Sent: Wednesday, 2 October 2019 12:06 PM
To: standards management
Subject: FSANZ Submission Form Received (Internet) - Danisco Singapore Pte Ltd
Attachments: Technical information for Xylanase to be used in brewing and cereal based beverages.pdf

Application/Proposal Number:	A1174 - Xylanase from Trichoderma reesei as a PA (Enzyme): to permit the use of Xylanase from Trichoderma reesei as a Processing Aid.
Organisation Name:	Danisco Singapore Pte Ltd
Organisation Type:	Food Manufacturer
Representing:	DuPont Industrial Biosciences, DuPont Australia
Street Address:	21 Biopolis Road, #06-21, Nucleos, South Tower
Postal Address:	21 Biopolis Road, #06-21, Nucleos, South Tower
Contact Person:	
Contact Number:	
Email Address:	
Submission Text:	Dupont wishes to add "brewing and cereal based food beverage production" as the approved application for the enzyme Xylanase from Trichoderma reesei, subject of A1174. This request would allow broader application of the enzyme, while would lower manufacturing cost, and gives more choices of raw material in brewing, and cereal based beverage industry.

	The additional application will not change the safety conclusion of the Xylanase enzyme as a processing aid. Therefore, DuPont hope FSANZ could consider accepting Dupont's request.

Technical information of Xylanase for application in brewing and cereal based beverage production

Contents:

1 Identity

1.1 Xylanase

The systematic name of the principle enzyme activity is 4-β-D-xylan xylanohydrolase. Other names used are endo-1,4-β-xylanase, endo-(1 → 4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo- β-1,4-xylanase; endo-1,4- β-D-xylanase; 1,4- β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase; β-D-xylanase, Xylanase X3.

The enzyme Xylanase is derived from *Trichoderma reesei* which is genetically modified to overexpress the xylanase gene from *Aspergillus niger* (var. tubingensis).

- EC number: 3.2.1.8
- CAS number: 9025-57-4

Please also refer to Appendix A of original submission.

1.2 Other enzymes

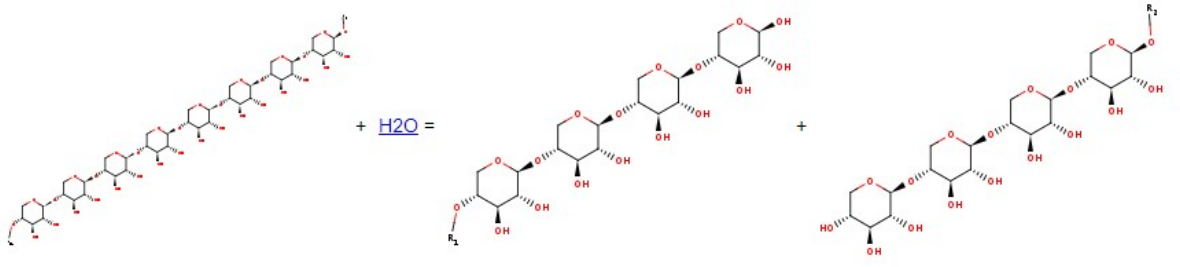
Downstream processing concentrates and purifies the enzyme product. The resulting enzyme preparation will not be totally pure and traces of other enzyme activities (e.g. protease) might be found but their level will be very low.

2 Chemical and physical properties

2.1 Substrate specificity

Endo-1,4-beta-xylanase (IUBMB 3.2.1.8) catalyses the reaction of endohydrolysis of (1->4)-beta-D-xylosidic linkages in xylans. The substrate(s) for Xylanase: xylans, including arabinoxylans.

Reaction catalyzed by endo-1,4-beta-xylanase (3.2.1.8)



(Xylbeta(1-4))n + H2O = (Xylbeta(1-4))n-m + (Xylbeta(1-4))m

Please also refer to Appendix A of original submission.

2.2 Activity

The activity of the Xylanase is defined in GPU. The substrate employed in the assay is azurine-crosslinked wheat arabino-xylan. This substrate hydrates in water but is water insoluble. Hydrolysis of the substrate by an endo-xylanase produces water-soluble fragments, and the rate of these fragments can be related directly to the enzyme activity by spectroscopy.

Xylanase preparations' enzyme activity will depend on the final product. An example product has the xylanase activity range of 170000-230000 units/g. Please also refer to Appendix A of original submission.

2.3 Temperature optimum

Enzyme activity of Xylanase was analysed at various temperatures under pH4.8 using method as described in Appendix A3. Results are shown in Figure 1.

Temperature optimum was determined to be 50°C with high relative activity between 45-55°C. The relative activity reduced significantly at 60°C.

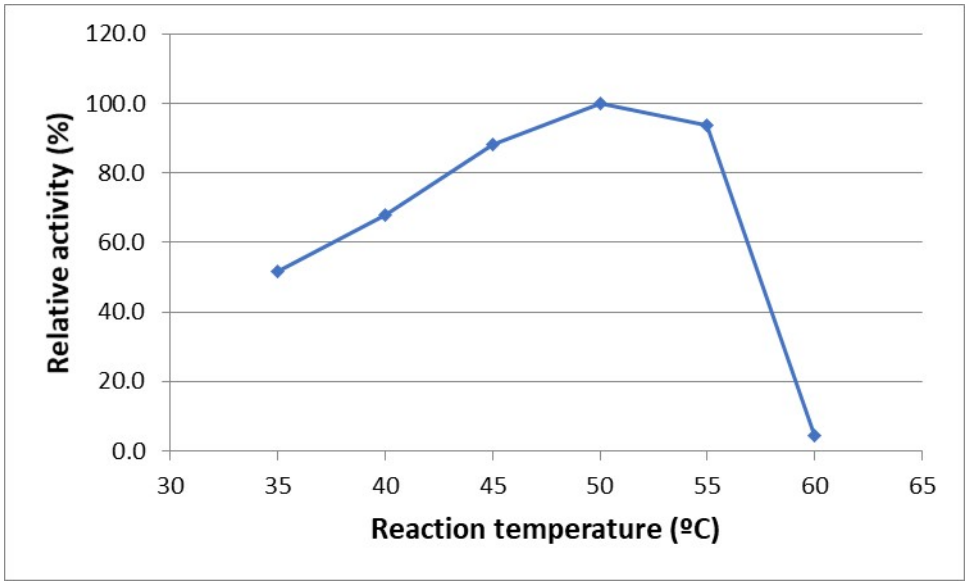


Figure 1: Temperature profile of Xylanase (pH4.8). Activity is shown as relative to the highest measured value.

Please also refer to Appendix A of original submission.

2.4 Thermal stability

Thermal stability of the Xylanase was determined by measuring residual xylanase activity after incubation at various temperatures for 10 minutes in McIlvaine buffer pH 4.8. Results are shown in Figure 2.

Xylanase retained over 80% of activity after 10 minutes of incubation at 40°C, 45°C and 50°C. A significant reduction was observed when the temperature is 55°C and above. Less than 10% of remaining activity was observed after incubation at 65°C for 10 minutes.

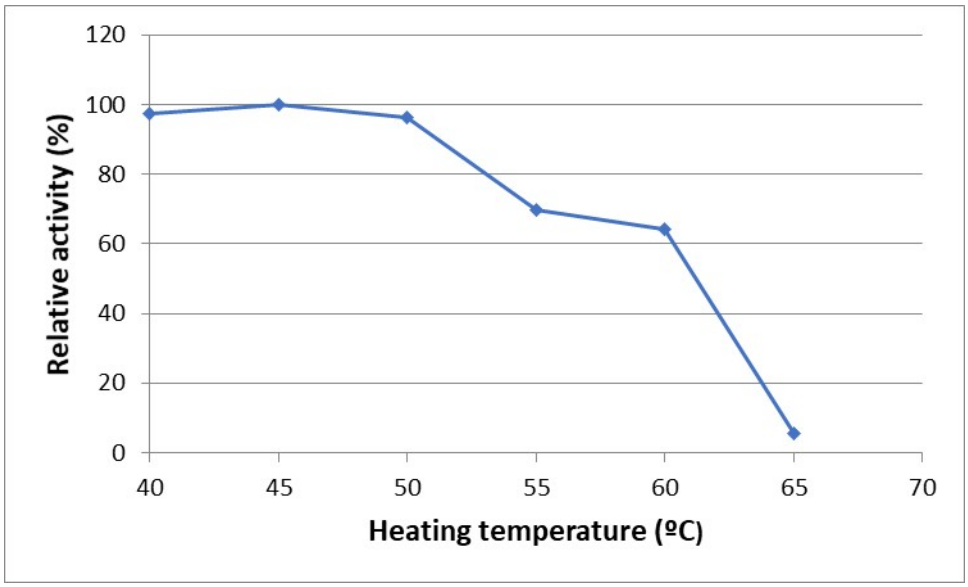


Figure 2: Activity was determined after 10 minutes of incubation at pH 4.8.

Please also refer to Appendix A of original submission.

2.5 pH optimum

Enzyme activity of Xylanase was determined at various pH values at 40 ºC. Results are shown in Figure 3.

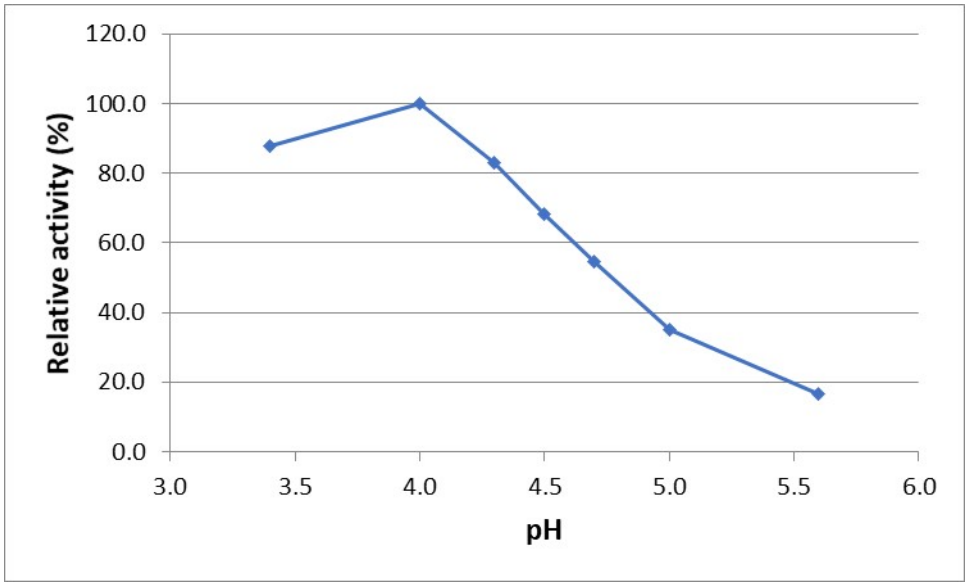


Figure 3: pH profile of Xylanase. Activity is shown as relative to the highest measured value

Maximum activity is observed at pH 4.0, with high relative activity between pH 3.5 – 4.5. Enzyme demonstrated activity in the range from pH 3.5 to 5.5.

Please also refer to Appendix A of original submission.

2.6 Storage stability

Food enzymes are not sold as such, but formulated into various enzyme preparations in order to obtain standardized and stable products. The stability thus depends on the type of formulation, not on the food enzyme as such.

According to Standard 1.2.5 of Food Standards Code, the date of minimum durability or use-by-date is indicated on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be mentioned on the label.

The figure below shows storage stability of an example commercial product of Xylanase. As seen in the figure, at 20°C, the enzyme is stable for 52 weeks with close to 90% remaining activity.

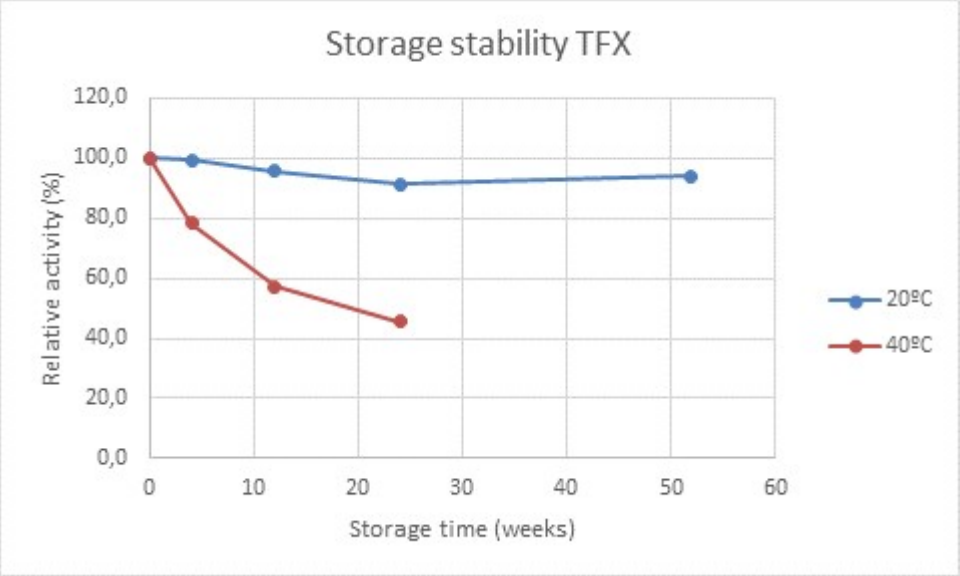


Figure 4. Storage stability of Xylanase

Please also refer to Appendix A of original submission.

3 Efficacy and benefits of the Xylanase enzyme preparation

3.1 Description

As noted above, the function of Xylanase is to catalyse the endo-hydrolysis of the (1,4)-beta-D-xylosidic linkages that are present in xylans, including arabinoxylan.

In general, the technological need of the enzymatic conversion of (arabino) xylans with the help of Xylanase can be described as: endo-hydrolysis of glycolsyl compounds resulting in mono- and oligosaccharide. In addition to usage in bakery as described in the original submission, Xylanase can also be used in following food manufacturing process.

Brewing and Cereal based beverage production:

- Increase flexibility in the choice of raw materials, including faster and more predictable lautering or mash filtration, and increased filtration rate and reduced need for beer filtration aids.
- Higher extract yield due to the improved processing, and thereby less use of raw materials.
- Decrease wort viscosity for brewing.

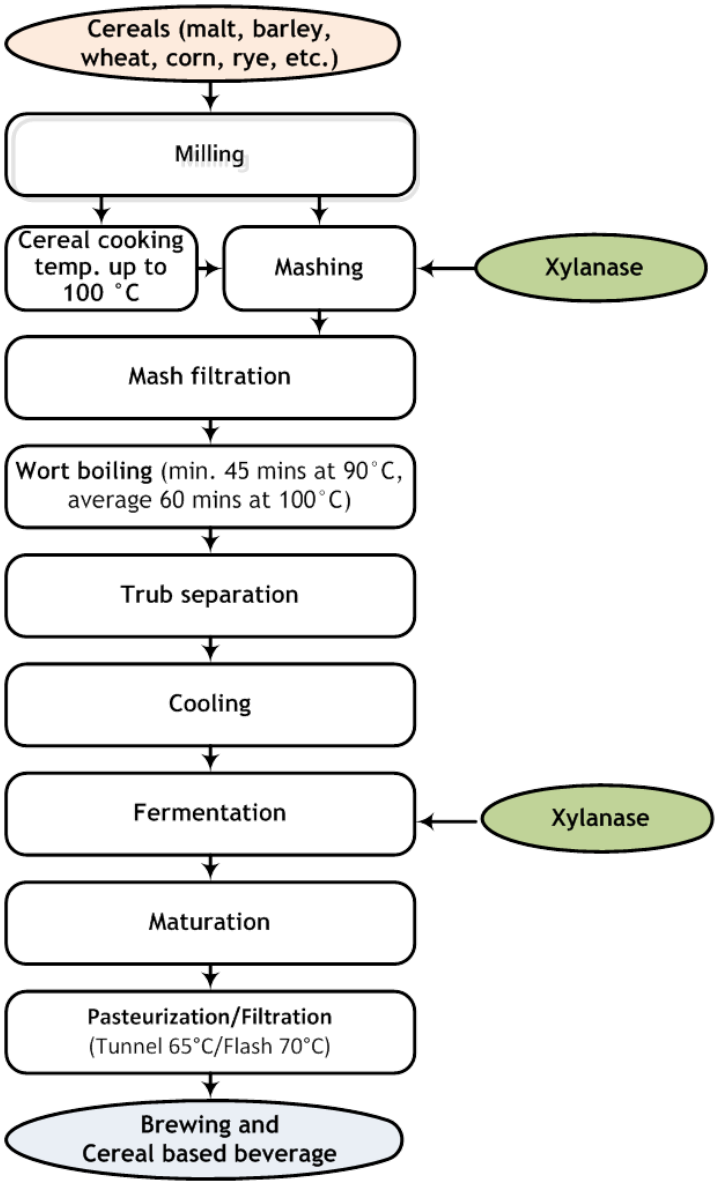
- Increase access of indigenous malt enzymes to starch granules and decrease water retention of spent grains.

In all of these applications, the enzyme product will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

3.2 Efficacy examples

Brewing and Cereal based beverage production:

Xylanase is typically added in mashing step or to the adjunct before addition of the adjunct to the mash tun. Xylanase is therefore denatured already in the consecutive lautering or mash filtration step. Xylanase may also be added during the fermentation step. In this case Xylanase will be denatured during the pasteurization step. Please refer to manufacturing flow chart below.



When barley is used as raw material to replace malt for brewing or cereal based food and beverage production, presence of arabinoxylan normally increases viscosity, and reduce filtration capacity. As seen in Figure 7 below, use of enzyme could significantly reduce high molecular weight (HMW) β-glucan in mashes, which will reduce viscosity, increases filtration rate, and eventually improve yield. High consistent beer filtration throughput will also minimizes energy consumption. The reduction on HMW is consistently observed when barley is used from 20% to 50%. Xylanase, used in combination with a β-glucanase, provides further reduction compared to market reference enzyme sample. For example, when 50% barley is used as raw material, HMW β-glucan level can reach to about 800 mg/L, but is deceased to less than 100 when market reference enzyme is used. When Xylanase is used with β-glucanase, β-glucan level is below 20 mg/L.

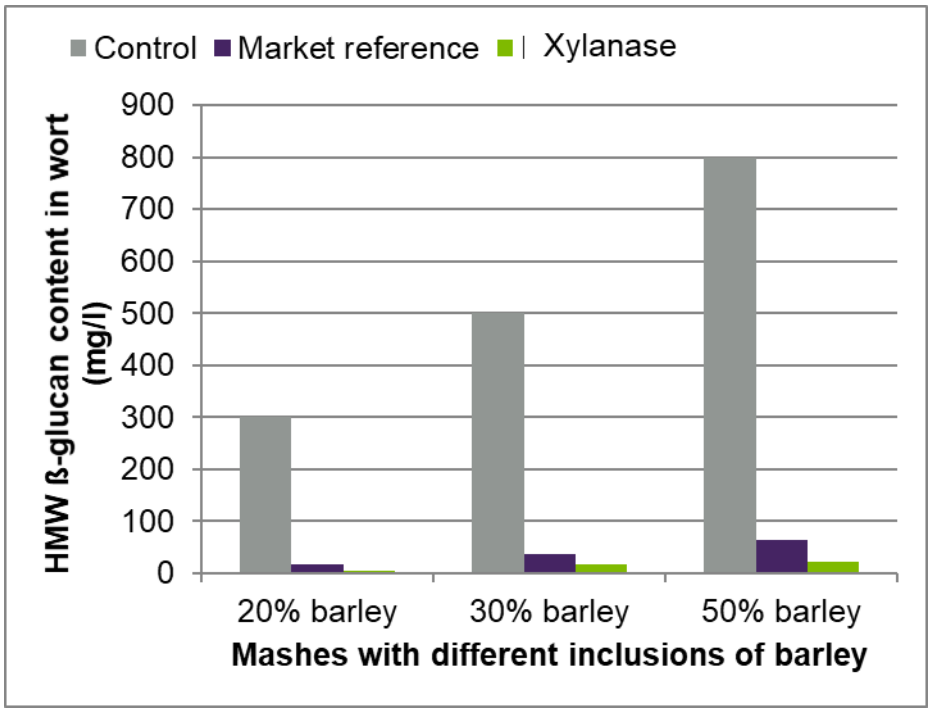


Figure 7. HMW ß-glucan reduction with Xylanase (in combination with beta-glucanase)

Table 1 below further compared the performance of Xylanase (in combination with a beta-glucanase) and a market reference enzyme sample. Xylanase (in combination with a beta-glucanase) will increase extraction yield from 14.5 to 14.8. HMW ß-glucan was reduced from 54-48 mg/L. Total pentosan was reduced from 2410 to 1910 mg/L, meaning less off-flavor potential.

Table 1. Wort Analysis

	Market Reference	Xylanase (in combination with a beta-glucanase)
Extract (°P)	14.5	14.8
HMW ß-glucan (mg/l)	54	48
Total pentosan (mg/l)	2470	1910

4 Manufacturing process

The manufacturing process for the production of Xylanase will be conducted in a manner similar to other food and feed production processes. It is conducted in accordance with food good manufacturing practice (GMP) and the resultant product meets the general requirements for enzyme preparations of the Food Chemicals Codex, Sixth Edition (FCC 2008) and the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA, 2006).

The manufacturing process is a three-part process consisting of fermentation (growth of organism and production of enzyme), recovery (separation of cell mass from enzyme and concentration/purification of enzyme) and formulation/ drying (preparation of a stable enzyme formulation). The production process follows standard industry practices (see, Enzyme Applications, 1994; Aunstrup et al, 1979; and Aunstrup, 1979).

Please also refer to Appendix A of original submission.

4.1 Raw materials

The raw materials used in the fermentation and recovery process for the Xylanse enzyme concentrate are standard ingredients used in the enzyme industry. All the raw materials conform to the specifications of the Food Chemical Codex, 6th edition (FCC 2008), except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. DuPont IB uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E of original submission. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

Please also refer to Appendix A of original submission.

4.2 Fermentation

Xylanase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *T. reesei* described in Appendix B of original submission. The fermentation is an aerobic process and requires continuous addition of air to the fermenter. 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 conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

The fermentation process consists of three operations: laboratory propagation of the culture, seed fermentation and primary fermentation. These processes, except for the laboratory propagation are carried out in sealed vessels carefully designed to prevent both the release of the production organism and/or the entry of other microorganisms.

A new lyophilized stock culture vial of the *T. reesei* production organism is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

The fermentation media is sterilised at 121°C for at least 20 minutes. The medium is sampled for microbiological testing prior to inoculation. The fermentation takes place at controlled temperatures.

All stages of the production process are controlled to ensure that the final product conforms to specifications. The culture liquid is sampled at intervals during fermentation for microbiological and enzyme activity tests. Operational parameters such as temperature, pH, air flow, agitation and oxygen content are monitored and controlled to desired values/ranges throughout the fermentation. In addition, at all stages, microbial growth is checked for correct morphological development of the microorganism and for the lack of contamination. Once the fermentation is completed, the fermentation broth is transferred to processing tanks.

Please also refer to Appendix A of original submission.

4.3 Recovery

The purpose of the recovery process is to separate the biomass, purify, concentrate, and stabilise the desired enzyme, i.e. Xylanase.

Separation of the cell debris from the liquid from the fermentation broth is achieved by either filtration or centrifugation, or a combination of both. Exactly which cell separation technique is used is dependent upon the manufacturing site. The broth may be treated with flocculating agents to maximize separation and is then fed into the filter or the centrifuge. The relatively solids free stream then passes a polishing filter to further clarify the liquid and achieve clear, cell-free filtrate.

The liquid containing the enzyme is concentrated via ultrafiltration, which removes low molecular weight compounds. Diafiltration may follow ultrafiltration to help reach the activity target, remove colour and smaller particles, and carbon treatment may additionally be used to reduce colour. The final recovery step is a polish filtration using either microfiltration membranes, fine filtration aids such as diatomaceous earth or sterile filtration pads.

The ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier, or stabilised by e.g. glycerol to produce a liquid product.

Please also refer to Appendix A of original submission.

4.4 Formulation

The ultrafiltrated concentrate is then formulated and analysed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the FCC.

Please also refer to Appendix A of original submission.

5 Specification for identity and purity

5.1 Purity criteria

Appropriate GMP controls and processes are used in the manufacture of Xylanase to ensure that the finished product does not contain any impurities of a hazardous or toxic nature.

The specifications for the Xylanase enzyme preparation meet or exceed the requirements for enzyme preparations as set forth in the Food Chemical Codex, 6th Edition (2008) and by the Joint FAO/WHO Expert Committee on Food additives (JECFA 2006). Please also refer to Appendix A of original submission.

5.2 Allergens

An example allergen declaration of the enzyme preparation used for brewing is included below.

Yes	No	Allergens	Description of components
	(X)	Wheat	Glucose (used in fermentation)*
	X	Other cereals containing gluten	
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
	(X)	Soybeans	Soy flour (used in fermentation)*
	X	Milk (incl. lactose)	
	X	Nuts includes: almond, Hazelnut, Cashew-nut, Brazilian-nut, Macadamia, Walnuts, Pecan, Pistachio, Pinoli and Chestnuts	
	X	Celery	
	X	Mustard	
	X	Sesame seeds	
	X	Sulphur dioxide and sulphites (>10mg/kg)	
	X	Lupin	
	X	Molluscs	
	X	Natural Latex	

The table indicates the presence (as added component) of the following allergens and products thereof (according to US Food Allergen and Consumer Proctection act (FALCPA), 2004 and Directive 2000/13/EU as amended).

*Danisco has determined that fermentation nutrients are outside the scope of US and EU food allergen labeling requirements [1], [2]. [1]
Position paper sent by ETA to the FDA on September 12, 2005 (www.enzymetechnicalassoc.org/Allergen%20psn%20paper-2.pdf).
[2] Summarized in the position paper of the Association of Manufacturers and Formulators of Enzyme products: http://www.amfep.org/documents/AmfepstatementScopeAllergyLabellingDirf

Even though wheat is uses in the fermentation process, the amount of final wheat proteins or protein fragments in the final food product is determined to be *de minimis* and not pose a risk to the final consumer as explained in the letter below.

Risk Assessment: DuPont IB Enzyme Products

Product Stewardship and Regulatory
Dupont Industrial Biosciences
925 Page Mill Road
Palo Alto, CA 94304
www.dupont.com

January 15, 2018

As noted by AMFEP, food enzymes are in most cases used as processing aids in the production of food ingredients or final foods. Most commercial food enzymes are produced by fermentation with the help of selected microorganisms. For the growth of such microorganisms, a variety of nutrients can be used, which may include some allergenic substances (e.g., soy protein). During the fermentation process most nutrients are consumed by the microorganisms and further down-stream processing typically includes washing and filtration steps. Thus, the final food enzyme will typically not contain significant residual amounts of the nutrients used during fermentation.

Labelling provisions for food enzymes and food enzyme preparations are established by Regulation (EC) No 1332/2008(3). Article 11 of said Regulation states that food enzymes and food enzyme preparations which are *not* intended for the final consumer shall be labelled, *where relevant*, with information about the presence of substances that are listed as substances with allergenic or intolerance effects. It is the responsibility of the food enzyme manufacturer to comply with the labelling provisions for food enzymes and food enzyme preparations. AMFEP, therefore, recommends that the relevance of labeling allergenic substances when added during fermentation should be addressed in a *risk assessment.*

Risk Assessment: Enzymes produced in bacterial fermentation processes

DuPont Industrial Biosciences (IB) employs soy protein as a nutrient raw material in various bacterial fermentation processes to produce enzymes. Consequently, DuPont IB has conducted a risk assessment of soy protein added during bacterial fermentations. Enzyme samples were shipped to a third-party laboratory for analysis of soy protein using a soy allergen ELISA test kit (ELISA Systems brand, ESSOYPRD-48 test kit). All samples were below the limit of detection for the assay of <2.5 ppm. Based on these data, it can be concluded that there is essentially no residual soy parent protein left in the enzyme products. Considering a recommended use rate of 0.1% of our enzyme product to our customers and a worst case of 2.5 ppm parent soy protein remaining in the fermentation mix, the amount of soy protein in the final food would be 2-3 ppb (worst case). The Voluntary Incidental Trace Allergen Labelling (VITAL) program (http://allergenbureau.net/vital/) in Australia specifies a reference dose of 1 mg soy protein (the eliciting dose for an allergic reaction in 1% of the population), below which only extremely sensitive allergic persons will experience an adverse reaction (Allen, K.J. et al., Allergen reference doses for precautionary labelling (VITAL 2.0): Clinical implications, J. Allergy Clin. Immunol., 133:156-164). Protein levels are in the ppb (i.e., µg) range in the final food processed with our enzyme product (worst case).

Risk Assessment: DuPont IB Enzyme Products

ELISAs are unable to measure small protein fragments in the fermentation mix. Therefore, any residual soy protein fragments remaining in the fermentation mix can't be quantified. Small soy protein fragments that may survive the fermentation process will not pose a risk to the consumer for the following reasons:

1) as noted by the Food Allergy Research and Resource Program at the University of Nebraska, 'if any residual but undetected fragments of the food allergen remain, the relevance of any such residual material to food allergenicity is unproven' (http://www.enzymeassociation.org/wp-content/uploads/2013/11/Expert-Enzymes2013.pdf);

2) very small amounts of enzymes are needed and used by food processors therefore further diluting any residual protein fragments that are remaining (leading to levels in ppm range or less). Therefore, any *de minimis* amount of protein fragments in the final enzyme product will not pose a risk to the consumer.

Risk Assessment: Residual protein levels in glucose and sorbitol products derived from wheat

DuPont IB uses glucose as a nutrient raw material in various fermentation processes to produce enzymes and glucose or sorbitol can be added to enzyme formulations. DuPont IB purchases both glucose and sorbitol that may be derived from wheat from commercial sources. To determine if the disclosure of wheat on the PD is necessary for enzyme products using glucose or sorbitol derived from wheat, a risk assessment was conducted. The risk assessment focused on measuring the amount of total protein remaining in various glucose and sorbitol products. Samples were measured for total protein levels using Thermo Scientific Coomasie Plus assay (Bradford assay)—colorimetric method since this assay has the highest tolerance to glucose. All samples of sorbitol and glucose contained less than 3 ppm (<LOD) total protein except for one glucose sample from wheat that was 10 ppm. Further, gluten levels were below quantification level of 5 ppm, based on ELISA analysis.

For this assessment, a worst case was assumed in which all sorbitol and glucose products contain 10 ppm wheat protein. Additionally, it is assumed that all the protein ends up in the enzyme product following recovery (worst case). Such residual levels of protein or potential fragments, however, will not pose a risk to the consumer for the following reasons:

1) In our fermentation process, the glucose syrup would be diluted approximately 50% in the fermentation mix. Therefore, the fermentation mix would contain 5 ppm wheat protein. 5 ppm total wheat protein in the enzyme product results in 5 ppb protein in the final food processed with 0.1% enzyme product, a *de minimis* amount of protein.

   10 ppm total wheat protein in sorbitol or glucose results in 2.5-3 ppm protein in our enzyme formulations, which means 2-3 ppb protein in the final food processed with 0.1% enzyme product, a *de minimis* amount of protein. Based on these various sources, the highest contribution of wheat protein would result in 5 ppb wheat protein in the final food (worst case);

Risk Assessment: DuPont IB Enzyme Products

2) as noted above by the Food Allergy Research and Resource Program at the University of Nebraska, 'if any residual but undetected fragments of the food allergen remain, the relevance of any such residual material to food allergenicity is unproven';

3) the Voluntary Incidental Trace Allergen Labelling (VITAL) program (http://allergenbureau.net/vital/) in Australia specifies a reference dose of 1 mg cereal protein, (the eliciting dose for an allergic reaction in 1% of the population), below which only extremely sensitive allergic persons will experience an adverse reaction (Allen, K.J. et al., Allergen reference doses for precautionary labelling (VITAL 2.0): Clinical implications, J. Allergy Clin. Immunol., 133:156-164). Protein levels are in the ppb range (i.e., µg) in the final food processed with our enzyme product (worst case).

Based on the above risk assessments, DuPont IB concludes that the amount of final soybean or wheat proteins or protein fragments in the final food product to be *de minimis* and not pose a risk to the final consumer.

Prepared and reviewed by:

Gregory S. Ladics, Ph.D., DABT, Fellow ATS

Technical Fellow

DuPont Industrial Biosciences

(302) 451-5748

Gregory.s.ladics@dupont.com

6 Dietary Risk Analysis

6.1 Application areas

Xylanase will be used in baking, brewing and cereal based beverages production.

Consequently, according to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), Xylanase will be used in:

- 7 Bread and Bakery Products
- 14 Non-alcoholic and alcoholic beverages

6.2 Level of use

Xylanase is intended for use in baking, brewing and manufacturing of cereal based beverage. The xylanase enzyme preparation is used at the minimum level required to achieve the

desired effect, in accordance with the principles of current Good Manufacturing Practice (GMP).

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune this process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and Xylanase is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

The dosage of a food enzyme depends on the activity of the enzyme protein (in this case Xylanase) present in the final food enzyme preparation (i.e. the formulated food enzyme). However, the activity Units as such do not give an indication of the amount of food enzyme actually added. Microbial food enzymes contain – apart from the enzyme protein in question – also some substances derived from the producing microorganism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids[1] (TOS, FAO/WHO, 2006). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS.

The table below shows the range of recommended use levels for each application where the food enzyme may be used.

Application	**Raw material (RM)**	**Recommended use levels (mg TOS/kg RM)**	**Maximal recommended use levels (mg TOS/kg RM)**
Baking	Flour	0.5 - 55	55
Brewing	Cereals	0.5 - 35	35
Cereal based beverage	Cereals	0.1 – 6	6

6.3 Level of residues in food

As discussed above, Xylanase may be used in the manufacture of a wide variety of foods and beverages. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological

[1] In the case of food enzymes, which are – per legal definition – not formulated, TOS is the same as Dry Matter minus ash. The amount of ash (e.g. mineral salts used in the fermentation) does generally not exceed a few percent.

requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages:

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	**0.0125**	**0.025**

In addition to the assumptions from the Budget Method, it is assumed that beer and cereal beverages are consumed in the same amount as soft drinks.

The recommended use levels of the enzyme Xylanase are given in section 6.2, based on the raw materials used in the various food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food or beverage is obtained per kg raw material and it is assumed that all the TOS will end up in the final product.

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food or beverage	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Beverages	Brewing and cereal beverage production	Cereal	35	Beer, cereal beverage etc.	0.17	6.0
Solid food	Baking	Flour	55	Bread etc.	0.71	39.05

The Total TMDI can be calculated on basis of the **maximal** values found in food and beverage multiplied by the average consumption of food and beverage/kg body weight/day. Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
39.05x0.0125=0.488	6.0x0.025=0.15	**0.635**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the specific enzyme Xylanase from *Trichoderma reesei*.
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food as well as in beverage, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process, (except for alcohol distillation where it can safely be assumed that nothing of the TOS will end up in the final product. Therefore, this use was excluded from the calculation);
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass *et al.*, 1997).

6.4 Safety assessment

Xylanase is an enzyme produced from *T. reesei* which was genetically modified to express the xylanase gene from *A. niger* var. *tubingensis*.

DuPont IB has determined by scientific procedures that production organism *T. reesei* is safe as a production organism as it pertains to the DuPont IB *T. reesei* Safe Strain Lineage (see Appendix B3, B4 of original submission) – more specifically the '*T. reesei* Host Strain #4' branch. For the determination of the safety of Xylanase, different endpoints of toxicity were investigated. Summarizing the results, the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions was observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1000 mg total protein/kg bw/day, equivalent to 1214.4 mg total organic solid (TOS)/kg bw/day.

Determination of the Margin of Safety

The margin of safety is calculated by dividing the NOAEL obtained from the 13-weeks oral (gavage) study in rats by the human exposure (worst case scenario). If the margin of safety is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of Safety} = \frac{\text{NOAEL (mg/kg bw/day)}}{\text{Human Exposure (mg/kg bw/day)}}$$

$$\text{Margin of Safety} = \frac{1214.4 \text{ mg TOS/kg bw/day}}{0.635 \text{ mg TOS/kg bw/day}}$$

$$\text{Margin of Safety} = 1912$$

6.5 Conclusion

The safety of Xylanase from *T. reesei* as a food processing aid in baking, brewing cereal based beverage manufacturing is assessed in a battery of toxicology studies investigating its mutagenic and systemic toxicity potential. A battery of genotoxicity assays was conducted and under the conditions of these assays Xylanase is not a mutagen, a clastogen, or an aneugen. Daily administration of Xylanase by gavage for 13 weeks did not result in overt signs of systemic toxicity. A NOAEL is established at 1000 mg total protein/kg bw/day corresponding to 1214.4 mg TOS/kg bw/day.

Based on a margin of safety of 1912 the proposed uses of the Xylanase in baking, brewing and cereal based beverage manufacturing processes are not a human health concern and are supported by existing toxicology data.

7 References

Aunstrup, K. 1979. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Goldsstein, L. pp. 28-68.

Aunstrup, K., Andersen, O., Falch, E. A., and Nielsen, T. K. 1979. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Douglass JS, Barraj LM, Tennant DR, Long WR, Chaisson CF (1997). Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants, 14, 791-802

Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition. 1994. Kroschwitz, J.I., Volume 9, pp. 567-620.

Food Chemicals Codex (FCC) 6th Edition. 2008. US Pharmacopeia, Rockville, Maryland.

Hansen, S.C. (1996). Acceptable daily intake of food additives and ceiling on levels of use. Food Cosmet. Toxicol., 4, 427-432.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing.