is given in Section 2.4 and 2.5.

2.3 Safety of host and recombinant strain

The species *Bacillus subtilis* and *Bacillus amyloliquefaciens* are difficult to separate on basis of classical phenotypic tests alone. Only after introduction of probabilistic identification methods it was decided that *Bacillus subtilis* and *Bacillus amyloliquefaciens* should be classified as two separate species (Priest et al., 1987). Information on the safety of *Bacillus subtilis* before that time consequently also includes information on the safety of *Bacillus amyloliquefaciens*.

Bacteria related to *Bacillus subtilis* are commonly found in soil and from there they are transferred to various environments such as plants, plant materials, foods, animals and marine and freshwater environments. *Bacillus subtilis*-related organisms grow aerobically at intermediate temperatures and pH. They secrete substantial amounts of hydrolytic enzymes such as amylases and proteases and take part in the breakdown of organic materials.

For several decades, *Bacillus subtilis/amyloliquefaciens* has been safely used in the commercial production of various food enzymes, such as amylase, protease, beta-glucanase and xylanase. Most countries that regulate the use of enzymes, such as the USA, France, Denmark, Australia and Canada, have accepted the use of enzymes from *Bacillus subtilis/amyloliquefaciens* in food applications.

This long experience of industrial use has resulted in a good knowledge of the safety characteristics of *Bacillus subtilis/amyloliquefaciens* and understanding of metabolic reactions.

The long industrial use and wide distribution of *Bacillus subtilis/amyloliquefaciens* in nature has never led to any pathogenic symptoms. Moreover, no case demonstrating invasive properties of the species has been found in the literature (de Boer and Diderichsen, 1991). *Bacillus subtilis/amyloliquefaciens* is therefore generally accepted as a **nonpathogenic** organism.

In 2000 the EU Scientific Committee on Animal Nutrition (SCAN, 2000), issued an opinion that Bacillus strains might be able to produce toxins, even though products from *Bacillus subtilis/amyloliquefaciens* have been used in food for many decades, and there was never any evidence that this species produces toxins. In reaction to the SCAN report the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) initiated a study on specific toxin-analyses with many Bacillus production strains,

including the parental strains of the DSM Bacillus strain lineages. The analyses showed that none of the industrial strain lineages has the potential to produce toxins under the conditions as described in the SCAN opinion (see Annex 2.3.1).Later such analyses were also done on the recombinant *Bacillus subtilis* xylanase production strain XAS-1 and it was confirmed that the strain is not producing any toxins (see Annex 2.3.2). Recently the successor of SCAN, the EFSA FEEDAP panel confirmed that industrial Bacillus enzyme production strains are non-toxicogenic.

The **non-toxicogenicity** of *Bacillus subtilis/amyloliquefaciens* has been confirmed by a large amount of toxicological tests on enzymes derived from the species, including recombinant strains (see Section 9 of this dossier)

The host, *Bacillus subtilis* strain BS154, was declared a suitable host strain for the construction of genetically modified organisms belonging to Group I safe micro-organisms and as GMO obtained by self-cloning (see Annex 2.3.3 and 2.3.4).

The recombinant *Bacillus subtilis* strain XAS-1 differs from its parent BS154 only in the presence of some additional copies of the xylanase (*xyn*A) gene. The Dutch Ministry of the Environment classified strain XAS-1 as a self-cloned micro-organism (see Annex 2.3.5). The French Committee on Genetic Modification has approved strain XAS-1 as group 1, class 1, confinement level 1 (see Annex 2.3.6). Therefore the strain can be used for large scale production of bacterial xylanase in the DSM factory in Seclin, France

Based on the genetic modification performed (see Sections 2.4 and 2.5), there are no reasons to assume that the recombinant production strain should be less safe than the host or its original classical parent. The only difference between the parental strain BS154 and the recombinant production strain XAS-1 is the over-expression of the enzyme xylanase.

The recombinant production strain is asporogenic and at the end of the fermentation, the cells are effectively killed off (see Section 3.2).

2.4 Genetic modification by which the host strain was obtained

The *Bacillus subtilis* host strain BS154 was obtained by a combination of classical mutation and genetic modification. The purpose was to create an asporogenic strain that is deficient in amylase, neutral protease and alkaline protease. The following steps were involved (see also Annex 2.2.1 of Section 2.2):

- *Bacillus subtilis* strain 168 (the Marburg strain, genotype: *trp*C2, see Section 2.2) was treated with UV radiation to give rise to an amylase negative mutant (Yuki,1967). This strain was designated as 1-85 (DS 3225, genotype: *trp*C2, *amy*$^-$).
- Also originally derived from *Bacillus subtilis* strain 168 through a series of classical mutations using UV radiation and transformations with specific vectors, is strain DB104, which is auxotrophic for histidine and deficient in neutral and alkaline protease (genotype: *his*$^-$, *npr*E, Δ*apr*A) (Spizizen, 1957; Okubo & Yanagida, 1968; Sandaie & Marui, 1976; Kawamura & Doi, 1984).
- Strain DB104 was treated with UV radiation to give rise to an asporogenic mutant (genotype: *spo*$^-$, frequency of revertants of $<1 \times 10^{-8}$), designated DB105 (genotype: *his*$^-$, *npr*E, Δ*apr*A, *spo*$^-$).
- The two parental strains DB105 and 1-85 were fused through protoplast fusion techniques creating strain FBS22.2 with the genotype *npr*E, *amy*$^-$, *spo*$^-$.
- This strain was subsequently transformed with a pE194-derived plasmid containing a deleted version of the alkaline protease gene region *apr*A. The biology of pE194 and related Bacillus plasmids, such as pUB110, is very well studied (see Annex 2.4.1). Homologous recombination events directed by the *apr*A flanking regions allowed exact deletion of the *apr*A coding region via integration of this plasmid into of the *apr*A region of the chromosome. Using an essentially similar approach, the neutral protease gene, still weakly expressed in FBS22.2, was deleted creating strain BS154. Both deletions as well as the loss of all plasmid sequences were confirmed by Southern blot analyses.

The final strain *B.subtilis* BS154 thus is deficient in amylase, neutral and alkaline protease and sporulation (genotype: *amy*,$^-$ Δ*npr*E, Δ*apr*A, *spo*$^-$).

2.5 Genetic modification by which the recombinant strain was obtained

The recombinant *Bacillus subtilis* xylanase production strain was obtained by transforming the host strain BS154 with vector pGBB01XAS-10 (see Annex 2.5.1 for the physical map). This vector consists of the following elements:

- The 27 base pairs long strong promoter from the α-amylase gene *amyQ* derived from the closely related species *Bacillus amyloliquefaciens*;
- The 639 base pairs long coding sequence of the *xynA* endo-1,4-β-xylanase gene of *Bacillus subtilis* strain 168. The gene sequence was published by Paice et al (1986) and encodes a precursor protein of 213 amino acids. The mature protein is 185 amino acids long and has an apparent molecular weight of 22 kDa.;
- The 137 base pairs long terminator sequence of the *Bacillus subtilis* strain 168;
- The 3,4 Kb pUB110 plasmid. This approved plasmid (see Annex 2.4.1) naturally occurs in Bacillus and contains a resistance gene for neomycin and bleomycin. The *xyn*A gene was inserted into the mobilisation (*mob*) gene of pUB110.

The complete DNA sequence of the plasmid is given in Annex 2.5.2.

The transformants, containing multiple copies of the pGB01XAS-10 self-replicating plasmid, were identified by their neomycin resistance and analysed for their overproduction of xylanase. From the selected transformant XASB154-10#18 (DS40843) single colonies were isolated. As production strains, the isolates XAS-1 (DS47435) and XAS-3 (DS49678, current production strain) were chosen.

2.6 Genetic stability and gene transfer

The growth, morphology and production characteristics of the recombinant xylanase production strain can easily be maintained under normal laboratory and production conditions. Abnormal colonies can be isolated at very low frequency. This is a normal phenomenon for highly specialised, industrial strains.

The recombinant xylanase production strain stably overproduces the xylanase for over 60 generations as observed by xylanase productivity measurements as well as by analysis of the strain for neomycin resistance, which is indicative of the presence of the plasmid. No experimental data are available regarding the plasmid copy number, but in analogy with other pUB110 derived plasmids this is estimated as 50-100 copies per cell. At the end of the fermentation more than 90% of all analysed colonies are neomycin resistant, indication the stability of the strain.

Enzyme production with genetically modified microorganisms always occurs under containment, which *a priori* minimizes the chances of gene transfer. Moreover, plasmid pUB110 contains an inactivated *mob* gene, due to the insertion of the *xyn*A gene (see Section 2.5). This results in a non-mobilizable genotype of the recombinant xylanase production strain, and therefore transfer of the vector to other bacteria is not possible.

3 MANUFACTURING PROCESS

Xylanase is produced by a controlled submerged fermentation of a selected, pure culture of *Bacillus subtilis* (see Section 2). The production process includes the fermentation process, recovery (downstream processing) and formulation of the product. An overview of the different steps involved is given in Annex 3.0.1.

3.1 Fermentation process

The fermentation process consists of two steps: inoculum fermentation and main fermentation. The whole process is performed in accordance with Good Manufacturing Practice (see Section 7).

Inoculum fermentation:

The inoculum fermentation consist of three pre-culture phases. During the first phase, a vial with a pure culture of *Bacillus subtilis* is suspended in a shake flask with growth medium containing:

- Glucose
- Yeast extract
- Antifoam (Clérol FBA3107).
- Finally, neomycin sulphate is added to the medium.

This first phase inoculum is allowed to grow under agitation (250 rpm) for 12 ±2 hours at 34°C.

Subsequently, 20 ml of the first phase inoculum is transferred aseptically to a fermentor of 6 litres (“puntbus”), containing the same medium. This second phase inoculum is also allowed to grow for 12 ±2 hours at 34°C.

Next, the content of the “puntbus” is transferred aseptically to an inoculum fermentor containing a culture medium consisting of:

- Yeast extract
- Glucose
- Mineral salts
- Antifoam (Clérol FBA3107)

The pH of the medium is controlled with ammonia/phosphoric acid.

The inoculum fermentation is run under the following conditions:

- Temperature : 31 ± 1°C

- Stirring : 160 rpm
- pH : 6.7 ± 0.1
- Aeration : 500 Nm^3/hour[1]
- Overpressure : 0.5 bar

After growth, this second phase inoculum is used to start the main fermentation.

Main fermentation:

Biosynthesis and excretion of xylanase by the bacterial cells occurs during the main fermentation. To produce the enzyme of interest, a submerged, aerobic fed-batch fermentation process is employed. The fermentor is equipped with devices for pH-, temperature-, oxygen- and antifoam control, a top-mounted mechanical agitator and a bottom air sparger.

The fermentation medium has been developed for both optimal growth of the *Bacillus subtilis* production strain and optimal production of xylanase. It contains the following food grade or equivalent components:

- yeast extract
- glucose syrup
- mineral salts (calcium chloride, potassium chloride, zinc sulphate and manganese sulphate)

Foaming is prevented by controlled addition of Clérol FBA3701. Ammonia and phosphoric acid are used for pH adjustment.

During growth, the fermentor is continuously fed by aseptically introducing sterilised fermentation medium according to a pre-set feeding programme.

Depending on the volume of the fermentor, the conditions during the main fermentation may vary as follows:

- Temperature	37°C ± 1°C
- Aeration[2]	4000 - 6000 Nm^3/h
- Counter pressure	1.0 – 1.2 bar
- Stirring	speed of stirrer to avoid oxygen limitation 100 ± 10 rpm

[1] 1 Nm^3/h is the amount of air in m^3 (as measured under Normal atmospheric conditions: 1 mole O_2 is equivalent to 22.4 l gas that is introduced into the fermentor per hour

[2] aeration (+ stirring) to achieve a sufficiently high dissolved oxygen concentration (DOC). The actual values of aeration and stirring depend on the architecture of the fermentor used.

- pH 7.5 ± 0.1

Growth of the production organism and increase of enzyme production in the main fermentation are regularly monitored by aseptic collection of samples that are analysed for xylanase activity and microbial purity. The fermentation is stopped after maximally 48 hours.

3.2

Downstream processing

The downstream processing consists of the following steps:

- ending of the fermentation and killing of the production organism
- mash flocculation
- removal of cell material
- ultrafiltration
- formulation

End of the fermentation and killing of the production organism:

After the fermentation has been stopped the broth is adjusted to pH 7.0 ± 0.2 with sodium hydroxide.

The bacterial cells are lysed by incubating them with 6.5 g lysozyme/kg broth during 4 hours. Subsequently, a so-called MEP solution (consisting of 125 g/l methyl paraben and 50 g/l ethyl paraben in 1,2-propanediol) is added (40 kg/tonne) at a temperature of 30 °C. The broth is kept 3 hours at that temperature and is subsequently cooled down to < 15 °C. This procedure is sufficient to kill the production organism.

Mash flocculation

A filter aid (Dicalite BF) and flocculation aids (calcium chloride and C577) are added to the broth, to achieve flocculation.

Removal of cell material

Before the mash is passed through a membrane filter press, the membrane is precoated with the filter aid Dicalite BF. A subsequent germ reduction filtration ensures the removal of all cell debris.

Ultrafiltration

The filtrate is then concentrated by ultrafiltration. During ultrafiltration molecules with a molecular weight of 5 000 – 10 000 Da (depending also on the shape of the molecules) and lower are partly removed from the broth.

The duration of the ultrafiltration varies from a few hours to more than a day, depending on the volume, viscosity and filterability of the liquid. The volume is reduced at least 20 times. Thus, the amount of low molecular weight molecules is reduced about 5 times after ultrafiltration.

Drying and formulation to granulates

The UF concentrate is co-dried and granulated with wheat flour. After drying the product is standardised with granulated wheat flour up to the required activity level.

4 CARRIERS AND OTHER ADDITIVES AND INGREDIENTS

4.1 Carriers, diluents, excipients, supports and other additives and ingredients

Apart from the enzyme in question, the xylanase preparation will also contain some substances derived from the micro-organism and the fermentation medium. These harmless contaminations consist of polypeptides, proteins and carbohydrates and salts.

The starting material for the formulation is the UF concentrate. Typically, its composition is as follows:

Enzyme activity	26920	NBXU/g
Water	81.9	%
Ash	2.8	%
Proteins (N x 6.25)	8.2	%

After spray drying of the UF-concentrate, the product is diluted and standardised with food grade granulated wheat flour (q.s.) to obtain commercial products with various enzyme concentrations, ranging from 5000-25000 NBXU/g.

4.2 Total Organic Solids (T.O.S.)

The Total Organic Solids of the xylanase preparation were calculated from 3 different batches of the UF concentrate:

Batch no.	RER402	RER505	BXY 6160	Mean
Activity (NBXU/g)	20600	29800	30360	26920
Ash (%)	2,35	3,00	2,95	2,77
Water (%)	85,3	79,9	80,6	81,9
TOS (%)	12,4	17,1	16,5	15,3
NBXU/g TOS	167000	174000	184000	175000

Based on the above figures it can be calculated that the formulated commercial product, with an activity of 5000-25000 NBXU/g, thus have TOS values ranging from 29-143 mg/g.

5 USAGE

5.1 Technological function of the enzyme

The xylanase from *Bacillus subtilis* described in this dossier is intended to be used as processing aid in the milling and baking industry. Xylanases are known to have a beneficial effect on dough strengthening and dough tolerance, due to the fact that the hemicellulose in the dough is partly broken down. This also results in a significantly increased volume of baked bread.

The beneficial effect of fungal xylanases in baking has been described extensively in literature (Kulp, 1968; Gruppen et al, 1993, Rouau, 1993, Rouau and Moreau, 1993, Rouau et al, 1994, Courtin et al, 1999). One of the disadvantages of fungal xylanases is that it may increase the stickiness of dough in industrial bakery applications. The xylanase from *Bacillus subtilis* does not have this disadvantage.

5.2 Types of foodstuffs in which the enzyme is intended to be used

The xylanase system is proposed for use in the preparation of baked goods, such as:

- standard bread,
- special bread,
- biscuits.

5.3 Maximum amount of enzyme preparation to be used in each foodstuff

Generally, enzyme preparations are used in “quantum satis”. The enzyme can be added either to the flour at the mill or to the ingredient mix at the bakery. The average dosage of xylanase, depending on the natural enzyme content of the flour, the baking process and the type of the final product, varies between 25-375 NBXU/kg flour for bread and 100-500 NBXU/kg flour for biscuits.

6 STABILITY AND FATE IN THE FOOD

6.1 Amount of enzyme preparation in the final food

The action of xylanase takes place in the dough during mixing, proofing, and in the early stage of baking. Since the inactivation temperature of xylanase is 60°C (see Section 1.2), no residual enzyme activity remains in the final food after baking.

Final food	**Enzyme use level in food ingredient**	**Amount of ingredient in final food**	**Residual amount of (denatured) enzyme in final food**	**Amount of TOS in final food**
Bread	25-375 NBXU/kg flour	67-91%	17 – 341 NBXU / kg bread	0.10 – 1.95 mg TOS / kg bread
Biscuits	100-500	30-80%	30 – 400 NBXU / kg biscuit	0.17 – 2.29 mg TOS / kg biscuit

6.2 Main reaction products and possible reaction products not considered normal constituents of the diet

Xylanases, as well as other enzymes, is already a normal constituent of flour (Kruger and Reed, 1988). Moreover, malt, which is worldwide used as a flour supplement, also contains xylanases (Preece and Mac Dougall, 1958; Dekker and Richards,1976). The addition of extra xylanases therefore does not lead to new or unintended reaction products in the flour.

Since the enzyme is inactivated during the baking process, no further reaction products will be formed during storage of the bread. The inactivated enzyme remains inert in the food as any other protein.

6.3 Possible effects on nutrients

Based on the information given in Section 6.2, there are no reasons to expect any possible effects on the nutrients in bread.

III GENERAL REQUIREMENTS AND SPECIFICATIONS

7 HYGIENE

7.1 Good Food Manufacturing Practice

For optimal enzyme production, it is very important that hygienic conditions during the whole fermentation process are strictly controlled. Microbial contamination would immediately result in less growth of the production organism and consequently in a low yield of the wanted enzyme(s).

In addition to the microbial hygiene, it is also important that the raw materials and processing aids used during fermentation are of sound quality and do not contain contaminants such as pesticides or a high amount of heavy metals, which might affect the optimal growth of the production organism and thus enzyme yield.

Of course, the quality of the stock culture and the strict control of parameters as pH, temperature and aeration during fermentation are also of the utmost importance for optimal enzyme production and yield.

Thus, the commercial self-interest of any enzyme producer demands a strictly controlled fermentation process.

Enzyme fermentation experience since 1922 in the DSM (formerly Gist-brocades) factory at Seclin, France, has resulted in a solidly established Good Food Manufacturing Practice within the framework of a certified ISO system.

Technical measures:

The batches of **primary seed material**, also called Working Cell Bank (WCB), are always prepared from the so-called Master Cell Bank (MCB) in Laminar Air-flow (down-flow) safety cabinets to ensure the absence of contamination. The batches are divided into a large number of vials for use in production over a long period of years without any changes in strain- and production properties. In theory, a batch is large enough to last for about 10 years, depending on the strain viability and the fermentation frequency and thus the market demand.

The WCB is preserved by deep-freezing using glycerol as protective agent and slow freezing (1°C per min.) to reduce cell damage to a minimum. The deep-frozen vials are stored at minus 75°C or in the vapour phase of liquid nitrogen.

The above procedures for preparation, preservation and storage are chosen to avoid degeneration and to secure genetic stability. All vials are clearly labelled and in revival of the culture, strict aseptic techniques are applied.

The **raw materials** used to make up the nutrient medium for the fermentation are added to mixing tanks and sterilised. The heat-treated nutrient solutions are then cooled for optimum cell growth and subsequently transferred to the fermentor.

The **fermentor** is a closed system. Air introduced into the fermentor is sterilised with a filter**.** Proper temperature conditions are maintained with cooling coils inside the fermentor.

Prior to inoculation, the fermentor is cleaned with solutions of food grade detergents, rinsed with water and then sterilised (empty) with steam.

All materials are pumped into the fermentor under overpressure via fixed connections, which are equipped with self-closing valves. In this way, the sterilised nutrient medium from the mixing tank and the complete biomass broth from the inoculum fermentation are transferred aseptically to the main fermentor.

Microbial contamination during **fermentation** is prevented by the use of a large inoculum, carefully chosen optimum growth conditions for the production organism, overpressure in the fermentation vessel, and the use of sterile air.

The germ filtration during **downstream processing** additionally ensures that the end product is free of microbial contamination.

Control measures:

A new WCB is prepared from the MCB as soon as the previous batch becomes depleted or the concentration of viable cells decreases.

After preparation of a new WCB, samples are checked for identity, viability and microbial purity, using different temperatures (25, 30 and 37°C) and media, by enrichment and viewing morphology (colony shape and microscopy). If all these parameters are correct, the strain is tested for production capacity, first on laboratory scale and later on large

scale production level. Only if the productivity and the product quality meet the required standards, the new WCB is accepted for further production runs.

The viability of the WCB is checked at least once a year.

The **raw materials** used in the fermentation process are checked to be of suitable purity and free of harmful substances. The ingredients used are tightly controlled to minimise contaminants that would inhibit growth of the production organism or enzyme production.

The Quality Control (QC) Department provides assurance that these materials comply with appropriate specifications.

During the **inoculum fermentation** manual samples are taken aseptically from a sampling port on the fermentor for analysis in the laboratory. Samples are checked for pH and microbiological quality.

During the **main fermentation** the correct temperature, pH and dissolved oxygen content are monitored and automatically adjusted (dissolved oxygen is only monitored) throughout the process to ensure optimal enzyme production and a consistent process and thus product.

During the main fermentation manual samples are taken aseptically from a sampling port on the fermentor for analysis in the laboratory. These samples are analysed for such parameters as microbial purity, pH, viscosity and enzyme activity. If microbial controls show that contamination has occurred, the fermentation will be discontinued.

Also during **downstream processing,** most particularly at the end of the UF-concentration samples are being taken and checked for activity, dry matter, pH, specific gravity and the level of microbial contamination.

After **granulation**, the semi-finished product is analysed for colour, xylanase activity, dry matter content, particle size and it is checked for microbiological contamination. Only if the product meets the in-process specifications, it will be accepted as a base to formulate the final commercial product.

The **final enzyme preparation** is analysed for the following parameters:

Parameter	**Norm**
Xylanase activity (EDX/g)	5,000 or 25,000
Colour	Off white to creamy

Dry matter (%)	>90%
Particle size	63 µm<90%<225 µm

In addition, according to the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives of the FAO/WHO (JECFA, 2006) and according to the French legislation (Arrêté 2006),, the xylanase preparations from *Bacillus subtilis* fulfil the following demands:

Parameter	**Norm**
Lead	≤ 5 mg/kg
Arsenic	≤ 3 mg/kg
Mercury	≤ 0.5 mg/kg
Cadmium	≤ 0.5 mg/kg
Mycotoxins	Absent by test
Antimicrobial activity	Absent by test
Standard plate count	≤ $5x10^4$/g
Coliforms	≤ 30/g
Salmonella	0/25 g
Escherichia coli	0/25 g
Staphylococcus aureus	0/g
Anaerobe sulphite reducing	< 30/g

7.2 Total Microbial Count

As is explained in Section 7.1 and proven in Section 8.2, the xylanase preparation complies with international purity standards and addition to foodstuff (in an amount of less than 1% on basis of w/w) will therefore not cause an increase in the total microbial count.

8 CONTAMINANTS

8.1 Heavy metals

As can be seen in the 3 Certificates of Analysis given in Annex 8.1.1, the xylanase preparations comply with the specifications for heavy metals as recommended by JECFA (see also Section 7.1).

8.2 Microbiological contaminants

As can be seen in the 3 Certificates of Analysis given in Annex 8.1.1, the xylanase preparations comply with the specifications for microbial contaminants as recommended by JECFA (see also Section 7.1).

8.3 Test for absence of the production strain

For proprietary reasons, it is very important for each enzyme producer that the final commercial product does not contain viable production organisms. In the case of xylanase production the cells are killed off during downstream processing (see Section 3.2), which ensures that the final product is free of the production organism *Bacillus subtilis.*

8.4 Test for absence of antibiotic activity

The enzyme preparation is tested to ensure the absence of antibiotic activity in accordance with the recommendation from JECFA. As can be seen in the 3 Certificates of Analysis given in Annex 8.1.1, the xylanase preparations do not contain antibiotic activity.

8.5 Test for absence of toxins

Bacillus species are not known to produce mycotoxins. According to JECFA, only enzymes from fungal origin have to be tested for mycotoxins. Besides this, *Bacillus subtilis* has been identified as an organism which is negative for toxin production (see Annex 2.3.2). Further analysis on the final enzyme preparations for (myco)toxins has therefore not been performed.

IV DOCUMENTATION FOR SAFETY IN USE

9 BASIC TOXICOLOGICAL REQUIREMENTS

The safety of several enzymes, including xylanase, from *Bacillus subtilis* has been evaluated by the FAO/WHO Joint Expert Committee on Food Additives (JECFA 1972, 1991, 1998 and 2005). Based on adequate toxicological data and the fact that *Bacillus subtilis* occurs ubiquitously and is a common contaminant of food, JECFA laid down an unlimited Acceptable Daily Intake (ADI) for all these enzymes.

In addition, an article on the safety of a xylanase expressed in a self-cloned strain of *Bacillus subtilis* was published by Harbak and Thygesen (2002).

Although the above data strongly suggest that xylanase from *Bacillus subtilis* is completely safe for human consumption, DSM has performed the following test programme to confirm the safety of its product:

- Subacute (14-day) oral toxicity study
- Subchronic (90-day) oral toxicity study
- Ames test
- Chromosomal aberration test, *in vitro*

The safety studies have been performed with the non-formulated UF concentrate of batch number JLL03001HMC, referred to as ‘tox-batch’. The tox-batch had an activity of 17400 NBXU/g.

All safety studies were performed according to internationally accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

As already mentioned in Section 2.3, the chance that xylanase derived from *Bacillus subtilis* being contaminated with toxins is negligible. Nevertheless, the amount of product given to the test animals was chosen high enough to detect any toxin if present.

Summarizing the results obtained from the several toxicity studies performed as given below, the following conclusions can be drawn:

- The tox-batch does not show any mutagenic or clastogenic activity under the given test conditions
- Based on the results of the sub-chronic oral toxicity study the No Observed Adverse Effect Level (NOAEL) of the tox-batch is 1000 mg/kg body weight/day, which equivalent to 17400 NBXU/kg body weight/day.

Based on the NOAEL and the amounts of inactive enzyme present in the final food (see Section 6), the Margin of Safety (MoS) for human consumption was calculated, using two different ways for the estimation of the daily consumption of the enzyme:

1. Theoretical Maximum Daily Intake (TMDI)

Using the Budget method (Douglass et al, 1977), the TMDI of inactive xylanase in food will be TMDI= (NBXU/kg food)/80[3] = 400/80 = 5 NBXU kg body weight/day.

2. Estimated Daily Intake (EDI) in the Netherlands

Final food	**Residual amount of (denatured) enzyme in final food (NBXU/kg)**	**90th percentile intake level (g food/per person/day)[1]**	**Estimated daily intake of (denatured) enzyme (NBXU/kg bw/day)[2]**
Bread	17 – 341 NBXU / kg bread	270	0.08-1.5
Biscuits	30 – 400 NBXU / kg biscuit	82	0.04-0.6
TOTAL			0.12-2.1

[1] 90th percentile is 2 times the intake level (CFSAN, 2006). Intake level of the average Dutch population taken from the Dutch food consumption survey (Voedingscentrum, 1998).

[2] Calculated for a person of 60 kg

The Margin of Safety (MoS) can be calculated by dividing the NOAEL by the TMDI or the Estimated Daily Intake. With an overall NOAEL of 17400 NBXU /kg body weight/day the following MoS can be calculated:

MoS via TMDI (Budget method):	3480
MoS via EDI, minimal:	8286
MoS via EDI, maximal:	145000

Regarding the height of the MoS, it was concluded that further testing of the safety of the product is not meaningful.

[3] This calculation is based on the assumption that daily consumption of xylanase containing foods will usually not exceed half of the assumed maximum total solid food intake (i.e. 12.5 g/kg bw/day (1/80 kg food/kg bw/day)). According to the Budget method, the maximum daily food intake is 25 g/kg bw/day (1/40 kg food/kg bw/day)

9.1 14-days oral toxicity (range finding study)

A sub-acute (14 days) oral toxicity study was performed at Rallis Research Centre, India, based upon the following guidelines:

- EEC directive 96/54/EEC, B7 Repeated dose (28 days) toxicity (oral), 1996
- OECD 704, Repeated dose 28-day Oral Toxicity Study in Rodents, 1995.

The RRC report 3635/03 (2003) and an amendment to the report (Amendment No.1 CHECK) are both presented in Annex 9.1.1.

During 14 days three groups of six male and six female Wistar rats received the tox-batch (batch JLL03001HMC) by oral gavage at dosages of 50, 200 and 1000 mg/kg body weight/day. A similarly constituted control group received water.

The following parameters were evaluated in all animals:

- Clinical signs
- Ophthalmoscopic examination
- Body weights
- Food consumption
- Hematology
- Clinical chemistry
- Organ weights of principal organs
- Macroscopic examination

No histopathological examination of organs and tissues was performed.

Results

50 mg/kg body weight/day	No treatment-related findings
200 mg/kg body weight/day	No treatment-related findings
1000 mg/kg body weight/day	Significant higher aspartate aminotransferase (AST) activity in females, significant increase in relative weight of kidneys in males

Conclusion

As no corresponding changes in other liver enzymes and organ weight are observed, the change observed for AST activity is considered incidental and of no toxicological relevance. The increased kidney weight is considered to be incidental and of no

toxicological importance, since the increase is small and other, related parameters (such as clinical chemistry values) are comparable among the treated and control group.

9.2 90-days oral toxicity

A sub-chronic (90 days) oral toxicity study was performed at Rallis Research Centre, India, based on the following guidelines:

- EEC Directive 87/302/EEC, B Sub-chronic Oral Toxicity test: 90-day repeated oral dose using rodent species, L133/8, 1987
- US-EPA Prevention, Pesticides and Toxic Substances (7101) Health effects Test Guidelines OPPTS 870-3100 “90-day Oral Toxicity in Rodents” [EPA 712-C-98-199], 1998
- OECD 408, Repeated Dose 90-day Oral Toxicity Study in Rodents, 1998

The RRC report 3636/03 (2003) is presented in Annex 9.2.1.

Ten male and ten female Wistar rats received the tox batch (batch JLL03001HMC) during 90 days at 0 (water), 50, 200 and 1000 mg/kg body weight/day by oral gavage.

The following parameters were evaluated in all animals (unless stated otherwise):

- Clinical signs
- Ophthalmoscopic examination
- Body weights
- Food consumption
- Hematology
- Clinical chemistry
- Organ weights of principal organs
- Macroscopic examination
- Histopathology of organs in control and top-dose group
- Microscopy of organs in control and top-dose group
- Microscopy of all lesions

Results

50 mg/kg body weight/day	No treatment-related findings
200 mg/kg body weight/day	No treatment-related findings
1000 mg/kg body weight/day	Significant higher relative neutrophil counts and lower relative lymphocytes counts in males, significant lower red blood cells (RBC) and

haematocrit (Ht) value in females, significant higher sodium level in females

Conclusion

Based on the absence of any functional disturbance and morphological changes in the treated animals, the changes in lymphocytes and neutrophils are considered to be of no toxicological relevance. The changes in RBC and Ht value are considered to be of no biological relevance as corresponding values as Mean Corpuscular Haemoglobin and Mean Corpuscular Haemoglobin Concentration were not changed. In addition, the haemoglobin concentration and total bilirubin level as a measure of RBC destruction remained unchanged. The increase in sodium level is, although statistically significant, only marginal and not accompanied by changes in other electrolytic parameters and therefore of no biological significance.

From the results of the 90-day study a No Observed Adverse Effect level of 1000 mg/kg body weight/day, i.e. the highest dose tested, was concluded.

9.3 Mutagenicity tests

AMES test

An Ames test was performed at TNO, The Netherlands, according to the following guideline:

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

The TNO report V5005/07 is presented in Annex 9.3.1.

The tox-batch (JLL 03001HMC) 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).

The test substance 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 both the absence and the presence of S9-mix and in all strains, the test substance 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.

It is concluded that the tox-batch is not mutagenic under the conditions employed in this study.

Chromosomal aberration test

In vitro chromosomal aberration was tested at TNO, The Netherlands, according to the following guideline:

- OECD guideline 473, Genetic toxicology: *In vitro* Mammalian Chromosome Aberration Test, adopted 21 July 1997

The TNO report V5002/04 is presented in Annex 9.3.2.

In a first chromosomal aberration test the treatment/harvest times were 4/24 hours (pulse treatment) in the presence of S9-mix. In the absence of S9-mix, the treatment/harvest times were 4/24 hours (pulse treatment) and 24/24 hours (continuous treatment).

In a second chromosomal aberration test the treatment/harvest times were 4/24 hours (pulse treatment) and 4/48 hours (pulse treatment) in the presences of S9-mix. In the absence of S9-mix the treatment/harvest times were 24/24 hours (continuous treatment) and 48/48 hours (continuous treatment).

The test substance was dissolved in RPMI-1640 medium prior to testing. In both performed chromosomal aberration tests, the highest concentration of the test substance, analyzed for the induction of chromosomal aberrations, was the maximum required concentration in the final culture medium.

In the two (independent) chromosomal aberration tests, in both the absence and presence of S9-mix, the test substance did not induce a statistically significant increase in the number of cells with structural chromosomal aberrations at any of the dose levels and time points analyzed, when compared to the negative control values.

The positive control substances mitomycin C (in the absence of the S9-mix) and cyclophosphamide (in the presence of the S9-mix) induced the expected increases in the incidence of structural chromosomal aberrations.

These data support the conclusion that, under the conditions used in both chromosomal aberration tests, the tox batch is not clastogenic for cultured human lymphocytes.

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