

GRAS Notice (GRN) No. 479

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July 2, 2013



VIA FEDERAL EXPRESS

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Beta-Glucanase, Cellulase, and Xylanase from *Talaromyces emersonii*

Dear Sir or Madam:

On behalf of DSM Food Specialties ("DSM"), we are submitting under cover of this letter three paper copies and one eCopy of DSM's GRAS notification of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* ("*T. emersonii*"). DSM has determined, through scientific procedures, that its beta-glucanase, cellulase and xylanase enzyme preparation from the DSM FBG lineage of *T. emersonii* is generally recognized as safe ("GRAS") for use as an enzyme for breaking down the glucans and xylans, which are starch-like compounds (polysaccharides) present in plants such as barley, in levels not to exceed good manufacturing practices.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, this use of beta-glucanase, cellulase and xylanase from *T. emersonii* is exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act, because the notifier has determined that such use is GRAS.

If you have any questions regarding this notification, or require any additional information to aid in the review of DSM's conclusion, please do not hesitate to contact me via email at gyingling@morganlewis.com or by telephone, (202)739-5610.

Sincerely,

(b) (6)

Gary L. Yingling

cc: DSM Food Specialties

**GRAS NOTIFICATION FOR BETA-
GLUCANASE, CELLULASE AND
XYLANASE FROM *TALAROMYCES*
*EMERSONII***

SUBMITTED BY:

DSM Food Specialties

PO Box 1

2600 MA Delft

The Netherlands

**GRAS NOTIFICATION FOR BETA-GLUCANASE, CELLULASE AND XYLANASE
FROM *TALAROMYCES EMERSONII***

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1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

DSM Food Specialties (“DSM”) manufactures an enzyme preparation of beta-glucanase, cellulase and xylanase, which is produced by submerged batch fermentation of a selected, pure culture from the DSM FBG lineage of *Talaromyces emersonii*. DSM produces the beta-glucanase, cellulase and xylanase preparation in liquid form, which is standardized with glycerol.

This enzyme preparation is for use in the food industry as a processing aid to reduce the wort viscosity and haze formation in cold beer during the storage.

Barley usually contains between 4-7% of beta-glucan and a smaller content of arabinoxylan. Most of the beta-glucan is present in the endosperm cell wall of the grain.

As raw barley (adjunct) and/or less modified malt makes up a significant proportion of the mash filtration problems of the wort become apparent. An excess of wort beta-glucan will increase the wort viscosity and thereby the mash run-off¹ times. A second problem related to the presence of beta-glucans is haze formation in (cold) beer, particularly in strong ales, and in the final filtration of the beer.

Beta-glucanase, cellulase and xylanase are able to degrade the polymeric beta-glucan into smaller less viscous molecules, thereby solving the filtration and haze problems. The thermostability of the beta-glucanase, cellulase and xylanase activities from *Talaromyces emersonii* offers the opportunity to apply the enzyme preparation in high temperature mash or in mashing programs having only short rests at lower temperatures. The enzyme preparation is applied in the mash tun (with malt or mixtures of malt and barley).

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, DSM has determined that its beta-glucanase, cellulase and xylanase enzyme preparation from the DSM FBG lineage of *Talaromyces emersonii* is a GRAS substance for the intended food applications and is therefore exempt from the requirement for premarket approval. Information on the enzyme preparation and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme preparation, its applicable conditions for use, DSM’s basis for its GRAS determination and the availability of supporting information can be found here in Section 1.

The production organism, from the DSM FBG lineage of *Talaromyces emersonii*, has a long history of safe use and is discussed in Section 2. Section 2 also describes the production microorganism and strain improvement that was performed. The safety studies outlined in Section 7 indicate that *Talaromyces emersonii* beta-glucanase, cellulase and xylanase show no evidence of pathogenic or toxic substances. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4, while Section 5 reviews the strictly hygienic composition, specifications

¹ Run-off: first step between wort and spent grains.

as well as the self-limiting levels of use for the enzyme preparation. Finally, Section 6 provides information on the mode of action, applications, use levels and enzyme residues in the final food product.

1.1. Name and Address of Notifier

NOTIFIER

DSM Food Specialties

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The Netherlands

MANUFACTURER

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PERSON RESPONSIBLE FOR THE DOSSIER

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1.2. Common or Usual Name of Substance

DSM's beta-glucanase, cellulase and xylanase enzyme preparation from *Talaromyces emersonii* is produced by submerged batch fermentation of a selected, pure culture of the DSM FBG lineage of *Talaromyces emersonii*. The common or usual name of the substance is "beta-glucanase, cellulase and xylanase". It is produced and sold in liquid form, which is standardized with glycerol.

1.3. Applicable Conditions of Use

The *Talaromyces emersonii* enzyme preparation is to be used during the production of beer and other fermented beverages. The enzyme preparation is applied during the mashing phase in the manufacture process. The enzyme activities are heat denaturated, and consequently inactivated, during the boiling stage. The use of beta-glucanase, cellulase and xylanase can thus be regarded as a processing aid because it has no function in the finished foodstuff.

1.3.1. Substances Used In

The *Talaromyces emersonii* enzyme preparation is to be used during the mashing phase followed by boiling step in manufacturing of beer (all types of beers) and other fermented beverages.

1.3.2. Levels of Use

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the type and quality of the raw materials used, and the process conditions. The levels of use expected to result in beneficial effect are described below.

The enzyme preparation contains a range of 10,000-80,000 BGF²/g and 78-624 XVU³/g. The dosage in brewing, standardized on BGF activity, is 100-500 g (10,000-80,000 BGF/g) per metric ton of grist (malt). About 200 g malt is used to produce 1 L beer. Therefore, a maximal level of 200-8,000 BGF will be present per liter beer.

The enzyme preparation is applied during the mashing phase in the manufacture process. However, this process contains a vigorous boiling step of 1-2 hours consecutive to the mashing phase, during which the enzyme is denaturated, and consequently inactivated.

1.3.3. Purposes

Beta-glucanases, cellulase and xylanases are used in the manufacture of different food products and animal feed and as subsidiary materials in biological research when it is necessary to cleave

² BGF = beta-glucanase fungique unit. Cellulase hydrolyses 1,4-linkages in β -D-glucans that also contains 1,3-linkages. So BGF units relate to both beta-glucanase and cellulase activities.

³ XVU = xylanase viscosimetric unit.

the beta-glycosidic linkages in (1,3-1,4)-beta-glucans and the 1,4-β-D-xylosidic linkages in xylans. Especially in the brewing industry the use of such hydrolyzing enzymes permits the application of larger proportions of raw grain in substitution for the use of malt, without this causing any trouble in the filtration due to high viscosity of the mash which may be caused by an increased amount of glucan and xylan compounds.

Barley usually contains between 4-7% of beta-glucan and a smaller content of arabinoxylan. Most of the beta-glucan and arabinoxylan are present in the endosperm cell wall of the grain. Beta-glucan is a mixed linked polysaccharide composed of glucose residues, linked by beta 1,3 and 1,4 bonds, the 1,4 bonds making up to 70 % of the total bonds in the beta-glucan molecule (Clarke, A.E. and Stone, B.A., 1966). Xylans are polysaccharides containing β-D-xylopyranosyl units linked by (1–4) glycosidic bonds. The water-soluble fraction of beta-glucan and xylan is responsible for viscosity.

As raw barley (adjunct) and/or less modified malt makes up a significant proportion of the mash, filtration problems of the wort become apparent. An excess of wort beta-glucan and xylan will increase the wort viscosity and thereby the mash run-off⁴ times. A second problem related to the presence of beta-glucans and xylans is haze formation in (cold) beer, particularly in strong ales, and in the final filtration of the beer.

Beta-glucanase, cellulase and xylanase are able to degrade the polymeric beta-glucans and xylans into smaller less viscous molecules, thereby solving the filtration and haze problems. The thermostability of the enzyme activities from *Talaromyces emersonii* offers the opportunity to apply the enzyme in high temperature mash or in mashing programs having only short rests at lower temperatures. The enzyme is applied in the mash tun (with malt or mixtures of malt and barley). The use of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* will also increase the yield in the case of low quality malts to increase the productivity in the brewhouse which is economically interesting.

The different steps in the brewery process are shown in Annex 10. The application of enzymes like beta-glucanase, cellulase and xylanase in brewery is comprehensively described (Briggs, D.E. *et al.*, 2004). The effect of the beta-glucanase, cellulase and xylanase preparation from *Talaromyces emersonii* is illustrated in Annex 11.

1.3.4. Consumer Population

Beta-glucanase, cellulase and xylanase are widely distributed in nature. They have been isolated from a variety of sources, such as fungi, yeasts, bacteria, plants and marine invertebrates (Müller, J.J. *et al.*, 1998; Wong, Y.-. and MacLachlan, G.A., 1980; Polizeli, M.L.T.M. *et al.*, 2005; Mawadza, C. *et al.*, 2000; McCarthy, T.C. *et al.*, 2005; Knowles, J. *et al.*, 1987). Since beta-glucanase, cellulase and xylanase are enzymes naturally present in nature and notably in plants and marine invertebrates consumed by human, DSM expects it will be digested as would any other protein occurring in food.

⁴ Run-off: first step between wort and spent grains.

In addition, the enzymes beta-glucanase, cellulase and xylanase have a long history of use in food processing. Those enzymes were already used for an increased filterability of beer in 1994 (Godfrey, T. and West, S., 1996). Beta-glucanase from *Talaromyces emersonii* is present in the list of enzymes used in food processing made by Pariza and Johnson in 2001 (Pariza, M.W. and Johnson, E.A., 2001), while xylanases and cellulases from several microorganisms are also present in the same list. Several GRAS notifications have been submitted and accepted by FDA with no questions for the use of xylanase, cellulase and beta-glucanase enzyme preparations (CFSAN / Office of Food Additive Safety, 2000, GRN 000054); (CFSAN / Office of Food Additive Safety, 2004, GRN 000149); (CFSAN / Office of Food Additive Safety, 2006, GRN 000195), (CFSAN / Office of Food Additive Safety, 2009). Several enzyme preparations of beta-glucanase, cellulase or xylanase have been evaluated by JECFA attributed an ADI 'not specified' for their use in several applications such as the preparation of fruit juices, beer and baking products (Joint FAO/WHO Expert Committee on Food Additives, 2006a, Joint FAO/WHO Expert Committee on Food Additives, 2006b), (Joint FAO/WHO Expert Committee on Food Additives, 2000, Joint FAO/WHO Expert Committee on Food Additives, 1992, Joint FAO/WHO Expert Committee on Food Additives, 1988).

As is shown in Section 6.4 of this dossier, the amount of denatured enzyme in the final product is expected to be maximally 200-8,000 BGF/L beer and the amount of enzyme TOS in the final product 0.11-4.56 mg/L beer (0.000011-0.000456%).

Since the enzyme preparation is present in food products at such low levels as an inactive protein, and because it is a naturally occurring substance in tissues commonly ingested by humans, it is clear that the consumer population will be unaffected by the presence of the enzyme preparation in food.

1.4. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, DSM has determined, through scientific procedures, that its beta-glucanase, cellulase and xylanase enzyme preparation from the DSM FBG lineage of *Talaromyces emersonii* is GRAS for use as an enzyme for breaking down the glucans and xylans, which are starchlike compounds (polysaccharides) present in plants such as barley, in levels not to exceed good manufacturing practices.

The enzyme preparation is applied during the mashing phase in the manufacture of beer and fermented beverages. The beta-glucanase, cellulase and xylanase activities in the enzyme preparations are heat denatured, and consequently inactivated, during the boiling stage of the brewing process.

1.5. Availability of Information for FDA Review

The data and information that are the basis for DSM's GRAS determination are available for the FDA's review and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Gary L. Yingling
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1111 Pennsylvania Avenue, NW
Washington, DC 20004-2541

2. PRODUCTION MICROORGANISM

2.1. Name and designation

The strain used for the production of the enzyme preparations containing beta-glucanase, cellulase and xylanase activities belongs to the species *Talaromyces emersonii*⁵.

2.2. Source of the organism

The original strain used for the production of enzyme preparations containing beta-glucanase, cellulase and xylanase activities, *Talaromyces emersonii* was originally isolated from Italian compost as a new interesting thermophilic fungus and identified as *Talaromyces emersonii*. *Talaromyces emersonii* is also found in the literature as *Penicillium emersonii* or *Geosmithia emersonii*. Based on genetical analyses, physiological parameters and extrolite profile the species was recently renamed *Rasamsonia emersonii* (Houbraken, J. *et al.*, 2012). In this dossier, the strain is named *Talaromyces emersonii*.

2.2.1. Information on reproductive cycles (sexual/asexual) of the recipient or classical production organism

The fungus *Talaromyces emersonii* is a filamentous eukaryotic fungus which can reproduce both by asexual as well as sexual processes. In the asexual propagation the vegetative mycelium forms spores in conidia on a conidiophore. When a fungus only is restricted to this asexual propagation this is called the anamorph. Sexual propagation takes place by means of asci which are often formed by karyogamy of two nuclei from different gametangia. Asci are usually enclosed in ascocarps (fruit-bodies). This sexual form of propagation is called the teleomorph. *Talaromyces emersonii* is the anamorph of *Penicillium emersonii* (Samson, R.A. and van Reenen-Hoekstra, E.S., 1988).

2.3. Strain improvement

The original production strain was acquired by Gist-brocades (now DSM) in 1993 from an external source and was used for large scale production of beta-glucanase and cellulase. Later strain *Talaromyces emersonii* FBG1 was derived as a single colony isolate from the original wild-type strain, to be able to produce kosher beta-glucanase, cellulase and xylanase (a genealogy is located in Annex 1). This production strain FBG1 (DS28601) has been identified by the Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands as *Talaromyces emersonii* Stolk, the teleomorph state of *Penicillium emersonii* (Annex 2).

Recently, the strains FBG1 and FBG2 and FBG210CE - resulting from classical strain improvement of FBG1 by mutagenesis and selection for higher enzyme productivity - were again identified by the CBS (Utrecht, the Netherlands) as *Rasamsonia emersonii* (= *Talaromyces emersonii*) (Houbraken, J. *et al.*, 2012) (Annex 3).

⁵ This species was recently renamed *Rasamsonia emersonii* (Houbraken, J. *et al.*, 2012).

2.4. The classical taxonomy

The formal classification of *Talaromyces emersonii* is:

Kingdom	:	Fungi
Division	:	Ascomycota
Subdivision	:	Pezizomycotina
Class	:	Euriotomycetes
Order	:	Eurotiales
Family	:	Tricocomaceae
Genus	:	Rasamsonia
Section	:	Emersonii
Species	:	<i>Talaromyces emersonii</i>

2.5. Molecular biological taxonomy

(Houbraken, J. *et al.*, 2012) have used molecular techniques to differentiate *Talaromyces emersonii* from related thermophilic micro-organisms.

2.6. Stability of parental or classical production organism in terms of relevant genetic traits

The *Talaromyces emersonii* FBG lineage is regarded as a lineage of genetically stable strains. The FBG strains have been maintained for almost 20 years under laboratory conditions without any significant degeneration in yield or appearance of morphological variants. After plating out, a low frequency of morphological dissimilar colonies are found, but this is a normal phenomenon for selected, high producing strains. The stability of the strains FBG1, FBG 2 and FBG210 does not differ from other DSM fungal production strains.

2.7. Nature of pathogenicity and virulence, infectivity, toxicity and vectors of disease transmission

Talaromyces emersonii strains described to date exclusively have been isolated from soil, air, plants or compost (Stolk, A.C., 1965; Raper, K.B., and Thom, C., 1949; Benjamin, C.R., 1955; Samson, R.A. and van Reenen-Hoekstra, E.S., 1988; Stolk, A.C. and Samson, R.A., 1972; Frisvad, J.C. *et al.*, 1990; Harrington, J. *et al.*, 1979)). (Cimon, B. *et al.*, 1999) so far have published the only report of a human case of chronic airway colonization by the teleomorph of *Talaromyces emersonii*, *Penicillium emersonii*. This however was in a patient suffering from cystic fibrosis, and filamentous fungi frequently are recovered from bronchial secretions of cystic fibrosis patients. In fact the patient also was infected with *Aspergillus fumigatus*. There are

absolutely no indications that would compromise the statement that the fungus *Talaromyces emersonii* is non-pathogenic for humans or animals.

(Frisvad, J.C. *et al.*, 1990) have evaluated the potential of the several *Talaromyces* species to produce secondary metabolites and showed that *Talaromyces emersonii* strains did not produce any secondary metabolites known as mycotoxins. More recently it was shown that all *Talaromyces emersonii* strains have the ability to produce a specific peptide-like secondary metabolite, secalonic acid.

To gain insight into the potential to produce toxins our beta-glucanase, cellulase and xylanase production strain *Talaromyces emersonii* FBG1 (DS28601) was tested for the production of secondary metabolites including (myco)toxins under various growth conditions. The strain showed the production of various metabolites, but no known mycotoxins were found (Annex 4). In addition a fermentation broth sample was tested. In the sample no known mycotoxins were found (Annex 5). More recent and more sophisticated analyses showed that the strains FBG1 and FBG2 have the ability to produce a variety of secondary metabolites including secalonic acid and the produced enzyme concentrates may contain trace amounts of this compound (Annex 6). To the best of our knowledge, there is no information suggesting the oral toxicity of these secondary metabolites. In addition, the presence of the secondary metabolites found in trace amounts in the fermentation broth would lead to levels in the final preparation well below the toxic levels reported in literature after i.p. or s.c. administration (Ciegler, A. *et al.*, 1980; Mayura, K. *et al.*, 1982). The strains of the FBG lineage have no potential to produce any of the well-known food mycotoxins, the trace amount of secondary metabolites are not toxicologically relevant, and the resulting enzyme preparation has been tested for safety resulting in an appropriate margin of safety (see section 7.4). Therefore, we conclude that the enzyme product is safe for application as a processing aid in food.

2.8. Natural habitat, geographic distributions and climatic characteristics of the original habitats

Talaromyces emersonii is a filamentous fungus commonly found in nature in environments with a high content of plant cell-wall material (soil, compost, wood-piles, and plant debris). The species is strongly thermophilic, with a minimum growth temperature somewhat below 30 °C, and optimum of 40-45 °C, and a maximum growth temperature of 55 °C. As the fungus likes high oxygen concentration it grows best at surfaces of the organic substrates. After growth the fungal mycelium produces specialized cells, called conidiophores, which in turn produces conidiospores. These spores can be spread widely by air turbulences. The fungus is amongst the dominant thermophiles surviving in all heat treated compost piles (exposure to 70 °C for at least 48 hours) (Harrington, J. *et al.*, 1979).

Many organisms are involved in the degradation of complex organic material including *Talaromyces emersonii*. Not much is known about all the competitive and symbiotic interactions possible. The effect of our production organism(s) on these types of interactions are not considered important as only negligible amounts of mycelia may be released into the environment. This is due to contained use in fermentation and down-stream processing.

2.9. Good Industrial Large scale Practice GILSP

DSM Food Specialties (formerly Gist-brocades Food Specialties Division) has produced since 1994 beta-glucanase and cellulase and xylanase activities with strains of the *Talaromyces emersonii* FBG strain lineage at various large scales, up to 200 m³, at its enzyme production facility in Seclin, France. No adverse effects on the environment or health to the personnel employed in the fermentation plants have been observed. At DSM (formerly Gist-brocades), only submerged fermentations are used, during which no conidiospores are formed. Consequently no health problems associated with exposure to *Talaromyces* spores are encountered.

Built-in biological barriers, without interfering with optimal growth in the reactor or fermenter, confer limited survivability and replicability, without adverse consequences, in the environment.

The FBG strains do not have any built in barriers to prevent survival in nature. The strains are, however, selected for good growth and efficient enzyme production under artificial conditions and this leads in general to reduced survivability.

3. ENZYME IDENTITY

3.1. Enzyme Identities

The enzyme preparation contains the main activities beta-glucanase, cellulase, xylanase, and several subsidiary activities.

Key enzymes and protein chemical characterization of the enzymes are:

Beta-glucanase:

- Systematic name : 3-(1,3-1,4)-beta-D-glucan 3(4) glucanohydrolase
- Other names : Glucanase, Laminarinase
- Accepted name : endo-1,3(4)-beta-glucanase
- IUPAC/IUB Number : EC 3.2.1.6
- CAS number : 62213-14-3

Cellulase:

- Systematic name : 4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase
- Other names : endo-1,4- β -D-glucanase; β -1,4-glucanase; β -1,4-endoglucan hydrolase; cellulase A; cellulysin AP; endoglucanase D; alkali cellulase; cellulase A 3; celludextrinase; 9.5 cellulase; avicelase; pancellase SS; 1,4-(1,3;1,4)- β -D-glucan 4-glucanohydrolase
- Accepted name : cellulase
- IUPAC/IUB Number : EC 3.2.1.4
- CAS number : 9012-54-8

Xylanase

- Systematic name : 1,4-Beta-D-xylan xylanohydrolase
- Recommended name : endo-1,4-beta-xylanase
- Other names : Xylanase , endo-1,4-xylanase
- EC number : EC 3.2.1.8
- CAS number : 9025-57-4

3.2. Principal enzymatic Activities

Beta-glucanase:

Beta-glucanase catalyses the endo-hydrolysis of the 1,3- or 1,4-linkages in beta-D-glucans, when the glucose residue whose reducing group is involved in the linkage to be hydrolysed, is itself substituted at C-3 atom.

In the literature (Murray, P. *et al.*, 2001) the beta-glucanase activity is expressed in international units IU units. One IU unit of endo-beta-glucanase activity is defined as the amount of enzyme that yields 1 μmol reducing sugar (as glucose equivalence) in 1 min under the conditions of the assay.

The US Food Chemical Codex (FCC) gives a method to measure the beta-glucanase activity of enzyme preparations derived from *Aspergillus niger* var. and *Bacillus subtilis* var. This method is based on a 15-min. hydrolysis of lichenin substrate at 40°C and at pH 6.5. The enzyme activity of this method is expressed as Beta- Glucanase Units (BGU). One BGU is the quantity of enzyme that will liberate reducing sugar (as glucose equivalence) at a rate of 1 $\mu\text{mol}/\text{min}$ under the conditions of the assay.

The biochemical properties of beta-glucanase have been investigated by several scientists. The most complete review regarding beta-glucanase isolated from *Talaromyces emersonii* can be found in the following scientific publications (Murray, P. *et al.*, 2001); (McCarthy, T. *et al.*, 2003).

Beta-glucanase is active (>50% of max) between pH 2.5 – 6.5 with a maximum activity around pH 4.3 at 60°C. The temperature optimum at pH 4.7 lies around 75°C and inactivation of the beta-glucanase activity occurs after 30 minutes at 90°C.

The literature data suggested that the activity of beta-glucanase from *Talaromyces emersonii* lies within a broad pH range of 2.5-8, with an optimum at pH=4.8. The enzyme is active at temperatures from 40-100°C and has an optimum ~80°C (Murray, P. *et al.*, 2001). Beta-glucanase from *Talaromyces emersonii* is more thermostabile than beta-glucanases from other sources (*Bacilli*, *Trichoderma* and *Aspergillus*) (Briggs, D.E. *et al.*, 2004). The half-life of beta-glucanase from *Talaromyces emersonii* at 65°C and 75°C is respectively 84 and 37 minutes. The specific activity of the pure enzyme has been described to be between 1651.8 IU per mg protein using barley-beta-glucan (BBG) as a substrate and 1962.7 IU per mg protein using lichenan from *Usnea barbata* substrate (Murray, P. *et al.*, 2001).

Although the enzyme is active at 100°C for a few minutes, it will be completely inactivated during the 1-2 h boiling of the wort.

Cellulase:

Cellulase is responsible for the endohydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans and the hydrolysis of 1,4-linkages in beta-D-glucans that contain 1,3-linkages.

The cellulase assay can be found in the Food Chemicals Codex (FCC). The FCC assay measures the release of reducing sugars by the action of the enzyme on a cellulase substrate. One unit of activity liberates 1 micromole of reducing sugar (expressed as glucose equivalents) in one

minute under conditions described (50 °C, pH 4.8, 10 minutes). The sample activity is then related to a standard with a stated carboxymethylcellulase activity.

The optimum of the cellulase activity lies around pH 4.25 (at 60°C) and more than 50% of activity is shown between pH 3 and pH 6. The temperature optimum of the cellulase activity lies at 75°C (pH 4.7).

Although the enzyme is active at 100°C for a few minutes, it will be completely inactivated during the 1-2 h boiling of the wort.

Beta-glucanase and cellulase DSM activity method

Both enzymes beta-glucanase and cellulase are playing important role in the application such as brewing and therefore beta-glucan is used as substrate to analyze both: beta-glucanase and cellulase activities in one assay.

DSM developed method allows to measure beta-glucanase and cellulase activities using beta-D-glucan as substrate. Since beta-D-glucan contains both 1,4-linkages and 1,3-linkages it will be hydrolysed by both enzymes, beta-glucanase and cellulase. In literature (Murray, P. *et al.*, 2001) often beta-D-glucan is used as substrate for beta-glucanase but it will be also substrate for cellulase. Therefore cellulase is mixed with beta-glucanase and it is difficult from literature to distinguish those enzymes.

Internal DSM proteomics studies showed presence of both enzymes in the enzyme cocktail.

Method to measure both beta-glucanase and cellulase is given in Annex 7. The enzyme activity in this method is expressed as Beta-Glucanase Fungique (BGF) unit. One beta-glucanase fungique (BGF) unit is the amount of enzyme per ml reaction mixture (15 ml substrate and 2 ml enzyme solution) that causes a change in viscosity of the substrate with a speed giving a slope of 0.147 per minute under the conditions of the test.

The BGF units will characterize both beta-glucanase and cellulase activities.

Xylanase

The enzyme catalysed the endo-hydrolysis of 1,4-beta-D-xylosidic linkages in xylans. The enzyme activity is widespread among Bacilli and fungi. The optimum of the xylanase activity lies around pH 4.0 (at 50°C) and more than 50% of activity is shown between pH 3 and pH 6. The temperature optimum of the xylanase activity lies at 80°C (pH 5.6). The characteristics of the xylanase(s) from *Talaromyces emersonii* can be found in literature (Tuohy, M.G. and Coughlan, M.P., 1992).

The enzyme activity of xylanase is expressed in so-called XVU Units. One XVU Unit corresponds to the quantity of enzyme that causes a variation in the viscosity of a 1 ml incubation mixture with an apparent constant of 5 per minute under the conditions of the assay (pH 4.6 and 42 °C).

The method developed by DSM and used for determination of the xylanase activity in products is given in Annex 8.

Although the enzyme is active at 100⁰C for a few minutes, it will be completely inactivated during the 1-2 h boiling of the wort.

4. MANUFACTURING PROCESS

4.1. Overview

Beta-glucanase, cellulase and xylanase from DSM is produced by a controlled submerged batch fermentation of a selected, pure culture of *Talaromyces emersonii* (see Section 2.1). 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 9.

4.2. Raw Materials

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. This is confirmed by the toxicological studies performed (see Section 7.4 of this dossier). The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

The fermentation medium used has been developed for optimum production of enzymes (in this case beta-glucanase, cellulase and xylanase) by the *Talaromyces emersonii* host.

The antifoam used in the fermentation (ethoxylated propoxylated glycerol oleate) is used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998.

4.3. Fermentation process

Beta-glucanase, cellulase and xylanase from DSM is produced by a controlled submerged batch fermentation of a pure culture of *Talaromyces emersonii*. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms.

The fermentation process consists of four steps: two successive pre-culture fermentation steps, followed by the seed fermentation and the main fermentation. The whole process is performed in accordance with Good Food Manufacturing Practice (see Section 5.2).

Biosynthesis of beta-glucanase, cellulase and xylanase occurs during the main fermentation. To produce the enzymes of interest, a submerged, aerobic batch fermentation process is employed, using a stirred tank fermentor.

Growth of the production organism and increase of enzyme production are checked at the end of the main fermentation by analysis of aseptically collected samples.

4.4. Recovery process

The cell material is separated from the enzymes by means of a simple membrane filtration process. Subsequently, the remaining particles are removed with a polish filtration and a germ reduction filtration, and then concentrated by ultrafiltration (UF).

4.5. Formulation and standardization process

In order to obtain a liquid enzyme preparation, the UF concentrate is standardized with 40-45 % glycerol to the desired final enzyme activity, followed by a polish filtration and another germ reduction filtration. Sodium benzoate is added as stabilizing agent at level 1-3 g/kg of the final product.

Quality Control of Finished Product

The final beta-glucanase, cellulase and xylanase preparation from *Talaromyces emersonii* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives (JECFA) of the FAO/WHO in 2006 and the FCC (8th edition). These specifications are described in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1. Formulation

The common starting material for all formulations is the ultra-filtrate concentrate. Typically, its composition falls within the following ranges:

Enzyme activities	100,000-500,000 BGF/g (both cellulase and beta-glucanase), 1,170-1,560 XVU/g
Water (%)	80-90
Ash (%)	0-2
Proteins (Nx6.25, %)	5-10

Apart from the enzyme complex, the beta-glucanase, cellulase and xylanase preparation will also contain some substances derived from the microorganism and the fermentation medium. These harmless constituents consist of polypeptides, proteins, carbohydrates and salts.

In order to obtain a final formulation, the ultra-filtrate concentrate is stabilized with 40-45% glycerol and diluted with water to an activity of $\geq 10,500 \pm 5\%$ BGF/g. The sodium benzoate 1-3 g/kg of total composition is used as stabilizing agent.

The Total Organic Solids of the beta-glucanase cellulase and xylanase preparation were calculated from 4 commercial batches as well as the 'tox-batch', on the ultra-filtrate concentrate:

Calculation of the TOS					
Batch number	Water (%)	Ash (%)	TOS (%)	Activity (BGF/g)	BGF/mg TOS
OP 8015	91.6	1.8	6.6	112000	1697
OP 8016	90.8	1.5	7.7	151000	1961
OP 8018	89.9	1.4	8.8	128000	1455
OP 8017	93.8	0.9	5.3	92000	1736
('tox-batch') 612020901	73.0	0.6	26.4	509750	1931
Mean					1756

The TOS values of the final standardized enzyme preparations can be easily calculated on basis of values presented in the table above and taking the dilution factor into account. For instance, a formulated commercial product with an activity of 10,500 BGF/g will have a TOS value of about 5.98 mg/g enzyme preparation, while a formulated product with an activity of 80,000 BGF/g will have a TOS value of about 45.6 mg/g enzyme preparation.

5.2. General Production Controls and Specifications (Good Manufacturing Practice)

Commercial demands require a strictly controlled fermentation process.

The enzyme fermentation factory at Seclin, France, which has fermentation experience since 1922, has acquired the ISO 9001-2000 certification.

5.2.1. Technical measures

The batches of **primary seed material** are prepared, preserved and stored in such a way that contamination and degeneration is avoided and genetic stability is secured. The vials are clearly labeled and strict aseptic techniques are applied during the recovery of the culture.

Only sterilized **raw materials** are used to prepare the nutrient medium for the fermentation.

The **fermentor** is a contained system. Only sterilized air is used in the fermentation. Membrane valves, air filters and seals are regularly checked, cleaned and replaced if necessary. Prior to inoculation, the fermentor is cleaned, rinsed and sterilized. The sterilized nutrient medium and the complete biomass broth are transferred aseptically to the main fermentor. The methods used effectively prevent microbial contamination during fermentation.

The preparation of sterile media and the cleaning of the equipment are laid down in Quality Assurance documents and strictly followed.

Microbial contamination is prevented during **downstream processing** by several germ reduction filtrations. The filters are thoroughly cleaned for each production run.

5.2.2. Control measures

After preparation of a new batch of **primary seed material**, samples are checked for identity, viability and microbial purity. If these parameters are correct, the strain is tested for production capacity. Only if the productivity and the product quality meet the required standards, the new batch of primary seed material will be accepted for further production runs. Each time a vial from such a certified batch of primary seed material is used for production, the viability, purity and identity of the strain is checked.

The **raw materials** used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

At regular intervals during the **seed fermentation** manual samples are taken aseptically for analysis of pH, and microbiological quality in the laboratory.

During the **main fermentation** the dissolved oxygen content, pH, temperature, viscosity and microbial quality are monitored. If microbial controls show that significant contamination has occurred, the fermentation will be discontinued.

Also during **downstream processing** samples are being taken and checked for the level of microbial contamination. If these checks show that significant contamination has occurred, the downstream processing will be discontinued.

The finished product is subjected to extensive controls and complies with JECFA and FCC specifications:

Parameter	Norm
Heavy metals	< 30 mg/kg (as Pb)
Lead	< 5 mg/kg
Cadmium	< 0.5 mg/kg
Mercury	< 0.5 mg/kg
Arsenic	< 3 mg/kg
Standard plate count	< 10 ⁴ CFU/g
Coliforms	< 15 CFU/g
Salmonella	0/25 g
<i>Escherichia coli</i>	0/25 g
Anaerobe sulphite reducing	< 30 CFU/g
<i>Staphylococcus aureus</i>	0/g
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

6. APPLICATION

6.1. Mode of Action

The beta-glucanase, cellulase and xylanase enzymes preparation is to be used in beer and fermented beverages.

Barley usually contains between 4-7% of beta-glucan and a smaller content of arabinoxylan. Most of the beta-glucan and arabinoxylan are present in the endosperm cell wall of the grain. Beta-glucan is a mixed linked polysaccharide composed of glucose residues, linked by beta 1,3 and 1,4 bonds, the 1,4 bonds making up to 70 % of the total bonds in the beta-glucan molecule. Xylans are polysaccharides containing β -D-xylopyranosyl units linked by (1-4) glycosidic bonds. The water-soluble fraction of beta-glucan and xylan is responsible for viscosity.

As raw barley (adjunct) and/or less modified malt makes up a significant proportion of the mash filtration problems of the wort become apparent. An excess of wort beta-glucan and xylan will increase the wort viscosity and thereby the mash run-off times. A second problem related to the presence of beta-glucans and xylans is haze formation in (cold) beer, particularly in strong ales, and in the final filtration of the beer.

Beta-glucanase, cellulase and xylanase are able to degrade the polymeric beta-glucans and xylans into smaller less viscous molecules, thereby solving the filtration and haze problems. The thermostability of the enzyme activities from *Talaromyces emersonii* offers the opportunity to apply the enzyme in high temperature mash or in mashing programs having only short rests at lower temperatures. The enzyme is applied in the mash tun (with malt or mixtures of malt and barley). The use of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* will also increase the yield in the case of low quality malts to increase the productivity in the brewhouse which is economically interesting.

During the boiling stage (typically a 1-2 hour production step, (Briggs, D.E. *et al.*, 2004), the enzymes will be completely inactivated by denaturation. The use of beta-glucanase, cellulase and xylanase can thus be regarded as a processing aid, having no function anymore in the finished foodstuff.

The different steps in the brewery process are shown in Annex 10. The application of enzymes like beta-glucanase, cellulase and xylanase in brewery is comprehensively described (Briggs, D.E. *et al.*, 2004). The effect of the beta-glucanase, cellulase and xylanase preparation from *Talaromyces emersonii* is illustrated in Annex 11.

6.2. Use Levels

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the type and quality of the raw materials used, and the process conditions. The levels of use expected to result in beneficial effect are described in section 1.3.2.

6.3. Enzyme Residues in the Final Food

6.3.1. Residues of inactive enzyme in beer and fermented beverages application

The enzyme preparation is applied during the mashing phase in the manufacture of beer and fermented beverages. The beta-glucanase, cellulase and xylanase activities in enzymes preparations are heat denaturated, and consequently inactivated, during the boiling stage (see Annex 10). So, beer and fermented beverages the consumer buys does not contain active enzyme.

Based on the information given in Sections 1.3.2 and 5.1, the following calculation can be made:

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
Beer	1,000-40,000 BGF/kg malt	20%	200-8,000 BGF/L beer	0.11-4.56 mg/L beer

6.3.2. Possible Effects on Nutrients

The reaction products of the enzymatic conversion of beta-glucan and xylan are (glucose and di-, tri-, tetra and polysaccharides composed of glucose and xylose residues. All is being subject to further degradation by yeast during the fermentation stage. This process also occurs by native beta-glucanases, cellulases and xylanases originated from the malting stage. So, the use of exogeneous enzyme activities does not introduce new reaction products.

Therefore, there is no basis to believe that glucose and xylose residues will have a significant effect, if any, on processed foods or on the human body.

7. SAFETY EVALUATION

7.1. Safety of the Production Strain

The species *Talaromyces emersonii* is considered by the various biosafety expert groups as a biosafety level 1 micro-organism. In Germany the “Berufsgenossenschaft der Chemischen Industrie” has classified the organism as a group 1 organism. The Dutch competent authorities for GMO have included the species on the list of approved hosts to construct GM strains which are safe to be used for large scale productions under conditions not exceeding the GILSP level of physical containment. In their conclusion to add *Talaromyces emersonii* to the list they note that several *Talaromyces* species have the ability to produce mycotoxins, however *Talaromyces emersonii* does not have this ability. In addition they point out that although the species can grow at human body temperature, only one case has been reported in literature of an infection with *Talaromyces* in a patient. And this patient was immunocompromised and also infected with *Aspergillus fumigatus*. This can be considered as an opportunistic infection; for workers safety the organism is classified as biosafety level 1 and thus is safe. Strains of *Talaromyces emersonii* deposited at ATCC carry a note stating that the strains can be used under biosafety level 1 conditions.

Strains from the FBG strain lineage have been used in laboratories and in large scale GILSP production systems at DSM (Gist-brocades) for several decades without any documented health incidents.

The current production strains FBG210 has been analysed for its ability to produce mycotoxins. In the test it was shown that the production strain does not produce any known food mycotoxins of concern. The strain has the ability to produce secondary metabolites (similar to almost any filamentous fungus) as seen in a very sensitive screening analysis but only trace amounts of these compounds could be detected in the fermentation broth. The strains of the FBG lineage have no potential to produce any of the well-known food mycotoxins, the trace amount of secondary metabolites are not toxicologically relevant, and the resulting enzyme has been tested for safety resulting in an appropriate margin of safety (see chapter 7.4). Therefore we conclude that the production strain *Talaromyces emersonii* FBG210 is a safe production strain.

7.2. Safety of the Beta-glucanase, Cellulase and Xylanase Enzyme Preparation

The beta-glucanase, cellulase and xylanase enzyme preparation from *Talaromyces emersonii* is added to the grist during the production of beer and fermented beverages. The beta-glucanase, cellulase and xylanase activities but also the arabinofuranosidase activities has a positive effect in beer and fermented beverages production since they help to make the substrate for beta-glucanase and xylanase more available.

The enzymes beta-glucanase, cellulase and xylanase have a long history of use in food processing. GRAS notifications have been submitted and accepted by FDA with no questions for the use of xylanase enzyme preparation from *Fusarium venenatum* (CFSAN / Office of Food Additive Safety, 2000, GRN 000054), beta-glucanase enzyme preparation from *Trichoderma harzianum* (CFSAN / Office of Food Additive Safety, 2004, GRN 000149), cellulase enzyme preparation from *Myceliophthora thermophila* (CFSAN / Office of Food Additive Safety, 2009)

and mixed beta-glucanase and xylanase enzyme preparation from *Humicola insolens* (CFSAN / Office of Food Additive Safety, 2006, GRN 000195) for use in wine and beer. Several enzyme preparations of beta-glucanase, cellulase or xylanase have been evaluated by JECFA, such as beta-glucanase from *Aspergillus niger* and from *Trichoderma harzianum* (Joint FAO/WHO Expert Committee on Food Additives, 2006a, Joint FAO/WHO Expert Committee on Food Additives, 2006b), cellulase from *Penicillium funiculosum*, *Trichoderma longibrachiatum* and *Trichoderma reesei* (Joint FAO/WHO Expert Committee on Food Additives, 2000; Joint FAO/WHO Expert Committee on Food Additives, 1992; Joint FAO/WHO Expert Committee on Food Additives, 1988) or xylanase from *Thermomyces lanuginosus* and from *Bacillus subtilis* (Joint FAO/WHO Expert Committee on Food Additives, 2003; Joint FAO/WHO Expert Committee on Food Additives, 2004) who have all been attributed an ADI 'not specified' for their use in several applications such as the preparation of fruit juices, beer and baking products. DSM enzyme preparation from *Talaromyces emersonii* is authorized as processing aid in beer manufacture in the United Kingdom since 1982. Its use as processing aid in beer was also approved in Australia, in France, in Brazil and in China.

Beta-glucanase, cellulase and xylanase are widely distributed in nature. It has been isolated from a variety of sources, such as fungi, yeasts, bacteria, plants and marine invertebrates (Müller, J.J. *et al.*, 1998; Wong, Y.-. and MacLachlan, G.A., 1980; Polizeli, M.L.T.M. *et al.*, 2005; Mawadza, C. *et al.*, 2000; McCarthy, T.C. *et al.*, 2005; Knowles, J. *et al.*, 1987). Since beta-glucanase, cellulase and xylanase are enzymes naturally present in nature and notably in plants and marine invertebrates consumed by human, DSM expects it will be digested as would any other protein occurring in food.

The enzyme preparation beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* was evaluated according the Pariza & Johnson Decision Tree and accepted. The decision tree is based on the safety evaluation methodology published by Pariza and Foster in a 1983 article, which was extended by the IFBC into the decision tree format and published in 1991. In 2001, Pariza and Johnson published an update. DSM's decision tree analysis, based on the most recent update of the decision tree, is described in Annex 12.

However, in order to confirm the assumption that beta-glucanase, cellulase and xylanase would not have any toxic properties and to further establish the toxicological safety of the use of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* in food, a number of toxicity studies have been performed on the ultra-filtrate concentrate.

An Ames test, an acute, subacute and subchronic oral toxicity study in rats were performed in the seventies on the enzymes preparation from *Talaromyces emersonii* FBG1 by third parties. The NOEL of the subchronic toxicity study was > 0.5 ml/kg/day (equivalent to 3700 Units/kg/day). In the eighties an Ames test and a 90-day subchronic oral toxicity study in rat were performed with a limit concentration of 3500 mg/kg/day (~ 42000 BGU/kg/day). From these studies it was concluded that the enzyme preparation showed absence of mutagenicity and the highest dose tested in the 90-day toxicity study was regarded as the NOAEL.

However, due to some modifications in the production process during the years and lack of cytogenicity data, new toxicity studies were performed to confirm the safety of the product:

- Ames test,

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

The results of these studies are summarized in Section 7.4.

7.2.1. Allergenicity

Proteins have the potential to cause allergic responses in process operators (worker's safety) and in consumers (food allergy). Several cases of occupational allergy consecutive to the inhalation of aerosols containing beta-glucanase, cellulase or xylanase have been reported (Martel, C. *et al.*, 2010, Martel, C. *et al.*, 2010). Inhalation of aerosols containing beta-glucanase, cellulase or xylanase from *Talaromyces emersonii* by operators in the production of beer or fermented beverages should therefore be prevented. First aid measures and potential health hazards related to inhalation sensitization have been described in the product MSDS's (see Annex 13).

Regarding food allergy, in theory, consumers could be sensitized or react allergic to enzymes in food. However, since exposure of consumers to enzymes used as processing aid in food is very low, and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, the likelihood of allergic sensitization by consumers to these proteins is virtually zero. The absence of food allergenicity has been confirmed by an extensive literature survey of producers' files, in which no cases have been found of people that have been sensitized or that reacted allergic by ingestion of food prepared with various enzymes (see Annex 14). Even people who ingest high daily doses of enzymes as digestive aids are not reported to have gastrointestinal allergy to enzymes even after many years of daily intake. Recently, it was concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindslev-Jensen, C. *et al.*, 2006).

In addition, beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* has a long history of safe use in food. To the best of our knowledge, no report exists on allergic reactions due to the ingestion of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii*. This strain is not listed in the World Health Organization/International Union of Immunological Societies allergen nomenclature.

7.3. Safety of the Manufacturing Process

The manufacture of the beta-glucanase, cellulase and xylanase is performed under the food GMP requirements and in addition the HACCP principles are followed. This is also described in Section 4. Moreover it is indicated that ingredients are used that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

Beta-glucanase, cellulase and xylanase preparation meets the general and additional quality requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex.

7.4. Summary of toxicology studies

This section describes the studies performed to evaluate the safety of using DSM's beta-glucanase, cellulase and xylanase preparation.

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

The batch used for the toxicity studies (referred to as "the tox-batch") was produced by the procedure used for the commercial preparation of the enzyme. The production process - performed according to the requirements of ISO9002 - includes the fermentation process, recovery (down-stream processing) and formulation of the product. The purification process produced the final, non-standardised ultra-filtrate concentrate (tox-batch), which was characterised by chemical and microbial analysis. The initial enzyme activities of the tox-batch are approximately 92,000 BGF/g (both beta-glucanase and cellulase) and 520 XVU/g with a TOS value of 5.3%.

Levels used in the 90-day oral gavage studies were chosen to provide a sufficient margin of safety towards expected exposure (see Section 7.5).

7.4.1. 90-day oral toxicity in rats

The sub-chronic oral toxicity of the tox-batch was examined in a 90-day toxicity study with groups of 20 male and 20 female Wistar rats, performed by Huntingdon Life Sciences Ltd, Suffolk, England. The rats received daily the tox-batch by gavage, at dosages of 100, 400 or 1600 mg/kg body weight/day for 13 weeks. The control group received the vehicle (water obtained by reverse osmosis) alone. Clinical signs, body weight, food consumption, food conversion efficiency, haematology, blood chemistry, ophthalmoscopic examination, organ weights, macroscopic and microscopic pathology were studied.

Results

Treatment of CD rats with tox-batch for 13 weeks at dosages of 100, 400 or 1600 mg/kg bw/day did not result in any treatment-related deaths. There were no general clinical signs associated with treatment, organ weights were not affected and ophthalmoscopic, macroscopic and microscopic examination did not reveal any abnormalities associated with the administration of the test material.

The males receiving 1600 mg/kg bw/day had a slightly lower overall bodyweight gains (6% less) than the Controls, and they ate 4 to 12 g food/animal/week less than the control, but these effects were not significant.

When compared with the Controls, slightly longer prothrombin times (0.7 seconds longer) were recorded for both sexes at the highest dose with slightly longer activated partial thromboplastin times (3.6 seconds longer) also seen in the females. Changes in clotting times may be related to changes in hepatic metabolism. However, given that there are no macroscopic or microscopic treatment-related findings recorded in the livers of these animals and that the liver weights are unremarkable, these slight changes in haematology are of only minor importance. These effects

were only noted in the group which received a dose much higher than the anticipated human exposure (see Chapter 7.5), and resulted in no overt clinical manifestations such as premature death or anatomic abnormalities.

Slightly low aspartate amino-transferase activities were observed in females at the highest dose (71 U/L compared to 85 U/L for the Controls). High levels of aspartate amino-transferase activity are markers of liver damage in rats, low activity levels are considered to be of no toxicological significance.

Conclusion

The administration of the tox-batch to CD rats at dosages of 100, 400 or 1600 mg/kg bw/day resulted in no treatment-related effects in animals receiving 100 or 400 mg/kg bw/day. A few slight effects were noted in animals receiving 1600 mg/kg bw/day, but these were not considered to be of toxicological significance.

The No-Observed-Adverse-Effect-Level (NOAEL) for this study is therefore considered to be 1,600 mg/kg bw/day, corresponding to 84.8 mg TOS/kg bw/day.

7.4.2. Genotoxicity: Bacterial mutation assay (Ames test).

The mutagenic potency of the tox-batch was studied by Huntingdon Life Sciences Ltd, Eye, Suffolk, England in four histidine-dependent *Salmonella typhimurium* mutant strains TA 98, TA 100, TA 1535 and TA 1537 and one tryptophan-dependent *Escherichia coli* mutant WP2uvrA strain (CM891). Experiments were performed in the absence and presence of S9-mix, a rat liver-derived metabolic activation system.

Concentrations of up to 10 mg /ml were tested in the main mutation tests. Other concentrations used were a series of dilutions of the highest concentration (separated by *ca* half-log₁₀ intervals). The concentrations are expressed in terms of the dry matter content of the enzyme preparation. Negative (i.e purified water) and positive controls were run simultaneously with the test substance.

Results

No signs of toxicity were observed towards the tester strains in either mutation test.

No evidence of mutagenic activity was seen at any concentration of the tox-batch in either mutation test, while the concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It is concluded that the tox-batch shows no evidence of mutagenic activity in this bacterial system.

7.4.3. *In vitro* mammalian chromosome aberration test in human lymphocytes

The tox-batch was examined for its potential of induction of chromosomal aberrations in cultured human peripheral lymphocytes in the presence and absence of S9-mix. Two independent chromosomal aberration experiments were conducted by Huntingdon Life Sciences Ltd, Suffolk, England. Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Negative (i.e. solvent) and positive controls were run simultaneously with the test substance.

(i) Experiment 1

In both the absence and presence of S9-mix, the tox-batch was incubated with the cells during 3 hours in concentrations 1250, 2500 and 5000 µg/ml, and recovered after 17 hours.

(ii) Experiment 2

In the absence of S9-mix, the tox-batch was incubated with the cells during 20 hours (continuous treatment) in concentrations 1000, 3000 and 4000 µg/ml. In the presence of S9-mix, the tox-batch was incubated with the cells during 3 hours (pulse treatment) in concentrations 1250, 2500 and 5000 µg/ml, and recovered after 17 hours.

In the first test, in both the absence and presence of S9 mix, the tox-batch caused no statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations, at any dose level, when compared with the solvent control.

In the second test in the absence of S9 mix, but only when gap-type aberrations were included, the tox-batch caused a statistically significant increase in the proportion of cells with chromosomal aberrations at 4000 µg/ml ($P < 0.01$). However, due to the questionable nature of gap-type aberrations and the absence of a dose response relationship, this is not considered to be of biological significance.

In the second test, in the presence of S9 mix, the tox-batch caused no statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations, at any dose level, when compared with the solvent control.

A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No increases in the proportion of polyploid cells were seen in either test.

The positive control substances gave the expected statistically significant increase in the incidence of structural chromosomal aberrations.

It is concluded that the tox-batch has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system.

7.5. Estimates of Human Consumption and Safety Margin

On the basis of the information given in Section 6.3 the following estimation of the human consumption can be made:

Final food	Residual amount of (inactive) enzyme in final food (BGF/L beer)	Amount of TOS in final food	95 th percentile intake level (mL food/person/day) ¹	Estimated daily intake of TOS (mg/kg bw/day) ²
Beer	200-8,000	0.11-4.56 mg/L beer	360	0.00066-0.027

¹ Intake level of beer and ale based on (Wilson, J.W. *et al.*, 1997). 90th percentile is approximately 2 times the intake level and 95th percentile approximately 4 times the intake level (US Food and Drug Administration, 2006). As alcohol intake is usually thought to be underreported in surveys (US Food and Drug Administration, 2006), the 95th percentile is taken here as worst-case scenario.

² Calculated for a person of 60 kg.

This estimate is conservative as the mashing step will be followed by a vigorous boiling step of 1-2 hours that will denature and inactivate the enzymes.

The 90-day oral toxicity study showed a NOAEL of 1,600 mg enzyme preparation/kg bw/day, corresponding to 84.8 mg TOS/kg bw/day. Thus the Margin of Safety lies between 3,140 and 128,500.

7.6. Results and Conclusion

Results of the toxicity and mutagenicity tests described in Section 7.4 demonstrate the safety of DSM's beta-glucanase, cellulase and xylanase preparation, which showed no toxicity or mutagenicity across a variety of test conditions. The data resulting from these studies is consistent with the long history of safe use for *Talaromyces emersonii* in food processing and the natural occurrence of beta-glucanase, cellulase and xylanase in foods, and in accordance with the conclusions found in a review of relevant literature. Based upon these factors it is DSM's conclusion that beta-glucanase, cellulase and xylanase preparation from *Talaromyces emersonii* is GRAS for the intended conditions of use.

8. LIST OF REFERENCES

- Benjamin, C.R. (1955) *Ascocarps of Aspergillus and Penicillium.*, Mycologia 47 669-687
- Bindslev-Jensen, C., Skov, P.S., Roggen, E.L., Hvass, P. and Brinch, D.S. (2006) *Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*, Food Chem. Toxicol. 44 (11) 1909-1915
- Briggs, D.E., Boulton, C.A., Brookes, P.A. and Stevens, R. (2004) *Supplementary enzymes. In: Brewing Science and practice.* Woodhead Publishing 46-49
- CFSAN / Office of Food Additive Safety. (2000, GRN 000054) *Xylanase derived from Fusarium venenatum carrying a gene encoding xylanase from Thermomyces lanuginosus*, Agency Response Letter
- CFSAN / Office of Food Additive Safety. (2004, GRN 000149) *Beta-glucanase enzyme preparation from Trichoderma harzianum.*, Agency Response Letter
- CFSAN / Office of Food Additive Safety. (2006, GRN 000195) *Mixed beta-Glucanase and xylanase enzyme preparation from Humicola insolens.*, Agency Response Letter
- CFSAN / Office of Food Additive Safety. (2009) Agency Response Letter to GRAS Notice no. GRN 000292: *Cellulase enzyme preparation derived from a genetically modified strain of Myceliophthora thermophila*, available at:
<http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=292>
- Ciegler, A., Hayes, A.W. and Vesonder, R.F. (1980) *Production and biological activity of secalonic acid D.* Applied and Environmental Microbiology 39 (2) 285-287
- Cimon, B., Carrere, J., Chazalette, J.P., Vinatier, J.F., Chabasse, D. and Bouchara, J.P. (1999) *Chronic airway colonization by Penicillium emersonii in a patient with cystic fibrosis.* Med. Mycol. 37 291-293
- Clarke, A.E. and Stone, B.A. (1966) *Enzymic hydrolysis of barley and other beta-glucans by a beta-(1-4)-glucan hydrolase.* Biochem J. 99 (3) 582-588
- Frisvad, J.C., Filtenborg, O., Samson, R.A. and Stolk, A.C. (1990) *Chemotaxonomy of the genus Talaromyces.* Antonie Van Leeuwenhoek 57 179-189
- Godfrey, T. and West, S. Industrial enzymology (second edition). Macmillan Press Ltd, United Kingdom (1996) 609-123-124
- Harrington, J., Kirwan, L. and Stuart, M.R. (1979) *Heat tolerance of thermophilic and thermotolerant fungi in composted conifer bark.* J. Lifer Sci. R. Dubl. Soc. 1 99-105

Houbraken, J., Spierenburg, H. and Frisvad, J.C. (2012) *Rasamsonia*, a new genus comprising thermotolerant and thermophilic *Talaromyces* and *Geosmithia* species. *Antonie Van Leeuwenhoek* 101 403-421

Joint FAO/WHO Expert Committee on Food Additives, 31st meeting, Geneva, 1987, *Cellulase from Trichoderma reesei*, WHO Food Additives Series 38,52 (1988), available at: <http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/>

Joint FAO/WHO Expert Committee on Food Additives, 39th meeting, Rome, 1992, *cellulase from Trichoderma longibrachiatum*, WHO Food Additives Series 52 (1992), available at: <http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/>

Joint FAO/WHO Expert Committee on Food Additives, WHO 31th meeting, Rome, *cellulase from Penicillium funiculosum*, WHO Food Additives Series 52 (2000), 3, available at: <http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/>

Joint FAO/WHO Expert Committee on Food Additives, 61st meeting, Rome, 2003, *Xylanase from Thermomyces lanuginosus expressed in Fusarium venenatum*, WHO Food Additives Series 52 (2003), add 11,

Joint FAO/WHO Expert Committee on Food Additives, 63th meeting, Geneva, 8-17 June 2004, *Xylanase from Bacillus subtilis expressed in Bacillus subtilis*, WHO Food Additives Series 52 (2004), add 12,

Joint FAO/WHO Expert Committee on Food Additives. (2006a) *beta-glucanase from Aspergillus niger, var.* Combined Compendium of Food Additive Specifications

Joint FAO/WHO Expert Committee on Food Additives. (2006b) *beta-Glucanase from Trichoderma harzianum*, Combined Compendium of Food Additive Specifications

Knowles, J., Lehtovaara, P., Penttilä, M., Teeri, T., Harkki, A. and Salovuori, I. (1987) *The cellulase genes of Trichoderma*, *Antonie Van Leeuwenhoek* 53 (5) 335-341

Martel, C., Nielsen, G.D., Mari, A., Rask Licht, T. and Poulsen, L.K. (2010) *Biobibliographic review on the potential of microorganisms, microbial products and enzymes to induce respiratory sensitization*. Scientific/technical Report Submitted to EFSA CFP/EFSA/FEEDAP/2009/02 1-95

Mawadza, C., Hatti-Kaul, R., Zvauya, R. and Mattiasson, B. (2000) *Purification and characterization of cellulases produced by two Bacillus strains*, *J. Biotechnol.* 83 (3) 177-187

Mayura, K., Hayes, A.W. and Berndt, W.O. (1982) *Teratogenicity of secalonic acid D in rats*. *Toxicology* 25 311-322

- McCarthy, T., Hanniffy, O., Savage, A.V. and Tuohy, M.G. (2003) *Catalytic properties and mode of action of three endo-beta-glucanases from Talaromyces emersonii on soluble 1,3-1,4-beta-linked-glucans*, International Journal of Biological Macromolecules 33 141-148
- McCarthy, T.C., Lalor, E., Hanniffy, O., Savage, A.V. and Tuohy, M.G. (2005) *Comparison of wild-type and UV-mutant β -glucanase-producing strains of Talaromyces emersonii with potential in brewing applications*, Journal of Industrial Microbiology and Biotechnology 32 (4) 125-134
- Müller, J.J., Thomsen, K.K. and Heinemann, U. (1998) *Crystal structure of barley 1,3-1,4-beta-glucanase at 2.0 Å resolution and comparison with Bacillus 1,3-1,4-beta-glucanase*, J. Biol. Chem. 273 (6) 3438-3446
- Murray, P., Grassick, A., Laffey, C.D., Cuffe, M.M., Higgins, T., Savage, A.V., Planas, A. and Tuohy, M.G. (2001) *Isolation and characterisation of thermostable endo-beta-glucanase active on 1,3-1,4-beta-D-glucans from the aerobic fungus Talaromyces emersonii CBS 814.70*. Enzymes and Microbial Technology 29 90-98
- Pariza, M.W. and Johnson, E.A. (2001) *Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century*, Regul. Toxicol. Pharmacol. 33 (2) 173-186
- Polizeli, M.L.T.M., Rizzatti, A.C.S. and Monti, R. (2005) *Xylanases from fungi: properties and industrial applications*. Appl Microbiol Biotechnol 67 577-591
- Raper, K.B., and Thom, C. (1949) *A manual of the Penicillia*. Williams and Wilkins Comp. Baltimore U. S. A.
- Samson, R.A. and van Reenen-Hoekstra, E.S. (1988) *Introduction to food-borne fungi*. CBS, Baarn and Delft
- Stolk, A.C. (1965) *Thermophilic species of Talaromyces Benjamin and Thermoascus Miede*. Antonie Van Leeuwenhoek 31 262-276
- Stolk, A.C. and Samson, R.A. (1972) *The genus Talaromyces. Studies on Talaromyces and related genera II*. Studies in Mycology 2 1
- Tuohy, M.G. and Coughlan, M.P. (1992) *Production of thermostable xylan-degrading enzymes by Talaromyces emersonii*, Bioresource Technology 39 131-137
- US Food and Drug Administration. (2006) *Estimating Dietary Intake of Substances in Food*. Center for Food Safety & Applied Nutrition, CFSAN/Office of Food Additive Safety.
- Wilson, J.W., Wilkinson Enns, C., Goldman, J.D., Tippet, K.S., Mickle, S.J., Cleveland, L.E. and Chahil, P.S. (1997) *Data tables: combined results from USDA's 1994 and 1995 Continuing Survey of Food Intakes by Individuals and 1994 and 1995 Diet and Health Knowledge Survey*, ARS Food Surveys Research Group

Wong, Y.-. and MacLachlan, G.A. (1980) *1,3-beta-D-glucanases from Pisum sativum seedlings*,
Plant Physiol. 65 222-228

9. LIST OF ANNEXES

- 1 Genealogy / strain lineage
- 2 Taxonomic identification of FBG1 strain
- 3 Taxonomic identification of FBG1, FBG2 and FGB210 strains
- 4 Analyses of FBG1 strain for toxic metabolite production
- 5 Analyses of FBG1 fermentation broth for toxic fungal metabolites
- 6 Analyses of *Talaromyces emersonii* strains and fermentation broth for the presence of secondary metabolites
- 7 Method of analysis of BGF (beta-glucanase and cellulase) activity: DSM method
- 8 Method of analysis of XYL activity: DSM method
- 9 Flow diagram of manufacturing process
- 10 Brewing process
- 11 Effect of beta-glucanase, cellulase and xylanase enzyme preparation on filterability, viscosity and beta-glucan content
- 12 Safety evaluation using the Pariza & Johnson decision tree of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii*
- 13 MSDS of Filtrase NL[®]: enzyme preparation of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii*
- 14 Report from the Amfep Working Group on Consumer Allergy: risk from enzyme residues in food (August 1998)

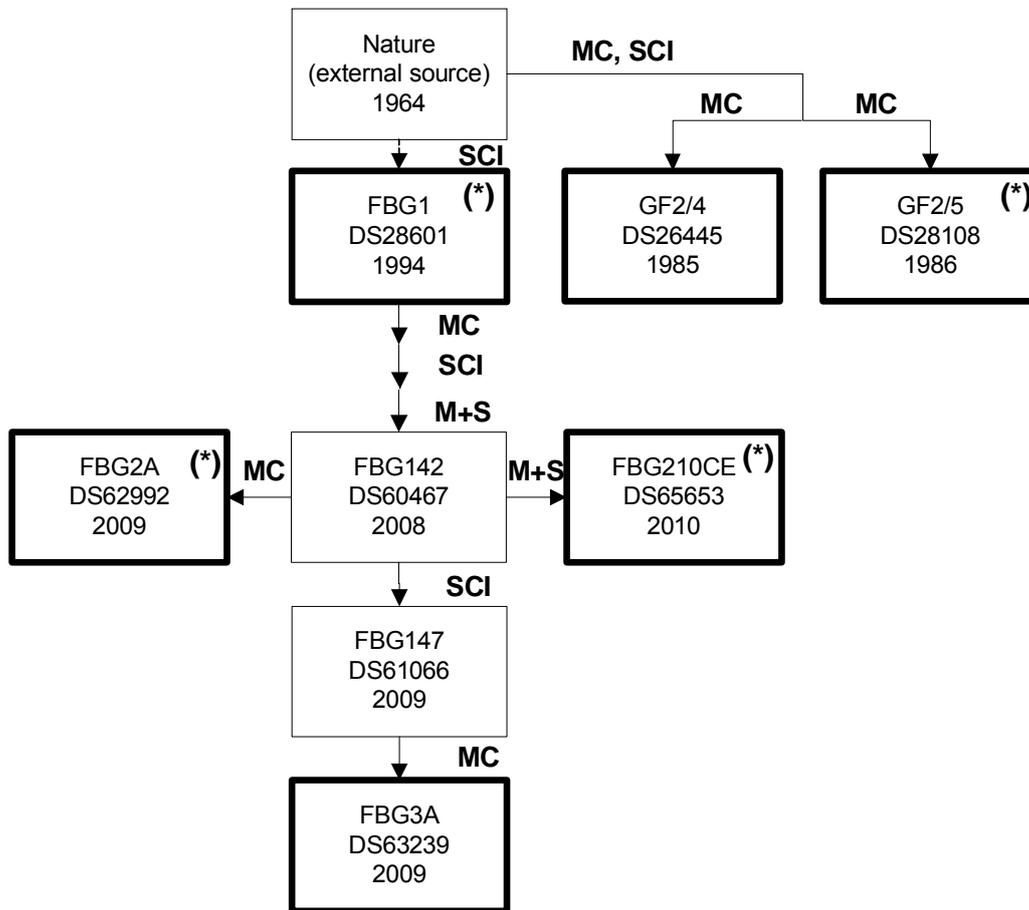
ANNEX 1
Genealogy / strain lineage

Product: Fungal β -Glucanase, cellulase & xylanase (BGF)

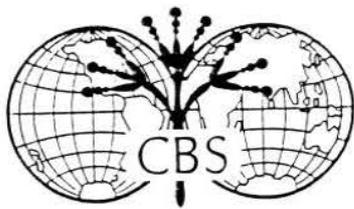
Strain: *Rasamsonia emersonii*
(formerly *Talaromyces emersonii*)

Print date: 28 May 2013

M+S	<input type="checkbox"/> Mutation and Selection
MC	<input type="checkbox"/> Mass Culture
SCI	<input type="checkbox"/> Single Colony Isolate
rDNA	<input type="checkbox"/> using r-DNA techniques
(*)	<input type="checkbox"/> Identified (externally)
Bold 	<input type="checkbox"/> Production strain



ANNEX 2
Taxonomic identification of FBG1 strain



De Heer C. de Vogel
R&D Stamconservering
Gist Brocades
Postbus 1
2600 MA DELFT

Baarn, 10-3-1997

Onze ref.: Det.: 20 RAS/as

Uw ref.: order 6522653

DETERMINATIE DIENST

Hieronder vindt U de uitslag van het door U gevraagde onderzoek.

ARO-1 (DS6047) = *Aspergillus niger* v. Tieghem

PEC-4 (DS2825) = *Aspergillus niger* v. Tieghem

FBG-1 (DS28601) = *Penicillium emersonii* Stolk. (De teleomorph is in de cultuur niet waargenomen!)

DXL-1 (DS31362) = *Disporotrichum dimorphosporum* (von Arx) Stalpers

Tevens treft U de rekening aan voor deze identificaties.

Hoogachtend,

(b) (6)

Drs. R.A. Samson

CENTRAALBUREAU VOOR SCHIMELCULTURES (CBS)
Institute of the Royal Netherlands Academy of Arts and Sciences

Mr. C. de Vogel
Research and Development / Strain conservation
Gist-brocades
P.O. box 1
2600MA Delft

Baam, 10-03-1997

Our ref: Det.: 20 RAS/as

Your ref.: order 6522653

Taxonomic identification service

Below you will find the result of the requested investigation

- ARO-1 (DS6047) *Aspergillus niger* v. Tieghem
- PEC-4 (DS2825) *Aspergillus niger* v. Tieghem
- FBG-1 (DS28601) *Penicillium emersonii* Stolk (the teleomorph was not observed in the culture)
- DXL-1 (DS31362) *Disporotrichum dimorphosporum* (von Arx) Stalpers

The invoice for the taxonomic identifications is attached.

Sincerely,

Dr. R.A. Samson

ANNEX 3
Taxonomic identification of FBG1, FBG2
and FGB210 strains



Centraalbureau voor Schimmelcultures

Fungal Biodiversity Centre

Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

DSM Food Specialties
Attn. Mr. C. Van der Weijden
P.O. box 1
2600 MA Delft

Utrecht, 22 november 2011

CBS IDENTIFICATION SERVICE

Uw ref.: **FBG1 (DS28601), FBG2A (DS62992) en FBG210CE (DS65653)**

Onze ref.: **Det 11-120**

Wanneer u contact met ons opneemt graag ons referentienr gebruiken.

Hierbij sturen wij u de resultaten van onze identificatie van de door u ingezonden stammen.

FBG1 (DS28601)	=	<i>Rasamsonia emersonii</i> (Stolk) Houbraken & Frisvad (voorheen <i>Talaromyces emersonii</i> Stolk)
FBG2A (DS62992)	=	<i>Rasamsonia emersonii</i> (Stolk) Houbraken & Frisvad (voorheen <i>Talaromyces emersonii</i> Stolk)
FBG210CE (DS65653)	=	<i>Rasamsonia emersonii</i> (Stolk) Houbraken & Frisvad (voorheen <i>Talaromyces emersonii</i> Stolk)

Tevens treft u een kopie van het artikel aan waarin deze naamswijziging in beschreven staat.

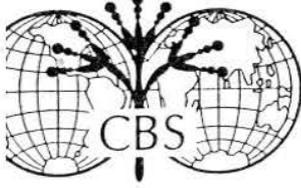
De factuur voor deze identificatie zal separaat naar u worden toegezonden.

Met vriendelijke groet,

(b) (6)

M. Meijer, Bsc.

ANNEX 4
**Analyses of FBG1 strain for toxic metabolite
production**



Report

Analyses of

one strain of

***Talaromyces emersonii* FGB-1 (DS28601)**

for

toxic metabolite production

Aim of the investigation

Fungi are known to produce many secondary metabolites. Some of these metabolites are considered mycotoxins. The aim of this investigation was to analyse culture extracts of one *Talaromyces emersonii* strain for toxic metabolites.

Cultivation and extracting of the strains and samples were performed at the Centraalbureau voor Schimmelcultures in Baarn. The extracts were sent to Prof. Jens C. Frisvad (University of Denmark, Lyngby), who analysed the metabolites and compared them with all important toxins which may be significant.

Methods

The following strain was examined:

Talaromyces emersonii FGB-1 (DS28601)

The strain was cultured on the following media which are given the most optimal expression for the production of secondary metabolites: Czapek yeast autolysate agar (CYA), Blakeslee malt agar (MEA), yeast extract sucrose agar (YES) and oatmeal agar (OA) (Frisvad and Filtenborg, 1989; Frisvad, 1993). All cultures were incubated for 10 days in darkness at 37°C.

For metabolite analysis, the contents of each plate were combined and extracted by the method described in Frisvad and Thrane (1987) and analysed by high performance liquid chromatography (HPLC) with diode array detection (DAD) (Frisvad and Thrane, 1993). For the samples of the fermentation products 1 gram of the dried powder was extracted in 25 ml EtOAc and 30 ml CHCl₃: MeOH (2:1) and stand overnight.

The metabolites found were compared to a spectral UV library made from authentic standards run at the same conditions (the maximal similarity was a match of 1000), and retention indices were compared with those of standards.

Results

The strain showed the production of several metabolites (see enclosed HPLC spectra), but no known mycotoxins were found.

Conclusions

When compared to a spectral UV library made from authentic standards of all important fungal toxins which may be of significance, no mycotoxins were found. References see Cole and Cox (1981) and Smith and Moss (1985).

References:

Cole, R.J. & Cox, R.H. (1981). Handbook of toxic fungal metabolites. Academic press, New York.

Frisvad, J.C. & Filtenborg, O. 1989. Terverticillate penicillia: chemotaxonomy and mycotoxin production. *Mycologia* **81**: 837-861.

Frisvad, J.C. & Thrane, U. 1987. Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). *Journal of Chromatography* **404**: 195-214.

Frisvad, J.C. & Thrane, U. 1993. Liquid column chromatography of mycotoxins. In: Betina, V. (ed.): *Chromatography of mycotoxins. Techniques and applications*. *Journal of Chromatography Library* **54**: 253-372. Elsevier, Amsterdam.

Smith, J.E. & Moss, M.O. (1985). *Mycotoxins. Formation, analysis and significance*. John Wiley & Sons, Chichester.

Baarn, 15 April 1997

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Dr. R.A. Samson

ANNEX 5
**Analyses of FBG1 fermentation broth for
toxic fungal metabolites**



CENTRAALBUREAU VOOR SCHIMMELCULTURES

Institute of the Royal Netherlands Academy of Arts and Sciences

Report

Analyses of

one sample of

Fermentation broth BGF batch 8016

for

toxic fungal metabolites

Centraalbureau voor Schimmelcultures,

P.O.Box 273, 3740 AG BAARN

1

In order of: Gist-brocades B.V.
P.O. Box 1
2600 MA Delft

Your ref.: SBG/SCU/98-755

Our ref: GBE/98-12

Aim of the investigation

Fungi are known to produce many secondary metabolites. Some of these metabolites are considered mycotoxins which are significant for food. The aim of this investigation was to analyse extracts of one fermentation broth for toxic metabolites.

Extracting of the sample was performed at Gist-brocades and subsequently at the Centraalbureau voor Schimmelcultures in Baarn, the Netherlands. The extract was sent to Prof. Jens C. Frisvad (Technical University of Denmark, Lyngby), who analysed the metabolites and compared them with all important toxins which may be significant in food.

Methods

The following sample was examined:

Fermentation broth BGF batch 8016

For metabolite analysis, the samples were extracted by the method described in Frisvad and Thrane (1987) and analysed by high performance liquid chromatography (HPLC) with diode array detection (DAD) (Frisvad and Thrane, 1993).

The metabolites found were compared to a spectral UV library made from authentic standards run at the same conditions (the maximal similarity was a match of 1000), and retention indices were compared with those of standards.

Results

The sample fermentation broth BGF batch 8016 contained no known metabolites (see enclosed HPLC spectra).

Conclusions

When compared to a spectral UV library made from authentic standards of all important fungal toxins which may be of significance in food, no important mycotoxins were found.

None of the metabolites found are considered significant mycotoxins (Cole and Cox, 1981; Smith and Moss, 1985).

References

Cole, R.J. & Cox, R.H. (1981). Handbook of toxic fungal metabolites. Academic press, New York.

Frisvad, J.C. & Thrane, U. 1993. Liquid column chromatography of mycotoxins. In: Betina, V. (ed.): Chromatography of mycotoxins. Techniques and applications. Journal of Chromatography Library 54: 253-372. Elsevier, Amsterdam.

Smith, J.E. & Moss, M.O. (1985). Mycotoxins. Formation, analysis and significance. John Wiley & Sons, Chichester.

Baarn, 18-12-1998

(b) (6)



Drs. E.S. Hoekstra

ANNEX 6
**Analyses of *Talaromyces emersonii* strains
and fermentation broth for the presence of
secondary metabolites**



Centraalbureau voor Schimmelcultures
Fungal Biodiversity Centre
Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

Report

**Analyses of two *Talaromyces emersonii* strains and two
fermentation broths on the presence of toxic
metabolites**

In order of: DSM Food Specialties
P.O. box 1
2600 MA Delft

Contact: Mr. C. Van der Weijden

Our ref.: TM 10.051

Your ref.: DBC/SCU/2010.1503/WEC

Analyses of: *Talaromyces emersonii* FBG1 (DS28601)
Talaromyces emersonii FBG2A (DS62992)
Fermentation broth 409019301
Fermentation broth 610003301

Aim of the investigation

Fungi are known to produce many secondary metabolites. Some of these metabolites are considered to be mycotoxins. The aim of this investigation was to analyze two *Talaromyces emersonii* strains (**FBG1** and **FBG2A**) and two fermentation broths (**409019301** and **610003301**) for toxic metabolites and to confirm the identity by molecular methods. Extractions of the strain and of the two fermentation broths were performed at the the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands. The extracts were sent to Dr. Michael Sulyok (University of Natural Resources and Life Sciences, Vienna), who analyzed the extracts on the presence of metabolites and compared them with all important toxins which may be significant.

Methods

Metabolite analyses (strain)

The following strains were examined:

***Talaromyces emersonii* FBG1 (DS28601)**

***Talaromyces emersonii* FBG2A (DS62992)**

The two *Talaromyces emersonii* strains were cultured on the following media which are given the most optimal expression for the production of secondary metabolites: Czapek Yeast Autolysate agar (CYA), Malt Extract Agar (MEA, Oxoid), Oatmeal Agar (OA) and yeast extract sucrose agar (YES) (Samson *et al.*, 2010). All Petri dishes were incubated for 14 days in darkness at 37°C. For metabolite analysis six agar plugs with fungal colonies of each plate were combined and extracted by the method described in Smedsgaard (1997).

Metabolite analyses (broths)

The following fermentation broths were examined:

409019301

610003301

Of each broth 600µl was taken and 600µl ethyl acetate was added, mixed and centrifuged. The supernatant was transferred to a HPLC vial for analysis by LC-MS/MS as described in Nielsen *et al.* (2009). The metabolites found were compared to a spectral UV library made from authentic

standards run at the same conditions (the maximal similarity was a match of 1000), and retention indices were compared with those of standards or known from literature.

Results and Conclusions

Metabolite analyses (strains)

The *Talaromyces emersonii* strain, **FBG1** produced a pattern of secondary metabolites characteristic for *Talaromyces* species (including secalonic acid D, brevianamide F, emodin and malformin).

The *Talaromyces emersonii* strain, **FBG2A** produced a pattern of secondary metabolites characteristic for *Talaromyces* species (including secalonic acid D, brevianamide F, emodin and malformin).

Metabolite analyses (broths)

In fermentation broth sample **409019301** traces of secalonic acid D and brevianamide F were detected.

In fermentation broth sample **610003301** traces of secalonic acid D and brevianamide F were detected.

The concentration of secalonic acid D and brevianamide F was lower in fermentation broth sample **610003301** than in **409019301**.

References

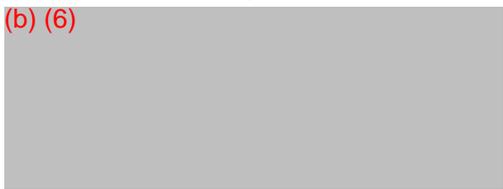
Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC.(2009).Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal Bioanal Chem.* 395:1225-1242

Samson RA, Houbraken, J., Frisvad, JC, Thrane U, Andersen B (2010). *Food and Indoor fungi.* CBS Laboratory Manual series 2, 390 pp.

Smedsgaard, J. (1997). Micro scale extraction procedure for standardized screening of fungal metabolite production in culture. *Journal of Chromatography A* 760: 264-270.

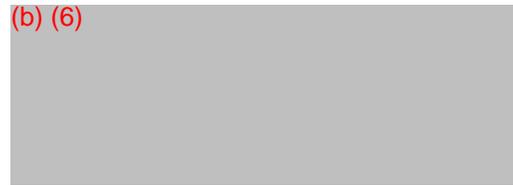
Utrecht, 21 July 2011

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Dr. R.A. Samson

(b) (6)

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Ing M. Meijer

Note: The HPLC spectra of the analysis carried out in this investigation are filed in the Centraalbureau voor Schimmelcultures archive and can be available for inspection.

ANNEX 7
**Method of analysis of BGF (beta-glucanase
and cellulase) activity: DSM method**

Analysis Service and solutions	METHOD OF ANALYSIS	No : 1993 Version : 3 Page : 1 of 10
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Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

Product: Fungal betaglucanase Standard preparations	Validated method NO	Date of issue:
---	----------------------------	----------------

AUTHORIZATION

Compiled by	W. W. van Kan	Date:
Approved by expert	G. Hermans	Date:
Approved by QA/QC Analysis	M.M. Immerzeel	Date:
Approved by Team Lead Service Lab Delft	M. Steenbeek	Date:
Approved by external QA/QC (if applicable)	n.a.	Date:
Approved by external QA/QC (if applicable)	n.a.	Date:

Analysis Service and solutions	METHOD OF ANALYSIS	No : 1993 Version : 3 Page : 2 of 10
--	---------------------------	---

Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

1 SAFETY AND ENVIRONMENT

Restrictions for working with chemicals and ML-I samples are mentioned in the work instructions concerning management, storage and use of chemicals, the handling of dangerous substances and standard rules for ML-I laboratories. These restrictions are also applicable for material that has been in contact with ML-I samples.

When working with strong acids, bases, carcinogenic matters and toxic matters etc. take all necessary precautions.

When working with highly concentrated enzyme preparations take all necessary precautions. Avoid inhalation of dust and prolonged contact with unprotected skin.

2 PRINCIPLE

2.1 Application

This method is applicable for the determination of the fungal betaglucanase activity in e.g. fungal betaglucanase standard preparations that are used for the relative fungal betaglucanase activity determination.

2.2 Description of the method

The reduction of the viscosity of a betaglucan solution of pH 5.60 and 45 °C, caused by betaglucanase enzyme activity is measured using an Ubbelohde viscosimeter. The reduction in viscosity is a measure for the enzyme activity.

2.3 Unit definition

One **BetaGlucanase Fungique** (BGF) unit is the amount of enzyme per ml reaction mixture (15 mL substrate and 2 mL enzyme solution) that causes a change in viscosity of the substrate with a speed giving a slope of 0.147 per minute under the conditions of the test.

2.4 Measuring range

The measuring range of this method is 3.4 to 6.8 BGF per mL.

2.5 Summary of the validation report

Not applicable.

Analysis Service and solutions	METHOD OF ANALYSIS	No : 1993 Version : 3 Page : 3 of 10
--	---------------------------	---

Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

3 APPARATUS AND CONDITIONS

3.1 Apparatus

- Bath with boiling water : GFL
- Water bath, adjusted at 45.0 \pm 0.2 $^{\circ}$ C : Thamson, TV 4000
- Viscosimeter, with a circulation flow constant of approximately 0.03 : Ubbelhode No. 1C
- Stopwatches, readable to within seconds : Hanhart
- Balance, accurately to within 0.001 g : Mettler PE 160
- Balance, accurately to within 0.0001 g : Mettler AE 200
- Diluter, provided with 0.5 and 5.0 mL cylinders : Hamilton, Microlab 500
- Reagent tubes, 20 x 200 mm

Or equivalent apparatus.

3.2 Conditions

Not applicable.

4 MATERIALS

4.1 Chemicals

- Potassium di-hydrogen phosphate (KH_2PO_4), p.a. : Merck, 1.04873
- Ortho-phosphoric acid, 85 $\%$, p.a. : Merck, 1.00573
- Betaglucan from Barley, Viscosity 20 \pm 30 cst. : Magazyme, P-BGBM
- Sodium hydroxide solution 1 mol/L : Merck, 1.09137

Or equivalent quality.

4.2 References, standards and controls

Not applicable.

4.3 Reagents

- Water:

Ultra High Quality water, resistance \geq 18.2 mega Ohm/cm and TOC \leq 500 μ g/L.

- Phosphate buffer 1.0 mol/L, pH 5.00:

Dissolve 13.6 g potassium di-hydrogen phosphate in approximately 80 mL water in a 100 mL volumetric flask. If necessary heat while dissolving and allow cooling to ambient temperature. Adjust

Analysis Service and solutions	METHOD OF ANALYSIS	No : 1993 Version : 3 Page : 4 of 10
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Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

the pH to 5.00 by adding phosphoric acid solution 1 mol/L or sodium hydroxide solution 1 mol/L. Make up to volume with water and mix. This solution may be kept for 2 months.

- Phosphoric acid 1.0 mol/l:

Slowly and while stirring continuously add 67 mL ortho-phosphoric acid 85% to approximately 500 mL water in a 1 L volumetric flask. Make up to volume and mix. This solution may be kept for 2 months.

- Betaglucan substrate solution:

Use a reserved batch of betaglucan, with a known substrate batch factor. Dissolve 1.0 g Betaglucan in approximately 30 mL water in a 100 mL glass vial. Stir for approximately 1 hour. Next place the solution in the bath with boiling water for 5 minutes and allow cooling to ambient temperature. Add 10.0 mL phosphate buffer 1 mol/l, pH 5.00 and quantitatively transfer to a 100 mL volumetric flask with water. Make up to volume with water and mix.

Check the pH of the solution. It must be 5.60 \pm 0.05. Prepare a new solution when pH is out of range. Only use a freshly prepared solution.

In case of the calibration of a standard preparation, prepare two substrate solutions at the same time (Sa and Sb).

5 PROCEDURE

5.1 Preparation

Not applicable.

5.2 Pretreatment reference

Not applicable.

5.3 Pretreatment standard

Not applicable.

5.4 Pretreatment control

Not applicable.

5.5 Pretreatment samples

Dilute the samples with water to an activity between 3.4 and 6.8 BGF/mL. In case of a calibration of a standard preparation, perform the calibration according to the schedule in the appropriate work instruction (Introduction and management of enzyme standards, controls and substrates).

Analysis Service and solutions	METHOD OF ANALYSIS	No : 1993 Version : 3 Page : 5 of 10
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Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

Allow the standard preparation to be calibrated to attain room temperature. Weigh, accurately to within 0.0001 gram, and in duplicate an amount of standard corresponding to 10 000 BGF in a 100 mL volumetric flask. Dissolve in water by stirring on a magnetic stirrer. Make up to volume with water and mix. Dilute 0.250 mL of these solutions with 4.75 mL water in a centrifuge tube and mix.

5.6 Preparation measurement

Allow the Ubbelohde viscosimeter to equilibrate at 45.0 °C for at least 20 minutes.

5.7 Measurement

Place a reagent tube containing 15.0 mL substrate in the 45.0 °C water bath and allow equilibrating for at least 20 minutes. At time T = 0 minutes (stopwatch 1) add 2.00 mL sample solution to the equilibrated substrate, mix on a tube shaker and fill the reservoir of the viscosimeter through tube 3 to a level between the marks (see annex 1). Measure the viscosity of this solution every three minutes for a 15 minutes period (= 5 times) as follows:

At T = approximately 2.5 minutes close tube 1 (with finger) and execute suction on tube 2 to fill tube 2 up to the pre-run sphere. Stop suction, remove finger from tube 2 and start stopwatch 2 when the liquid reaches the upper timing mark (M1). At this moment read the time on stopwatch 1 (Rt 1) (**do not stop stopwatch 1!**) Allow the liquid to run down to the lower timing mark (M2). Stop stopwatch 2 when the lower timing marker is reached (Vt 1).

Repeat this handling every three minutes giving Rt 2 to 5 and Vt 2 to 5.

For each substrate execute the same measurement as mentioned above using a mixture of 15.0 mL substrate and 2.00 mL water as the blank (Vt_b).

Also execute the same measurement as mentioned above using 17.0 mL water as the blank (Vt_w).

6 CALCULATION

For each of the five measuring points calculate the reaction time in seconds (accurately to 0.01 seconds) by means of $T1 = \{Rt\ 1 - (Vt\ 1)\}$, $T2 = \{Rt\ 2 - (Vt\ 2)\}$ etc.

Calculate the average fall time of the five measuring point of the substrate blank in seconds: $Vt_{bl} = (Vt\ 1\ to\ 5) / 5$.

Calculate the average fall time of the five measuring points of the water in seconds: $Vt_w = (Vt\ 1\ to\ 5) / 5$.

For each of the five measuring points calculate X by dividing the average fall time of the substrate blank by the fall time of the measuring point corrected for the fall time of water:

$$X = Vt_{bl} / (Vt - Vt_w)$$

For each of the measuring point plot the T (y axis) against the corresponding X (x-axis), giving a linear relation according to $y = ax + b$ (see annex 2).

From this line calculate the slope P.

Analysis Service and solutions	METHOD OF ANALYSIS	No : 1993 Version : 3 Page : 6 of 10
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Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

Calculate the enzyme concentration in the incubation mixture as follows:

$$C = (W / 100) \times (0.250 / 5.00)$$

Calculate the enzyme activity of the sample as follows:

$$(P \times 60 / 0.147) \times (17/2) \times (1/C) \times Sf = \text{BGF units per g}$$

Where:

- Rt reaction time [seconds]
- Vt fall time between timing marks [seconds]
- Vt_{bl} Average fall time substrate blank [seconds]
- Vt_w Average fall time water [seconds]
- W sample weight [g]
- 100 volume of volumetric flask [mL]
- 17/2 correction for incubation mixture
- 0.250 / 5.00 dilution of sample
- P slope of graph [seconds⁻¹]
- 60 from seconds to minutes
- 0.147 factor from unit definition
- C sample concentration in incubation mixture [g/mL]
- Sf substrate batch factor of substrate used.

7 ASSESSMENT

7.1 Requirements

The sample must have an activity within the measuring range permitted.

7.2 Actions

Repeat the analysis of the sample with an adjusted dilution when the result is outside the measuring range

7.3 Authorisation

After a training period by a for this method authorised laboratory technician, a technician will be authorised for this method when he/she succeeds on performing the test single-handed, whereby the standards and selected samples meet the criteria mentioned above.

<p>Analysis Service and solutions</p>	<p>METHOD OF ANALYSIS</p>	<p>No : 1993 Version : 3 Page : 7 of 10</p>
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<p style="text-align: center;">Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.</p>

8 REFERENCES

This method is based on the DSM Seclin method CQA 4040.00 version 03 dated 20000406.

9 REMARKS

Not applicable.

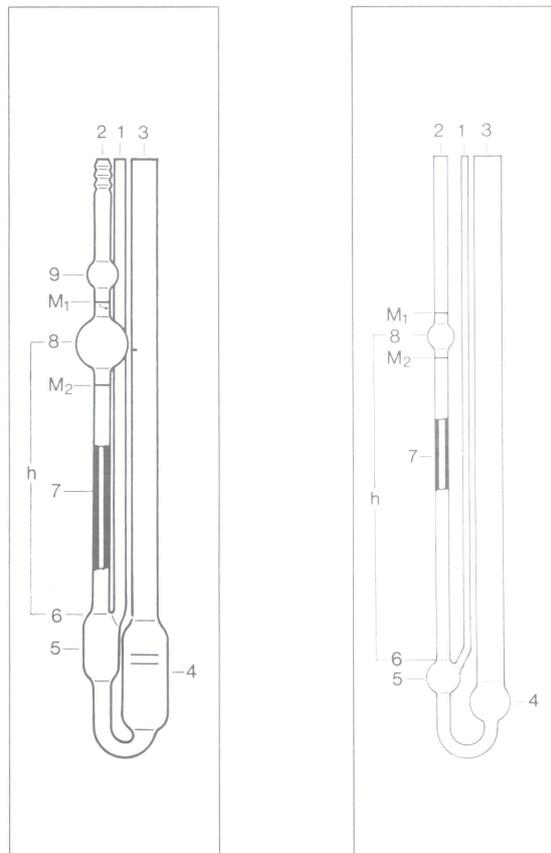
10 ANNEXES

Annex 1. Ubbelohde viscometer
Annex 2. Example of graph

<p>Analysis Service and solutions</p>	<p>METHOD OF ANALYSIS</p>	<p>No : 1993 Version : 3 Page : 8 of 10</p>
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<p align="center">Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.</p>
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Annex 1. Ubbelohde viscometer

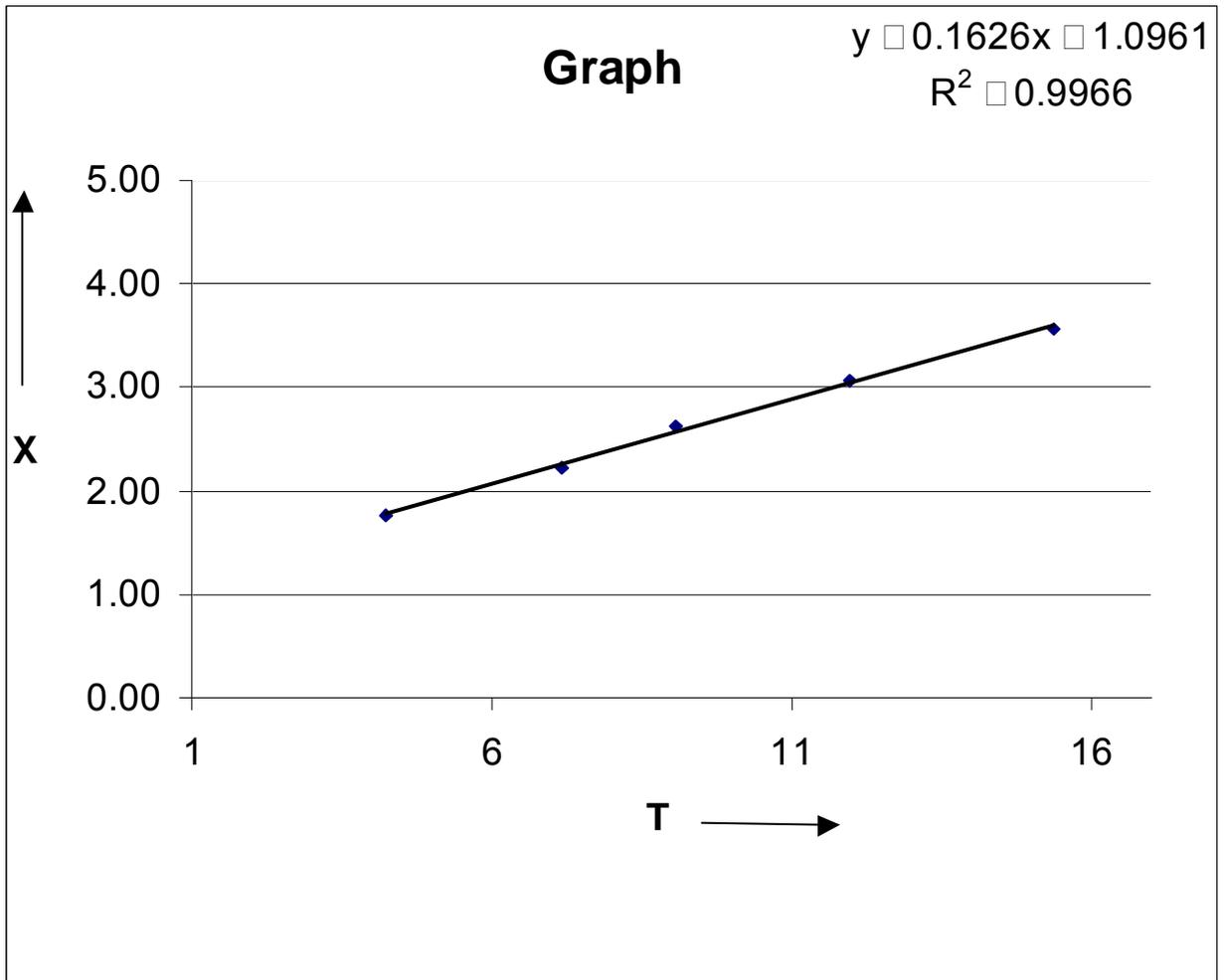


- 1 Ventilating tube
- 2 Capillary tube
- 3 Filling tube
- 4 Reservoir
- 5 Reference level vessel
- 6 Dome-shaped top part
- 7 Capillary
- 8 Measuring sphere
- 9 Pre-run sphere
- M1 Upper timing mark
- M2 Lower timing mark

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Title:
Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

Annex 2: Graph



Analysis Service and solutions	METHOD OF ANALYSIS	No : 1993 Version : 3 Page : 10 of 10
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Title: Determination of the fungal betaglucanase activity, manual absolute viscosimetric method with betaglucan as substrate.

HISTORY

Version	Description of the modification
1	First version This method was translated from the French method CQA 4040.00 version 03.
2	Potassium di-hydrogen phosphate p.a. must be art. Nr. 1.04873 instead of 1.05101
3	Method in new DBC format. Phosphate buffer 1.0 mol/l, pH 5.00 stability is mentioned.

ANNEX 8
Method of analysis for XYL activity: DSM
method

Unlimited. DSM		Type : INSTRUCTION	
Titre XVU - XYLANASE ACTIVITY DETERMINATION BY USING A VISCOSIMETRIC AUTO ANALYSER		Process/Emitting Unit : CQA	Revision index : 05
		Page 1 on 6	
		Replacement document : CQA 4 072 00 of 01/15//2005	
	Name	Date	Visa
Editor	Laboratory technicien	05/27/2010	EL
Approbator	E. DUPONT	09/23/2010	SB
Auditor	C. VAN NESPEN	09/28/2010	CVN

Modifications :

04/27/2010 ☒ **Complete Revision**

01/07/2005 ☒ **Integrally retyped**

12/03/1998 ☒ **General revision**

Date of IMPLEMENTATION : 09/28/2010

Date of PROOFREADING :

Titre
**XVU - XYLANASE ACTIVITY DETERMINATION BY
 USING A VISCOSIMETRIC AUTO ANALYSER**

Process/Emitting Unit :
CQA

Revision index :
05

Page 2 on 6

1 PRINCIPLE

The enzyme pentosanase is incubated with its substrate (rye Xylan) in a continuous flow autoanalyzer system. The hydrolysis of the gums rye extract causes a reduction in the viscosity which is measured in a continuous flow viscometer. Recording is in the form of peaks whose height is related to the activity of the sample.

2 REFERENCES

Instruction □ XVU □ valeur du standard xylanase □ (transl. : value of the standard xylanase)
 Instruction □ XVU □ valeur du contrôle xylanase □ (transl. : value of the control xylanase)
 Instruction □ XVU □ substrat xylane □ (transl. : substrate xylane)

3 ACTIVITY

This is the number of units XVU per gram or per milliliter of enzyme preparation.

4 EQUIPMENT

- Balance
- pH meter
- diluter
- magnetic stirrer
- autoanalyzer viscosimetric comprising the following modules :
 - . sampler : sampling 60 s, rinsing 120 s
 - . pump
 - . thermostated water bath at 42°C □ 0,2°C
 - . viscosimeter continuous flow (see diagram in annex 1)
 - . recorder
- microwave oven
- glass cups.

5 MANIFOLD

See diagram in annex 2.

6 REAGENTS

Reagents are quality "pure for analysis".
 The water is deionized water or equivalent quality.

6.1 Diluant : sodium chloride solution 1 %

Place in a flask of 1 liter, 100 ml of a stock solution of 10% NaCl (dissolution of a vial of 1 kg in 10 liters of water) to 1 liter with water. Add 1 ml of Triton X405

Unlimited. DSM	Type : INSTRUCTION	
Titre XVU - XYLANASE ACTIVITY DETERMINATION BY USING A VISCOSIMETRIC AUTO ANALYSER	Process/Emitting Unit : CQA Page 3 on 6	Revision index : 05

6.2 Enzymatic solutions

Weigh an amount (Xg) of enzyme preparation in a flask or beaker.
Complete the volume with a solution of 1□ NaCl without triton.
Perform other dilutions using a diluter with diluent □ Triton (see 6.1).
We will work between the first and the last point of the standard range, i.e around 0.15 XVU/ml.
Use glass cups.

6.3 Calibration solutions

See instruction □ XVU - valeur du standard xylanase □ (transl. : value of the standard xylanase)

6.4 Control solution

See instruction □ XVU - valeur du contr□le xylanase □ (transl. : value of the control xylanase)

6.5 Substrate

See instruction □ XVU - substrat xylane □ (transl. : substrate xylane)

7 PROCEDURE

Before each analysis, it is imperative to clean the entire pumping system.
Place all pumping lines in water and then start up the complete chain in the correct order.
Start by turning on the sample changer, prime the pump and then switch it on.
Attach the hose to the sensor and turn on the water bath (adjust it to the correct dosage temperature and check the water level of the bath so that the coils are fully submerged).
Finally, turn the recorder on by setting the pen.

After about 10 minutes of passage of the water, place the lines substrate / buffer (2 gray pipes) in a NaOH solution at 1:10 and leave about 5 minutes.
Rinse with water about 5 minutes and then place in a nitric acid solution at 1:10, let also about 5 minutes.
Finally rinse these two lines in the water at least 10 minutes.

All pumping lines have been in water for at least 10 minutes and are passing through the system.
Check at the recorder that the button "VAR" is up and that the sensitivity is 2000 mV.

Then press the zero of the recorder, set the pen to "0" to the left on the graph with the zero button, release this button and reposition the pen to "0" using the potentiometer of the pressure sensor (it must be normally at about 5.0).
Leave a few minutes to check that the line is relatively stable.

Depending on the dosage, the placement of the three lines differs (refer to paragraph "Pumping lines of reagents" for each analysis).

Wait until all reagents pass and when equilibrium is reached the pen on the graph should be between 40 and 80, if the substrate has a proper viscosity.
Press again the zero key on the recorder, set the pen to "100" (on the right of the graph) using the zero button.
Release the zero, press the "VAR" key, if necessary change the sensitivity to 1000 mV (depending on the response of the substrate), and place the pen at about 90 with the potentiometer of the pressure sensor.

Leave as such a few minutes to check the stability of the baseline and then start the 3 strong peaks (fill in 3 cups of the standard solution 5 □ highest concentration).

When the first peak of the strong peaks occurs, adjust it using the button "VAR" to about 20 on the graph scale and then verify that the two other peaks are correct.
Then wait for the baseline returns to its initial position (i.e. between 80 and 100 on the graph).

Titre
**XVU - XYLANASE ACTIVITY DETERMINATION BY
 USING A VISCOSIMETRIC AUTO ANALYSER**

Process/Emitting Unit :
CQA

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Fill in the cups of the various solutions and place them on the sampler rack.

Position of the samples in the system

Place the sample cups on the tray as follows:

- the five points of the standard range (S1 - S2 - S3 - S4 and S5 □ from the lowest concentration to the highest)
- first control
- samples to be assayed
- second control
- set the 5 points of the standard range in the same order.

Remarks

It is essential to begin and end with a standard range.
 It is recommended to place two cups per sample and control.

When the whole series is over and all the peaks are present, it is necessary to wait until the baseline has returned to its original position before releasing the three lines in the water.

When the baseline returns to zero of the graph (on the left), leave for another twenty minutes on the water to get a good rinse, then stop the devices in reverse order of start (recorder - bath - remove the hose from the sensor - stop the pump - defuse it and finally shut down the sample changer).

8 PUMPING LINES OF REAGENTS

Green line in the diluant.
 Two grey lines in the substrate.

9 CALCUL

On the computer open the Excel spreadsheet corresponding to the dosage.
 Enter the data from the weight-out sheet.
 Draw a baseline on the graph taking into account the assay drift.
 Measure the height of each peak using a ruler, and assign the value of these peaks on the Excel sheet.
 Check that the controls are good before putting the results in the computer software.

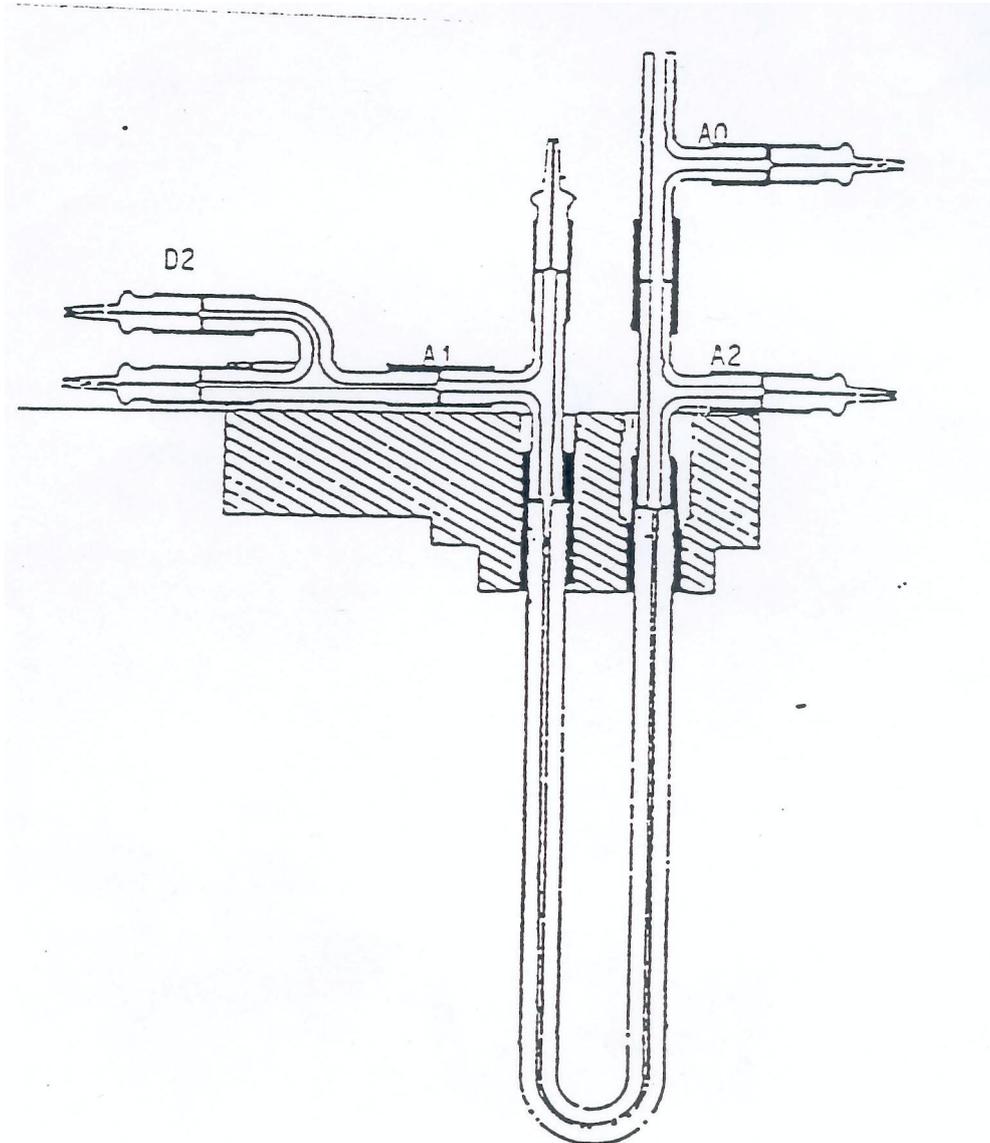
Titre
**XVU - XYLANASE ACTIVITY DETERMINATION BY
USING A VISCOSIMETRIC AUTO ANALYSER**

Process/Emitting Unit :
CQA
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05

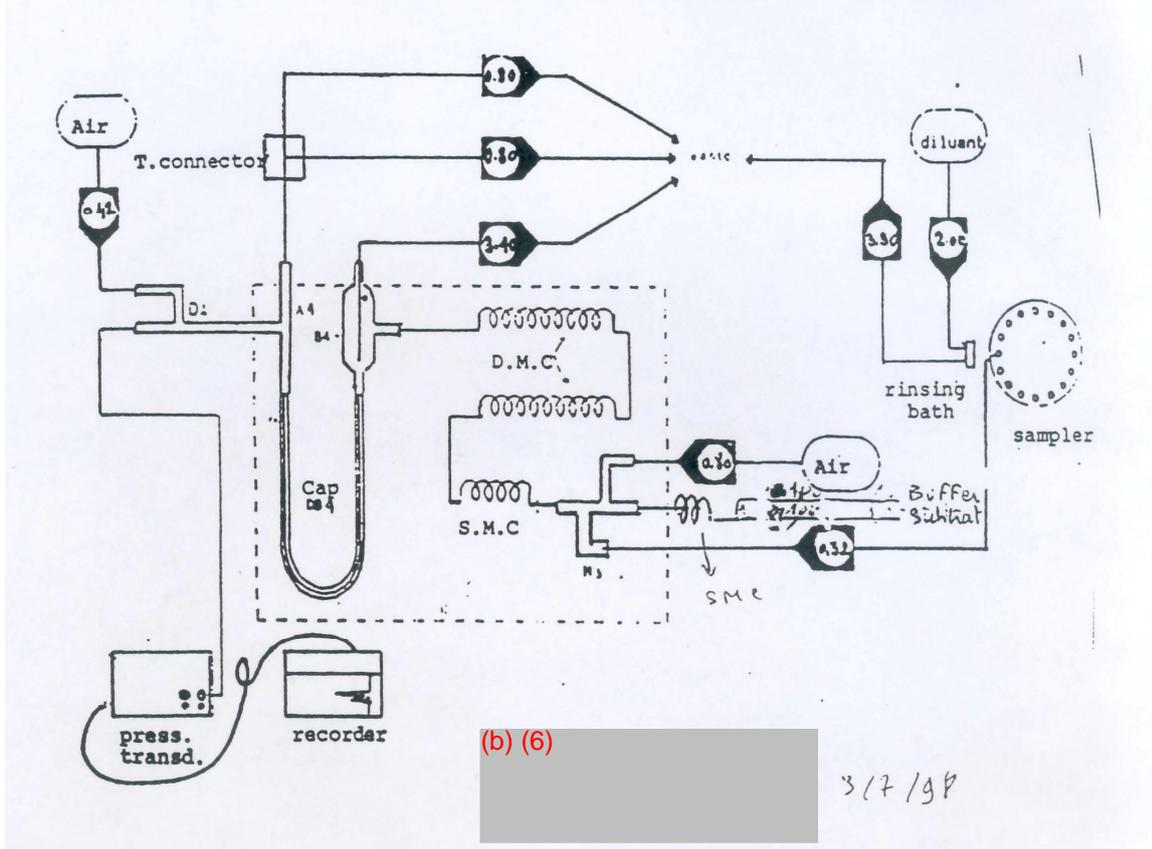
ANNEX 1

SCHEME OF THE VISCOSIMETER CONTINUOUS FLOW



ANNEX 2

MANIFOLD ENDOXYLANASE

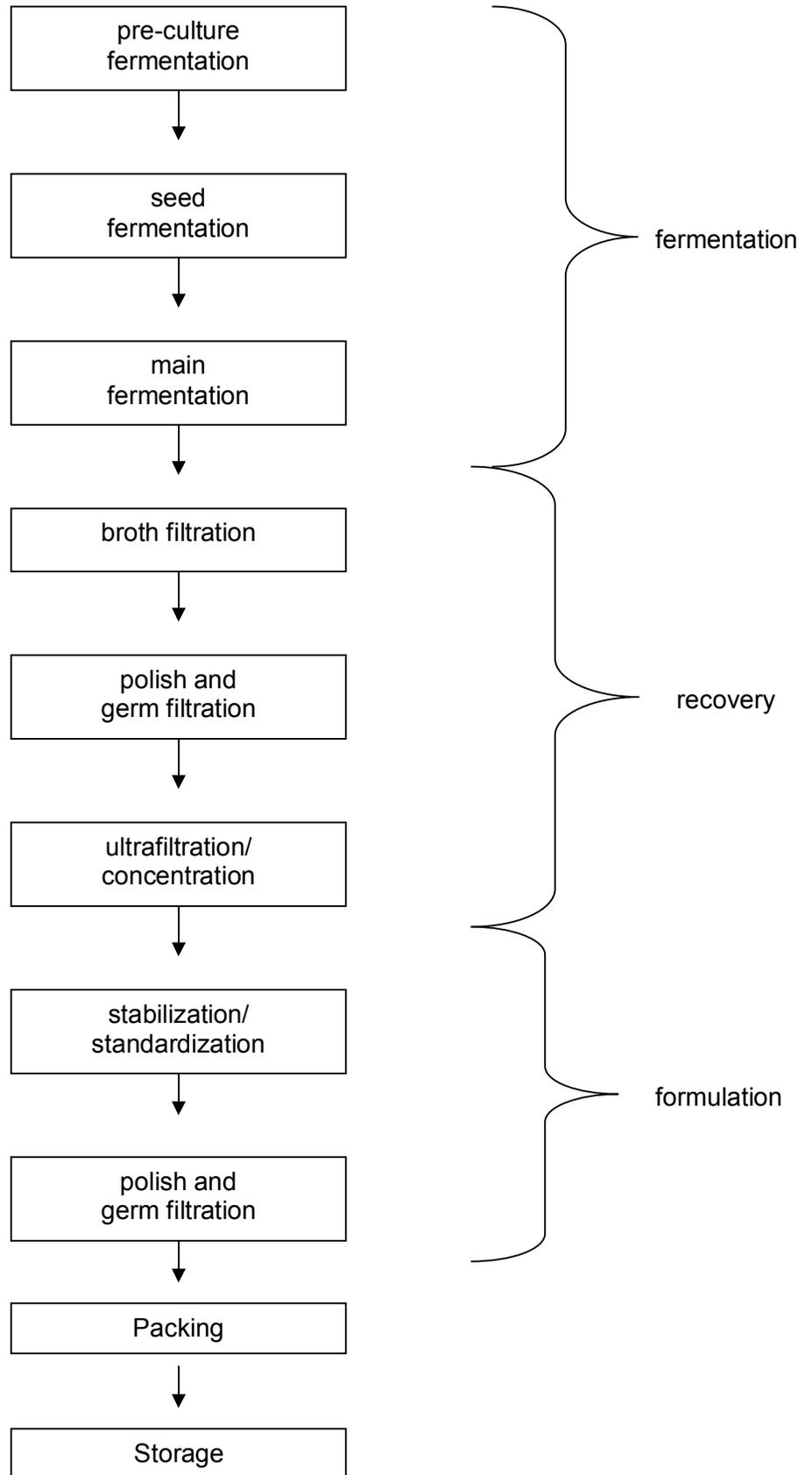


(b) (6)

3/7/98

ANNEX 9
Flow diagram of manufacturing process

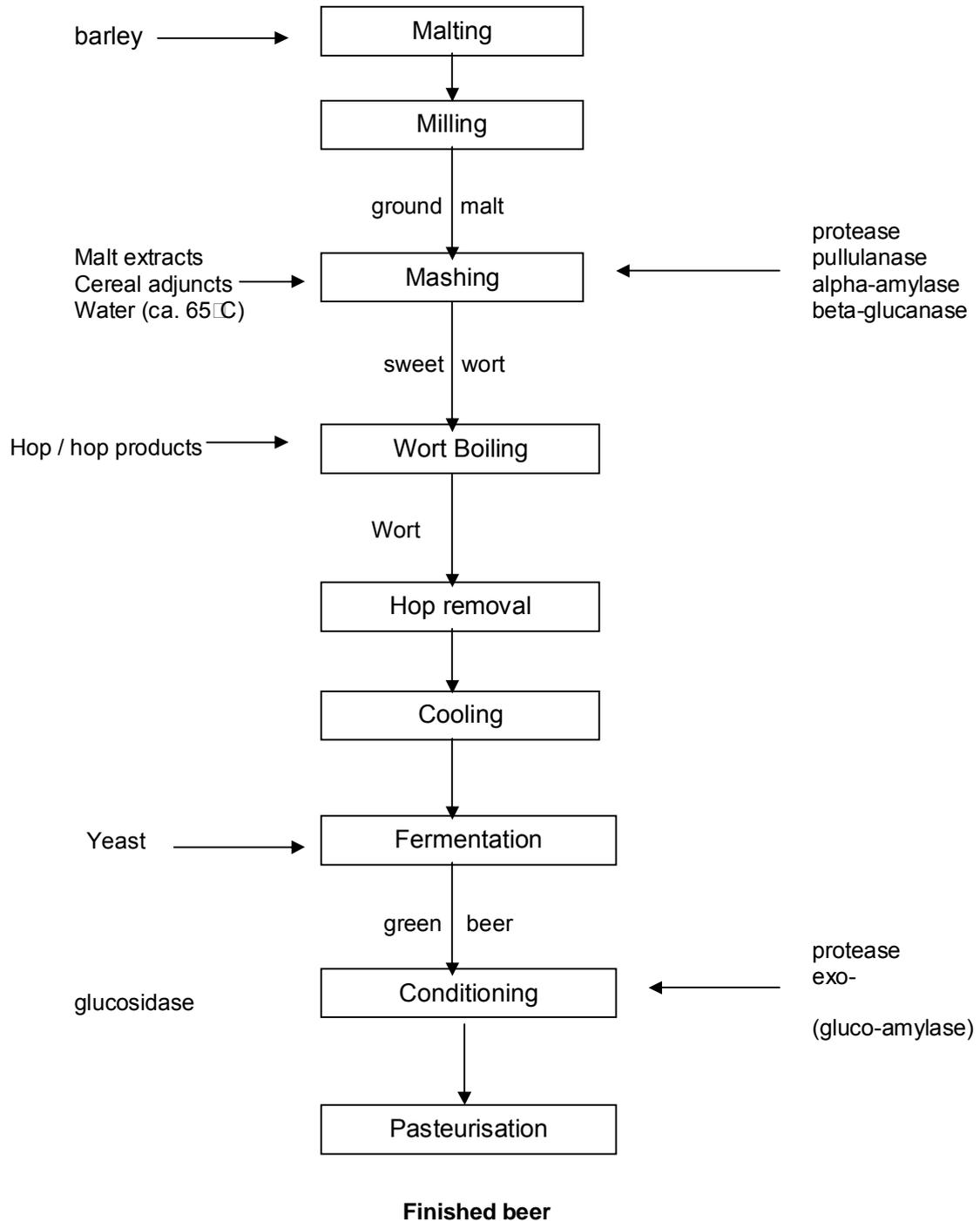
Manufacturing process of beta-glucanase



ANNEX 10

Brewing process

The Brewing process¹



¹ Overview taken from Ministry of Agriculture Food and Fisheries, Food Additive Committee report (MAFF/FAC/REP/35).

The process of wort boiling inactivates any enzyme present by heat denaturation

Pasteurisation temperatures are insufficient to inactivate more than a small proportion of the enzymes present.

ANNEX 11
**Effect of beta-glucanase, cellulase and
xylanase enzyme preparation on filterability,
viscosity and beta-glucan content**

Effect of beta-glucanase, cellulase and xylanase from *Talaromyces. emersonii* (Filtrase □ NL) on filterability, viscosity and beta-glucan content.

Example 1: Analysis of the wort, Filtrase NL dosage 400 g/ton

	Control	Filtrase NL
Filtered volume (ml) after 2 min.	35	52
4 min.	56	85
6 min.	72	112
8 min.	88	134
10 min.	98	150
12 min.	106	162
60 min.	188	240
Viscosity (mpa/s)	2.95	2.68
Beta-glucan content (mg/l)	228	□ 50

Example 2 : Analysis of the wort after Filtrase NL addition

Filtered volume (ml)	Control	Filtrase NL 200 g/t	Filtrase NL 300 g/t	Filtrase NL 500 g/t
After 2 min.	25	32	34	35
Id. 4 min.	38	58	58	60
Id. 6 min.	48	74	74	78
Id 8 min.	56	86	88	92
Id 10 min.	62	100	100	106
Id 12 min	68	108	108	115
Id 60 min.	120	192	192	202
Viscosity (mpa/s)	2.85	2.53	2.49	2.41
Beta-glucan content (mg/l)	NA	NA	NA	NA

ANNEX 12
Safety evaluation using the Pariza & Johnson
decision tree of beta-glucanase, cellulase and
xylanase from *Talaromyces emersonii*

Memo

DSM Food Specialties B.V.
Global Regulatory Affairs Nutrition Cluster

Alexander Fleminglaan 1
2613 AX Delft
P.O. Box 1
2600 MA Delft
The Netherlands

Date
May 28th, 2013

From Méline Rumelhard To [to] Cc [cc]

Subject: safety evaluation using the Pariza and Johnson decision tree of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* DFS/REG00058745

Introduction

The "Decision Tree for evaluation of the relative safety of food ingredients derived from genetically modified organisms" was published in 1991. This publication from the International Food Biotechnology Council (IFBC) was an extension, based on an earlier publication by Pariza and Foster in 1983¹. The 1991 IFBC Decision Tree was updated by Pariza and Johnson in 2001².

The enzyme preparation beta-glucanase, cellulase and xylanase derived from *Talaromyces emersonii* has been evaluated according the P&J Decision Tree. The result is described below.

Decision Tree

1. Is the production strain genetically modified?
NO
If yes, go to 2. If no, go to 6.
2. Is the production strain modified using rDNA techniques?
If yes, go to 3. If no, go to 5.
3. Issues related to the introduced DNA are addressed in 3a-3e.
 - 3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?
If yes, go to 3c. If no, go to 3b
 - 3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?
If yes, go to 3c. If no, go to 12.
 - 3c. Is the test article free of transferable antibiotic resistance gene DNA?

¹ Pariza M.W. and Foster E.M. J. Food Protection **46**. (1983), 453-468

² Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. **33** (2001) 173-186)

If yes, go to 3e. If no, go to 3d.

3d. Does (Do) the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animals?

If yes, go to 12. If no, go to 3e.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

If yes, go to 4. If no, go to 12.

4. Is the introduced DNA randomly integrated into the chromosome?

If yes, go to 5. If no, go to 6.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects, which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?

If yes, go to 6. If no, go to 7.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?

NO

Many strains of this strain lineage exist, for which safety data are available, that can be or have been tested through the P&J Decision Tree evaluation scheme.

If yes, the test article is ACCEPTED. If no, go to 7.

7. Is the organism nonpathogenic?

YES

Talaromyces emersonii strains described to date exclusively have been isolated from soil, air, plants or compost. There are no indications that would support any pathogenic concerns of the fungus, hence *Talaromyces emersonii*, is considered non-pathogenic for humans or animals.

If yes, go to 8. If no, go to 12.

8. Is the test article free of antibiotics?

YES

Beta-glucanase, cellulase and xylanase enzyme preparation is free from antimicrobial activity.

If yes, go to 9. If no, go to 12.

9. Is the test article free of oral toxins known to be produced by other members of the same species?

YES

It has been shown that *Talaromyces emersonii* strains do not produce any secondary metabolites known as mycotoxins. Moreover, using liquid column chromatography (Frisvad and Thrane, 1993), our betaglucanase, cellulase and xylanase production strain *T. emersonii* FBG-1 was tested for the production of secondary metabolites including (myco)toxins under various growth conditions known to induce the production of mycotoxins. The *T. emersonii* strain did produce some secondary metabolites; none of them were mycotoxins. Of all important fungal toxins, which may be of significance in food, the strain is not able to produce any (Cole and Cox 1981; Smith and Moss, 1985; Samson, 1997). Moreover, no known mycotoxins could be detected in a product (or fermentation broth) sample used for the safety studies (FBG1 strain) using the same analytical techniques (Hoekstra, 1998). Finally, no known mycotoxins could be detected in a ccUF sample of beta-glucanase, cellulase and xylanase from *T. emersonii* FGB210.

If yes, go to 11. If no, go to 10.

10. Are the amounts of such toxins in the test article below levels of concern?
NA
If yes, go to 11. If no, go to 12.
11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?
YES
The safety of beta-glucanase, cellulase and xylanase from *Talaromyces emersonii* has been tested in a 90-day oral toxicity study. The NOAEL of 1,600 mg enzyme preparation/kg bw/day, equivalent to 84.8 mg TOS/kg bw/day leads to a margin of safety of more than 3,140, which is considered sufficient to ensure the consumer's safety.
If yes, the test article is ACCEPTED. If no, go to 12.
12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.
NA

ANNEX 13
MSDS of Filtrase NL[®]: enzyme preparation
of beta-glucanase, cellulase and xylanase
from *Talaromyces emersonii*

SAFETY DATA SHEET



FILTRASE® NL

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1 Product identifier

Product name : FILTRASE® NL
 Internal code : WW14299
 Synonyms : Liquid enzyme (enzyme protein).
 Chemical formula : Not applicable.

1.2 Relevant identified uses of the substance or mixture and uses advised against

Recommended use : This product is an enzymatic preparation used in the food industry.

1.3 Details of the supplier of the safety data sheet

Supplier : DSM Food Specialties B.V.
 P.O. Box 1
 2600 MA Delft
 The Netherlands
 Telephone no.: +31 15 279 2865
 Fax no.: +31 15 279 3670
 e-mail address of person responsible for this SDS : Info.Worldwide@dsm.com

1.4 Emergency telephone number

Emergency telephone number : +31 15 279 2380

SECTION 2: Hazards identification

2.1 Classification of the substance or mixture

Product definition : Mixture

Classification according to Directive 1999/45/EC [DPD]

The product is classified as dangerous according to Directive 1999/45/EC and its amendments.

Classification : R42
 Physical/chemical hazards : Based on the available data of this product no hazardous properties are known.
 Human health hazards : May cause sensitisation by inhalation.
 Environmental hazards : Based on the available data of this product no hazardous properties are known.
 See Section 16 for the full text of the R-phrases declared above.

2.2 Label elements

Hazard symbol or symbols :



Indication of danger : Harmful
 : R42- May cause sensitisation by inhalation.
 Safety phrases : S23- Do not breathe spray.
 : S36/37- Wear suitable protective clothing and gloves.
 : S45- In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
 Hazardous ingredients : endo-1,3(4)- β -glucanase

2.3 Other hazards

Other hazards which do not result in classification : Not available.

SECTION 3: Composition/information on ingredients

Substance/mixture : Mixture

Product/ingredient name	Identifiers	%	Classification	
			67/548/EEC	Regulation (EC) No. 1272/2008 [CLP]
glycerol	EC: 200-289-5 CAS: 56-81-5	45 - 60	Not classified.	Not classified.
endo-1,3(4)-β-glucanase	EC: 263-462-4 CAS: 62213-14-3	1 - 10	R42 See Section 16 for the full text of the R-phrases declared above.	Resp. Sens. 1, H334 See Section 16 for the full text of the H statements declared above.

There are no additional ingredients present which, within the current knowledge of the supplier and in the concentrations applicable, are classified as hazardous to health or the environment, are PBTs or vPvBs or have been assigned a workplace exposure limit and hence require reporting in this section.

IUB number : beta-Glucanase: 3.2.1.6

Occupational exposure limits, if available, are listed in Section 8.

SECTION 4: First aid measures

4.1 Description of first aid measures

- Eye contact** : Rinse with plenty of running water. Get medical attention if symptoms occur.
- Inhalation** : Remove to fresh air. Prevent cooling of the person. Keep victim at rest in half-upright position. If not breathing, give artificial respiration. Get medical attention.
- Skin contact** : Rinse with plenty of running water. Remove contaminated clothing and shoes. Get medical attention if symptoms occur.
- Ingestion** : If swallowed, rinse mouth with water (only if the person is conscious). Get medical attention if symptoms occur.
- Protection of first-aiders** : No action shall be taken involving any personal risk or without suitable training. If it is suspected that fumes are still present, the rescuer should wear an appropriate mask or self-contained breathing apparatus. It may be dangerous to the person providing aid to give mouth-to-mouth resuscitation.

4.2 Most important symptoms and effects, both acute and delayed

Potential acute health effects

- Eye contact** : No known significant effects or critical hazards.
- Inhalation** : May cause sensitisation by inhalation.
- Skin contact** : No known significant effects or critical hazards.
- Ingestion** : No known significant effects or critical hazards.

Over-exposure signs/symptoms

- Eye contact** : May cause eye irritation. (redness).
- Inhalation** : The inhalation of airborne droplets or aerosols may cause irritation of the respiratory tract. May cause sensitisation by inhalation. Sensitive individuals may develop asthma on inhalation of this material.
- Skin contact** : Prolonged or repeated skin contact may be irritating.
- Ingestion** : There is no known acute effect after over-exposure to this product.

4.3 Indication of any immediate medical attention and special treatment needed

- Notes to physician** : Treat symptomatically. Contact poison treatment specialist immediately if large quantities have been ingested or inhaled.
- Specific treatments** : No specific treatment.

SECTION 5: Firefighting measures

5.1 Extinguishing media

Small fire

- Suitable** : Use dry chemical or CO₂.

Large fire

- Suitable** : Use extinguishing media suitable for surrounding materials.

5.2 Special hazards arising from the substance or mixture

- Hazards from the substance or mixture** : No specific hazard.
- Hazardous combustion products** : In case of fire, may produce toxic and/or corrosive decomposition products.

5.3 Advice for firefighters

- Special protective actions for fire-fighters** : Fire water contaminated with this material must be contained and prevented from being discharged to any waterway, sewer or drain.
- Special protective equipment for fire-fighters** : Wear suitable protective clothing. Self-contained breathing apparatus.

SECTION 6: Accidental release measures

6.1 Personal precautions, protective equipment and emergency procedures

- For non-emergency personnel** : No action shall be taken involving any personal risk or without suitable training. Evacuate surrounding areas. Keep unnecessary and unprotected personnel from entering. Do not touch or walk through spilt material. Avoid breathing vapour or mist. Provide adequate ventilation. Wear appropriate respirator when ventilation is inadequate. Put on appropriate personal protective equipment.
- For emergency responders** : If specialised clothing is required to deal with the spillage, take note of any information in Section 8 on suitable and unsuitable materials. See also Section 8 for additional information on hygiene measures.

- 6.2 Environmental precautions** : No special measures required.

6.3 Methods and materials for containment and cleaning up

- Small spill** : Take up with suitable material. Place in a suitable container. Clean up affected area with a large amount of water.
- Large spill** : Prevent entry into sewers, basements or confined areas. Dyke if necessary. Absorb spill with inert material (e.g. dry sand or earth) and place in a chemical waste container. Recycle, if possible.

- 6.4 Reference to other sections** : See Section 1 for emergency contact information.
See Section 8 for information on appropriate personal protective equipment.
See Section 13 for additional waste treatment information.

SECTION 7: Handling and storage

The information in this section contains generic advice and guidance. The list of Identified Uses in Section 1 should be consulted for any available use-specific information provided in the Exposure Scenario(s).

7.1 Precautions for safe handling

- Protective measures** : Preferably use in closed systems. Use with adequate ventilation. Use suitable protective equipment. Avoid contact with eyes, skin, respiratory tract and clothing.
- Advice on general occupational hygiene** : Eating, drinking and smoking should be prohibited in areas where this material is handled, stored and processed. Workers should wash hands and face before eating, drinking and smoking. Remove contaminated clothing and protective equipment before entering eating areas. See also Section 8 for additional information on hygiene measures.

- 7.2 Conditions for safe storage, including any incompatibilities** : Keep in a cool and dry place.
- Store between the following temperatures: 4 and 8 °C.

Packaging materials

- Suitable** : Polyethylene, high density (PEHD).

7.3 Specific end use(s)

- Recommendations** : Not available.
- Industrial sector specific solutions** : Not available.

SECTION 8: Exposure controls/personal protection

The information in this section contains generic advice and guidance. The list of Identified Uses in Section 1 should be consulted for any available use-specific information provided in the Exposure Scenario(s).

8.1 Control parameters

Occupational exposure limits

Product/ingredient name	Exposure limit values
glycerol	ACGIH TLV (United States, 2/2010). TWA: 10 mg/m ³ 8 hour(s). Form: Inhalable fraction

Recommended monitoring procedures : If this product contains ingredients with exposure limits, personal, workplace atmosphere or biological monitoring may be required to determine the effectiveness of the ventilation or other control measures and/or the necessity to use respiratory protective equipment. Reference should be made to European Standard EN 689 for methods for the assessment of exposure by inhalation to chemical agents and national guidance documents for methods for the determination of hazardous substances.

Derived effect levels

No DELs available.

Predicted effect concentrations

No PECs available.

8.2 Exposure controls

Appropriate engineering controls : Use only with adequate ventilation.

Individual protection measures

- Hygiene measures** : When using do not eat, drink or smoke. Wash hands after handling compounds and before eating, smoking and using the lavatory and at the end of the day.
- Eye/face protection** : Full-face mask
- Hand protection** : Chemical-resistant, impervious gloves complying with an approved standard should be worn at all times when handling chemical products if a risk assessment indicates this is necessary. >8 hours (breakthrough time): Nitril rubber, butyl rubber, neoprene, Viton®, PVC. Replace damaged gloves.
- Skin and body** : Wear suitable protective clothing.
- Respiratory protection** : Self-contained breathing apparatus. - air fed respirator .
- Environmental exposure controls** : Emissions from ventilation or work process equipment should be checked to ensure they comply with the requirements of environmental protection legislation. In some cases, fume scrubbers, filters or engineering modifications to the process equipment will be necessary to reduce emissions to acceptable levels.

Advice on personal protection is applicable for high exposure levels. Select proper personal protection based on a risk assessment of the actual exposure situation.

SECTION 9: Physical and chemical properties

9.1 Information on basic physical and chemical properties

- Physical state** : Liquid.
- Colour** : Colourless to brown. (product colour may vary from batch to batch)
- Odour** : Slight fermentation odour.
- Odour threshold** : Not available.
- pH** : 4 to 4.5 (Concentration 100%)
- Melting point** : Not available.
- Initial boiling point and boiling range** : Not available.
- Softening range** : Not available.
- Flash point** : Not available.
- Evaporation rate** : Not available.
- Flammability (solid, gas)** : Not available.
- Burning time** : Not applicable.
- Burning rate** : Not applicable.
- Upper/lower flammability or explosive limits** : Not available.
- Vapour pressure** : Not available.
- Vapour density** : Not available.
- Relative density** : Not available.
- Density (g/cm³)** : Not available.
- Bulk density** : Not available.
- Solubility** : Easily soluble in the following materials: cold water.
- Solubility in water** : Not available.
- Solubility at room temperature** : Not available.
- Partition coefficient: n-octanol/water** : Not available.

Auto-ignition temperature : Not available.
 Decomposition temperature : Not available.
 Viscosity : Not available.
 Explosive properties : Not available.
 Oxidising properties : Not available.

9.2 Other information

Remarks : More detailed information with regard to the color and pH can be requested from the supplier.

SECTION 10: Stability and reactivity

10.1 Reactivity : No specific test data related to reactivity available for this product or its ingredients.
 10.2 Chemical stability : Stable under recommended storage and handling conditions (see section 7).
 10.3 Possibility of hazardous reactions : Under normal conditions of storage and use, hazardous reactions will not occur.
 10.4 Conditions to avoid : No special recommendations.
 10.5 Incompatible materials : No special recommendations.
 10.6 Hazardous decomposition products : No specific data.

SECTION 11: Toxicological information

11.1 Information on toxicological effects

Acute toxicity

Product/ingredient name	Result	Species	Dose	Exposure
glycerol	LD50 Oral	Rat	12600 mg/kg	-
endo-1,3(4)- β -glucanase	LD50 Oral	Rat	>5000 mg/kg	-

Conclusion/Summary : Not available.

Irritation/Corrosion

Conclusion/Summary

Eyes : Not available.

Skin : Not available.

Respiratory : Not available.

Sensitisation

Conclusion/Summary

Skin : Not available.

Respiratory : Not available.

Mutagenicity

Product/ingredient name	Test	Experiment	Result
endo-1,3(4)- β -glucanase	OECD 471 Bacterial Reverse Mutation Test	Experiment: In vitro Subject: Bacteria	Negative

Conclusion/Summary : Not available.

Carcinogenicity

Conclusion/Summary : Not available.

Reproductive toxicity

Conclusion/Summary : Not available.

Teratogenicity

Conclusion/Summary : Not available.

Potential acute health effects

Eye contact : No known significant effects or critical hazards.

Inhalation : May cause sensitisation by inhalation.

Skin contact : No known significant effects or critical hazards.

Ingestion : No known significant effects or critical hazards.

Symptoms related to the physical, chemical and toxicological characteristics

Eye contact : No specific data.

Inhalation	: Adverse symptoms may include the following: wheezing and breathing difficulties asthma
Skin contact	: No specific data.
Ingestion	: No specific data.
General	: Once sensitized, a severe allergic reaction may occur when subsequently exposed to very low levels.
Carcinogenicity	: No known significant effects or critical hazards.
Mutagenicity	: No known significant effects or critical hazards.
Teratogenicity	: No known significant effects or critical hazards.
Developmental effects	: No known significant effects or critical hazards.
Fertility effects	: No known significant effects or critical hazards.

SECTION 12: Ecological information

12.1 Toxicity

Product/ingredient name	Result	Species	Exposure	Effects
glycerol	Acute LC50 54 ml/L Fresh water	Fish - Oncorhynchus mykiss - 0.9 g	96 hours	Mortality

Conclusion/Summary : Not available.

12.2 Persistence and degradability

Conclusion/Summary : Not available.

12.3 Bioaccumulative potential

Product/ingredient name	LogP _{ow}	BCF	Potential
glycerol	-1.76	-	low

12.4 Mobility in soil

Soil/water partition coefficient (K_{oc}) : Not available.

Mobility : Not available.

12.5 Results of PBT and vPvB assessment

PBT : Not applicable.

vPvB : Not applicable.

12.6 Other adverse effects : No known significant effects or critical hazards.

Remarks : The preparation is believed not to be dangerous to the environment with respect to mobility, persistence and degradability, bio-accumulative potential, aquatic toxicity and other data relating to eco-toxicity.

SECTION 13: Disposal considerations

The information in this section contains generic advice and guidance. The list of Identified Uses in Section 1 should be consulted for any available use-specific information provided in the Exposure Scenario(s).

13.1 Waste treatment methods

Product

Methods of disposal : Waste must be disposed of in accordance with national and local environmental regulations.

Hazardous waste : The classification of the product may meet the criteria for a hazardous waste.

Packaging

Methods of disposal : The generation of waste should be avoided or minimised wherever possible. Waste packaging should be recycled. Incineration or landfill should only be considered when recycling is not feasible.

Special precautions : This material and its container must be disposed of in a safe way. Care should be taken when handling emptied containers that have not been cleaned or rinsed out. Empty containers or liners may retain some product residues. Avoid dispersal of spilt material and runoff and contact with soil, waterways, drains and sewers.

SECTION 14: Transport information

	ADR/RID	ADN/ADNR	IMDG	IATA
14.1 UN number	Not regulated.	Not regulated.	Not regulated.	Not regulated.
14.2 UN proper shipping name	-	-	-	-
14.3 Transport hazard class(es)	-	-	-	-
14.4 Packing group	-	-	-	-
14.5 Environmental hazards	No.	No.	No.	No.
14.6 Special precautions for user	Not available.	Not available.	Not available.	Not available.
Additional information	-	-	-	-

14.7 Transport in bulk according to Annex II of MARPOL 73/78 and the IBC Code : Not available.

SECTION 15: Regulatory information

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture

EU Regulation (EC) No. 1907/2006 (REACH)

Annex XIV - List of substances subject to authorisation

Substances of very high concern

None of the components are listed.

Annex XVII - Restrictions on the manufacture, placing on the market and use of certain dangerous substances, mixtures and articles

Not applicable.

15.2 Chemical Safety Assessment : Not applicable.

SECTION 16: Other information

Classification according to Regulation (EC) No. 1272/2008 [CLP/GHS]

Resp. Sens. 1, H334

Procedure used to derive the classification according to Regulation (EC) No. 1272/2008 [CLP/GHS]

Classification	Justification
Resp. Sens. 1, H334	Calculation method

Full text of abbreviated H statements : H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.

Full text of classifications [CLP/GHS] : Resp. Sens. 1, H334 RESPIRATORY SENSITIZATION - Category 1

Full text of abbreviated R phrases : R42- May cause sensitisation by inhalation.

Full text of classifications [DSD/DPD] : Not applicable.

Alterations compared to the previous version : Alterations compared to the previous version are marked with a little (blue) triangle.

Abbreviations and acronyms : ATE = Acute Toxicity Estimate
CLP = Classification, Labelling and Packaging Regulation [Regulation (EC) No. 1272/2008]
DNEL = Derived No Effect Level
EUH statement = CLP-specific Hazard statement
PNEC = Predicted No Effect Concentration
RRN = REACH Registration Number

Sources of key data : Literature data and/or investigation reports are available through the manufacturer.

Internal code : WW14299

Training advice : Handling of this substance or preparation is restricted to skilled personnel only. Safe handling of enzymes is detailed in 'AMFEP Guide to the Safe handling of Enzymes' (www.amfep.org).

Notice to reader

The information contained in the Safety Data Sheet is based on our data available on the date of publication. The information is intended to aid the user in controlling the handling risks; it is not to be construed as a warranty or specification of the product quality. The information may not be or may not altogether be applicable to combinations of the product with other substances or to particular applications.

The user is responsible for ensuring that appropriate precautions are taken and for satisfying themselves that the data are suitable and sufficient for the product's intended purpose. In case of any unclarity we advise consulting the supplier or an expert.

History

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ANNEX 14
Report from the Amfep Working Group on
Consumer Allergy: risk from enzyme
residues in food (August 1998)

WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

AMFEP

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TBS Safety Consulting ApS

Copenhagen, August 1998

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 - 3.6. The consumption of enzymes for medical purposes and as digestive aids
 - 3.6.1. *Medical uses*
 - 3.6.2. *Digestive aids*

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- 5.0. **Bibliography**

Summary

In recent years, claims have been made by the media and some consumer organisations that enzyme residues in bread and other foods can result in allergic responses in the consumers of that food.

AMFEP established an Expert Group to evaluate whether residual enzymes in foods are an allergy risk for consumers. The Expert Group was asked to investigate existing scientific data and to report the results of the findings.

The main questions were whether enzymes in, for example, bread can sensitise a consumer of the bread, and subsequently if the presence of the enzyme residue could induce symptoms of allergy.

A further question was if a person with existing allergy to common allergens could develop allergy symptoms upon eating foods containing residual enzymes by cross reaction. This is not uncommon in the case of food allergy.

The literature survey was made to search for general food allergy, epidemiology and to find cases of food related enzyme allergy. In addition a survey of enzyme producers' files was carried out to look for adverse reactions to food enzymes.

High daily doses of industrial enzymes in are prescribed for patients with insufficient function of the pancreas. The literature on adverse events was reviewed and telephone interviews were undertaken with authorities and university hospital departments to check if experience of enzyme related gastrointestinal allergy were observed but not published.

Studies of common food allergy indicate a relatively low prevalence of about 2% of populations in Europe and the United States. There is however, a significant discrepancy between the perception of being allergic to foods (15%) and those that can be verified as food allergy (2%).

Yet, there are no firm data of the doses required to sensitise a person via the gastrointestinal tract, but the doses required to induce sensitisation seem to be very high. Indeed, patients with insufficient enzyme production of the pancreas need to take industrial enzymes in doses 100.000 - 1 million times higher than the amounts found in food.

There are no published cases of people that have been sensitised by the ingestion of food with residual enzymes, and even people who ingest high daily doses of enzymes as digestive aids are not reported to have gastrointestinal allergy to enzymes, even after many years of daily intake.

There are a few case histories of people who had reactions to papain, extracted from the papaya fruit. Papain in powder form is used as a meat tenderiser in some countries. It is unclear if the sensitisation in these cases occurred by inhalation of the powder or by ingestion of the meat with the papain.

One case history described a person who reacted with hay-fever upon eating a lactase tablet. This case was incomplete in describing the possible source of sensitisation.

There are 2 cases of people with baker's asthma and allergy to α -amylase, and wheat flour who developed symptoms after the ingestion of bread. The symptoms were somewhat more pronounced after bread prepared with α -amylase than bread without. One case with occupational allergy to α -amylase reacted upon ingestion of a very high test-dose of pure α -amylase, but not at lower doses. Four other persons with occupational α -amylase allergy did not react at any dose.

The question of cross reactions between common moulds and enzymes produced in related moulds was described in a double blind placebo controlled food challenge study of asthma patients with allergy to *Aspergillus fumigatus*. This mould is closely related to *Aspergillus oryzae* and - *niger* which are used for the production of industrial α -amylase. None of the test persons could be challenged to elicited symptoms by eating bread prepared with enzymes.

The expert group concludes that there are no scientific indications that the small amounts of enzymes in bread and other foods can sensitise or induce allergy reactions in consumers.

Employees with respiratory occupational enzyme allergy should be informed that in rare cases, symptoms may be induced by ingestion of food with residual enzymes. Enzyme residues in bread or other foods do not represent any unacceptable risk to consumers.

1.0. Introduction

Since the late 80's, and particularly since 1992 it has been repeatedly claimed that enzyme residues in foods may represent a hazard to consumers in the form of allergies, and that a certain percentage of the population are at risk of having allergy reactions to enzymes in bread and other foods.

In particular it has been claimed that consumers were at risk of developing severe allergy symptoms caused by α -amylase. The public was somewhat alarmed and there have been complaints, questions and other reactions of concern to bakers and other suppliers.

The media's interest was based on results from a study by Schata¹, published only as a 1/2-page abstract which does not allow for scientific evaluation.

However the issue was effectively raised within the public, and industry had no data with which to make a response.

Since 1992, the issue of allergy risk in consumers have emerged from time to time on television in the TV and the printed media. The general issue as it has emerged over these years is that there is a concern in the public that enzymes are unsafe, and as far as the bakers and the flour improvers are concerned, require and request data to oppose the allegations.

An additional concern is the possible cross reaction between enzymes produced by fermentation of certain moulds which may be related to common moulds. In theory, a person with a preexisting allergy to *Aspergillus sp.* might react to enzymes from e.g. *Aspergillus niger* or *A. oryzae*.

2.0 Background

2.1 General

In the public mind there is some confusion about the frequency of allergy, and in particular on food allergy. However, in the scientific community there seem to be consensus of the following:

- The frequency of common allergy (all allergies included) is 20 - 30%, in most populations around the world. The figure is increasing. Part of the increase may be due to higher awareness and improved diagnostic methods, however, a true increase cannot be ruled out.
- The frequency of occupational allergy in bakers is 8 - 27%. About 30 - 35%, of the bakers with occupational allergy to flour have an additional respiratory allergy to α -amylase and/or other baking enzymes.
- There is a reasonably good documentation of the frequency of food allergy in the general population at 1 - 2%. However, the frequency of perceived food allergy in the general population is 12 - 16%
- Food allergy does not differ from inhalation allergies with regard to the biological mechanisms taking place in the immune system. Any 'true' allergy is based on **a l l e r g y a n t i b o d i e s (I g E)**. Allergy antibodies are produced by the white blood cells called lymphocytes after the allergen has been introduced to these cells by inhalation or by ingestion. This process is called 'sensitisation'.
- Sensitisation then, is merely the event of the body recognising the foreign allergenic protein and reacting to it by producing allergy antibodies specifically recognising the particular allergen.
- Sensitisation is not a disease.
- It only becomes an allergic disease if the person develop symptoms related to exposure to the particular allergen.
- Not all sensitised people exhibit symptoms of allergy have allergy-symptoms.

2.2 Occupational respiratory allergy

allergy caused by inhalation of airborne particles of proteins, incl. Enzymes

Fungal enzymes, bacterial enzymes and extracted plant and animal enzymes are equally capable of inducing respiratory allergy - Papain and Bromelain²⁻⁴, Trypsin⁵, protease's from the skin yeast *Candida albicans*⁶, from bacteria/ subtilisins^{7,8}, fungal amylases^{9,10}, bacterial amylases¹¹, fungal hemicellulases¹², lipases¹³, xylanases and cellulases^{14,15} are all examples of industrial enzymes known to induce allergic sensitisation and respiratory occupational allergy. This is a feature characterised by highly purified enzyme protein products rather than the origin or the methods of production.

They all share the structural and biological properties that may cause sensitisation when inhaled.

The classical food allergens are also capable of inducing respiratory allergy when they are brought into a dust- or aerosol form and inhaled. Soya¹⁶, eggs^{17,18}, milk¹⁹ and fish²⁰ are just examples. Soya may be one of the best described examples of epidemic inhalation allergy to an allergen also well recognised as a food allergen²¹.

3.0. Food allergy

3.1. Allergy caused by ingestion of proteins in foods

Eight percent of children under 3 years of age are allergic to food²². In, and in this age group, milk, egg, fish and soya are examples of common allergens. Many of these allergies disappear with age, but food allergy is seen also in older children and in adults. The overall frequency of verified food allergy is 1 - 2% of the population²²⁻²⁵.

Food allergy is the adverse reaction to food characterised by allergic sensitisation to food proteins and elicitation of symptoms by ingestion of the same food proteins.

Symptoms

The symptoms of food allergy are gastrointestinal with vomiting and diarrhoea, sometimes accompanied by urticaria, asthma or hay-fever. Generalised very severe reactions occur in rare cases.

Many food allergies are very mild, with symptoms of itching and burning sensation in the mouth. This is also a feature of most of the well known cross-reactions between common inhalation allergens and foods. An example can be found in patients with a birch pollen allergy who also react to e.g. fresh apples, without having a specific allergy to apples. Another well known cross reaction is that of latex and bananas. There are a number of such cross reactions between common pollen allergens and certain foods.

Types of food allergens

Examples of 'true food allergens' are proteins in milk, egg, soya, wheat, fish, nuts and, peanuts and a few more. There are others, but only about 10 food allergens account for more than 95% of severe cases. However the list of food allergens is extremely long and a large number of food allergens only give rise to allergy in sporadic cases.

The common features of food allergens are largely shared by those of respiratory allergens. However, foods are very often treated by cooking and other physico-chemical means that may destroy part of the protein structure and thereby its allergenic properties.

Properties of food allergens

The molecular weights of allergens are typically in the range of 10 -70 (90) kDa.

They have a number of 'epitopes', i.e. sequences of 8 - 16 amino acids. These are the structural 'units' which can be identified by the immune system and lead to production of specific IgE (sensitisation). In the sensitised individual the specific IgE readily recognises the epitopes on the particular protein, resulting in allergy symptoms. Some of these epitopes are described in literature²⁶⁻²⁸.

Food allergens are stable to digestion and most also to heating by cooking, and in most cases, food allergens can represent a very large proportion of the food itself. Enzymes are not well described with regard to neither their fate after ingestion nor their allergenic properties after cooking.

The TNO Institute performed a study⁵⁸ on native α -amylase from *Aspergillus oryzae* in a gastrointestinal model simulating the physiological events in the stomach.

The results indicate that about 92%, of the epitopes of the α -amylase are destroyed and about 8%, of the epitopes on the α -amylase are intact at the delivery from the stomach to the duodenum.

However, it can be expected that the proteolytic pancreatic enzymes will reduce even further, the remaining 7 – 8%, of the α -amylase during the passage through the duodenum.

Doses at which food allergy occurs

The doses and other conditions necessary to sensitise an individual are not well known. It is believed that the sensitising doses must be considerably higher than doses required for elicitation of symptoms in patients already sensitised. There are many examples of sensitised people reacting to trace amounts of allergens in the food - some of them with fatal outcomes.

It is therefore understandable that there is some focus on hidden allergens like traces of milk, nuts and peanuts in other foods.

Steinman²⁹ wrote a leading article in the August 1996 issue of *J. Allergy Clin. Immunol.* regarding hidden allergens in food. It is representative of the concern in the medical profession and in the public. He suggested a number of preventive measures including labelling in clear language. His article does not mention enzymes.

Food produced by GMO's

Genetically Modified Organisms (GMO's), and enzymes produced by GMO's have raised concern in general and also specifically for enzymes used in food processing.

Scientists in the fields of gene technology³⁰⁻³³ and allergy seem to agree that gene technology and the results thereof expressed in foods should not cause concern with regard to allergy risk. However, gene technology does bring about new proteins, and it is important to be aware that some of these new proteins may be allergenic.

Genetically modified proteins may, or may not share allergenic properties with traditional allergens. This would relate to the nature of the protein as it does in all other circumstances, and there are no examples of involuntary (or voluntary) changes of allergenicity of proteins in food.

A possibility may be that in the future, gene technology may be used as a tool to produce less allergenic proteins. This might be a future example of voluntary change of allergenicity.

Enzymes produced by GMO's have been on the market in some countries for many years. Enzyme producers have not experienced any difference in allergenicity of these enzymes as compared to traditional extracted or fermented enzymes. They appear to have the same sensitising potential as are capable of sensitising exposed employees at the same rate as traditional enzymes.

3.2. Epidemiology of Food Allergy

In a survey of 5000 households in the USA carried out in 1989, 1992 and again in 1993²⁵ it was found that 13.9 -16.2% of the households reported at least one member to be allergic to foods.

A study of food allergy in a random sample of 1483 adults in Holland²³ showed that 12.4% reported allergy to foods, but by controlled tests only 2.4% could be confirmed by Double Blind Placebo Controlled Food Challenge (DBPCFC).

In Spain, 3034 patients from the outpatient allergy clinics at two hospitals were tested for food allergy²⁴. The patients were tested by skin prick, RAST and open food challenge. They found 0.98% positive to one or more foods.

When looking at food additives, the same pattern emerges. In a survey of a population sample in the UK, 7% claimed to have reactions to food additives. Double blind challenge tests could verify only 0.01 - 0.23% to be true reactions to food additives³⁴.

The frequencies of confirmed food allergy in different countries in Europe and the USA are quite uniform at 1 - 2.5% of the populations.

A number of explanations to the discrepancy of perception and verified cases has been offered. There are indications that the public attribute a number of conditions to 'something in the food' and consider themselves allergic without ever having it tested.

A certain number of perceived food allergy may be induced by members of the medical profession, conducting less efficiently controlled test programs. In some cases, patients are declared food allergic solely based on skin prick tests -which may well over-diagnose food-reactions. High focus on food allergy in the media combined with personal and psychological conditions may also play a role. Actually some specialists in food allergy consider the psychological disorders the most important differential- diagnosis from food allergy.

A diagnosis must rest upon a combination of a medical history and objective tests to confirm or reject the tentative diagnosis. In the field of food-related allergies, the diagnostic test systems have been difficult to establish. However, the Double Blind Placebo Controlled Food Challenge (DBPCFC)^{35,36}, is the method of choice to confirm or reject indications of food allergy that may derive from the patient's perception and in many cases also from skin prick testing.

The experience from food allergy centres is that objective test programs to confirm or reject a suspected 'food allergy', requires skin- and blood tests and up to 6 placebo controlled challenges to be reliable.

Therefore a diagnosis of food-related allergy, based solely on medical history and a skin prick test is not good clinical practice and must be regarded un-ethical

3.3. Enzymes in food

In theory, enzyme sensitisation and allergy symptoms may be induced by direct ingestion of consumer products containing enzyme residues may occur

The tendency in recent years to focus on allergy and food allergy in particular may explain part of the marked discrepancy between the public perception of allergy to food - and the relatively few cases that can be verified in controlled clinical tests.

Papain is relatively widely used as a meat tenderiser, often supplied in a powder form to apply to the meat before cooking.

In 1983 Mansfield and co-workers³⁷ published a case story of a person who had allergic symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients³⁸ with allergy of which 5 had a positive skin prick test to Papain.

The 5 papain positive were subjected to oral challenge with papain and all had positive reactions to the challenge.

Unfortunately, the challenge was only single blinded, and there is no report of occupational exposure or the use of powdered meat tenderisers that may have caused respiratory sensitisation.

In one other case story by Binkley³⁹, described below in the section 3.6.2, it can't be totally excluded that sensitisation took place by ingestion of a food product containing relatively high amounts of industrial produced enzymes.

A recent review by Wüthrich⁴⁰ of enzymes in food concluded that orally ingested enzymes are not potent allergens and that sensitisation to ingested enzymes is rare as is also the case of reactions to bread in bakers with occupational allergy to enzymes.

The member companies of AMFEP have not registered, experienced or heard of consumers that have become sensitised to enzymes or enzyme residues in consumer products by ingestion.

It has not been possible to verify the claims in the media of such cases, and they seem as yet un-substantiated as examples of enzyme allergies in consumers. The patients presented and the symptoms and tests described are not documented, merely describing sensations and feelings, however presented as facts.

A large proportion of adverse reactions to food must be ascribed to digestive disorders such as intolerance to for example gluten and lactose, which are not allergic reactions.

3.4. The Theory of cross reactions

people sensitised with common moulds might react to enzymes produced in related moulds

The theory that people with allergy to common moulds which are related to those used for the fermentation of enzymes might react to enzyme residues in food was one of Schata's¹ claims and was given relatively high coverage in the media.

The theory could not be readily rejected as cross-reactions are relatively common in allergy. A number of food allergy reactions are merely cross reactions than caused by primary sensitisation.

The most commonly used moulds for fermenting enzymes are *Aspergillus oryzae* or *A. niger*.

According to the theory, people with allergy to *Aspergillus*-moulds would be a high risk population. *Aspergillus* allergy occurs in less than 0.5%, of the population.

A study by Cullinan⁴¹ was conducted with the objective of testing if patients with a well-documented allergy to the widely distributed common mould *Aspergillus fumigatus* reacted upon the ingestion of bread prepared with enzymes of *Aspergillus* origin. The study was a double blind placebo controlled food challenge study on 17 *Aspergillus* allergic people.

The 17 test persons all had allergy antibodies to *Aspergillus fumigatus*, but in addition, 6 also reacted at the skin prick test to the enzymes produced in *A. oryzae* or *A. niger*.

Each patient was challenged with bread baked with the 2 enzymes in standard doses and with placebo bread baked without enzymes. Allergy symptoms and a number of general physiological parameters were monitored before, during and for 24 hours after the challenge.

No allergic reactions were seen upon ingestion of enzyme containing bread as compared to placebo bread.

This study clearly demonstrates that patients who must be considered at the highest risk for cross reactions to baking enzymes do not react with clinical symptoms when they eat enzyme containing bread containing enzymes.

It is a general experience that once a person is sensitised, even very small amounts of the allergen can elicit allergy symptoms.

In the case of baking enzymes it seems well documented that even patients with severe asthma caused by *Aspergillus fumigatus* did not react to the baking enzymes produced in *A. oryzae* and *A. niger*.

3.5. Food related reactions in occupationally sensitised people

The situation of possible reactions to enzymes in bread in patients with occupational allergy to enzymes

There are a few papers describing cases of allergy symptoms elicited by the ingestion of enzymes in people who have occupational allergy to enzymes:

Kanny & Moneret-Vautrin,⁴² and Baur & Czuppon⁴³ each describes one patient who since late childhood, has had asthma and occupational asthma with allergy to flour and enzymes for several years. Both patients were tested for elicitation of symptoms by ingestion of bread baked with and without enzymes. Kanny & Moneret-Vautrin's patient was tested in a blinded design, Baur's patient in an open, non-controlled programme. In both cases the result was elicitation of respiratory symptoms after challenge with bread baked with enzymes. Baur's patient also had a slight reaction to bread without enzymes, however not as pronounced as the reaction after the enzyme containing bread.

Losada et al⁴⁴ investigated occupational allergy to α -amylase in a pharmaceutical plant and found a number of employees sensitised to α -amylase. None reported reactions related to ingestion of bread. Five patients, all positive to α -amylase were given oral doses of native α -amylase in doses up to 10 mg.

At this dosage, one of the 5 test persons reacted with respiratory- and generalised allergy symptoms. Four did not react.

Baur et al⁴⁵ described the possible background for consumer sensitisation to α -amylases in bread. 138 subjects, of which 98 were allergic, and 11 bakers with occupational allergy were tested. The bakers reacted to α -amylase as may be expected. None of the atopics and none of the control persons reacted to skin prick test with α -amylase. Two atopics had weak RAST to native α -amylase and one reacted also to heated ce-amylase. Reactions to other related compounds, for example *Aspergillus* was not tested.

Tarlo and co-workers⁴⁶ reported results of testing for papain allergy in 330 allergy patients. - Seven had positive RAST and Skin prick test but none of them had any gastrointestinal or other allergic symptoms to papain.

The elicitation of gastrointestinal symptoms upon respiratory sensitisation is also reported for flours. One example is reported by Vidal et al⁴⁷ and describes a man with occupational asthma after exposure to flours and other grain dusts. He was sensitised to barley, and experienced gastrointestinal reaction upon ingestion of foods and beverages made from barley.

Enzyme producers and other companies handling concentrated enzymes do see cases of employees being sensitised to baking enzymes. These would be the people at the highest risk of reacting to enzyme residues in bread.

However, none of the members of AMFEP had any reports of sensitised employees who had experienced allergy symptoms in connection to ingestion of bread, and there are no reports of α -amylase sensitised employees avoiding bread.

Cases of people with occupational allergy to flours and food-related reactions to ingestion of flours/bread do occur. One case report describes a person with asthma to barley dust and also with reaction to beverages and foods produced from barley.

The conclusion from these reports of people with pre-existing occup. allergy to α -amylase is:

- Allergic reactions after ingestion of enzyme containing foods are described in 3 individuals.
- The 3 cases are people with definite occupational respiratory allergy to flour and an additional sensitisation to α -amylase. It means they are most probably sensitised by inhalation of flour dust and enzyme dust and not by eating bread or other foods with enzyme residues in it.

3.6 *The consumption of enzymes for medical purposes and as digestive aids:*

Many people around the world eat enzymes for medical purposes or for convenience as digestive aids.

In many countries enzymes are used routinely as digestive aids by healthy people. The number of people in the world, frequently eating enzyme preparations must be counted in millions.

A number of diseases require the daily addition of enzyme preparation to the food to compensate the patient's insufficient production of digestive enzymes.

3.6.1. *Medical uses:*

Medical use of enzyme preparations are subject to clinical trials, the results of which are normally reported to the health authorities, and such adverse effects are described in the pharmacopoeia/registry of drugs.

Patients with chronic pancreatitis suffer from insufficient production of digestive enzymes from the pancreas. They are dependent on daily intake of enzymes, some of these produced from *Aspergillus* and other moulds, some extracted from animal glands. The doses of these enzymes are in the order of gram's a day. - we have not been able to identify published documentation of allergy to enzymes in these patients, and the drug registry's does not even mention allergy as an adverse effect.

Proteolytic enzymes and mixtures of different enzymes are commonly used for treatment of a number of physical lesions and also for a number of more special conditions⁴⁸⁻⁵⁰.

The enzymes are administered in the form of tablets with mixtures of enzymes and in doses of 6 to 600 mg per day, in some cases several times more.

We have not been able to find any evidence of sensitisation or allergy symptoms caused by the ingestion of enzymes from these enzyme preparations. One example is the use of enzymes given as tablets for the treatment of non-articular rheumatism. Uffelmann⁵¹ describes a double blind study of 424 patients, of which 211 received enzyme treatment. The daily doses of the mixed enzyme preparations was 240 mg Lipase, 240 mg Amylase, 1,44 g Papain, 1,08 g Bromelain and 2.4 g Pancreatin,. This dosage was given for 8 weeks and no serious adverse effects and no allergy reactions were reported.

Patients with Cystic Fibrosis suffer a hereditary disease characterised by severe lung symptoms and insufficient production of digestive pancreatic enzymes. They too are dependent of daily intake of grain-doses of enzymes. - There are a few reports of parents

and hospital staff who have become sensitised by inhalation of dust from these enzyme preparations⁵²⁻⁵⁴. This of course might also happen to the Cystic fibrosis patients when they handle the enzyme preparations themselves. However no cases of enzyme allergy in Cystic Fibrosis patients have been described, but there are reports of allergy to common food allergens⁵⁵.

An informal telephone survey on unpublished cases of enzyme allergy to European Cystic fibrosis Centres, resulted in only one possible case. The patient was a boy who reacted with vomiting after administration of the enzyme preparation containing amylase, protease and lipase. - The enzyme treatment had been stopped because of suspected allergy to the enzymes. However, testing for specific allergy antibodies by Maxisorp RAST⁵⁶ did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed⁵⁷

3.6.2. Digestive aids one possible case of allergy to digestive aid enzymes

In some cultures the use of digestive enzymes after large meals is very common. Enzymes for this purpose are 'over the counter' (OTC) drugs. We have found no studies of possible allergy to enzymes in these populations. That may be irrelevant if no-one ever thought of the possibility that enzymes might be the cause of allergic symptoms had not been considered. - However, with millions of people using enzymes frequently, some cases of adverse effects in the form of allergic symptoms would be expected to emerge and be described in the literature. In most patients with allergic reactions, symptoms would appear immediately or very shortly after the intake.

Binkley³⁹, described a case of allergic reaction to ingested lactase. This patient had a respiratory allergy with positive skin prick test reaction to *Aspergillus sp.*

He had had two incidents with allergic reactions in the form of swelling and burning of the mouth and throat after ingestion of Lactaid tablets. The lactase was produced from fermentation of *Aspergillus oryzae*. Skin prick test with extracts of Lactase tablets gave a very strong positive reaction. He had not taken Lactaid tablets previous to the first experience of symptoms, but he had taken milk products containing lactase from *Saccharomyces fragilis* and from *Kluyveromyces lactis*. Although highly unlikely, it may be speculated if these may cross react with Lactaid. In this case it seems unlikely that sensitisation was caused by the Lactaid tablets as the symptoms appeared the first time he ever took Lactaid. It could be a 'cross reaction' based on sensitisation to yeast-produced lactase and symptoms elicited by the ingestion of Lactaid. Another possibility may be a cross reaction from his pre-existing *Aspergillus sp.* allergy.

This case may be regarded a possible but not verified case of oral sensitisation to enzymes in food.

A few other consumers have claimed allergy to these OTC drugs but thorough testing has not verified allergy to enzymes in any of these cases.

With the background of the very high awareness of food related allergy in the populations, the widespread use of digestive aid and medical uses of enzymes should have attracted interest if allergy to ingested enzymes were of importance. However, up to now, only the single case mentioned above have been described.

To evaluate the risk of sensitisation from ingestion of enzymes and eventually experience of symptoms, we are aware of only the one case that may have become sensitised by ingestion.

This has to be related to the total number of people world-wide who ingest enzymes for short periods of time as part of a medical treatment, and to those who are dependent of daily intake of high amounts of digestive enzymes.

4.0. Conclusion

The working group has studied the available literature on these subjects and came to the conclusion that from a scientific point of view there is no indication that enzyme residues in bread or in other foods may represent an unacceptable risk for consumers.

Lack of scientific data is not evidence of lack of risk, and the working group realises that evidence of 'no risk' is extremely difficult or impossible to generate.

The group wish to stress that a 'zero-risk' can never be proved by science, and it must be anticipated that even an extremely low risk (e.g. 1 in 50 or 100 millions) of verified allergy to enzymes in food may well be perceived as a significant and unacceptable risk by the public in which more than 10% believe they are allergic to food.

Scientific data are of high value as the credible background for promotion to the public, to trade organisations and individual customers and for an ongoing dialogue with opinion leaders and consumer organisations.

It is the opinion of the group that many cases of perceived allergy to enzymes may be attributed to insufficient diagnostic procedures employed by members of the medical profession.

A minimum requirement for establishing a diagnosis of food related enzyme allergy should be a well conducted DBPCFC.

5.0. Bibliography

1. Schata M. Allergische reaktionen durch alpha-amylase in backmitteln. *Allergologie* 1992;57:s0(abstract).
2. Baur X. Studies on the specificity of human IgE-antibodies to the plant proteases papain and bromelain. *Clin Allergy* 1979;9:451-457.
3. Wuthrich B. [Proteolytic enzymes: potential allergens for the skin and respiratory tract?] *Proteolytische Enzyme: Potente Allergene fur Haut- und Respirationstrakt? Hautarzt* 1985;36:123-125.
4. Gailhofer G, Wilders Truschnig M, Smolle J, Ludvan M. Asthma caused by bromelain: an occupational allergy. *Clin Allergy* 1988; 18:445-450.
5. Colten HR, Polakoff PL, Weinstein SF, Strieder DJ. Immediate hypersensitivity to hog trypsin resulting from industrial exposure. *N Engl J Med* 1975;292:1050-1053.
6. Akiyama K, Shida T, Yasueda H, Mita H, Yamamoto T, Yamaguchi H. Atopic asthma caused by *Candida albicans* acid protease: case reports. *Allergy* 1994;49:778-781.
7. Flood DF, Blofeld RE, Bruce CF, Hewitt JI, Juniper CP, Roberts DM. Lung function, atopy, specific hypersensitivity, and smoking of workers in the enzyme detergent industry over 11 years. *Br J Ind Med* 1985;42:43-50.
8. Pepys J, Mitchell J, Hawkins R, Maio JL. A longitudinal study of possible allergy to enzyme detergents. *Clin Allergy* 1985; 15:101-115.
9. Alvarez MJ, Tabar AI, Quirce S, Olaguibel JM, Lizaso MT, Echechipia S, Rodriguez A, Garcia BE. Diversity of allergens causing occupational asthma among cereal workers as demonstrated by exposure procedures. *Clinical and Experimental Allergy* 1996;26:147-153.
10. De Zotti R, Larese F, Bovenzi M, Negro C, Molinari S. Allergic airway disease in Italian bakers and pastry makers. *Occup Environ Med* 1994;51:548-552.
11. Bernstein DI, Bernstein IL, Gaines WG, Jr., Stauder T, Wilson ER. Characterization of skin prick testing responses for detecting sensitization to detergent enzymes at extreme dilutions: inability of the RAST to detect lightly sensitized individuals. *J Allergy Clin Immunol* 1994;94:498-507.
12. Baur X, Weiss W, Sauer W, Fruhmarm G, Kimin KW, Ulmer WT, Mezger VA, Woitowitz HJ, Steurich FK. [Baking ingredients as a contributory cause of baker's asthma] Backmittel als Mitursache des Backerasthmas. *Dtsch Med Wochenschr* 1988; 113:1275-1278.
13. Bossert J, Fuchs E, Wahl R, Maasch HJ. Occupation sensitization by inhalation of enzymes diaphorase and lipase. *Allergologie* 1988;11:179-181.
14. Losada E, Hinojosa M, Moneo I, Dominguez J, Diez Gomez ML, Ibanez MD. Occupational asthma caused by cellulase. *J Allergy Clin Immunol* 1986;77:635-639.
15. Tarvainen K, Kanerva L, Tupasela O, Grenquist Norden B, Jolanki R, Estlander T, Keskinen H. Allergy from cellulase and xylanase enzymes. *Clin Exp Allergy* 1991;21:609-615.
16. Aceves M, Grimalt JO, Sunyer J, Anto JM, Reed CE. Identification of soybean dust as an epidemic asthma agent in urban areas by molecular marker and RAST analysis of aerosols. *J Allergy Clin Immunol* 1991;88:124-134.

17. Oehling A, Garcia B, Santos F, Cordoba H, Dieguez I, Fernandez M, Sanz ML. Food allergy as a cause of rhinitis and/or asthma. *J Investig Allergol Clin Immunol* 1992;2:78-83.
18. Valero A, Lluch M, Amat P, Serra E, Malet A. Occupational egg allergy in confectionary workers. *AllergyNet* 1996;51:588-592.
19. Bemaola G, Echechipia S, Urrutia I, Fernandez E, Audicana M, Fernandez de Corres L. Occupational asthma and rhinoconjunctivitis from inhalation of dried cow's milk caused by sensitization to alpha-lactalbumin. *Allergy* 1994;49:189-191.
20. Droszcz W, Kowalski J, Piotrowska B, Pawlowicz A, Pietruszewska E. Allergy to fish in fish meal factory workers. *Int Arch Occup Environ Health* 1981;49:13-19.
21. Ferrer A, Torres A, Roca J, Sunyer J, Anto JM, Rodriguez Roisin R. Characteristics of patients with soybean dust-induced acute severe asthma requiring mechanical ventilation [published erratum appears in *Eur Respir J* 1990 Jul;3(7):846]. *Eur Respir J* 1990;3:429-433.
22. Burks AW, Sampson H. Food allergies in children. *Curr Probl Pediatr* 1993;23:230-252.
23. Jansen JJ, Kardinaal AF, Huijbers G, Vlieg Boerstra BJ, Martens BP, Ockhuizen T. Prevalence of food allergy and intolerance in the adult Dutch population. *J Allergy Clin Immunol* 1994;93:446-456.
24. Joral A, Villas F, Garmendia J, Villareal O. Adverse reaction to food in adults. *J Invest Allergol Clin Immunol* 1995;5:47-49.
25. Altman DR. Public perception of food allergy. *J Allergy Clin Immunol* 1996;97:124-151.
26. O'Neil C, Helbling AA, Lehrer SB. Allergic reactions to fish. *Clin Rev Allergy* 1993;11:183-200.
27. Shimojo N, Katsuki T, Coligan JE, Nishimura Y, Sasazuki T, Tsunoo H, Sakamaki T, Kohno Y, Niimi H. Identification of the disease-related T cell epitope of ovalbumin and epitope-targeted T cell inactivation in egg allergy. *Int Arch Allergy Immunol* 1994;105:155-161.
28. Mole LE, Goodfriend L, Lapkoff CB, Kehoe JM, Capra JD. 'Me amino acid sequence of ragweed pollen allergen Ra5. *Biochemistry* 1975;14:1216-1220.
29. Steinman HA. Hidden allergens in foods. *Journal of Allergy and Clinical Immunology* 1996;98:241-250.
30. Lehrer SB, Homer WE, Reese G. Why are some proteins allergenic? - Implications for Biotechnology. *Cri Rev in Food Science and Nutr* 1996;36:553-564.
31. Metcalfe DD, Fuchs R, Townsend R, Sampson H, Taylor S, Fordham J. Allergenicity of foods produced by genetic modification. 1995;(Abstract).
32. Metcalfe DD. Public perception of food-allergy problems is high, experts say. *Food Chemical News* 1994;April 25:45-46.
33. Fuchs R, Astwood JD. Allergenicity Assessment of Foods Derived from Genetically Modified Plants. *Food Technology* 1996;February:83-88.
34. Young E, Stoneham M, Petrukevitch A, Barton J, Rona R. A population study of food intolerance. *The Lancet* 1994;343:1127-1130.
35. Bock SA, Sampson HA, Atkins FM, Zeiger RS, Lehrer S, Sachs M, Bush RK, Metcalfe DD. Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. *J Allergy Clin Immunol* 1988;82:986-997.

36. Ortolani C, Pastorello EA, Ansaloni R, Incorvaia C, Ispano M, Pravettoni V, Rotondo F, Scibilia J, Vighi G. Study of nutritional factors in food allergies and food intolerances. 1997;EUR 16893 en:1-196.(Abstract).
37. Mansfield LE, Bowers CH. Systemic reaction to papain in a nonoccupational setting. *J Allergy Clin Immunol* 1983;71:371-374.
38. Mansfield LE, Ting S, Haverly RW, Yoo TJ. The incidence and clinical implications of hypersensitivity to papain in an allergic population, confirmed by blinded oral challenge. *Ann Allergy* 1985;55:541-543.
39. Binkley KE. Allergy to supplemental lactase enzyme. *J Allergy Clin Immunol* 1996;97:1414-1416.
40. Wuthrich B. Enzyme als ingestive Allergene. *Allergie für die Praxis* 1996;4:74-91.
41. Cullinan P, Cook A, Jones M, Cannon J, Fitzgerald B, Newman Taylor AJ. Clinical responses to ingested fungal alpha-amylase and hemicellulase in persons sensitized to *Aspergillus fumigatus*? *Allergy Eur J Allergy Clin Immunol* 1997;52:346-349.
42. Kanny G, Moneret Vautrin DA. alpha-Amylase contained in bread can induce food allergy. *J Allergy Clin Immunol* 1995;95:132-133.
43. Baur X, Czuppon AB. Allergic reaction after eating alpha-amylase (Asp o 2)-containing bread. A case report. *Allergy* 1995;50:85-87.
44. Losada E, Hinojosa M, Quirce S, Sanchez Cano M, Moneo I. Occupational asthma caused by alpha-amylase inhalation: clinical and immunologic findings and bronchial response patterns. *J Allergy Clin Immunol* 1992;89:118-125.
45. Baur X, Sander I, Jansen A, Czuppon AB. [Are amylases in bakery products and flour potential food allergens?] Sind Amylasen von Backmitteln und Backmehl relevante Nahrungsmittelallergene? *Schweiz Med Wochenschr* 1994;124:846-851.
46. Tarlo SM, Shaikh W, Bell B, Cuff M, Davies GM, Dolovich J, Hargreave FE. Papain-induced allergic reactions. *Clin Allergy* 1978;8:207-215.
47. Vidal C, Gonzalez Quintela A. Food-induced and occupational asthma due to barley flour. *Ann Allergy Asthma Immunol* 1995;75:121-124.
48. Stauder G, Pollinger W, Fruth C. Systemic enzyme therapy. A review of the new clinical studies. *Allgemeinmedizin* 1990;19:188-191.
49. Rahn HD, Kilic M. The effectiveness of a hydrolytic enzyme in traumatology. The results of two prospective, randomized, double-blind studies. *Allgemeinmedizin* 1990;19:183-187.
50. Ransberger K, Stauder G. Use of catabolic enzymes for controlling the acquired immune deficiency syndrome (AIDS) and its precursors (LAS, ARC). 1991;5,002,766:1-8.(Abstract).
51. Uffelmann K, Vogler W, Fruth C. The use of a hydrolytic enzyme in extra-articular rheumatism. *Allgemeinmedizin* 1990;19:151-153.
52. Moss RB. Drug allergy in cystic fibrosis. *Clin Rev Allergy* 1991;9:211-229.
53. Sakula A. Bronchial asthma due to allergy to pancreatic extract: a hazard in the treatment of cystic fibrosis. *Br J Dis Chest* 1977;71:295-299.
54. Lipkin GW, Vickers DW. Allergy in cystic fibrosis nurses to pancreatic extract. *Lancet* 1987;Feb 14:

55. Lucarelli S, et al. Food allergy in cystic fibrosis. *Minerva Paediatr* 1994;46:543-548.
56. Poulsen LK et al. Maxisorp PAST Allergy 1989,44:173-180.
57. Dab I. - personal communication. Not published.
58. TNO Nutrition and Food Research Institute. Stability of fungal and bacterial (-amylase in gastric compartment of TIM. 1998, in prep.

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