ORIGINAL SUBMISSION

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AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

AB Enzymes
an ABF Ingredients company

GRN000628

December 30, 2015

Office of Food Additive Safety (HFS-255),
Center for Food Safety and Applied Nutrition,
Food and Drug Administration,
5100 Paint Branch Parkway, College Park, MD 20740.

RE: GRAS NOTICE FOR endo-1,4-β-xylanase enzyme preparation from a genetically modified *Trichoderma reesei* strain

Pursuant to proposed 21 C.F.R § 170.36, AB Enzymes GmbH is providing in electronic media format (determined to be free of computer viruses), based on scientific procedures – a generally recognized as safe (GRAS) notification for endo-1,4-β-xylanase enzyme preparation from a genetically modified *Trichoderma reesei* strain used as a processing aid in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles, and brewing, grain processing, and potable alcohol production. The mannanase enzyme preparation described herein when used as described above and in the attached GRAS notice is exempt from the premarket approval requirements applicable to food additives set forth in Section 409 of the Food, Drug, and Cosmetic Act and corresponding regulations.

Please contact the undersigned by telephone or email if you have any questions or additional information is required.

Candice Cryne
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December 30, 2015

RE: GRAS Notification – Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) AB Enzymes GmbH hereby claims that endo-1,4-β-xylanase enzyme preparation from a genetically modified *Trichoderma reesei* strain produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:
Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt, Germany

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance.*
endo-1,4-β-xylanase enzyme preparation from a genetically modified *Trichoderma reesei* strain

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use.*
This dossier is specifically submitted for use of endo-1,4-β-xylanase used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles, brewing, grain processing, and potable alcohol production. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices.

Proposed 21C.F.R. § 170.36 (c)(iv) *Basis for GRAS determination.*
This GRAS determination is based upon scientific procedures.

Proposed 21C.F.R. § 170.36 (c)(v) *Availability of information.*
A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times at a specific address set out in the notice or will be sent to FDA upon request.

(b) (6)

12/30/2015

Candice Cryne
Regulatory Affairs Specialist

Date

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

endo-1-4, β-xylanase from a genetically modified strain of *Trichoderma reesei*

AB ENZYMES GmbH

DECEMBER 30, 2015

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

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938 (April 17, 1997)), AB Enzymes GmbH ("AB Enzymes") has determined that the endo-1,4-β-xylanase enzyme preparation from a genetically modified *Trichoderma reesei* strain is a GRAS substance for the intended applications based on scientific procedures and is therefore exempt from the requirement for premarket approval. Information on the enzyme 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, its applicable conditions for use, AB Enzymes' basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1.

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from harmful sequences.

Section 3 shows the enzymatic activity of the enzyme, along with comparison to other similar enzymes. The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4. Section 5 reviews the hygienic measurements, composition and specifications as well as the self-limiting levels of use for endo-1,4-β-xylanase. Section 6 provides information on the mode of action, applications, and use levels of endo-1,4-β-xylanase and enzyme residues in final food products. The safety studies outlined in Section 7 indicate that the endo-1,4-β-xylanase enzyme preparation from *T. reesei* shows no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1 Name and Address of Notifier

Notifier:

AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

Manufacturer:

Roal Oy[1]
Tykkimäentie 15
FIN-05200 Rajamäki
Finland

Person(s) Responsible for the Dossier:

Candice Cryne
AB Enzymes GmbH
Toronto, Ont.
Canada
Candice.cryne@abenzymes.com

[1] ROAL is a Joint Venture between Associated British Foods (UK) and Altia OY (Finland). Manufacturing and research and development activities are performed for AB Enzymes by ROAL Oy in Finland. ROAL coordinates its R&D activities independently while taking into account the market requirements reported by their sole distributor AB Enzymes GmbH.

1.2 Common or Usual Name of Substance

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. The enzyme is known as **endo-1,4-ß-xylanase** and more commonly as xylanase; also designations endo-(1→4)-β-xylan 4-xylanohydrolase; 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 are used.

1.3 Applicable Conditions of Use

For an enzyme to perform a technological function in the final food, certain conditions have to be met, such as the enzyme must be in its native, non-denatured form, and must be free to move, a substrate must be present and conditions such as pH, temperature and water content must be favourable for the particular enzyme.

1.4 Food Products Used in

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions. This dossier is specifically submitted for use of endo-1,4-β-xylanase used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles, brewing, grain processing, and potable alcohol production. A further description of the enzyme in these food technology applications will be given in subsequent sections.

1.5 Levels of Use

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg RM)
Baking and other cereal based products	Flour	1-10	10
Brewing	Cereals	1-5	5
Grain processing	Cereals	1-10	10
Potable alcohol production	Cereals	1-5	5

1.6 Purposes

Endo-1,4-ß-xylanase (IUB 3.2.1.8) catalyses the hydrolysis of xylosidic linkages in an arabinoxylan backbone (and other ß-1,4-linked xylans) resulting in depolymerisation of the arabinoxylan into smaller oligosaccharides. Like most of the food enzymes, endo-1,4-β-xylanase performs its technological function during food processing. Endo-1,4-β-xylanase enzyme preparation is produced by a genetically modified *Trichoderma reesei* strain expressing a xylanase gene deriving from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*).

1.7 Technological Need

In principle, the enzymatic conversion of (arabino)xylans with the help of endo-1,4-β-xylanase can be of benefit in the processing of all vegetable based foods and food ingredients which naturally contain xylans.

In general, the technological need of the enzymatic conversion of arabinoxylans with the help of endo-1,4-β-xylanase can be described as: degradation of a component (the substrate arabinoxylan) which causes technical difficulties due to its high viscosity in processing of raw materials containing this component.

Endo-1,4-β-xylanase is naturally present in many vegetable raw materials, including wheat, barley and malt. The natural enzymatic conversion of (arabino)xylans in such materials is of technological benefit in

several industrial food manufacturing processes. However, the levels of endogenous endo-1,4-β-xylanase are often inadequate and vary from batch to batch of raw material, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, industrial endo-1,4-β-xylanase is used during food processing.

For more than 25 years, xylanases have been used extensively in various industrial food applications *(Beg et al. 2001)*.

Typical uses of xylanase in food processing are:

- Starch processing
- Beverage alcohol (distilling) processes
- Brewing processes and other cereal based beverage processes
- Baking processes and other cereal based processes

In all the above processes, the endo-1,4-β-xylanase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

Endo-1,4-β-xylanase is acting on one family of the components of the plant cell wall, and is often used together with other enzymes (enzyme systems) which modify other components of the plant cell walls. In particular endo-1,4-β-xylanase is often applied together with β-glucanase and cellulase (e.g. for brewing, grain processing, starch processing and beverage alcohol processing), or with amylase (baking processes and other cereal based processes).

1.8 Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, AB Enzymes GmbH has determined, through scientific procedures, that the endo-1,4-β-xylanase enzyme preparation from a *Trichoderma reesei* strain object of this dossier, is GRAS for use in baking.

1.9 Availability of Information for FDA Review

A notification dossier providing a summary of the information that supports this GRAS determination is enclosed herein. The dossier includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration (FDA) for review and copying at reasonable times at a specific address set out in the notice or will be sent to FDA upon request. Please direct all inquiries regarding this GRAS determination to:

Candice Cryne

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Canada

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2. PRODUCTION MICROORGANISM

2.1 Donor, Recipient Organism and Production Strain

The *Trichoderma reesei* host strain is genetically modified with a xylanase gene deriving from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*).

Name of the enzyme protein:	**Endo-1,4-ß-xylanase**
Production strain:	*Trichoderma reesei* RF5427

Donor:

The endo-1,4-ß-xylanase described in this dossier was derived from *Thermopolyspora flexuosa* (*Nonomuraea flexuosa*) DSM43186 which is an actinomycete known to produce thermo-tolerant xylanases. Actinomycetes are gram positive bacteria that can grow in filamentous form. They are common soil organisms.

Nonomuraea flexuosa DSM 43186 is currently named as *Thermopolyspora flexuosa (Goodfellow et al. 2005)*. It was previously named as *Actinomadura flexuosa*, *Microtetraspora flexuosa or Nonomuria flexuosa (Zhang et al. 1998)*. As the name *Nonomuraea flexuosa* has been used in our publications on xylanases deriving from this strain, in this dossier both names *Thermopolyspora flexuosa* and *Nonomuraea flexuosa* are used for the donor organism. The taxonomic lineage of *Thermopolyspora* is shown below (according to http://www.uniprot.org/taxonomy/103836):

Genus:	Thermopolyspora
Species:	*Thermopolyspora flexuosa*
Subspecies (if appropriate):	not applicable
Generic name of the strain:	DSM 43186 (ATCC 35864)
Previous or other name(s) (if applicable):	*Nonomuraea flexuosa*
Commercial name:	Not applicable. The organism is not sold as such.

The native xylanase gene was isolated from the genomic library of *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* DSM43186 by using a plate activity assay (lambda ZAP Express, Stratagene).

Recipient Organism:

The recipient strain used for the genetic modifications in constructing RF5427 was *Trichoderma reesei* strain RF4847.

The *T. reesei* recipient is a classical mutant strain originating from *T. reesei* QM6a. The identification of the strain as *T. reesei* has been confirmed by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands. It was identified based on the sequences of Internal Transcribed Spacer 1 and 2 and the 5.8S gene and Translation Elongation Factor 1α *(Kuhls et al. 1996)*.

T. reesei is an aerobic filamentous fungus (an ascomycete). It grows in mycelium form but starts to sporulate when cultivation conditions do not favor growth (e.g. due to lack of nutrients). *T. reesei* is a mesophilic organism which means that it prefers to grow at moderate temperatures. The cultures are typically fast growing at about 30° C (above 20°C and below 37°C). *T. reesei* prefers acidic to neutral pH (about 3.5 to 6) for growth. The colonies are at first transparent or white on agar media such as potato dextrose agar (PDA). The conidia are typically forming within one week of growth on agar in compact or loose tufts in shades of green. Sporulation is induced by daylight. Yellow pigment may be secreted into the agar by the growing fungal colonies, especially on PDA.

The taxonomic classification of the *T. reesei* is: *Hypocreaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota*, Fungi, according to Index *Fungorum* database.

Roal Oy has been using *T. reesei* as an enzyme producer since the 1980´s without any safety problems. AB Enzymes GmbH filed a GRAS notice for pectin lyase enzyme preparation produced with *T. reesei* containing a gene from *Aspergillus niger* and FDA had no question and designated it as GRAS (Notice No. GRN 000032, *Appendix #1*). Further, recently AB Enzymes GmbH has submitted GRAS notices for enzymes produced with genetically engineered *T.reesei* strains, specifically GRN 000524 (phospholipase

A2 enzyme preparation from *T. reesei* carrying a PLA2 gene from *Aspergillus fumigatus*), GRN 000566 (β-Mannanase enzyme preparation from a self-cloned *T. reesei*), GRN 000558 (pectin esterase enzyme preparation from *T. reesei* carrying a pectin esterase gene from *Aspergillus tubingensis)*, and GRN 000557 (polygalacturonase enzyme preparation from *T. reesei* strain expressing the gene encoding polygalacturonase from *Aspergillus tubingensis Mosseray* RH3544). *T. reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production (e.g. cellulases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries). Currently, various *Trichoderma reesei* enzymes and enzymes produced in recombinant *T. reesei* strains are also used in the brewing process (β-glucanases), as macerating enzymes in fruit juice production (pectinases, cellulases, hemicellulases), as a feed additive to livestock (xylanases, endoglucanases, phytases) and for pet food processing. *T. reesei* - wild type or genetically modified - is widely accepted as safe production organism for a broad range of food enzymes.

Production Strain:

The construction and the safety assessment of the production strain *Trichoderma reesei* RF5427 has been described in notifications to the Finnish competent authority, Gene Technology Board, prepared according to Directive 90/219/EEC and the Finnish Gene Technology Law 377/1995.The transformed production strain containing the xylanase gene is *Trichoderma reesei* strain RF5427 which is deposited in the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS114044.

Trichoderma reesei RF5427 was constructed for xylanase production. The production strain (RF5427) differs from its recipient strain (RF4847) in its high xylanase production capacity due to expression of the xylanase from the expression cassettes integrated into the RF5427 genome (see section 2.2). RF5427 secretes high amounts of the recombinant xylanase into its culture supernatant, resulting to high xylanase activity in the cultivation broth. The heterologous xylanase is the main component of the enzyme mix produced by RF5427. In addition to the heterologous xylanase, the RF5427 strain produces endogenous *Trichoderma* cellulases, e.g. cellobiohydrolases and endoglucanases.

The techniques used in transforming and handling *T. reesei* have been previously described *(Karhunen et al. 1993) (Penttilä et al. 1987)*. The production organism also meets the criteria for safe production microorganism (Pariza, Johnson 2001) (Decision Tree Analysis - *Appendix #2*). *T. reesei* strains are non-pathogenic and non-toxigenic and have been shown not to produce fungal toxins or antibiotics under conditions used for industrial enzyme production. Further they are considered as safe hosts for other harmless gene products *(Nevalainen et al. 1994; Olempska-Beer et al. 2006; Blumenthal 2004)*. The seed culture for the fermentation is inoculated with spores that have been stored at -80 ^{0}C. No additional growth cycles have been performed after the *T. reesei* RF5427 strain deposition to the culture collection.

2.2 Genetic Modification

Trichoderma reesei strain RF5427 was constructed for production of *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* derived xylanase by introducing the encoding gene into the genome of the *Trichoderma reesei* strain RF4847. The xylanase gene is included in the expression cassette, the linear 8622 bps *Eco*RI fragment, isolated from the expression plasmid.

Expression Cassette:

The *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* derived xylanase gene is expressed under the strong *T. reesei cbh1/cel7a* promoter, and the DNA sequence encoding the *T. reesei* CBHII/Cel6A tail/hinge is used as a carrier protein to improve the production of the recombinant xylanase. Standard molecular biology methods were used in the construction of the expression plasmid. The expression cassette fragment used in fungal transformation does not contain any vector derived sequences as it is isolated from the expression plasmid by restriction digestion and purification from an agarose gel, It is free from any harmful sequences and contains the following genetic materials:

- *T. reesei cbh2/cel6a* signal sequence and tail/hinge carrier encoding sequences: *T. reesei cbh2/cel6a* gene codes for cellobiohydrolyse II (CBHII) that degrades cellulose to cellobiose. The PCR-synthesized *cbh2/cel6a* signal and tail (A)/hinge (B) region is fused from its 5´-end to the *cbh1/cel7a* promoter and from its 3´-end to the xylanase gene (mature active enzyme form

encoding region). The fusion sequence between the *cbh2* tail/hinge and xylanase *N*-terminal sequences encodes four additional amino-acids, RDKR, representing the Kex2 protease cleavage site. The produced CBHII tail/hinge carrier protein is not a biologically active enzyme.

- *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* derived xylanase gene: a gene fragment encoding the amino acids of the mature active xylanase protein was used in the expression cassette.

- *Aspergillus nidulans amdS* gene: the gene has been isolated from *Aspergilus nidulans* VH1-TRSX6 *(Kelly, Hynes 1985)*. *Aspergillus nidulans* is closely related to *Aspergillus niger*, which is used in industrial production of food enzymes. The gene codes for an acetamidase that enables the strain to grow on acetamide as a sole nitrogen source. This characteristic has been used for selecting the transformants. The product of the *amdS* gene, acetamidase, can degrade acetamide and is not harmful or dangerous. The *amdS* marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 20 years.

Of the above genetic materials, the *Thermopolyspora flexuosa* derived gene and *Aspergillus nidulans amdS* gene are not naturally present in the RF4847 genome.

The DNA fragments that have been transformed to *T. reesei* host strain RF4847 are well characterized, the sequences of the genes are known, and the fragments are free from any harmful sequences.

Southern blot analyses were performed to the genome of the production strain RF5427. Results indicated that four to five copies of the expression cassettes were integrated in the genome of strain RF5427.

2.3 Stability of the Transformed Genetic Sequence

T. reesei strains are widely used in biotechnological processes because of their known stability. The transformed DNA does not contain any antibiotic resistance genes. The inserted DNA does not include

any mobile genetic elements. Additionally, it should be highlighted that *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome *(Kubicek et al. 2011; Martinez et al. 2008)*. This results to low risk of transfer of genetic material.

The fermentation process starts always from the identical replica of the RF6199 seed ampoule. Production preserves at -80oC ("Working Cell Bank") are prepared from the "Master Cell Bank" (culture collection maintained at -150°C) in the following manner: A Petri dish is inoculated from the culture collection preserve (spore suspension) in such a way that single colonies deriving from one spore, each, can be selected upon germination. Altogether at least 20 individual colonies are inoculated into three parallel slants in which strains are grown and let to germinate. Spores from one parallel slant, representing each of the individual colonies, are inoculated into shake flasks. The shake flasks constitute the culture stage.

A so-called productivity test is performed, i.e. shake flask cultivation being completed; the enzymatic activity is measured, which must correspond to a given value. If this value is not reached, the culture is discarded. This test serves to determine the characteristic metabolic efficiency of each strain (isolate), i.e. to establish its identity. The productivity test is redone in fermentor cultivations for the chosen isolates (out of at least 20) that showed the best productivity in the shake flask cultivations. The working cell bank ampoules with glycerol solution are then prepared from the slantswhose productivity tests show the highest results. The suspensions thus obtained are frozen and stored divided into 0.5 ml aliquots at -80°C.

The annual production starts from these production preserves. Six of them are thawed for inoculation of six shake flasks and subsequent inoculation of the first process bioreactor is from these flasks. Mutation frequencies are low and in case mutations would occur, they only occur in the vegetative state during cell division. Owing to the above-described procedure, this vegetative state of the cultures is reduced to an inevitable minimum during production.

Potential changes in the genome of the production strain could theoretically occur during the propagation in the fermentation process. Therefore, Southern blot analysis was performed after fermentation process of the RF5427 strain. The results revealed that the recombinant xylanase gene stays genetically stable in *T. reesei* genome over necessary time that is needed for industrial fermentation process of the RF5427 production strain.

Therefore, the production strain RF5427 is stable in terms of genetic traits. The genetic materials in the expression cassettes have been integrated as part of the genome and are as stable as any natural gene. The integrated genetic materials are not acting as mobilisable elements and they do not contain mobilisable elements. Additionally, it should be highlighted that *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome *(Kubicek et al., 2011)*.

Additionally, the stability is also followed as equal production of the xylanase activity in a number of fermentation batches performed for the RF5427. The activity measurements from parallel successful fermentations showed that the productivity of the RF5427 strain remains unchanged. The data of the analysis of enzyme activities from preparations from three different fermentation batches of the recombinant RF5427 strain is presented in *Appendix #3*.

2.4 Good Industrial Large Scale Practice (GILSP)

The *T. reesei* RF5427 endo-1,4-ß-xylanase enzyme production strain complies with all criteria for a genetically modified GILSP organism.

In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH, 1998) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes produced by *T. reesei* are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to

consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[2], if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

As a result, *T. reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD *(ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT 1992)*.

The host organism is non-pathogenic, does not produce adventitious agents under the fermentation conditions employed and has an extended history of safe industrial use (see Section 7.1). Indeed, the *T. reesei* RF4847 strain originates from the wild type strain QM6a from which it was developed by conventional mutagenesis programs. The wild type *T. reesei* strains have been isolated only at low altitudes and within a narrow belt around the equator *(Kubicek et al. 2008)*. The mycoparasitism-specific genes have been shown to be lost in *T. reesei (Kubicek et al. 2011)*.

Overall, industrial microorganisms modified to produce high levels of enzymes, in fermentation conditions (e.g. no competitive microorganisms, optimal nutrients and aeration that are not present in the natural environment) are not expected to have any competitive advantage against other microorganisms in nature, which themselves are well-adapted in their natural environment. The fitness of the industrial strains to survive is very likely reduced by their high performance characteristic: most of the energy is needed for the production of proteins in high amounts.

The possible transfer of the recombinant DNA, if accidentally released into the environment, would not have any harmful or pathogenic effects on environmental processes. The DNA fragments used in the construction of the expression cassette are well characterized and do not contain any undefined or harmful fragments: the recombinant DNA originates from *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* DSM 43186 (ATCC 35864), which is a soil actinomycete and from *Aspergillus nidulans* which is also a common species in soils. It can be concluded that the DNA fragments in the expression cassette

[2] Reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce

or their corresponding gene products are not biologically harmful and are common in surroundings. Also, the recombinant DNA used for transformation does not contain any antibiotic resistance markers.

Therefore, the *T. reesei* RF5427 production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the OECD. The production organism has been approved by the Finnish competent authorities for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment.

2.5 Absence of the Production Organism in the Product

The down-stream process following the fermentation includes unit operations to separate the production strain. The procedures are executed by trained staff according to documented standard operating procedures complying with the requirements of the quality system.

The *T. reesei* RF5427 is recovered from the fermentation broth by a widely used process (includes several filtering steps) that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch using an internal Roal method. This method has been validated in-house. The sensitivity of the method is 1 cfu/20 ml in liquid and 1 cfu/0,2 gram in dried semifinals. It is also important to notice that when the product is dried the drying step gives an efficient way to kill *Trichoderma* strains, as the temperature is ca. 75°C of the air leaving the dryer, and fungi are not very tolerant to heat.

2.6 Structure and amount of vector remaining in the production strain

Trichoderma reesei RF5427 strain does not harbor any bacterial vector DNA. The pALK1502 expression cassette used for transformation was cleaved from the pUC19 vector backbone by restriction enzyme digestion followed by isolation of the expression cassette from an agarose gel.

A Southern blot hybridization experiment using the pUC19 vector as a labeled probe and genomic DNA of the production host RF5427 was performed to confirm that no vector DNA is included in the genome of RF5427. It produced negative results (no hybridization), demonstrating that no part of the plasmid

vector removed to generate the linear transforming *Eco*RI DNA fragment was introduced into the *Trichoderma* production host.

2.7 Absence of Transferable rDNA Sequences in the Enzyme Preparation

The endo-1,4-beta-xylanase is produced by an aerobic submerged microbial fermentation using a genetically modified *Trichoderma reesei* strain. All viable cells of the production strain, RF5427, are removed during the down-stream processing through a known acceptable filtration method.

After this the final product does not contain any detectable number of fungal colony forming units or recombinant DNA. Three separate enzyme samples (dried semi-final concentrates) were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. No recombinant DNA (rDNA) of the production strain was shown to be present above the detection limits. Please refer to *Appendix #3*.

2.8 Absence of Antibiotic Genes and Toxic Compounds[3]

As noted above, the transformed DNA does not contain any antibiotic resistance genes. Further, the production of known mycotoxins according to the specifications elaborated by the General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(Food and Agriculture Organization of the United Nations 2006)* has been also tested from the fermentation products of the *T. reesei* strain RF5427. Adherence to specifications of microbial counts is routinely analysed. Several production batches produced by the production strain *T. reesei* RF5427 (3 dried semi-final concentrates) were analyzed and no antibiotic or toxic compounds were detected (*Appendix #3)*.

[3] The Food Chemicals Codex ("FCC", 9th edition), states the following: "Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants."

3. ENZYME IDENTITY

3.1 Enzyme Identity

Systematic name	Endo-1,4-ß-xylanase
Common names	Endo-1,4 (3)-beta-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
Enzyme Commission No.	EC 3.2.1.8
CAS number	9025-57-4

3.2 Molecular weight

The enzyme has a calculated molecular weight of 23.8 kDa (without glycosylation).

3.3 Enzymatic Activity

The main enzymatic activity of *T. reesei* RF5427 enzyme preparation is endo-1,4-ß-xylanase. This food enzyme catalyses the hydrolysis of xylosidic linkages in an arabinoxylan backbone (and other ß-1,4-linked xylans) resulting in depolymerisation of the arabinoxylan into smaller oligosaccharides.

Xylans are constituents of hemicellulose, a structural component of plant cell walls. Arabinoxylans (also known as pentosans) are highly branched xylans that occur e.g. in wheat and rye flour. Consequently, the substrate for endo-1, 4-β-xylanase occurs naturally in vegetable based foods and can be found in various plant materials including the cell walls and endosperm of cereals, such as wheat and barley.

The methods to analyse the activity of the enzyme is company specific and is capable of quantifying endo-1,4,-β-xylanase activity as defined by its IUBMB classification. The enzyme activity of the xylanase produced by RF5427 is given in BXU unit (1 BXU unit is equivalent to the amount of enzyme that products, under standard conditions (pH 5.3, 50°C), one nmol of reducing sugar, i.e. xylose, from birch xylan in one second). As international units (IU), one BXU is equivalent to 0.06 micromol/minute.

3.4 Secondary Enzymatic Activities

Food enzymes are biological concentrates containing, apart from the desired enzyme protein (expressing the activity intended to perform a technological purpose in a certain food process, also called 'main enzyme activity'), other organic substances.

These other substances may include various enzyme activities (defined as 'side activities' due to their lower relative amount compared to the amount of the main desired enzyme protein) derived from the producing microorganism. Like all living cells, microorganisms produce a variety of enzymes responsible for the hundreds of metabolic processes that sustain their life. As microorganisms do not possess a digestive system, many enzymes are excreted to digest the material on which the microorganisms grow. Most of these enzymes are hydrolases that digest carbohydrates, proteins and lipids (fats). These are the very same activities that play a role in the production of fermented food and in the digestion of food by – amongst others – the intestinal micro flora in the human body. In addition, if a food raw material contains a certain substrate (e.g. carbohydrate, protein or lipid), then, by nature, it also contains the very same enzymatic activities that break down such a substrate; e.g. to avoid its accumulation. Consequently, the presence in food of such enzyme activities and of the potential reaction products is not new and should not be of any safety concern. In addition, it is generally accepted that the enzyme proteins themselves do not pose any safety concern either.

During the production of food enzymes, the main enzyme activity is normally not separated from the other substances present. Consequently, the food enzyme contains a number of other enzymes excreted by the microbial cells or derived from the fermentation medium. Other strains of *Trichoderma reesei,* selected to produce other main enzyme activities, will produce and excrete the same set of enzymatic activities, albeit in various amounts. Consequently, the food enzymes from *Trichoderma reesei* which are approved and used in food processes already for many years, will also contain these activities. These activities are of no safety concern and their fate in the final food will be the same as that of the main enzyme activity. Thus, apart from xylanase, the food enzyme also contains other enzymatic side activities in small amount, which are naturally and typically produced by the production organism *Trichoderma reesei*. Those include ß-glucanase and cellulase.

4. MANUFACTURING PROCESS

4.1 Overview

Like all food enzymes, endo-1,4,-β-xylanase described in this dossier is manufactured in accordance with current Good Manufacturing Practices for Food (cGMPs) and the principals of Hazard Analysis of Critical Control Points (HACCP). Compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services in Finland.

T. reesei RF5427 endo-1,4,-β-xylanase described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. A manufacturing flow-chart is given in *Appendix #4*.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a vast majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

4.2 Fermentation

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation
- Main fermentation

4.2.1 Raw materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL Oy. The safety is further confirmed by toxicology studies (Section 7.4). The raw materials conform to either specifications set out in the Food Chemical Codex, 9th edition, 2014 (*Appendix #5*) or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation

(EC) No 1881/2006 setting maximum limits for certain contaminants in food. The raw materials used for the formulation are of food grade quality.

The antifoam agents and flocculants used in the fermentation and recovery processes are used as described in the Enzyme Technical Association submission to FDA on antifoam and flocculants (April 24, 1998). The maximum use levels of antifoam and flocculants are used below ≤0.15% and ≤1.5% respectively.

4.2.2 Materials used in the fermentation process (inoculum, seed and main fermentation)

- Potable water
- A carbon source
- A nitrogen source
- Salts and minerals (Ammonium sulphate, Monopotassium phosphate)
- pH adjustment agents
- Foam control agents

4.2.3 Inoculum

A suspension of a pure culture of *T. reesei* RF5427 is aseptically transferred to a shake flask (1 liter) containing fermentation medium.

In order to have sufficient amount of biomass, the process is repeated several times. When a sufficient amount of biomass is obtained the shake flasks are combined to be used to inoculate the seed fermentor.

4.2.4 Seed fermentation

The inoculum is aseptically transferred to a pilot fermentor and then to the seed fermentor. The seed fermentation is run at a constant temperature and a fixed pH. At the end of fermentation, the inoculum is aseptically transferred to the main fermentation.

4.2.5 Main fermentation

Biosynthesis of the endo-1,4,-β-xylanase product by the production strain *T. reesei* RF5427 occurs during the main fermentation.

The content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium. The fermentation in the main fermentor is run as normal submerged fermentation under well-defined process conditions (pH, temperature, mixing, etc.).

The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When these conditions are met, the fermentation is completed.

4.3 Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein,
- to concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

During fermentation, the enzyme protein is secreted by the producing microorganism into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

This Section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

- Pre-treatment
- Primary solid/ liquid separation
- Concentration
- Polish and germ filtration

The nature, number and sequence of the different types of unit operations described below may vary, depending on the specific enzyme production plant.

4.3.1 Materials

Materials used, if necessary, during recovery of the food enzyme include:

- Flocculants
- Filter aids
- pH adjustment agents

Potable water can also be used in addition to the above mentioned materials during recovery.

4.3.2 Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation. Typical amount of filter aids is 2.5 %.

4.3.3 Primary solid/liquid separation

The purpose of the primary separation is to remove the solids from the enzyme containing fermentation medium. The primary separation is performed at defined pH and temperature ranges in order to minimize loss of enzyme activity.

The separation process may vary, depending on the specific enzyme production plant. This can be achieved by different operations like centrifugation or filtration.

4.3.4 Concentration

The liquid containing the enzyme protein needs to be concentrated in order to achieve the desired enzyme activity and/or to increase the ratio enzyme activity/TOS before formulation. Temperature and pH are controlled during the concentration step, which is performed until the desired concentration has been obtained.

4.3.5 Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

4.4 Formulation and Packaging

Following formulation, the final product is defined as a 'food enzyme preparation.' Food enzymes can be sold as dry or liquid preparations, depending on the final application where the enzyme is intended to be used. For all kinds of food enzyme preparations, the food enzyme is standardized and preserved with food ingredients or food additives which are approved in the USA according to ruling legal provisions.

For the manufacture of dry food enzyme preparations, the food enzyme is typically spray dried. The dried food enzyme is then standardized to the desired/ declared activity with food grade ingredients.
In order to reduce the dust formation for health and safety purposes, dust-binding agents (food grade) are being added during manufacturing of final products.

The enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general testing requirements for Food Enzyme Preparations, and released by Quality Assurance (see section 4.5). The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations. Labels conform to relevant legislation.

4.5 Quality Control of Finished Product

The final enzyme product complies with the recommended General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(Food and Agriculture Organization of the United Nations 2006)* and the Monograph "Enzyme Preparations" Food Chemicals Codex (FCC) 9th edition (2014) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

Property	Requirement
Total viable counts	< 50000 g^{-1}
Yeasts and fungi	each < 1000 g^{-1}
E. coli	not present in 25 g
Salmonella	not present in 25 g
Coliform counts	< 30 g^{-1}
Arsenic	< 3 ppm
Lead	< 2 ppm
Heavy metals	< 30 ppm
Antibacterial Activity	not detectable
Mycotoxins[4]	No significant levels

Three different batches of endo-1,4,-β-xylanase were assessed based upon the above specifications and are summarized in *Appendix #3*.

[4] See JECFA specifications, ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, page 64: Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

The composition of the enzyme concentrate that is the starting material for all enzyme formulations has the following ranges:

Constituent	Value	Unit
Xylanase activity	4,303 333	BXU/g
Moisture	5.9	%
Ash	1.6	%
Total Organic Solids[5]	93	%

5.2 General Production Controls and Specifications

In order to comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account and controlled during production as described below:

Identity and purity of the producing microorganism:

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore it is essential that the identity and purity of the microorganism is controlled.

Production of the required enzyme protein is based on a well-defined Master (MCB) and Working Cell Bank (WCB). A Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. The WCB is derived from the MCB as described in section 2.3. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

[5] % Total Organic Solids: = 100-(Ash+Moisture+diluents)

Microbiological hygiene:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination might result in decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

- Hygienic design of equipment:
    - all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentors, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid are used as cleaning agents. After cleaning, the vessels are inspected manually; all valves and connections not in use for the fermentation are sealed by steam at more than 120°C; critical parts of down-stream equipment are sanitized with disinfectants approved for food industry
- Sterilization of all fermentation media:
    - all the media are sterilized with steam injection in fermentors or media tanks (at 121°C for at least 20 min at pH 4.3 – 4.8.).
- Use of sterile air for aeration of the fermentors:
    - Air and ammonia water are sterilized with filtration (by passing a sterile filter).
- Hygienic processing:
    - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
    - Maintaining a positive pressure in the fermentor
- Germ filtration

In parallel, hygienic conditions in production are furthermore ensured by:

- Training of staff:
    - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- Pest control
- Inspection and release by independent quality organization according to version-controlled specifications
- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

Chemical contaminants:

It is also important that the raw materials used during fermentation are of suitable quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In addition to these control measures in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high quality product (cGMPs). The whole process is controlled with a computer control system (Metso DNA) which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- CO2

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

Deviations from the pre-defined values at any of the preceding steps will lead to adjustment or actions ensuring an optimal enzyme products are achieved or to rejection of the product.

6. APPLICATION

6.1 Mode of Action

Like any other enzyme, endo-1,4-β-xylanase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product or products. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

The substrates for endo-1,4-β-xylanase are (arabino) xylans. Xylans are polysaccharides belonging to the so-called pentosans (polymers of C5-sugars). Xylans form part of the hemicellulose complexes found in the cell walls of plant cells. Consequently, the substrate for endo-1,4-β-xylanase occurs naturally in vegetable based foods. Arabinoxylans are xylans branched with arabinose. They can be found in an array of different molecular weights in various plant materials including the cell walls and endosperm of cereals, such as wheat, barley and malt.

The function of endo-1,4-β-xylanase is to catalyse the hydrolysis of the (1→4)-β-D-xylosidic linkages that are present in the centre (endo) of xylans, including arabinoxylan. Like the substrate, the enzyme also occurs by nature in plants such as papaya, wheat and barley. Xylanases are a widespread group of enzymes produced by bacteria, algae, fungi, protozoa, gastropods and anthropods. A wide variety of different xylanases are produced by the organisms and many of the organisms also produce multiple xylanases with different structures and properties. For a review, see e.g. *Collins et al. (2005)*.

The reaction products of the hydrolysis of arabinoxylans by endo-1,4-β-xylanase are (1→4)-β-D-arabinoxylan oligosaccharides of variable lengths. Like the substrate and the enzyme, these reaction products are also natural constituents of cereal-containing foodstuffs.

Such enzyme activity is widely present in nature and in particular in food ingredients. The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

6.2 Application

This dossier is specifically submitted for the use of endo-1,4-β-xylanase in baking processes and other cereal based processes, brewing, grain processing and potable alcohol production. Below, the benefits of the use of industrial endo-1,4-β-xylanase in those processes are described. The beneficial effects are of value to the food chain because they lead to better and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production

economy and environmental benefits such as the use of less raw materials and the production of less waste.

Baking processes:

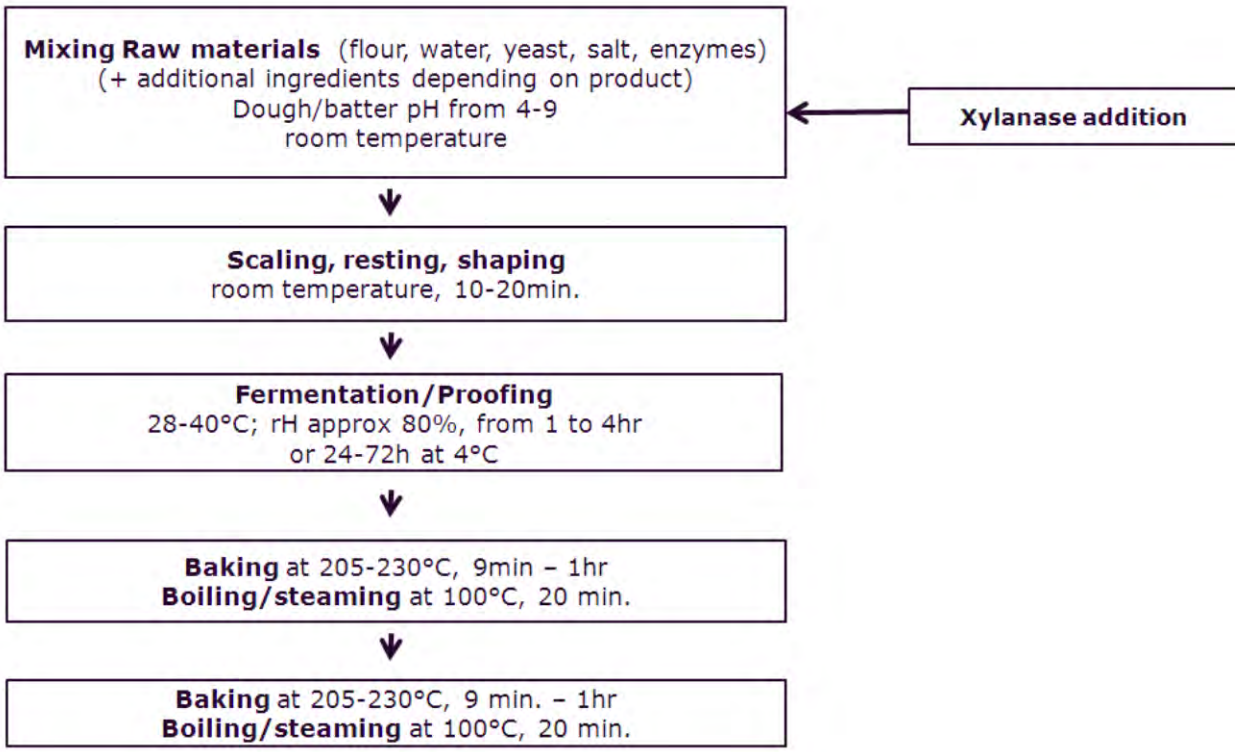
Endo-1,4-β-xylanase can be used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles.

Arabinoxylans provide functional properties during bread making due to their ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of the water-unextractable arabinoxylans with the help of endo-1,4-β-xylanase results in solubilized arabinoxylans with lower molecular weights, which improves the functional baking properties of these polysaccharides.

The benefits of the conversion of arabinoxylans with the help of endo-1,4-β-xylanase in baking are:

- Facilitate the handling of the dough (improved extensibility and stability, reduced stickiness leading to reduced losses of dough)
- Improve the dough's structure and behaviour during the baking step
- Ensure a uniform and slightly increased volume and an improved crumb structure of the bakery product, which might otherwise be impaired by processing of the dough
- Reduce batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits

The process flow of baking is presented below:



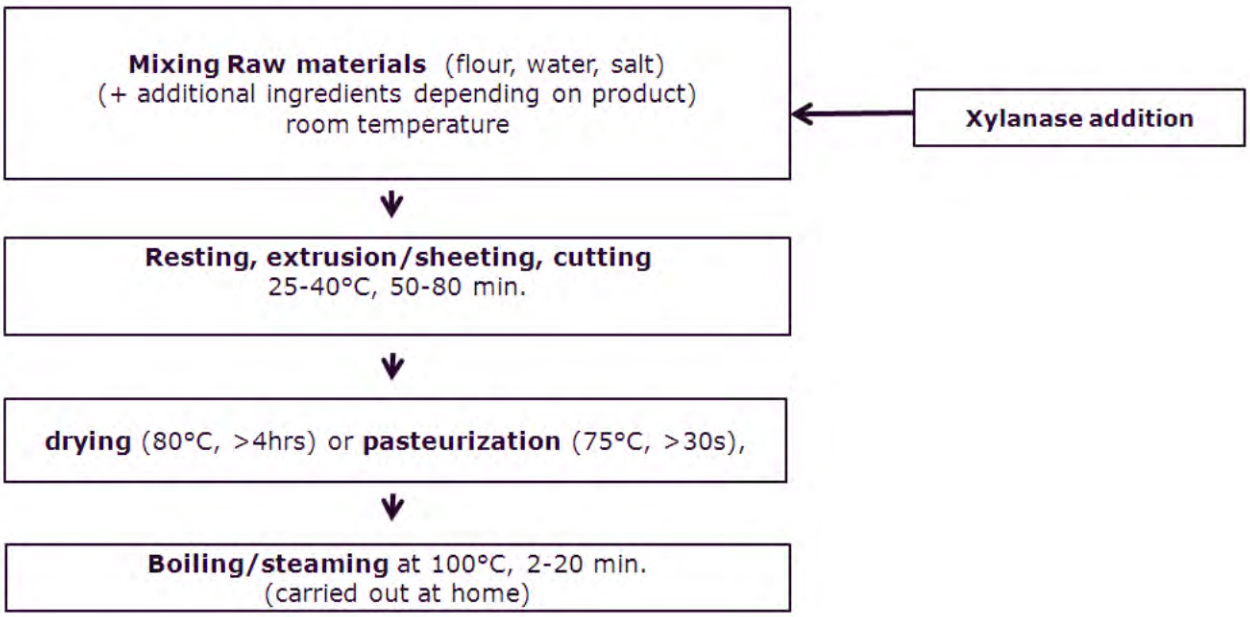
Endo-1,4-β-xylanase can also be used in the processing of other cereal based products such as, but not limited to, pasta, noodles and snacks, where they can improve the dough processability and accelerate the drying step, thereby shortening the process time. Arabinoxylans provide functional properties during pasta, noodle and snack making due to their ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of arabinoxylans with the help of endo-1,4-β-xylanase improves the functional properties of these polysaccharides.

The benefits of the action of endo-1,4-β-xylanase are:

- Facilitate the handling of the dough
- Increase firmness and reduce oil absorption in instant noodles
- Reduce checking (formation of hair line cracks)
- Accelerate the drying step, thereby shortening the process time.

Furthermore, endo-1,4-β-xylanase has been used in baking and other cereal based products for over 25 years *(Beg et al. 2001)*.

The process flow of pasta and noodles is present below:



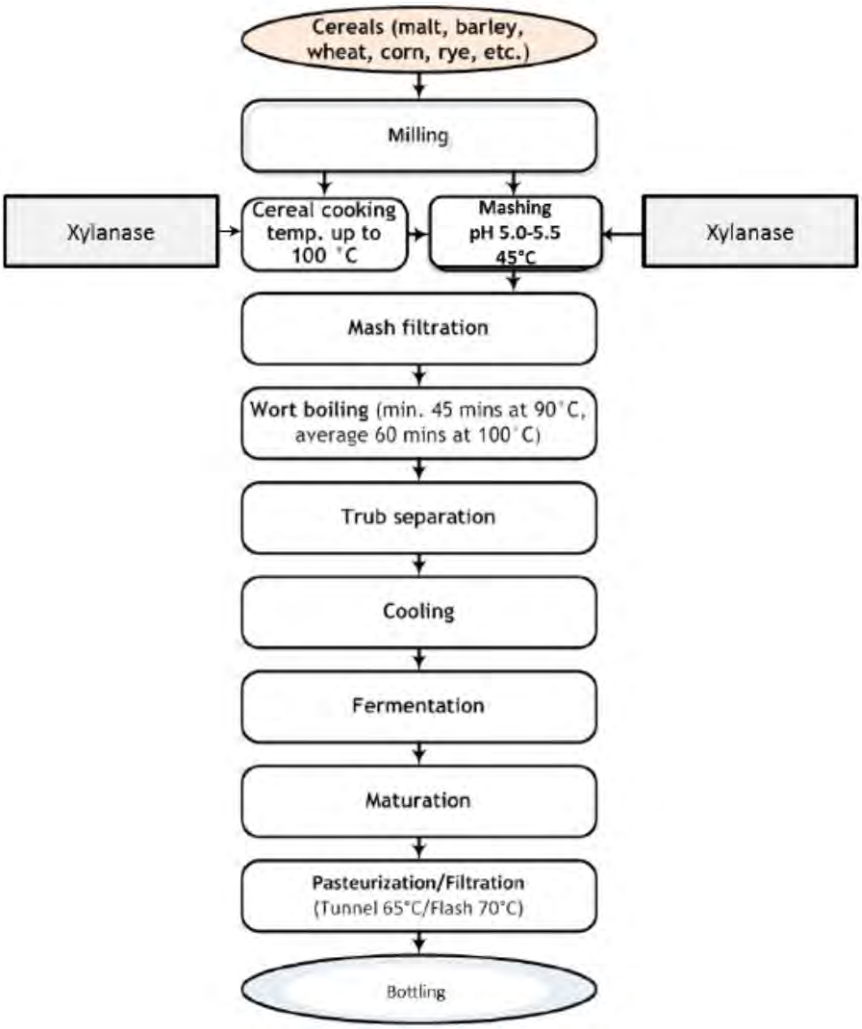
Brewing and other cereal base beverages:

During beer production, the xylans present in the cell walls of the grain are partly responsible for wort and beer viscosity - which impairs wort (lautering or mash filtration) and beer filtration.

The benefits of the conversion of (arabino)xylans with the help of xylanase in brewing are:

- Decreased wort viscosity
- Faster and more predictable lautering or mash filtration
- Faster and better beer filterability
- Improved extraction yield
- Reduced consumption of beer filtration aids

The process flow of brewing presented below shows the typical application of the food enzyme and shows the conditions under which the food enzyme is used.



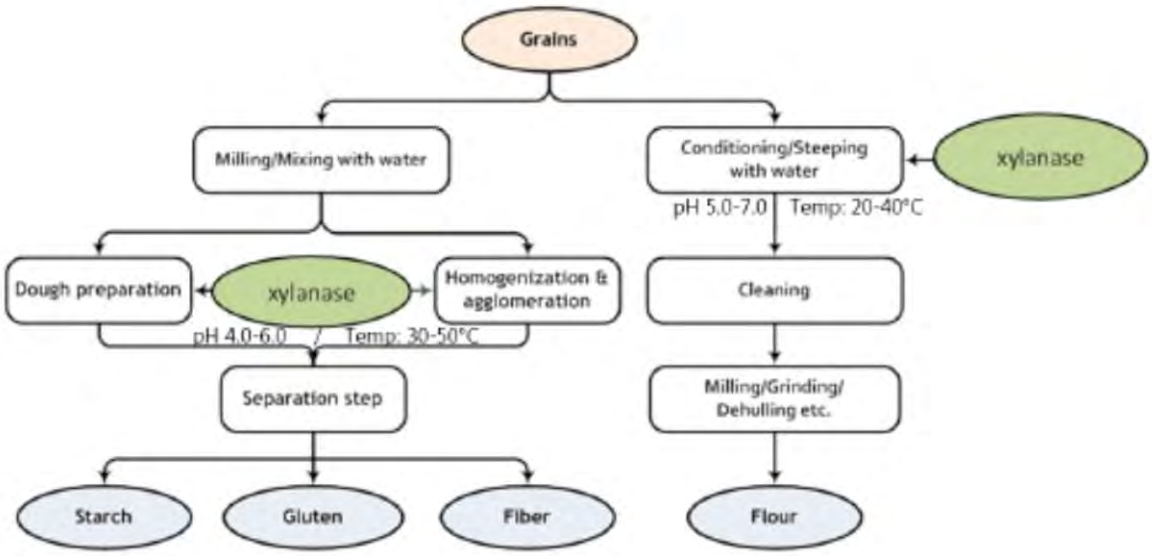
Grain processing

Cereals are highly complex structures causing technical difficulties during processing when milled and when fractionated to starch, gluten and fibres. Enzyme systems that act on the cereal components, including xylans, are used to ensure smooth and efficient processing, facilitate the separation (by opening the grain structure) and ensure high quality of the polysaccharide and gluten fractions. Grain processing also covers milling and peeling. Insufficiently hydrolysed grain cell wall components reduce the effectiveness of the mechanical treatments such as milling and peeling.

The benefits of the conversion of (arabino)xylans with the help of xylanase in Grain processing are:

- Reduced viscosity of the wheat flour batter, facilitating gluten and starch separation
- Improved gluten and starch purity due to greater extraction yield of the high value fraction and efficient removal of arabinoxylan
- Energy savings due to less use of process water, lower evaporator costs and decreased production time.
- Degradation of cell wall components increasing effectiveness of the mechanical treatments such as milling and peeling.

The process of grain processing is presented below:



Xylanase is typically added in grain processing during the initial steps such as conditioning, homogenization and dough preparation. The result of the grain processing is food ingredients such as flour or cereal fractions such as starch, gluten, fiber. Xylanase is not necessarily inactivated during grain processing process, but the resultant food ingredients (separated fractions) are further used in other food processes where the enzyme will be inactivated.

Use of the fractions obtained after grain processing:

Flour is used as a food ingredient in baking process. The starch fraction might be used as a food ingredient in other food applications such as baking and dairy, or for technical applications (e.g. for paper production), and for ethanol production or alternatively as animal feed. Starch might also be processed into glucose, maltose high fructose and other syrups which are themselves used in a number of food products.

The fibre fraction is used in baking as well as for animal feed.

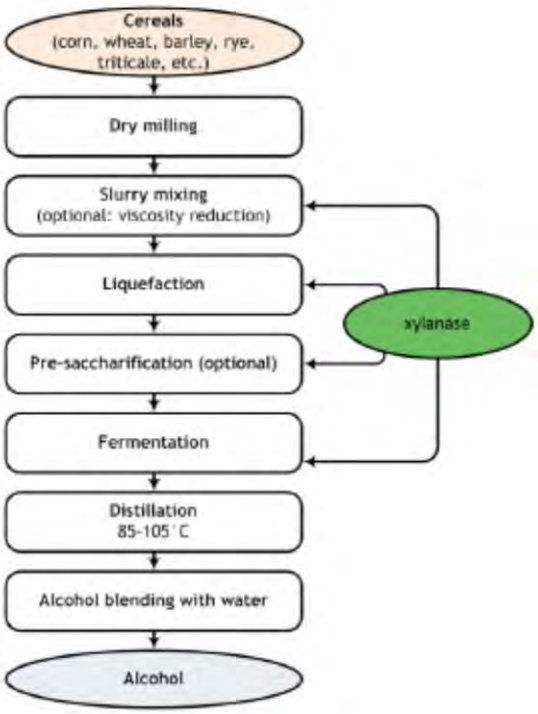
The gluten fraction is mostly used in baking to improve the properties of the flour. Gluten might also be used in other food applications such as meat processing.

In Potable alcohol production the high levels of xylans, cellulose, lichenin and beta-D-glucans results in high viscosity due to the water-binding capacity. High viscosity has negative effects on alcohol production because it limits solid concentration in mashing and reduces efficiency in the mixing, separation and filtration processes.

Xylanase is used in distilling industrial applications prior to the liquefaction of highly concentrated mashes. The benefits of the conversion of (arabino)xylans with the help of xylanase in potable alcohol production are:

- Decrease viscosity of grain mashes
- Better processing (solid/liquid separation, resulting in higher solid concentration during mashing; increase fermentable sugars and improve mass transfer during fermentation)
- Reduce fouling in the heat exchangers and distilling equipment
- Increase flexibility in the choice of raw materials and allow to use more grain and less water
- Potential higher alcohol (ethanol) yield as result of better processing, and thereby less use of raw materials.
- Reduce fuel consumption due to better heat transfer

The process flow of potable alcohol production presented below shows the typical application of the food enzyme and shows the conditions under which the food enzyme is used.



6.3 Use Levels

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 Practices. 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 his 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 endo-1,4-β-xylanase is used according to the QS principle. A food producer who would add much higher doses than needed would experience untenable costs as well as negative technological consequences.

Food enzymes also contain substances derived from the producing microorganism and the fermentation medium, and the presence of all organic material is expressed as Total Organic Solids[6] (TOS). This distinguishes the proportion of the enzyme preparation derived from the source material from that contributed by diluents, and other additives and ingredients.

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 endo-1,4-β-xylanase from *Trichoderma reesei* RF5427 may be used:

Application and Raw Material		**Recommended use levels (mg TOS/kg RM)**	**Maximal recommended use levels (mg TOS/kg RM)**
Baking and other cereal based products	Flour	1-10	10
Brewing	Cereals	1-5	5
Grain processing	Cereals	1-10	10
Potable alcohol production	Cereals	1-5	5

[6] 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.

6.4 Enzyme Residues or Activity in the Final Food

Like any other enzyme, endo-1,4-β-xylanase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

In general, enzymes perform their technological function during food processing. Like the endogenous enzymes present in food, they do not perform any technological function in the final food. The reasons why the enzymes do not typically exert enzymatic activity in the final food could be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer, such as:

- the enzyme protein must be in its 'native' (non-denatured) form, AND
- the substrate must still be present, AND
- the enzyme must be free to move (able to reach the substrate), AND
- conditions like pH, temperature and water content must be favourable

As noted in section 3.4, endo-1,4-β-xylanase from *T. reesei* RF5427 is inactivated at temperatures above 90°C, thus heating will inactivate the enzyme. Furthermore, under a lack of substrate and favourable pH conditions, the enzyme will be denatured (not active) in the final food application and would be similar to other endogenous enzymes in food.

In baking, the xylanases exert their function during dough or batter handling in order to contribute to an improved and consistent baking process. The xylanases are denatured by heat during the baking or steaming step.

In other cereal based processes such as pasta and noodles, the xylanases exert their function during dough handling thereby facilitating processing and reducing drying time and oil uptake during frying (in some noodle types). The enzymes are denatured by heat during the drying, boiling or steaming step.

Based on the conditions of use described herein and the activity of endo-1,4-β-xylanase under such conditions (see section 3.3), it can be concluded that the enzyme endo-1,4-β-xylanase does not exert any (unintentional) enzymatic activity in the final food. Indeed, it is inactivated during regular baking processes, where temperatures inside the dough reach between 95° and 100°C for a period of at least 10-15 minutes.

In addition to this denaturation process, it should be noted that other reasons are met why the enzyme does not exert any (unintentional) enzymatic activity in the final food. The remaining water content (water activity) within baked goods is much too low to support any hydrolytic enzymatic activity in the baking matrix. Furthermore the accessible xylan substrate for the enzyme is completely consumed during the dough making process, which leads to a situation that the enzyme by no means could still be functional after baking.

In other cereal based processes such as pasta and noodles, the xylanases exert their function during dough handling thereby facilitating processing and reducing drying time and oil uptake during frying (in some noodle types). The enzymes are denatured by heat during the drying, boiling or steaming step.

In the brewing and cereal based beverage industry, xylanase is typically added in mashing step or to the adjunct before the addition of the adjunct to the mash tun. xylanase is therefore denatured in the subsequent wort boiling.

When the starch is processed into syrups or alcohol, the remaining xylanase is denatured during the starch liquefaction step (which is typically in excess of 100°C). When liquified starch is fermented towards alcohol (ethanol), ethanol is recovered by distillation and separated from non-volatile solids including residual enzymes, i.e. no residues of the enzymes in the alcohol.

Also when starch is used as a food ingredient (e.g. in baking processes), xylanase is denatured during the baking process in the oven.

For application of gluten in baking xylanase will be denatured when the bread is baked in the oven and in meat processing xylanase will be denatured in the processing steps (70-122°C depending on processed meat product).

Fibres are typically use in baking during which xylanase will be denatured when the bread is baked in the oven.

In potable alcohol production, xylanase is added prior to the liquefaction. In Potable alcohol production, solids are separated from the fermentation slurry at the end of fermentation and any enzyme protein precipitate will be removed with the solids. The liquids are distilled. The distilled alcohol is subsequently filtered through a molecular sieve at temperatures well over boiling to adsorb further traces of water and water soluble proteins. Therefore, the enzymes will not be present/active in the end product due to distillation in the case of alcohol production.

Consequently, it can be concluded that the endo-1,4-β-xylanase does not exert any (unintentional) enzymatic activity in the final food.

6.5 Possible Effects on Nutrients

As the catalytic activity of the enzyme preparation is very specific, i.e., endo-1,4-β-xylanase - hydrolysis of the (1→4)-β-D-xylosidic linkages that are present in the centre (endo) of xylans, including arabinoxylan, it is not to be expected that the enzyme preparation will have any significant effect on other constituents or nutrients in food.

Like the substrate, the enzyme also occurs by nature in plants such as papaya, wheat and barley. Xylanases are a widespread group of enzymes produced by bacteria, algae, fungi, protozoa, gastropods and anthropods. A wide variety of different xylanases are produced by the organisms and many of the organisms also produce multiple xylanases with different structures and properties. For a review, see e.g. *Collins et al. (2005)*.

The reaction products of the hydrolysis of arabinoxylans by endo-1,4-β-xylanase are (1→4)-β-D-arabinoxylan oligosaccharides of variable lengths. Like the substrate and the enzyme, these reaction products are also natural constituents of cereal-containing foodstuffs.

Such enzyme activity is widely present in nature and in particular in food ingredients. The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

T. reesei is an industrially important filamentous fungus and has been used as producer of different hydrolases such as xylanase and cellulase for food, animal feed, and pulp and paper industries. It is also used as host for production of heterologous proteins in the same areas. Like many other organisms with a long safe history of industrial use, *T. reesei* strains have been and are being used by many commercial companies in the construction of production strains by genetic engineering.

Species belonging to *Trichoderma reesei* are common in soil as well as on vegetable debris. However, *T. reesei* strains have been isolated from soil (compost material) only at low altitudes and within a narrow belt around the *equator (+/-* 20 degrees altitude *(Kubicek et al. 2008)*. According to *Kuhls et al. (1996)*, *T. reesei* is a clonal, asexual derivative of the ascomycete *Hypocrea jecorina* and can be identified by PCR-fingerprinting assay and sequence analyses of the nuclear ribosomal DNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene *(Kuhls et al. 1996)*. All the *T. reesei* strains used for industrial enzyme production derive from the same wild type isolate, QM6a.

T. reesei is regarded as non-pathogenic and non-toxigenic. The safety of this organism as an enzyme producer has been reviewed by *Pariza, Johnson; Olempska-Beer et al.; Nevalainen et al.; Blumenthal (2001; 2006; 1994; 2004)* and deemed to be safe. The review article by *Nevalainen et al. (1994)* reveals that some species belonging to *Trichoderma* genus are able to secrete various types of antibiotics.

However, strains of *T. reesei* used in industrial applications are prove to be absent of antibiotic activities *(Hjortkjaer et al. 1986; Coenen et al. 1995).* Additionally, no genes have been introduced during the genetic construction that encodes antimicrobial resistance, and the absence of antibiotic activities was also confirmed from production batches (*Appendix #3*).

The transformed expression cassettes, fully characterized and free from potential hazards, are stably integrated into the fungal genome (see section 2.3) and are no more susceptible to any further natural mutations than any other genes in the fungal genome. Also, the transformation does not increase the natural mutation frequency. If there were any mutations in the genes affecting the relevant characteristics of the fungus, this would likely be noticed in the growth characteristics in the fermentation and/or in the product obtained, and no such changes have been observed. The possibility of mutations is further decreased by inoculating the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C. There is no indication that the genetic modification has a negative effect on the safety properties.

The endo-1,4-β-xylanase enzyme preparation from *T. reesei* RF5427, expressing the recombinant gene (xylanase) deriving from *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* was evaluated according to the Pariza and Johnson Decision Tree. The decision tree is based on the safety evaluation published by Pariza and Foster in 2001, adapted from their original evaluation in 1983. Based on the Pariza and Johnson decision tree analysis, AB Enzymes concludes that the endo-1,4-β-xylanase enzyme preparation is safe, see *Appendix #2*.

Therefore, it can be concluded that the *T. reesei* strain RF5427 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food processing.

7.2 Safety of endo-1,4-β-xylanase Enzyme

The xylanase enzyme has a long history of safe use in food processing, GRAS notifications have been submitted and accepted by FDA with no questions for the use of xylanase from *Fusarium venenatum*

(CFSAN / Office of Food Additive Safety, 2000, GRN 000054[7]), xylanase from *Talaromyces emersonii* (CFSAN / Office of Food Additive Safety, 2013, GRN 479[8]), mixed beta-glucanase and xylanase enzyme preparation from *Humicola insolens* (CFSAN / Office of Food Additive Safety, 2006, GRN 000195[9]) for use in wine and beer. Xylanase from *Thermomyces lanuginosus* and from *Bacillus subtilus* (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.

Non-exhaustive list of authorisations of authorised xylanases from production organisms other than *Trichoderma reesei*		
Authority	**Production organism**	**Reference**
JECFA	B. subtilis	
	Bacillus subtilis	JECFA Evaluations, 2004
	Fusarium venenatum	JECFA 52, 2004
	Humicola insolens (mixed xylanase and beta-glucanase)	JECFA 52, 2004
Australia/ New Zealand	Humiloca insolens	Standard 1.3.3 processing aids
	Aspergillus niger	
Canada	Aspergillus oryzae	B.16.100, Table V
	Bacillus subtilis	
	B. subtilis (as hemicellulase, pentosanase)	
USA[3]	Fusarium venenatum	GRAS Notice Inventory, GRN 54
	Humicola insolens	GRAS Notice Inventory, GRN 195
France	A. niger (endo xylanase)	Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées
	A. niger (as hemicellulase)	

[7] http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm153739.htm

[8] http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm396457.htm

[9] http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm154672.htm

	B. subtilis (as hemicellulase)	alimentaires | Legifrance
	B. subtilis (as xylanase)	
	Humicola insolens (as pentosanase)	
	A. oryzae	

It is generally accepted that known commercial enzyme preparations of *T. reesei* are not toxic and since endo-1,4-β-xylanase is a natural constituent in the environment, it is concluded that the endo-1,4-β-xylanase enzyme from *T. reesei* RF5427 is safe as for use as a food processing aid in various applications.

To further confirm that the endo-1,4-β-xylanase enzyme preparation does not have any toxic properties and to ensure the toxicological safety of the use of the enzyme preparation from *T. reesei*, the following studies were conducted:

- Sub-chronic (90 day) oral toxicity study
- Ames test
- Chromosomal aberration test, in vitro

Based upon the results of these studies, it can be concluded that the endo-1,4-β-xylanase enzyme preparation does not product adverse effects in rodents, nor was there any mutagenic or clastogenic activity detected, details are provided in section 7.4

7.2.1 Allergenicity

As some enzymes manufactured for use in food have been reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities, endo-1,4-β-xylanase may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the endo-1,4-β-xylanase residues in food (mainly baked goods) seems remote. In order to address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by *Bindslev-Jensen et al. (2006)* and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used*

in the food industry". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products *(Daurvin et al. 1998)*. The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

Thus, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.

Additional considerations supporting the assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens.
- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman *et al.*, 2008).
- In the case where proteins are denatured - which is the case for this endo-1,4-β-xylanase - due to the food process conditions (i.e baking process), the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins *(Valenta, Kraft 2002; Valenta 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006)*

- In addition, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic
- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

In order to specifically evaluate the risk that the xylanase enzyme will cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed. This test used a 80 amino acid (aa) sliding window search as well as conventional FASTA (overall homology), with the threshold of 35% homology and scanning with 6 mer for exact matches as recommended in the most recent literature *(Food and Agriculture Organization of the United Nations January/2001; Goodman et al. 2008; Ladics et al. 2007)*.

The sequence homology comparison tests were performed using "AllergenOnline" database (http://www.allergenonline.org), Allergen Database for Food Safety (http://allergen.nihs.go.jp/ADFS), Structural Database of Allergen Proteins (http://fermi.utmb.edu/SDAP/sdap_ver.html) and AllerMatch (http://www.expasy.org/cgi-bin/lists?allergen.txt).. According to the search results it can be concluded that the xylanase does not show relevant homology to any known allergen.

Accordingly, it is concluded that the endo-1,4-β-xylanase preparation is not a potential allergen and no further allergenicity studies are necessary.

Xylanases of fungal and bacterial origin have been used in food for decades. We have no knowledge of any reports of allergic reactions to the residues of endo-1,4-β-xylanase in food as well as to the residues of other enzymes used in food processing.

By analogy, and on the basis of the results obtained from a sequence homology comparison test, and on the fact that the enzyme is typically denatured during the food manufacturing process and that any residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is not likely that the endo-1,4-β-xylanase produced by *Trichoderma reesei* under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

7.3 Safety of the Manufacturing Process

T. reesei RF5427 meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex (2014) as described in section 4.5.

As described in Section 4, the *T. reesei* RF5427 enzyme production strain is produced in accordance with cGMPs using ingredients 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.

7.4 Safety Studies

This section describes the studies performed to evaluate the safety of the RF5427 endo-1,4-β-xylanase enzyme preparation. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

7.4.1 Summary of Safety Studies

The following studies were performed with the ultra-filtered enzyme concentrate *T. reesei* R5427:

- Bacterial reverse mutation test
- *In vitro* mammalian chromosome aberration test
- 13 week oral toxicity study in rats

The batch of endo-1,4-β-xylanase (test batch No "XT Mix Lims 2003-1463-1") tested in the toxicological studies outlined below is an ultra-filtrated concentrate, which is the most concentrated product before its formulation into a food enzyme preparation. The production process 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 composition and specifications of the toxicological batch is provided below and is representative of the commercial enzyme preparation:

Batch No	XT Mix Lims 2003-1463-1
Ash (%)	1.97
Water (%)	4.14
TOS (%)	93.89
Activity (BXU/g)	4 095 000
BXU/mg TOS	4 356
Protein (%)	75,70
Lead (mg/kg)	< 5
Salmonella sp. (per 25 g)	Not detected
Total coliforms (per g)	< 10
Escherichia coli (per 25 g)	Not detected
Antimicrobial activity	Not detected
Mycotoxins	Not detected

The safety of the endo-1,4-β-xylanase enzyme product was already evaluated by the FEEDAP and GMO Panels (Panel on Additives and Products or Substances used in Animal Feed and Panel on Genetically Modified Organisms) on behalf of the European Commission. The Panels concluded that the endo-1,4-

β-xylanase enzyme preparation of *Trichoderma reesei* CBS114044 can safely be used in animal feed and the Commission Regulation (EC) No 902/2009[10] has been published on 28 September 2009 authorising the enzyme preparation as a feed additive for weaned piglets, chickens for fattening, chickens reared for laying, turkeys for fattening and turkeys reared for breeding.

7.4.2 Results of the Safety Studies

Bacterial Reverse Mutation Test

Endo-1,4-β-xylanase enzyme preparation (approx. 94% TOS) was tested for mutagenic activity in *Salmonella typhimurium* strains TA 1535, TA 1537, Ta 98 and TA 100 and E*scherichia coli* WP2uvrA at concentrations ranging from 17 to 5000 µg/ml.

The test, based on OECD Guidelines No. 471 the European Commission Annex V Test Method B13 and B14, ICH guidelines CPMP/ICH/141/95 and CPMP/ICH/174/95 and USA EPA 712-C-98-247, was run at Charles River Laboratories, Edinburg, UK during 28 April – 23 July 2004.

There was no toxicity to the bacteria and no precipitation of the test item was observed in either mutation assay, in either the presence or the absence of a metabolizing enzyme mixture (S9 mix).

It was concluded that endo-1,4-β-xylanase was not mutagenic to *Salmonella typhimurium* or *E. coli* when tested in sterile, ultra-pure water up to a predetermined limit of 5000 µg/ml.

Chromosomal Aberration Test

Endo-1,4-β-xylanase enzyme material (approx. 94% TOS) was tested for clastogenic activity, with duplicate, Chinese hamster ovary cell cultures.

This study was conducted incorporating 2 independent tests. Ham's F-10 medium was the vehicle and cyclophosphamide and methylmethanesulfonate were the positive controls used in both tests. Xylanase was tested to the maximum permitted concentration of 5000 µg/ml in both tests.

[10] http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32009R0902

The test, based on OECD Guidelines No. 473, ICH guidelines and with the European Commission Annex V, Test Method B10 (updated) was run at Inveresk Laboratories Tranent, Scotland during 19 April – 23 June 2004.

No toxicity was noted in the cultures harvested at 24 h. In the cultures harvested at 48 h (absence of the metabolizing enzyme mixture - S9 mix) toxicity was noted at concentrations of 1250 – 5000 µL. There was no evidence that Xylanase induced structural chromosomal aberrations in either the presence or absence of S9 mix. Xylanase did not induce polyploidy in the cultures harvested 48 h post treatment.

It was concluded that endo-1,4-β-xylanase was not clastogenic when tested with Chinese hamster ovary cells *in vitro*. *In vivo* tests were not performed, as there was no *in vitro* mutagenicity detected.

90-Day Sub-Chronic Toxicity Study

The test was performed according to the following guidelines: OECD No. 408 and toxicological principles for the safety assessment of direct food additives and color additives used in food, US FDA, 1982.

The systemic potential of endo-1,4-β-xylanase (test batch XT Mix Lims 2003-1463-1) was assessed in a 13-week oral study in rats. The test was designed and performed in accordance with the OECD Guidelines No 408 adopted on 21.09.1998. The test was run at Inveresk Laboratories, Tranent Scotland during 28 June – 29 November 2004.

In this study, four groups of each 10 male and 10 female Sprague-Dawley rats were dosed daily for 13 weeks by gavage at levels of 0, 250, 500 or 1000 mg xylanase enzyme preparation/kg/day (raw enzyme preparation XT Mix with 94% TOS).

The animals were monitored daily for any signs of ill health or reaction to treatment. Detailed functional observations were performed weekly, with additional detailed functional observations performed during pre-treatment and during week 12 of treatment.

Body weights were recorded once during pre-treatment then daily throughout dosing. Food consumption was recorded once during pre-treatment and then weekly throughout treatment.

Water consumption was assessed visually on a weekly basis.

Ophthalmoscopic assessments were undertaken on all animals during pre-treatment and on all control and high dose animals during week 12.

Urine and blood samples were both collected for laboratory investigations during week 13. After 13 weeks of treatment, all surviving animals were killed and necropsied. All animals were given a detailed post mortem examination with major organs being weighed and/or placed in fixative. Tissues from all control and high dose animals and animals found dead were examined histologically. There were two premature decedents during the study. Histological examination of these animals indicated that their cause of death was not related to treatment with xylanase.

Daily oral dosing with endo-1,4-β-xylanase for 13 consecutive weeks was associated with a slight reduction in overall group mean body weight gain and an initial drop in food consumption performance in all treated female groups and in males treated at 500 or 1000 mg/kg/day. Softer than normal faeces were also noted throughout the study in all treated male groups and females dosed at 500 or 1000 mg/kg/day. Although these findings are regarded to be related to treatment with endo-1,4-β-xylanase, they are not considered to be of great toxicological significance.

There were no neurotoxic, other in-life, necropsy or histological findings that could be attributable to treatment with the test item.

In conclusion, a No Observed Adverse Effect Level (NOAEL) of 1000 mg endo-1,4-β-xylanase enzyme preparation/kg/day was derived, corresponding to an NOAEL of 940 mg TOS / kg bw / day.

7.5 Estimates of Human Consumption and Safety Margin

7.5.1 Estimate Dietary Exposure

The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method, originally known as the Danish Budget Method *(Douglass et al. 1997; Hansen 1966)*. This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological 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 (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Consumption of food patterns:

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 Section 6.3, the recommended use levels of endo-1,4-β-xylanase are given based on the raw materials used in the 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 and the wide variety of food products based on edible oils that are available to consumers. In the case of alcohol distillation, however, it is assumed that nothing of the TOS will end up in the final product due to the distillation process. Therefore, this application is not mentioned in the Table below.

Applications		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Maximal level in final food (mg TOS/kg food)
Liquid foods	Brewing	Cereals	5	Beer	0.17	0.85
	Grain Processing	Cereals	10	Starch, fibers, gluten used in – soft drinks and beverages	1.1	**11**
Solid foods	Baking and other cereal products	Flour	10	Bread, baked goods, etc.	0.71	10
	Grain processing	Cereals	10	Starch, fibers, gluten used in – soft drinks and beverages	1.1	**11**

* Assumptions behind ratios of raw material to final food*:*

<u>Baking</u>

- *Bakery products fall in the category of solid foods.*
- *Flour is the raw material for bakery product and the yield will vary depending on the type of final food produced. From 1 kg of flour you would have 4 kg of cakes, 1.4 kg of bread or 1.1 kg of cracker. Cracker may represent the most conservative input from the bakery processes. However, consumption of bread is higher than that of cracker, why bread is used as the assumption for the calculation of dietary exposure from bakery processes.*
- *The yield of 1.4 kg of bread per 1 kg of flour correspond to a RM/FF ratio of 0.71 kg of flour per kg bakery product is used.*

<u>Brewing and cereal drinks</u>

- *Brewing and cereal drinks add to the class of liquid foods.*

- *Raw materials used in brewing and cereal drink processes are various kinds of grist (e.g. malt, barley, wheat, sorghum and maize). Yields will vary dependent on the type of grist, process used and the type of drink produced.*
- *Beer production has a range of RM/FF from 14-28 kg of grist per 100 L of beer, with 80-90 % of all beers produced at a RM/FF ratio of 14-20 kg of grist per 100 L of beer. The same RM/FF ratio holds true for cereal beverage.*
- *The assumption used for calculation of dietary exposure is a yield of 100 L of drink per 17 kg of cereal corresponding to a RM/FF ratio of 0.17 kg grist per L of beer or cereal beverage.*

Grain processing

Food ingredients obtained from grain processing are typically Starch, Fibre, Gluten and Flour. These food ingredients can be use in the making of both solid and liquid final foods.

Grain processes might start with cereals (grains or grist) or flour as the raw material. Cereals contain starch in a range of 55-65%, fibre in the range of 6-18%1 and gluten in the range of 10-15%.

- *Starch: Typically 0.55 kg starch is produced per 1 kg cereal. The most considerable final food application is dairy and bakery with a maximum added starch content of 5%. Starch is also used in the less voluminous application area of confectionary, where it is used up to a content of 12%. Based upon the most considerable applications (bakery), the corresponding RM/FF ratio is 0.09 kg cereal per kg final food (same for dairy).*

  Starch can also be further processed into syrups (e.g. High Fructose Corn Syrup, HFCS), sweeteners and modified starch (Starch processing). Syrups and sweeteners are mainly used in liquid foods (soft drinks). With the assumptions expressed above (typically 0.55 kg starch is produced per 1 kg cereal) and assuming that typically 1 kg of sweetener is produced per 1 kg starch, and that soft drinks typically contain 10-14% w/v HFCS so on average 120 g HFCS per L, it can be concluded that the typical ratio of RM/FF is 0.21 kg cereals per L final beverage.
- *Fibre: Typically 0.12 kg fibre is produced per 1 kg cereal. Fibre is used in bakery and beverage products with a maximum added fibre content of 13% (total fibre content max. 25%). The corresponding RM/FF ratio is 1.1 kg cereal per kg final food.*

- *Gluten: Typically 0.10 kg gluten is produced per 1 kg cereal. Gluten is used in the production of bakery products with a maximum added gluten content of 10% in the final food. The corresponding RM/FF ratio is 1 kg cereal/kg final food.*

In respect to dietary exposure calculation, the worst case scenario, both in respect to solid and liquid food, is food ingredient Fibre with a RM/FF ratio of **1.1 kg cereal per kg final food.**

The Total Theoretical Maximum Daily Iintake (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.

The Total TMDI will consequently 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)
11 x 0.0125 = 0.1375	11 x 0.025 = 0.275	**0.4125**

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 endo-1,4-β-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, only THOSE foodstuffs were selected containing the highest theoretical amount of TOS. Thus, foodstuffs 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;
- 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)*.

7.5.2 Safety Margin

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions were observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 940 mg TOS/kg body weight/day.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI), the Total TMDI of the food enzyme is 0.4125 mg TOS/kg body weight/day.

Consequently, the MoS is:

MoS = 940 / 0.4125 = **2,279**

As is explained above, the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

8. CONCLUSION

Results of the toxicity and mutagenicity tests described in Section 7.4 demonstrate the safety of endo-1,4-β-xylanase preparation from *T. reesei* RF5427, 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 *T. reesei* derived enzymes and xylanases in food processing, and in keeping with the conclusions found in a review of relevant literature presented. Based upon these factors, as well as upon the limited and well characterized genetic modifications allowing for safe production of the enzyme preparations, it is AB Enzymes' conclusion that endo-1,4-β-xylanase enzyme preparation from *T. reesei* RF5427 is GRAS for the intended conditions of use described herein.

9. LIST OF APPENDICES

1 – GRAS Notice 32, April 20, 2000

2 – Pariza and Johnson, 2011 - Decision Tree

3 – RF5427 Analytical Report

4 – Flow Chart of Enzyme Manufacturing Process

5 – Food Chemical Codex #9, 2014

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U.S. Department of Health & Human Services

U.S. Food & Drug Administration

Food

Agency Response Letter GRAS Notice No. GRN 000032

CFSAN/Office of Premarket Approval

April 20, 2000

Gary L. Yingling
McKenna and Cuneo, L.L.P.
1900 K Street, N. W.
Washington, D.C. 20006-1108

Re: GRAS Notice No. GRN 000032

Dear Mr. Yingling:

The Food and Drug Administration (FDA) is responding to the notice, dated October 13, 1999, that you submitted on behalf of Rohm Enzyme GmbH in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received the notice on October 14, 1999 and designated it as GRAS Notice No. GRN 000032.

The subject of the notice is the pectin lyase enzyme preparation obtained from a *Trichoderma reesei* (formerly classified as *Trichoderma longibrachiatum*), which contains a recombinant gene encoding pectin lyase (also called pectin transeliminase) from *Aspergillus niger* var. *awamori*. The notice informs FDA of the view of Rohm Enzyme GmbH that the pectin lyase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid for the preparation of fruit and vegetable juices, purees and concentrates by various production processes, including the pulp wash processes and in-line pulp wash processes in the processing of citrus fruits. According to Rohm Enzyme GmbH, the pectin lyase preparation will be used for the degradation of the pectin to lower the viscosity and to clarify juices at a dose of 5 to 100 parts per million (ppm) to accomplish the intended technical effect in accordance with current good manufacturing practices.

The notice describes (1) published information pertaining to the safety of the various components of the production organism, including the host (*T. reesei*) and the donor (*Aspergillus niger*) organisms; (2) published information about the safety evaluation of microbial-derived food-grade enzyme preparations, including commercial pectinase enzyme preparations; (3) published information about the technical effect of the enzyme preparation; (4) published information related to the production process of the pectin lyase enzyme preparation; (5) scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing including enzymes derived from genetically modified microorganisms; and (6) unpublished studies conducted with the production strain and the pectin lyase enzyme preparation from *A. niger*.

The notice states that the pectin lyase preparation meets the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996) and the specifications for enzyme preparations provided by the Joint Expert Committee on Food Additives (JECFA; a joint committee of the Food and Agriculture Organization/World Health Organization).

Based on the information provided by Rohm Enzyme GmbH, as well as other information available to FDA, the agency has no questions at this time regarding the conclusion of Rohm Enzyme GmbH that the pectin lyase enzyme preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of the pectin lyase enzyme preparation. As always, it is the continuing responsibility of Rohm Enzyme GmbH to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the World Wide Web.

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

ANALYSIS OF SAFETY BASED ON PARIZA/JOHNSON DECISION TREE

Pariza and Johnson have published updated guidelines for the safety assessment of microbial enzyme preparations (2001)[1] from the 1991 IFBC Decision Tree[2]. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree as outlined in Pariza and Johnson, 2001. The outcome of this inquiry is that the xylanase from *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* expressed in *T. reesei* RF5427 is "ACCEPTED" as safe for its intended use.

Decision Tree:

1. **Is the production strain genetically modified?** *Trichoderma reesei* strain RF5427 was constructed for production of *Thermopolyspora flexuosa (Nonomuraea flexuosa)* AM24 xylanase by introducing the truncated *Thermopolyspora flexuosa (Nonomuraea flexuosa)* Nf xyn11A gene (referred to as am24) into the genome of the *Trichoderma reesei* strain *RF4847*.
   Yes go to #2;

2. **Is the production strain modified using rDNA techniques?** Yes go to #3a;

3.

3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food? Yes, Go to 3c;

3c. Is the test article free of transferable antibiotic resistance gene DNA? Yes, transferable DNA was not detected in xylanase manufactured using *T. reesei* and production process described herein. Additionally, no antibiotic resistance gene has been integrated. 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? Yes, inserted DNA is well characterized. Go to 4;

4. **Is the introduced DNA randomly integrated into the chromosome?** Yes, go to #5;

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[1] Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. Vol. **33** (2001) 173-186

[2] IFBC (International Food Biotechnology Committee), Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification, Regulatory Toxicology and Pharmacology. Vol. **12**:S1-S196 (1990).

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?** Yes, there is no concern for pleiotropic effects. Go to #6;

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes, *T. reesei* has been demonstrated as a safe production host and methods of modification have been well documented. Safety of this organism has been evaluated and confirmed through toxicological testing as described herein. **ACCEPTED**

Analytical report
RF5427
16.05.2012

Objective: **Chemical composition analysis of Xylanase from *Trichoderma reesei* strain RF5427**

Samples:
1. Dry semi-final concentrate: LIMS 2011-1345; batch no 254271588
2. Dry semi-final concentrate: LIMS 2010-1945; batch no 254270852
3. Dry semi-final concentrate: LIMS 2011-2317; batch no 254272050

Table 1. Enzyme activity, presence of production strain and antibiotic activity and microbiological quality of the product.

Batch	254271588	254270852	254272050
LIMS ID	2011-1345	2010-1945	2011-2317
Xylanase activity (BXU/g)	5240000	3160000	4510000
Presence of antibiotic activity	Not detected	Not detected	Not detected
Presence of production strain (in 20 ml)*	Not detected	Not detected	Not detected
Total viable count (cfu**/g)	1000	<1000	<1000
Escherichia coli (in 25g)	Not detected	Not detected	Not detected
Salmonella (in 25g)	Not detected	Not detected	Not detected
Total coliforms (cfu**/g)	<10	<10	<10

BXU: Assay of xylanase activity B-038G, Roal internal method
Antibiotic activity: Specifications for Identity and Purity of Certain food Additives, FAO Food and Nutrition Paper 49 (1990), Rome, Appendix A, p. 83.
Production strain: Detection of production strain (*Trichoderma reesei, Aspergillus*) in enzyme preparations M.-001A rev. C, Roal internal method
Total viable count: ISO 4833
E. coli: SFS 4089:1998 modified
Salmonella: NMKL 71:1999
Total coliforms: ISO 4832, modified
**Tested from end fermentation*
***cfu: colony forming units*

Table 2. Heavy metals (mg/kg)

Batch	254271588	254270852	254272050
LIMS ID	2011-1345	2010-1945	2011-2317
As	<0,5	0,5	<0,5
Cd	<0,05	<0,05	<0,05
Co	<0,5	<0,5	<0,5
Cr	1,0	1,3	0,7
Cu	0,9	0,7	1,0

Pb	<0,05	<0,05	0,05
Mn	1,5	9,1	3,8
Ni	0,5	0,8	0,5
Se	<1	<1	<1
Zn	2,2	6,3	3,9
V	1,2	3,2	3,4
Hg	<0,1	<0,1	<0,1

All: ISO 17294-2:2005

Table 3. Nutritional analysis

Batch	254271588	254270852	254272050
LIMS ID	2011-1345	2010-1945	2011-2317
Fat %	1,0	0,8	0
Protein %	80	58,6	72,7
Moisture %	5,9	6	5,7
Ash %	0,8	3	0,9
Carbohydrates %	18,2	31,6	20,7
Energy value (kJ/100 g)	1710	1563	1588
TOS % Total organic solids (proteins+carbohydrates+fats)	99,2	91,0	93,4

Fat: NMKL 131:1989 modified
Protein: AOAC 2000 2001.11 (4.2.11) modified
Moisture: AOAC 2000 950.46 (39.1.02) modified
Ash: NMKL 173:2005 modified
Carbohydrates: By difference 100% - (moisture+protein+fat+ash)%
Energy value: Calculated on the basis of contents of protein, fat and carbohydrate. Factors protein and carbohydrate 17 kJ/g, fat 38 kJ/g

Table 4. Mycotoxins (µg/kg)

Batch	254271588	254270852	254272050
LIMS ID	2011-1345	2010-1945	2011-2317
Aflatoxin B1	<0,05	<0,05	<0,05
Aflatoxin B2	<0,05	<0,05	<0,05
Aflatoxin G1	<0,05	<0,05	<0,05
Aflatoxin G2	<0,05	<0,05	<0,05

Analytical report
RF5427
16.05.2012

Sum of aflatoxins B1+B2+G1+G2	-	-	-
Sterigmatocystin	<10	<10	<10
Ochratoxin A	<2	<2	<2
Deoxynivalenol	<50	<40	<40
T2-Toxin	<20	<20	<20
HT-2-Toxin	<20	<20	<20
Fumonisin B2	<10	<10	<10
Zearalenone	<50	<50	<50

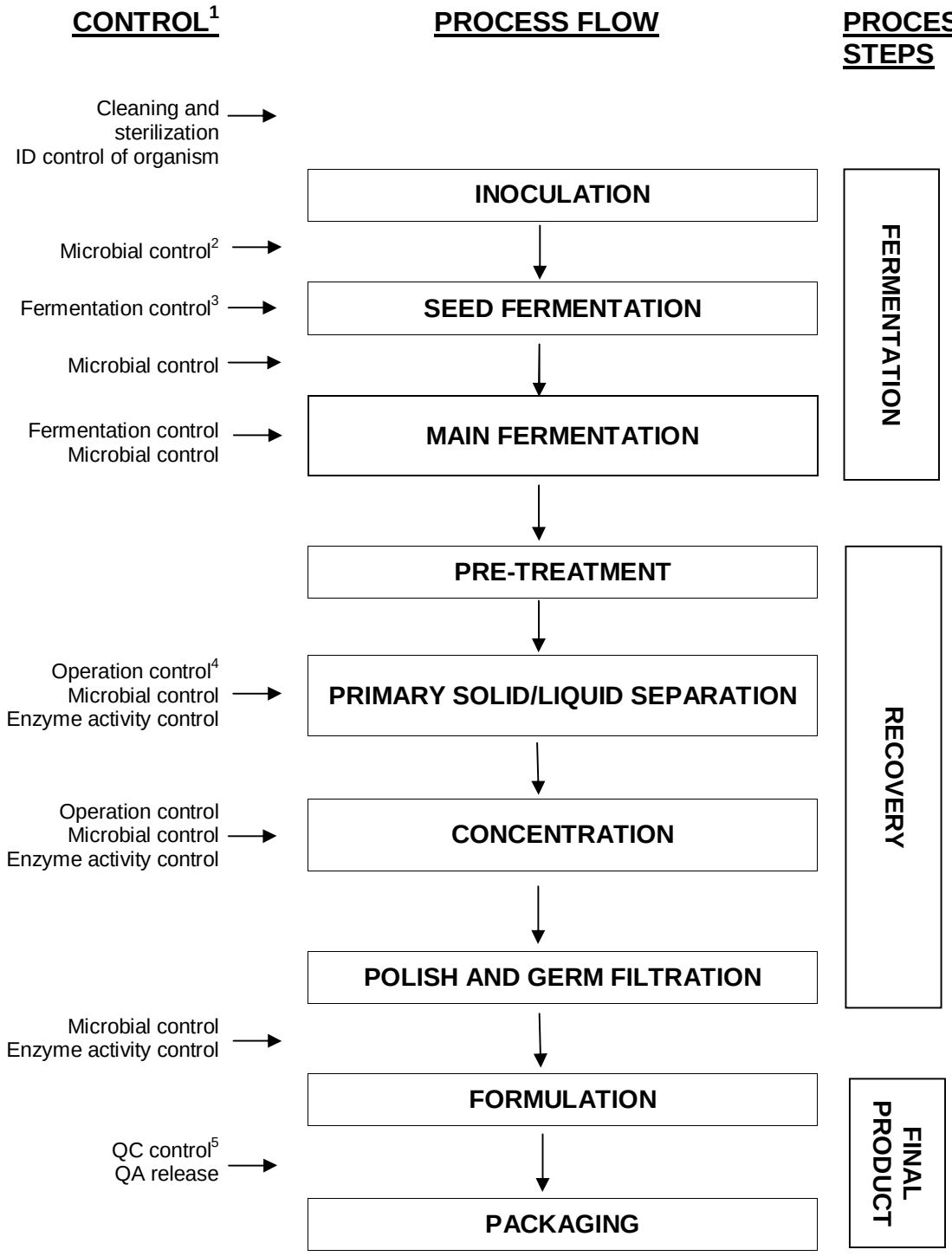
Aflatoxins: ASU 15.00-2; (= DIN 12955)
Sterigmatocystin: WEX 230, Extraction, LC-MS
Ochratoxin A: A. Thellmann DLR 93 (1), 1997
Deoxynivalenol: VDLUFA BD III; Kap. 16.12.1
T2- and HT-2Toxin: J.Agric.Food Chem. 2008 (56), 4968-4975 pp.
Fumonisin B2: EN 14352
Zearalenone: WES 128

Rajamäki, 16 May 2012

(b) (6)

Susanna Eerola
Laboratory manager

Production Process of Food Enzymes from Fermentation



[1] The controls shown on the flow chart may vary depending on the production set-up. Controls are conducted at various steps throughout the production process as relevant.

[2] Microbial control: Absence of significant microbial contamination is analyzed by microscope or plate counts

[3] During fermentation parameters like e.g. pH, temperature, oxygen, CO_2, sterile air overflow are monitored / controlled.

[4] Operation control in downstream processes cover monitoring and control of parameters like e.g. pH, temperature

[5] Final QC control will check that product does live up to specifications like e.g. enzyme activity as well as chemical and microbial specification.

Pages 000076-000542 have been removed in accordance with copyright laws. The list of the removed references can be found on pages 000066-000068.

SUBMISSION END