AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

AB Enzymes
an ABF Ingredients company

GRN 756

January 5, 2018

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: GR GRAS Notification for an endo-1,4-β- glucanase from a genetically modified *Trichoderma reesei*

AB Enzymes GmbH, we are submitting for FDA review, Form 3667, one paper copy, and the enclosed CD, free of viruses, containing a GRAS notification for **endo-1,4-β-glucanase**.

The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substances as discussed in GRAS final rule, 21 CFR Part 170.30 (a)(b), subpart E.

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

We look forward to your feedback.

Candice Cryne
Regulatory Affairs Manager
1 647-919-3964
Candice.cryne@abenzymes.com

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000756	1/24/2018
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Candice Cryne	Regulatory Affairs Manager

Organization *(if applicable)*: AB Enzymes GmbH

Mailing Address *(number and street)*: Feldbergstr. 78

City	State or Province	Zip Code/Postal Code	Country
Darmstadt		D-64293	Germany

Telephone Number	Fax Number	E-Mail Address
+49 (0)6151/3680-100	+49 (0)6151/3680-120	candice.cryne@abenzymes.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title

Organization *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

endo-1,4-β-glucanase enzyme preparation from Trichoderma reesei overexpressing endo-1,4-β-glucanase

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☒ Paper

If applicable give number and type of physical media

1 paper and CD copy

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

This dossier is specifically submitted for the use of endo-1,4-β-glucanase in brewing, grain processing and production of potable alcohol. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☒ Yes ☒ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Candice Cryne
(name of notifier)

has concluded that the intended use*(s)* of endo-1,4-β-glucanase enzyme preparation from Trichoderma reesei overexpressing endo-1,4-
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. AB Enzymes GmbH
(name of notifier)
agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Feldbergstr. 78, Darmstadt, Germany
(address of notifier or other location)

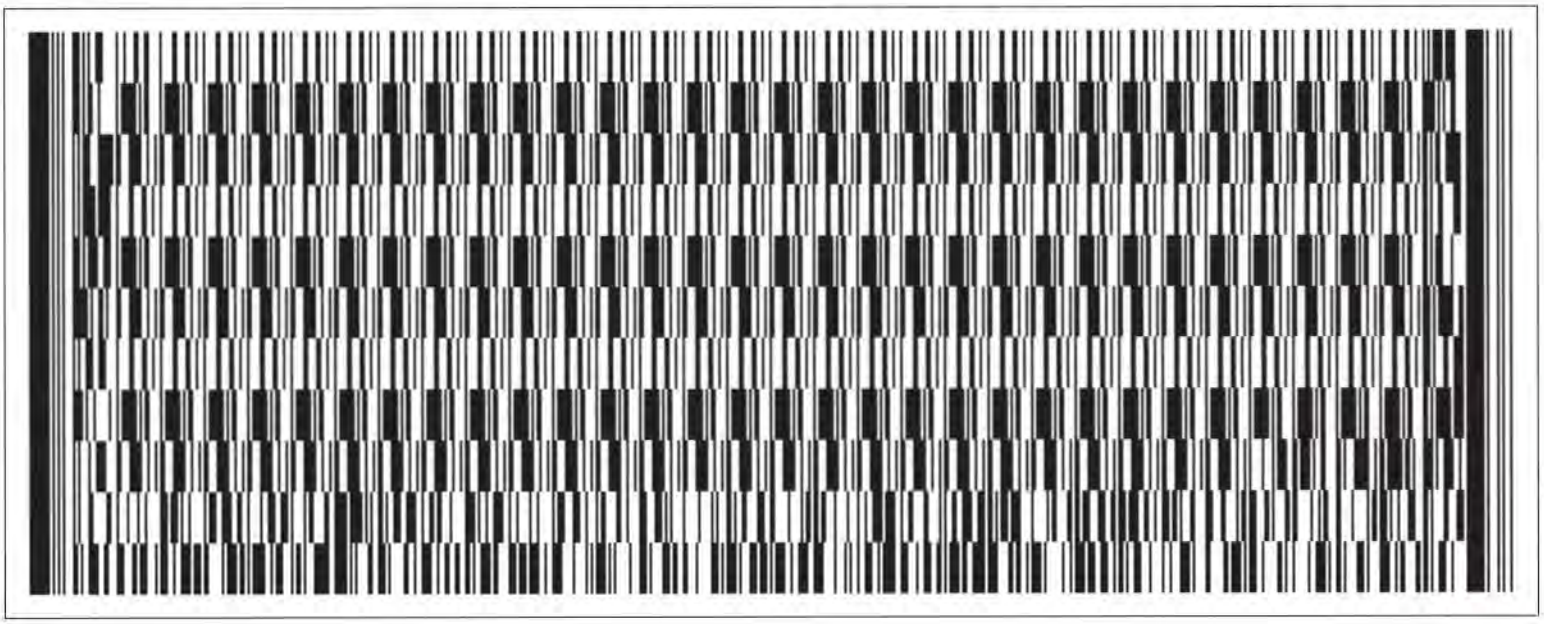
The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Candice Cryne Digitally signed by Candice Cryne Date: 2017.12.29 18:00:59 -08'00'	Candice Cryne	12/29/2017

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Cover Letter	Administrative
	GRAS Final Notice	Submission
	Appendices	Submission
	References	Submission



AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

GRAS Notification for an endo-1,4-β-glucanase from genetically modified *Trichoderma reesei*

AB ENZYMES GmbH

January 5, 2018

Contents

1 PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

Pursuant to 21 C.F.R. Part 170, subpart E, AB Enzymes GmbH, submits a Generally Recognized as Safe (GRAS) notice and claims that the use of endo-1,4-β-glucanase enzyme preparation from a genetically modified *Trichoderma reesei* produced by submerged fermentation is Generally Recognized as Safe under the conditions of its intended use; therefore, they are exempt from statutory premarket approval requirements.

The name and address of the notifier:
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt, Germany

Appropriately descriptive term:
endo-1,4-β-glucanase enzyme preparation from *Trichoderma reesei* overexpressing endo-1,4-β-glucanase.

Trade secret or confidential:
This notification does not contain any trade secret or confidential information.

Intended conditions of use:
This dossier is specifically submitted for the use of endo-1,4-β-glucanase in brewing, grain processing and production of potable alcohol. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximal limits set, just suggested dosages.

Statutory basis for GRAS conclusion:
This GRAS determination is based upon scientific procedures.

Premarket approval:
The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

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 (customary business hours) at a specific address set out in the notice or will be sent to FDA upon request (electronic format or on paper).

FOIA (Freedom of Information Act):

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to AB Enzymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

(b) (6)

Candice Cryne

Date: January 5, 2018

Regulatory Affairs Manager

2 PART 2 §170.230 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 Identity of the notified substance

The dossier concerns a **endo-1,4-β-glucanase** enzyme preparation from *Trichoderma reesei* overexpressing endo-1,4-β-glucanase. Throughout this dossier, endoglucanase will be used interchangable to describe endo-1,4-β-glucanase.

The identity of the enzyme in product was confirmed by N-terminal sequencing and mass spectrometry (LC-ESI-MS/MS).

2.1.1 Amino acid sequence and molecular mass

Depending on their physicochemical properties and on their origins, endo-1,4- β -glucanases often have a molecular mass in a range of 25-82 kDa (See Brenda[1]).

The number of amino acids with signal sequence is 459.

2.1.2 Common name of the enzyme

Name of the enzyme protein:	**endo-1,4-β-glucanase**
Synonyms:	endo-1,4-β-D-glucanase; β-1,4-glucanase; β-1,4-endoglucan hydrolase
EC (IUBMB) number:	3.2.1.4
Production strain:	*Trichoderma reesei* RF11412

2.1.3 Classification of the enzyme

IUBMB #	3..2.1.4
CAS number	9012-54-8

[1] http://www.brenda-enzymes.info/enzyme.php?ecno=3.2.1.4&Suchword=mass&organism%5B%5D=&show_tm=0

2.2 Identity of the Source

2.2.1 Recipient/Host Strain

The recipient strain used for the genetic modifications in constructing RF11412 *Trichoderma reesei* is a genetically modified derivative of a *Trichoderma reesei* mutant strain, originating from *Trichoderma reesei* QM6a. This strain has been shown to be genetically stable and it contains no genetic material from other species.

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. 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. 32). 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)*, GRN 000557 (polygalacturonase enzyme preparation from *T. reesei* strain expressing the gene encoding polygalacturonase from *Aspergillus tubingensis Mosseray* RH3544), GRN 653 (lysophospholipase enzyme preparation from *Aspergillus nishimurae* expressed in *Trichoderma reesei*.

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.

Therefore, the recipient can be described as followed:

Genus:	*Trichoderma*
Group:	*Trichoderma reesei*
Species or subspecies:	*n/a*
Commercial name:	Not applicable: it is not sold as such.

2.2.2 Donor:

Endo-1,4-β-glucanase (endoglucanase) gene, *egl1* described in this application derives from *Trichoderma reesei* wild type QM6a. The genetic construction (expression cassette) contains the native *egl1* gene. The *egl1* gene was synthesised from the QM6a genome using a standard polymerase chain reaction (PCR) method.

Genus:	*Trichoderma*
Species:	*Trichoderma reesei*

Subspecies (if appropriate): not applicable

Generic name of the strain: QM6a

2.3 Genetic modification

Trichoderma reesei strain RF11412 was constructed for overproduction of *Trichoderma reesei* EGI (Cel7B) endoglucanase. In constructing the strain RF11412, the pALK3717 expression cassette (the native *egl1* gene is under the control of the *T.reesei* promoter) was introduced into the genome of the *Trichoderma reesei* mutant strain.

The transformations of the mutant *T. reesei* strain with the pALK3717 expression cassette was performed as described in Penttilä et al. (1987) with the modifications described in Karhunen et al. (1993). The transformants were selected according to their ability to grow on acetamidase plates (*amdS marker* gene in pALK3717).

The plasmid vector (pUC19) was only used in constructing the expression cassette but was not introduced into the *T. reesei* recipient strain in fungal transformation.

pALK3717 expression cassette:

- *egl1* gene: the *egl1* gene (Penttilä et al., 1986; http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Trire2&id=56996) encodes the native *T. reesei* EGI protein.
- *Aspergillus nidulans amdS* gene and promoter: the *amdS* gene with its own promoter has been isolated from *Aspergilus nidulans* (Kelly and Hynes, 1985). The gene for construction of pALK3717 was isolated from the plasmid p3SR2 (Hynes et al., 1983). The *amdS* gene codes for an acetamidase which enables the strain to grow on acetamide as a sole nitrogen source. It has been widely used as a selection marker in fungal transformations without any disadvantage for more than 20 years.
- *T.reesei* promoter and terminator: the *egl1* gene is fused to *T. reesei* native *cbh1* promoter. This promoter is strong and is used to overexpress *egl1* transcription, to obtain high yields of EGI. For transcription terminator the native *T. reesei cbh1* terminator is used.

The DNA was sequenced and verified for the insertion and integration sites/flanking regions.

2.3.1 Stability of the Transformed Genetic Sequence

In practice, the fermentation process always starts from identical replicas of the RF11412 seed ampoule. Production preserves from the "Master Cell Bank" are used to start the fermentation process.

A Master 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. A MCB ampoule 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 MCB ampoule. The accepted MCB ampoule is used as seed material for the inoculation of the fermentations.

Mutations do not normally occur, and if so only 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 RF11412 strain. The results revealed that the genome of RF11412 after fermentation corresponds to that of the original RF11412 from culture collection. Thus, it can be concluded that the *egl1* gene integrated into the fungal genome stays genetically stable over necessary time that is needed for industrial fermentation process of the RF11412 production strain.

Additionally, the stability is also shown as equal production of the endoglucanase in a number of fermentation batches performed for the RF11412. The enzyme activity measurements from parallel successful fermentations showed that the fermentation batches of the RF11412 strain are similar. The data of the analysis of enzyme activities from preparations from three different fermentation batches of the recombinant RF11412 strain is presented in Appendix #1.

2.3.2 Structure and amount of vector and/or nucleic acid remaining in the GMM

Trichoderma reesei RF11412 strain does not harbor any vector DNA. The pALK3717 expression cassettes used for transformation were cleaved from the pUC19 vector plasmids by restriction enzyme digestion followed by isolation of the expression cassette from agarose gel.

A Southern blot hybridization experiment using plasmid with the pUC19 vector backbone as a labeled probe and genomic DNA of the production host RF11412 was performed to confirm that no vector DNA is included in the genome of RF11412. It produced negative result (no hybridization), demonstrating that no part of the plasmid vector removed to generate the linear transforming DNA fragments was introduced into the *Trichoderma* production host.

2.3.3 Demonstration of the absence of the GMM 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 RF11412 production strain is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch (Appendix #1), 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.

2.3.4 Inactivation of the GMM and evaluation of the presence of remaining physically intact cells

The RF11412 enzyme preparation is free from detectable, viable production organism. As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

It is also important to notice that the drying step gives an efficient way to kill *Trichoderma* strains, as the temperature is 75°C of the air leaving the dryer, and fungi are not very tolerant to heat.

2.3.5 Information on the possible presence of recombinant DNA

The endoglucanase is produced by an aerobic submerged microbial fermentation using a genetically modified *Trichoderma reesei* strain. All viable cells of the production strain, RF11412, are removed during the down-stream processing.

After this the final product does not contain any detectable number of fungal colony forming units or recombinant DNA. Two separate food enzyme samples (concentrates from industrial scale production) were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. No recombinant DNA (recDNA) of the production strain was shown to be present above the detection limits (Appendix #1).

2.3.6 Absence of Antibiotic Genes and Toxic Compounds

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. Adherence to specifications of microbial counts is routinely analysed. Three production batches produced by the production strain *T.reesei* RF11412 (concentrates) were analyzed and no antibiotic or toxic compounds were detected (Appendix #1).

2.4 ENZYME PRODUCTION PROCESS

2.4.1 Overview

The food enzyme is produced by ROAL Oy[2] by submerged fermentation of *Trichoderma reesei* RF11412 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

[2] See footnote 1

The enzyme preparation described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, measures are taken to comply with cGMPs and HACCP. The manufacturing flow-chart is presented in Appendix #2.

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.

2.4.2 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

2.4.3 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. The raw materials conform to either specifications set out in the Food Chemical Codex, 10th edition, 2016 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 maximum use levels of antifoam and flocculants are ≤0.15% and ≤1.5% respectively.

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

- Potable water
- A carbon source
- A nitrogen source

- Salts and minerals
- pH adjustment agents
- Foam control agents

2.4.5 Inoculum

A suspension of a pure culture of RF11412 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.

2.4.6 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.

2.4.7 Main fermentation

Biosynthesis of the enzyme by the production strain occurs during the main fermentation.

The fermentation in the main fermentor is run as normal submerged fermentation. The content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium.

As in all processes, additional fermentation medium is added during the fermentation. In order to control the growth of the production organism and the enzyme production, the feed-rate of this medium is based upon a predetermined profile or on deviation from defined set points.

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.

2.4.8 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 excreted 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.

2.4.9 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.

2.4.10 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 %.

2.4.11 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 a defined pH and a specific temperature range 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.

2.4.12 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. The filtrate containing the enzyme protein is collected for further recovery and formulation.

2.4.13 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.

2.4.14 Formulation and Packaging

Subsequently, the food enzyme is formulated. The resulting product is defined as a 'food enzyme preparation'.

The endoglucanase enzyme preparation from *T.reesei* RF11412 are sold mainly as liquid preparations. For all kinds of food enzyme preparations, the food enzyme is adjusted to a declared activity, standardized and preserved with food ingredients or food additives (food grade quality).

The food enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations, and

released by Quality Assurance. 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.

The Final enzyme preparation **does not contain any major food allergens** from the fermentation media.

2.4.15 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 by sub-culturing of one or more ampoules of the MCB. 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.

Microbiological hygiene:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination would immediately 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 which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of significant 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
- Aeration conditions

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.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

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.

2.4.16 Stability of the enzyme during storage and prior to use

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

The date of minimum durability or use-by-date is indicated on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be mentioned on the label.

2.5 Composition and specifications

2.5.1 Characteristics of the enzyme preparation

The characteristics of the enzyme preparation are:

Property	Requirement	
Activity	min.	330 kBU g^{-1}
Appearance	Liquid, Dark brown	
Density	1.0 – 1.1 g/ml	

2.5.2 Formulation of a typical enzyme preparation

Composition	
Constituent	**%**
endoglucanase	80.6
Sodium benzoate	0.5
Tap Water	18.9

2.5.3 Purity and identity specifications of the enzyme preparation

It is proposed that the food enzyme should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006):

Lead: Not more than 5 mg/kg

Salmonella sp.: Absent in 25 g of sample

Total coliforms: Not more than 30 per gram

Escherichia coli: Absent in 25 g of sample

Antimicrobial activity: Not detected

Mycotoxins: No significant levels[3]

[3] 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. Also see Section 3.2.1.2.4.

The confirmation that the food enzyme endoglucanase complies with these specifications is shown by the analyses on 3 different batches (see Appendix #1) and summarised below:

Batch #	***P160010B***	***C160115D***	***C160116E***
Lead (mg/kg)	<0.05	<0.05	<0.05
Salmonella sp. (per 25 g)	Not detected	Not detected	Not detected
Total coliforms (per g)	<1	<1	<1
Escherichia coli (per 25 g)	Not detected	Not detected	Not detected
Antimicrobial activity	Not detected*	Not detected	Not detected*

2.5.4 Composition of the enzyme preparation

Batch Number	**P160010B**	**C160115D**	**C160116E**	**Mean**
Ash (%)	0.36	0.27	0.27	*0.3*
Water (%)	77.8	78.5	77.9	*78.0*
Protein (%)	17.4	15.9	16.2	*16.5*
TOS (%)	21.8	21.2	21.8	*21.6*
Activity (BU/g enzyme concentrate)	464,000	510,000	558,000	*510,666*
Activity/mg TOS	**2,128**	**2,405**	**2,559**	***2,364***

The typical batch sizes range from 1 000 L to 150 000 L and are deeply depending on the market demand. Therefore, the frequency and the volume of production of the food enzyme vary.

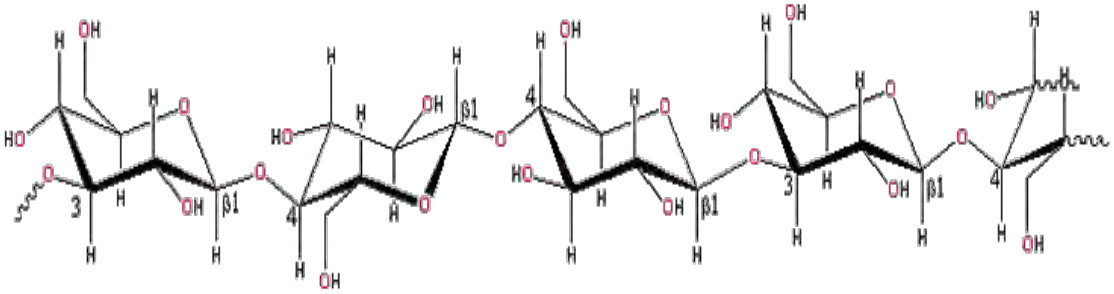
TOS values were calculated using the following formula: % TOS = 100 % - (% Ash + % Moisture + % Diluents) as recommended by JECFA. The 3 samples do not contain any diluents.

2.6 Enzymatic Activity

The main activity of the enzyme preparation is endo-1,4-β-glucanase (IUB 3.2.1.4), which has been identified in many sources, including plants, microorganisms and animals[4] (MANDELS, REESE 1957).

Endo-1,4-β-glucanase catalyzes the hydrolysis of endo (1,4)-beta-D-glucosidic linkages in beta-D-glucans, resulting in the breakdown of beta-D-glucans into smaller oligosaccharide units.

The substrates for endo-1,4-β-glucanase are cereal D-glucans (see Figure below).



These beta-glucans are polysaccharides of D-glucose monomers linked by beta-glycosidic bonds. Beta-D-glucans can be found in the bran of grains, such as oats and barley, baker's yeast and certain mushrooms (Whitaker et al. 2003). Consequently, the substrate for endo-1,4-β-glucanase occurs naturally in nature, and in particular in vegetable based foods and is therefore a natural part of the human diet.

Reaction products: as a result of the catalytic activity of endo-1,4-β-glucanase low levels of oligosaccharides composed of glucose residues are formed. As the beta -D-glucans containing plants themselves produce endo-1,4-β-glucanase during germination (Whitaker et al. 2003), these reaction products are naturally present in germinating vegetables. Consequently, also the reaction products occur naturally in foods.

[4] http://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.6&_sm_au_=iZs79QS7r9jq98t7

The method to analyse the activity of the enzyme is company specific and is capable of quantifying endo-1,4-β-glucanase activity as defined by its IUBMB classification. The enzyme activity is usually reported in BU/g.

2.6.1 Side activities of the enzyme protein which might cause adverse effects

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') - also some other substances. This is the reason why JECFA developed the TOS concept for food enzymes and why it is important that the source of a food enzyme is safe.

These other substances may include various enzyme activities (defined as 'side activities') 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.

The food enzyme is standardized on endo-1,4-β-glucanase activity. Apart from endo-1,4-β-glucanase, the production organism *Trichoderma reesei* produces endogenous *Trichoderma* cellulase.Those enzymes are, however, present in a small amount they are already present in the human diet and are not relevant from a safety point of view (see Appendix #1). Therefore there are no relevant side activities from an application and/or safety point of view.

As far as AB Enzymes is aware, the endo-1,4-β-glucanase described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

2.7 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-glucanase may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the endo-1,4-β-glucanase residues in food 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 was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers.
- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

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 enzyme proteins used in food being homologous to known food allergens[5].
- 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 enzyme due to the food process conditions, 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 2002; Valenta, Kraft 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).
- In addition, residual enzyme proteins 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 (Food and Agriculture Organization of the United Nations January/2001; Goodman et al. 2008).
- 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.

[5] The only enzyme protein used in food an known to have a weak allergenic potential is egg lysozyme

2.7.1 Allergenicity Search

In order to specifically evaluate the risk that endo-1,4-β-glucanase 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 alignment (overall homology), with the threshold of 35% homology as recommended in the most recent literature (Food and Agriculture Organization of the United Nations January/2001; Ladics et al. 2007; Goodman et al. 2008).

A sequence homology comparison test was then performed using "AllergenOnline" database (http://www.allergenonline.org), Allergen Database for Food Safety (http://allergen.nihs.go.jp/ADFS), and AllerMatch (http://www.expasy.org/cgi-bin/lists?allergen.txt). No indication of an allergenic potential of the endo-1,4-β-glucanase enzyme was detected. Accordingly, it is concluded that the endo-1,4-β-glucanase from T. *reesei* RF11412 is not a potential allergen and no further allergenicity studies are necessary.

Conclusion

Based on the results obtained from the bioinformatics approach to estimate potential allergenicity on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data, and based 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-glucanase produced by *Trichoderma reesei* RF11412 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

2.8 Technological purpose and mechanism of action of the enzyme in food

In principle, the enzymatic conversion of beta-glucans with the help of endo-1,4-β-glucanase can be used in the processing of all food raw materials which naturally contain beta-glucans .

Typical uses of endo-1,4-β-glucanase in food processing are baking, brewing, grain/starch processing and production of potable alcohol. These uses have been specifically approved for a number of years in Denmark and France, which together with the extensive use for decades in a number of EU countries justifies the technological need of endo-1,4-β-glucanase in these food processes.

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

The endo-1,4-β-glucanase from *T. reesei* RF11412 object of this dossier is specifically intended to be used in **brewing, grain processing and production of potable alcohol.**

Below, the benefits of the use of industrial endo-1,4-β-glucanase 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.

Brewing and other cereal base beverages:

In the brewing industry, endo-1,4-β-glucanase is added during mashing or to the adjunct before the addition of the adjunct to the mash tun, as shown in the brewing process flow given below. During the mashing step, pH is about 5.0-5. and temperature about 45-78°C, which corresponds to optimum pH- and temperature ranges for the endo-1,4-β-glucanase activity.

During beer production, the beta-D-glucans 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. Beta-D-glucans also cause haze problems, in particular during (cold) storage. Degradation of beta-D-glucans with the help of endo-1,4-β-glucanase into smaller, less viscous molecules solves these problems.

As endo-1,4-β-glucanase is acting on one family of the components of the plant cell wall, it is often used together with other enzymes (enzyme systems) which modify other components of the plant cell walls. In brewing, end endo-1,4-β-glucanase is often applied together with cellulase and xylanase.

The benefits of the conversion of beta-glucans with the help of endo-1,4-β-glucanase in brewing are:

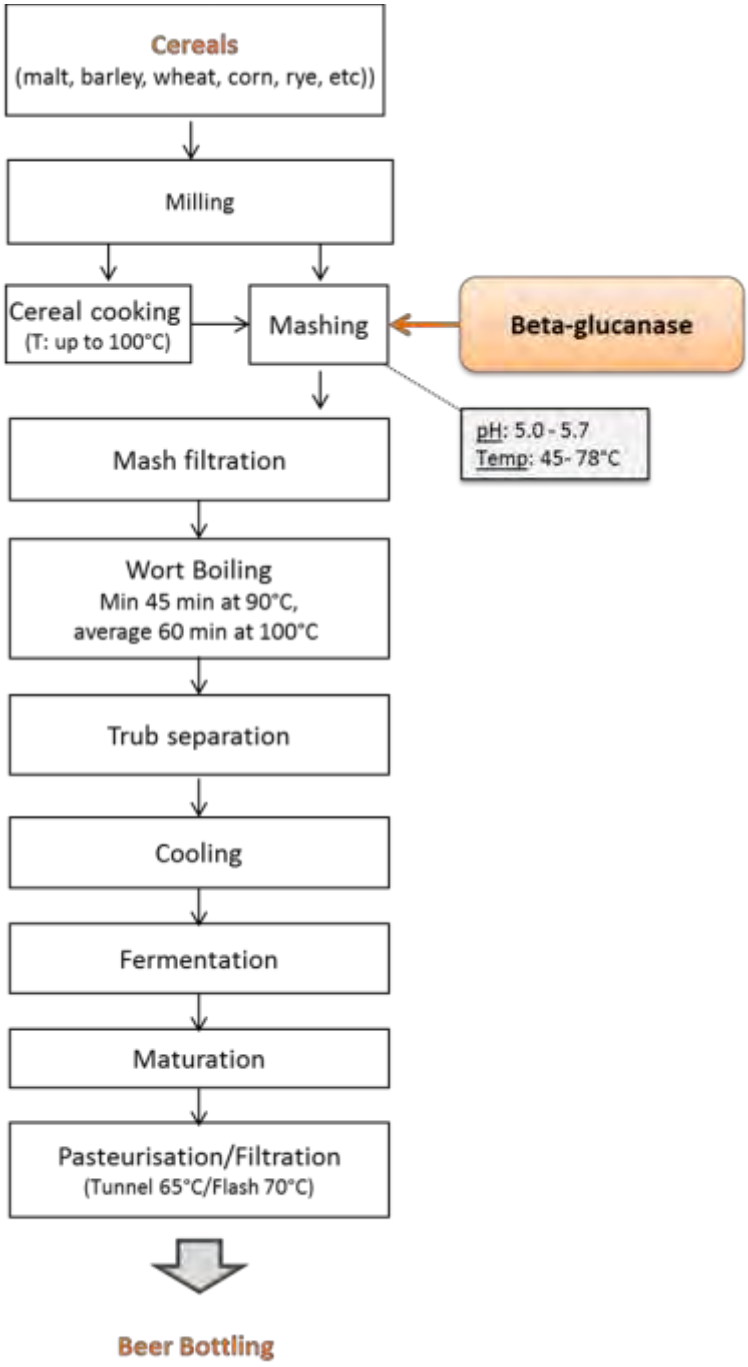
- Increase flexibility in the choice of raw materials.
- Decrease wort viscosity and beer turbidity
- Increase beer filtration rate and reduce need for beer filtration aids.
- Improve beer colloid stability as result of reduced haze caused by beta-D-glucans.
- Higher brewing yield due to the improved processing, and thereby less use of raw materials

Beneficial effects of the use of endo-1,4-β-glucanase in brewing and other cereal based raw materials:

The use of endo-1,4-β-glucanase does not specifically affect the characteristics of the beer, but by increasing the flexibility in the choice of raw materials such as i.e. wheat, wheat malt, sorghum and unmalted barley, allows making products which meet different customer's preferences for appearance and taste. In addition, it allows the brewer to use locally sourced malt rather than importing more expensive malt from traditional barley growing regions

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.

Process flow - Brewing processes and other cereal based beverage processes:



The fate of the enzyme protein during the brewing process:

In the brewing industry, endo-1,4-β-glucanase is added during mashing or to the adjunct before the addition of the adjunct to the mash tun. As the temperature is increased up 100°C for wort boiling, endo-1,4-β-glucanase is therefore inactivated at the end of the brewing process as it is similarly, when applied for other cereal based beverages such as e.g. malt extracts.

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 beta-glucans, 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 beta-D-glucans with the help of endo-1,4-β-glucanase in grain processing are:

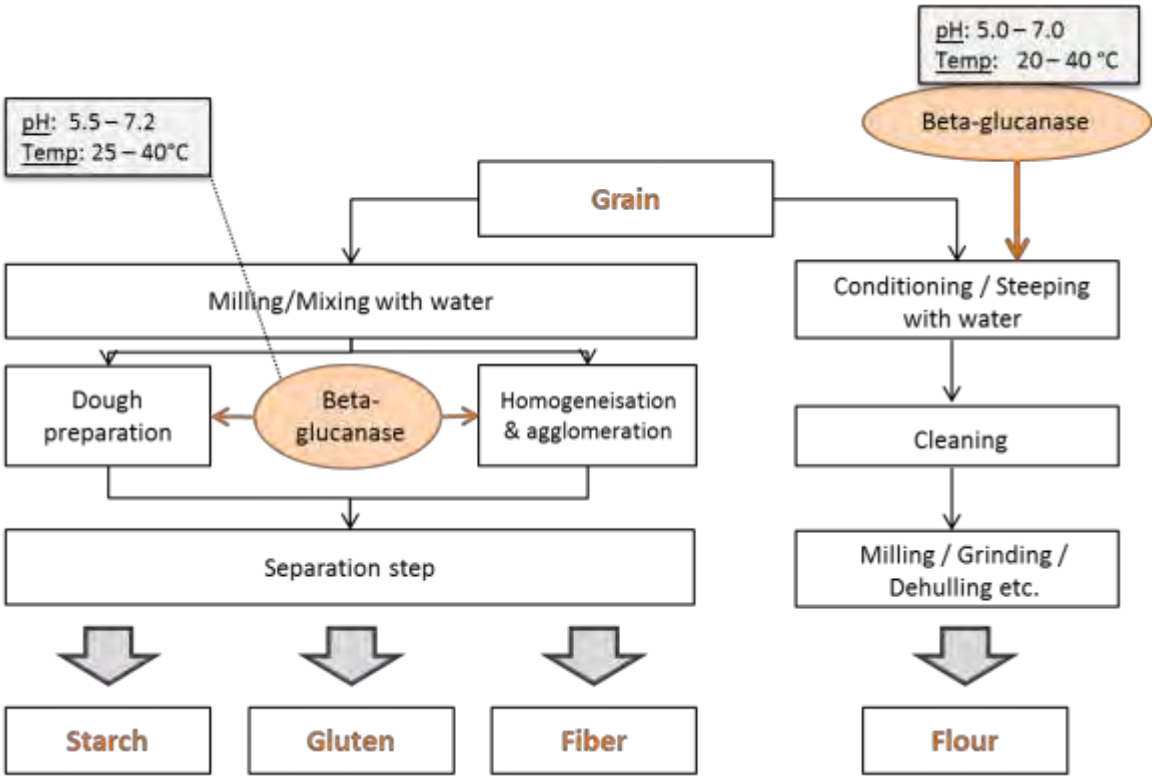
- Reduced viscosity of the wheat flour batter, facilitating gluten and starch separation.
- Improved gluten and starch purity due to greater extraction yield of the high value fraction and efficient removal of beta-D-glucans.
- Energy savings due to less use of process water, lower evaporator costs and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

Beneficial effects of the use of endo-1,4-β-glucanase in grain processing:

- As a result of efficient breakdown of beta-glucans, the use of endo-1,4-β-glucanase in grain processing leads to better starch/gluten separation, resulting in a higher yield of gluten in ingredient fractionation, and improved characteristics of gluten and starch fractions.

- Improved grain processing will lead to better production economy and environmental benefits such as the use of less raw materials, energy savings and production of less waste. This is also important from the perspective of consistent production performance: endo-1,4-β-glucanase is of specific benefit with other raw materials which might otherwise lead to a more cumbersome process (e.g. wheat beer often leads to a more a difficult and lengthy lautering as which can be avoided by the use of commercial beta-glucanases).

The process flow of grain processing is presented below:



Endo-1,4-β-glucanase is typically added in grain processing during the initial steps such as conditioning, homogenization and dough preparation.

When used in flour production process, the enzyme is added during the "conditioning/steeping with water" where the conditions are the following:

- pH: 5.0 – 7.0
- Temperature: 20 – 40°C

When used in starch/gluten/fiber separation process, the enzyme is added during "Milling/Mixing with water" step, where the conditions are the followings:

- pH: 4.0 – 6.0

- Temperature: 30 – 50°C

In any case, both pH and temperature conditions applied during the grain processing are food producer dependent (depending on the application itself and the production facilities) but are consistent with the measured pH and temperature optima.

The result of the grain processing is food ingredients such as flour or cereal fractions such as starch, gluten, fiber.

Use of the fractions obtained after grain processing

Flour is use as a food ingredient in baking process.

The starch fraction is mostly processed into glucose, maltose high fructose and other syrups which are used in a number of food products. Starch might also be used for other food applications such as baking, or technical applications (paper production) and the less valuable starch originating from the dough process is often used for ethanol production or alternatively as animal feed.

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.

Fate of the enzyme protein during grain processing

Endo-1,4-β-glucanase is not necessarily inactivated during grain processing process itself. However, any enzyme remaining in the gluten or starch fractions is at a very low level when comparing to what is initially added to the flour. In any case, any residual enzyme will be denatured when those gluten and starch fractions will be subsequently used in baking (gluten) or syrup production (starch), see below for further details.

When the starch is processed into syrups or alcohol, the remaining endo-1,4-β-glucanase 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), endo-1,4-β-glucanase is denatured during the baking process in the oven.

Gluten is typically used in baking but may also be used in other food applications such as meat processing. For application of gluten in baking endo-1,4-β-glucanase will be denatured when the bread is baked in the oven and in meat processing endo-1,4-β-glucanase will be denatured in the processing steps (70-122°C depending on processed meat product).

The fiber fraction is used in baking (where endo-1,4-β-glucanase will be denatured by heat) as well as for animal feed.

Fibres are typically use in baking during which endo-1,4-β-glucanase will be denatured when the bread is baked in the oven.

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

PRODUCTION OF POTABLE ALCOHOL

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.

Endo-1,4-β-glucanase is used in distilling industry being mainly added prior to the liquefaction of highly concentrated mashes in slurry mixing (in very rare cases, the enzyme may be added during saccharification / fermentation).

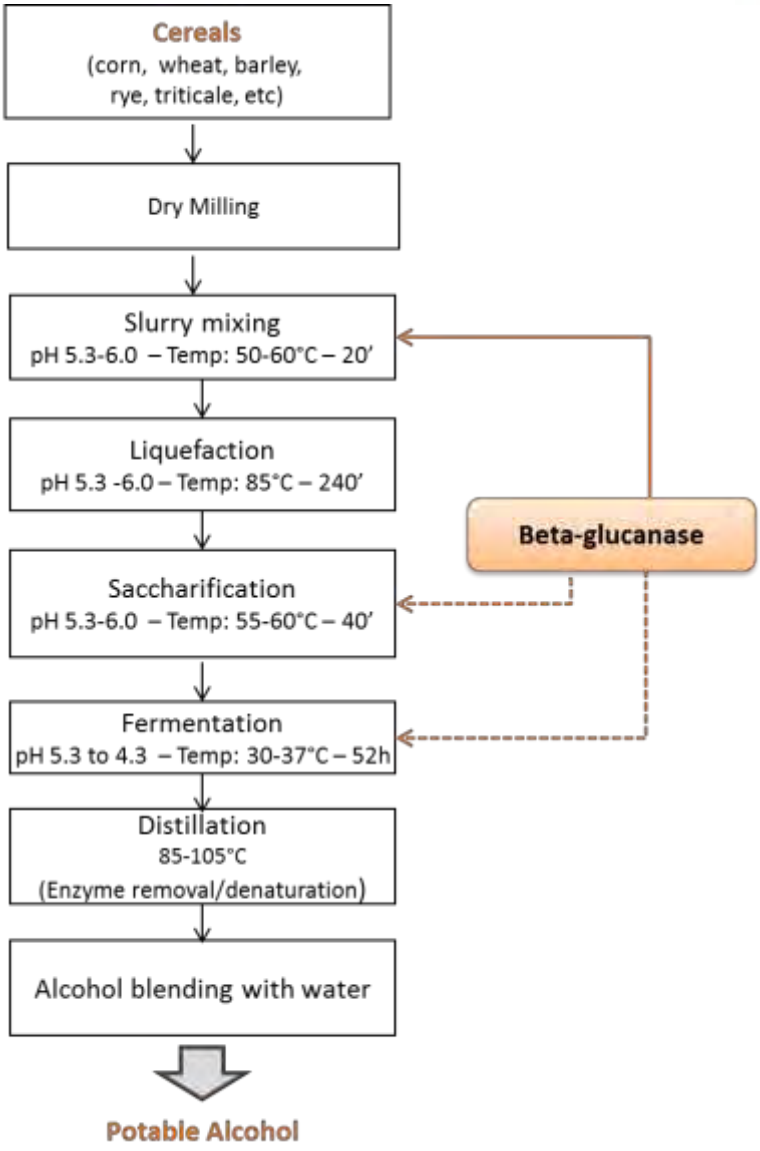
The benefits of the conversion of beta-glucans with the help of endo-1,4-β-glucanase in Potable alcohol production are:

- 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.

Beneficial effects of the use of endo-1,4-β-glucanase in potable alcohol production:

The main intention of the use of endo-1,4-β-glucanase is to facilitate the process of raw materials when dealing with Potable alcohol production and alcohol fermentation (improved fermentation process and increased ethanol yield). The effect of the enzymatic conversion is not noticeable in the final food.

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.



The fate of the enzyme protein during Potable alcohol production process:

In the Potable alcohol production industry, endo-1,4-β-glucanase is mainly added prior to the liquefaction (may rarely be added during saccharification and/or fermentation). In this process, 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 then 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 protein. Therefore, the enzymes will not be present/active in the end product due to distillation in the case of alcohol production.

2.9 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 Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions.

Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune 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-glucanase is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

The dosage of a food enzyme depends on the activity of the enzyme protein (in this case endo-1,4-β-glucanase) present in the final food enzyme preparation (i.e. the formulated food enzyme). However, the activity Units as such do not give an indication of the amount of food enzyme actually added.

Microbial food enzymes contain, apart from the enzyme protein in question, also some substances derived from the producing microorganism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS). From a safety point of view, the dosage on basis of TOS is more relevant. It must also be noted that the methods of analysis and the expression of the Units are company specific. Consequently, in contrast to when the amount is expressed in TOS the activity Units of a certain enzyme cannot be compared when coming from

different companies. Because of these reasons, the use levels are expressed in TOS in the Table below.

The Table below shows the range of recommended use levels for each application where the endo-1,4-β-glucanase is used:

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg RM)
Brewing	Cereals	2-12	12
Grain processing	Cereals	2-12	12
Production of potable alcohol	Cereals	2-12	12

3 PART 3 § 170.325 - DIETARY EXPOSURE

The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method *(Hansen 1966; Douglass et al. 1997)*. This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological 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):

Average consumption over the course of a lifetime/kg body weight/day 0.025	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

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 (see below the table) and it is assumed that all the TOS will end up in the final product.

Applications		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food (examples) (FF)	Ratio Raw Material / Final Food*	Maximal level in final food (mg TOS/kg food)
Liquid foods	Brewing	Cereals	12	Beer	0.17	2.04
	Grain processing	Cereals	12	Soft drinks (Starch derived syrups) and Beverages (fibers)	1.1	**13.2**
Solid foods	Grain processing	Cereals	12	Bakery products (Starch, Fibers, Gluten and Flour) Dairy products (Starch) Confectionnary (Starch, starch derived syrups)	1.1	**13.2**

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

- ***Brewing and cereal drinks***
  - *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%[6] 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 TMDI can be calculated on basis of the maximal values found in food and beverage (in this case, the enzyme won't be used in a process leading to liquid food, so the maximal value is

[6] http://wholegrainscouncil.org/whole-grains-101/fiber-in-whole-grains

found in baked products) multiplied by the average consumption of food and beverage/kg body weight/day.

The Total TMDI will consequently be calculated as follows:

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)
13.2 x 0.0125 = 0.165	13.2 x 0.025 = 0.33	**0.495**

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 use the endoglucanase from *Trichoderma reesei RF11412*;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food and in beverages, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS.
- Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- 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).

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL (see Section 7) by the Total Theoretical Maximal Daily Intake (TMDI). Total TMDI of the food enzyme is 0.495 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 1,000 / 0.495 = **2,020**

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 Margin of Safety in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

Conclusion:

The overall conclusion is that the use of the food enzyme endo-1,4-β-glucanase from *Trichoderma reesei* RF11412 in the production of food is absolutely safe. Considering the high safety factor – even when calculated by means of an overestimation of the intake via the Budget method – there is no need to restrict the use of the enzyme in food processing. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage.

4 PART 4 §170.240 – Self-limiting levels of use

This part is not applicable to this notified substance, see **Section 2.9** for further details regarding use levels.

5 PART 5 § 170.245 – Experience based on common use in food before 1958

This part is not applicable to this notified substance.

6 PART 6 § 170.250 – Part 6 of a GRAS notice: Narrative

The data and information contained in this GRAS notice provides a basis that the notified substance is safe under the conditions of its intended use described herein. In the following sub-sections, the safety of the enzyme, the genetic modification and toxicological studies are presented. The information is generally available and PART 6 § 170.250 does not contain any confidential information. This section provides the basis that the notified substance is generally recognized, among qualified experts, and study data, to be safe under the conditions of its intended use.

All available known information has been reviewed and AB Enzymes GmbH is not aware of any data or information that is, or may appear to be, consistent with our conclusion of the notified substance GRAS status.

6.1 Safety of the production strain

Production strain

The safety of *Trichoderma reesei* 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.

Pariza and Johnson Decision Tree

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations. 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. The outcome of this inquiry is that the endo-1,4-β-glucanase (overexpressed in *T. reesei*) enzyme preparation is "ACCEPTED" as safe for its intended use.

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

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 the endo-1,4-β-glucanase enzyme preparation 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;

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. If yes, the test article is **ACCEPTED**.

Thus, AB Enzymes concludes that the decision tree shows that the *T.reesei* production strain is **ACCEPTED.**

The original *T. reesei* isolate, QM6a (MANDELS, REESE 1957) is the initial parental organism of practically all currently industrially relevant food enzyme production strains, including our strain

RF11412. *Trichoderma reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production. Cellulases, hemicellulases, β-glucanases, pectinases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries.

Food enzymes deriving from *Trichoderma reesei* strains (including recombinant *T. reesei* strains) have been evaluated by JECFA and many countries which regulate the use of food enzymes, such as the USA, France, Denmark, Australia and Canada, resulting in the approval of the use of food enzymes from *Trichoderma reesei* in the production of various foods, such as baking, brewing, juice production, wine production and the production of dairy products.

For an extensive overview of countries that have accepted *Trichoderma reesei* as a safe production organism for a broad range of food enzymes, please see below:

Non-exhaustive list of globally authorised endo-1,4-β-glucanases from production organisms other than *Trichoderma reesei*		
Authority	**Production organism**	**Reference**
JECFA	*Aspergillus niger* *Humicol insolens* *Penicillium funiculosum* *Trichoderma harzianum*	Monograph (FNP 52 Add 2 (1993), JECFA 41st) Monograph (FNP 52 Add 11 (2003), JECFA 61th) Monograph (FNP 52 Add 8 (2000), JECFA 55th) Monograph (FNP 52 Add 8 (2000), JECFA 55th)
Australia/ New Zealand	*Aspergillus niger* *Aspergillus oryzae* *Bacillus amyloliquefaciens* *Bacillus subtilis* *Disporotrichum dimorphosporum* *Humicola insolens Talaromyces emersonii*	Standard 1.3.3 processing aids

Canada	*Humicola insolens*	B.16.100, Table V
France	*Bacillus amyloliquefaciens* *Bacillus subtilis* *Disporotrichum dimorthosporum* *Humicola insolens*	Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires | Legifrance
USA[7]	*Humicola insolens Trichoderma harzianum*	GRAS Notice Inventory, GRN195 GRAS Notice Inventory, GRN 149

Non-exhaustive list of globally authorised food enzymes (other than endo-1,4-β-glucanase) produced by *Trichoderma reesei*		
Authority	**Food Enzyme**	**Reference**
JECFA	Cellulase	FAS 30-JECFA 39/15 and FAS 22-JECFA 31/31
Canada	Cellulase Pentosanase Xylanase	B.16.100, Table V
Australia/ New Zealand	Cellulase Glucan 1-3 beta-glucosidase Hemicellulase complex Pectinase/Polygalacturonase	Standard 1.3.3 processing aids
USA[8]	Transglucosidase Protease Glucoamylase Pectinlyase	GRAS Notice Inventory, GRN 315 GRAS Notice Inventory, GRN333 GRAS Notice Inventory, GRN 372 GRAS Notices Inventory, GRN32

[7] GRAS affirmations and GRAS notifications

[8] GRAS affirmations and GRAS notifications

France	Alpha-amylase Cellulase Amyloglucosidase Xylanase Lysophospholipase	Arrêté du 19 octobre 2006

At Roal Oy, *Trichoderma reesei* has been used as enzyme producer for many years without any safety problems. *T. reesei* strains have indeed been cultivated in the production plant of Alko Oy/Roal Oy starting from year 1987 and the parental strain described here has been used from year 1995 on.

6.1.1 Pathogenicity and toxigenicity

Trichoderma reesei strains are non-pathogenic for healthy humans and animals (*Nevalainen et al. 1994)*. *Trichoderma reesei* is not present on the list of pathogens in the EU (Directive Council Directive2000/54/EC) and is present in major culture collections worldwide.

Trichoderma reesei is globally regarded as a safe microorganism:

- 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 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[9], if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

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

As a result, *Trichoderma reesei* can be used under the lowest containment level at large scale, GILSP, as defined by *(OECD, 1992)*.

The genus *Trichoderma* contains filamentous fungi which are frequently found on decaying wood and in soil. Industrial *T. reesei* strains have a long history of safe use and several of the *Trichoderma* based products have been approved for food and feed applications[10]. *T. reesei* is listed as a "Risk Group 1" organism according to German TRBA classification (Federal Institute for Occupational Safety and Health, www.baua.de) and as "Biosafety Level 1" organism by the American Type Culture Collection (www.atcc.org). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals. The DNA based identification methods have shown that *T. reesei* is taxonomically different from the other *Trichoderma* species of the section *Longibrachiatum (Druzhinina et al. 2005*).

Soome species belonging to *Trichoderma* genus are able to secrete various types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be devoid of antibiotic activities (*Coenen et al. 1995; Hjortkjaer et al. 1986)*. The absence of antibiotic activities, according to the specifications recommended by JECFA (Food and Agriculture Organization of the United Nations 2006), was also confirmed for RF11412. The analyzed data are presented in appendix #1.

Additionally, the original host *T. reesei* and the genetically modified strain do not carry any acquired antimicrobial resistance genes.

Conclusion: Based on the above mentioned available data, it is concluded that the organism *T. reesei*, has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. As an example, *T. reesei* strains have been cultivated in the production plant of Alko Oy/Roal Oy since 1987. During recent years, genetic engineering

[10] AMFEP. 2009. Association of Manufacturers and Formulators of Enzyme Products List of enzyme products on markets; http://amfep.drupalgardens.com/sites/amfep.drupalgardens.com/files/Amfep-List-of-Commercial-Enzymes.pdf

techniques have been used to improve the industrial production strains of *Trichoderma reesei* and considerable experience on the safe use of recombinant *Trichoderma reesei* strains at industrial scale has accumulated. From above, secondary metabolites are of no safety concern in fermentation products derived from *Trichoderma reesei*. Thus, *Trichoderma reesei* and its derivatives can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for other safe gene products.

6.1.1 Safety of the genetic modification

The genetic modification, e. g. integration of the expression cassette into the genome of the recipient strain *Trichoderma reesei*, results in the recombinant *Trichoderma reesei* strain RF11412. As described in Section #2, the production strain RF11412 differs from its original recipient strain in its high endoglucanase (EGI) production capability due to overexpression of the *egl1* gene from the *Trichoderma reesei* promoter.

egl1 gene

The gene for endoglucanase protein overproduced by RF11412 originates from *T. reesei* QM6a. The EGI produced by RF11412 is identical in its sequence and properties to the endoglucanase produced by the wild-type strain. Also, endoglucanase with identical sequence is already produced, although in low amounts, by the unmodified recipient strain. Endoglucanase proteins are not harmful for humans/animals as shown in safety evaluations by Hjortkjaer et al. (1986), Bindslev-Jensen et al. (2006) and Coenen et al. (1995). As the EGI protein is not toxic our evaluation of the genetically modified *T. reesei* strain is comparable to that of the recipient strain e.g. *T. reesei* strains and the enzyme mixes produced are non-pathogenic for healthy humans and animals.

AmdS

The origin of the *amdS* marker gene is *Aspergillus nidulans*. *A. nidulans* is closely related to *Aspergillus niger*, which is used in industrial production of food enzymes. The product of the *amdS* gene, acetamidase (AmdS) can degrade acetamide which enables the strain to grow on media without any other nitrogen sources. The *amdS* 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.

The original host *T. reesei* or the recipient strain does not carry any acquired antimicrobial resistance genes.

The transformed expression and deletion cassettes are fully characterized and free from potential hazards (as described above). The transformed DNAs are stably integrated into the fungal genome, 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. The possibility of mutations is decreased to its minimum by inoculating the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C and by keeping the vegetative state of the culture in an inevitable minimum during production.

No additional growth/mutagenesis cycles have been performed after the RF11412 strain deposition to the culture collection.

Therefore no reason can be seen that these genetic modifications should have a negative effect on the safety properties.

6.2 DATA FOR RISK ASSESSMENT

6.2.1 Toxicological testing

The safety of the endo-1,4-β-glucanase produced by the genetically modified *Trichoderma reesei* RF11412 is based on the historical safety of the strain lineage in section #2. The original *T.reesei* isolate, QM6a is the initial parental organism of practically all industrial used food enzyme production strains, including the *T.reesei* production strain RF11412. Thus, the parental *T. reesei* mutant of RF11412 is a classical mutant deriving from the natural isolate, *T. reesei* QM6a.

A final purified and concentrated fermentation product derived from the parental *T.reesei* mutant (cellulase enzyme concentrate) was tested for mutagenicity in a *Salmonella typhimurium* reverse mutation assay and for subacute toxicity in rats. Both tests were performed in accordance with the existing OECD and EU guidelines and GLP. The product did not show any mutagenicity in the *Salmonella typhimurium* assay with and without metabolic activation. In the study for subacute toxicity in rats, no treatment related effects occurred up to the highest concentration tested and the NOAEL was established to be 1000 mg / kg.

A so-called safe strain lineage can be established based upon repeated testing of member strains of the same lineage and their products in toxicological studies. New strains of the safe strain lineage can subsequently be evaluated using a decision tree approach, which was done for *T. reesei* (see Section 6.1). Previous members with the same recipient/host strain as *T.reesei* RF11412 have been toxicological tested (according to the latest OECD guidance) and have been accepted as GRAS #32 (*T. reesei* expressing a *A. tubigensis* pectin lyase), #558 (*T. reesei* expressing a *A. tubigensis* pectin esterase), and #557 (*T.reesei* expressing a *A. tubigensis* polygalacturonase).

Summarizing the results obtained from these toxicity studies, the following conclusions can be drawn for each of them:

- 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 1,000 mg TOS/kg body weight/day.

Because the host organism is safe based on previous toxicological evaluations and because the genetic modifications are well characterized, introducing genetic materials which do not encode and express any toxic substances, it is concluded that the use of the endo-1,4-β-glucanase from genetically modified *Trichoderma reesei* RF11412 as a processing aid in food processes would pose no significant risk to human health.

7 PART 7 §170.255 – LIST OF SUPPORTING DATA AND INFORMATION

This section contains a list of all the data and literature discussed in this dossier to provide a basis that the notified substance is safe under the conditions of its intended use as described in accordance with §170.250 (a)(1). All information presented in this section are publically available.

Appendices

1. Certificate of Analysis
2. Manufacturing Flow Chart

Publication bibliography

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Analytical report
RF11412
02.01.2018

Objective: **Chemical composition analysis of beta-glucanase from *Trichoderma reesei* strain RF11412**

Sample:
1. Liquid end fermentation concentrate batch P160010B
2. Liquid end fermentation concentrate batch C160115D
3. Liquid end fermentation concentrate batch C160116E

Table 1. Main and side enzyme activities

Batch	**P160010B**	**C160115D**	**C160116E**
Beta-glucanase activity (BU/g)	464000	510000	558000
Cellulase activity (CU/mg)	16500	15000	15600

BU: Assay of fungal beta-glucanase activity, Roal internal method B031
CU: Assay of cellulose activity, Roal internal method B029

Table 2. Antibiotic activity, RecDNA, presence of production strain and microbiological quality

Batch	**P160010B**	**C160115D**	**C160116E**
Antibiotic activity	not detected	not detected	not detected
Recombinant DNA	not detected	not detected	not detected
Presence of production strain	not detected	not detected	not detected
Escherichia coli (/25 g)	not detected[1)]	not detected[2)]	not detected[2)]
Salmonella (/25 g)	not detected	not detected	not detected
Total coliforms (cfu*/g)	<1	<1	<1
Staphylococcus aureus (/25 g)	not detected	not detected	not detected
Sulphite reducing anaerobes (cfu*/g)	<10	<10	<10

Antibiotic activity: Specifications for Identity and Purity of Certain food Additives, FAO Food and Nutrition Paper 65 (2006), Rome, Vol.4, p. 122.
rDNA: Qualitative PCR for rDNA in food and feed enzymes, validated for the specific sequences
Production strain: Detection of production strain (Trichoderma reesei, Aspergillus) in enzyme preparations, Roal internal method M001
E. coli: [1)] SFS 4089:1998, mod. [2)] ISO 16649-3:2015, mod.
Salmonella: NMKL 71:1999, mod.
Total coliforms: ISO 4832:2006, mod.
S.aureus: Eur. Pharmac. 6.3.
Sulphite reducing anaerobes: NMKL 56:2008
*cfu: colony forming units

Table 3. Nutritional analysis

Batch	**P160010B**	**C160115D**	**C160116E**
Protein %	17.4	15.9	16.2
Moisture %	77.8	78.5	77.9
Ash %	0.36	0.27	0.27
TOS %[1)]	21.8	21.2	21.8

Protein: NMKL 6, Kjeldahl
Moisture: NMKL 23
Ash: NMKL 173
1) Total organic solids TOS%= [100-(moisture % + ash %)]

Table 4. Heavy metals

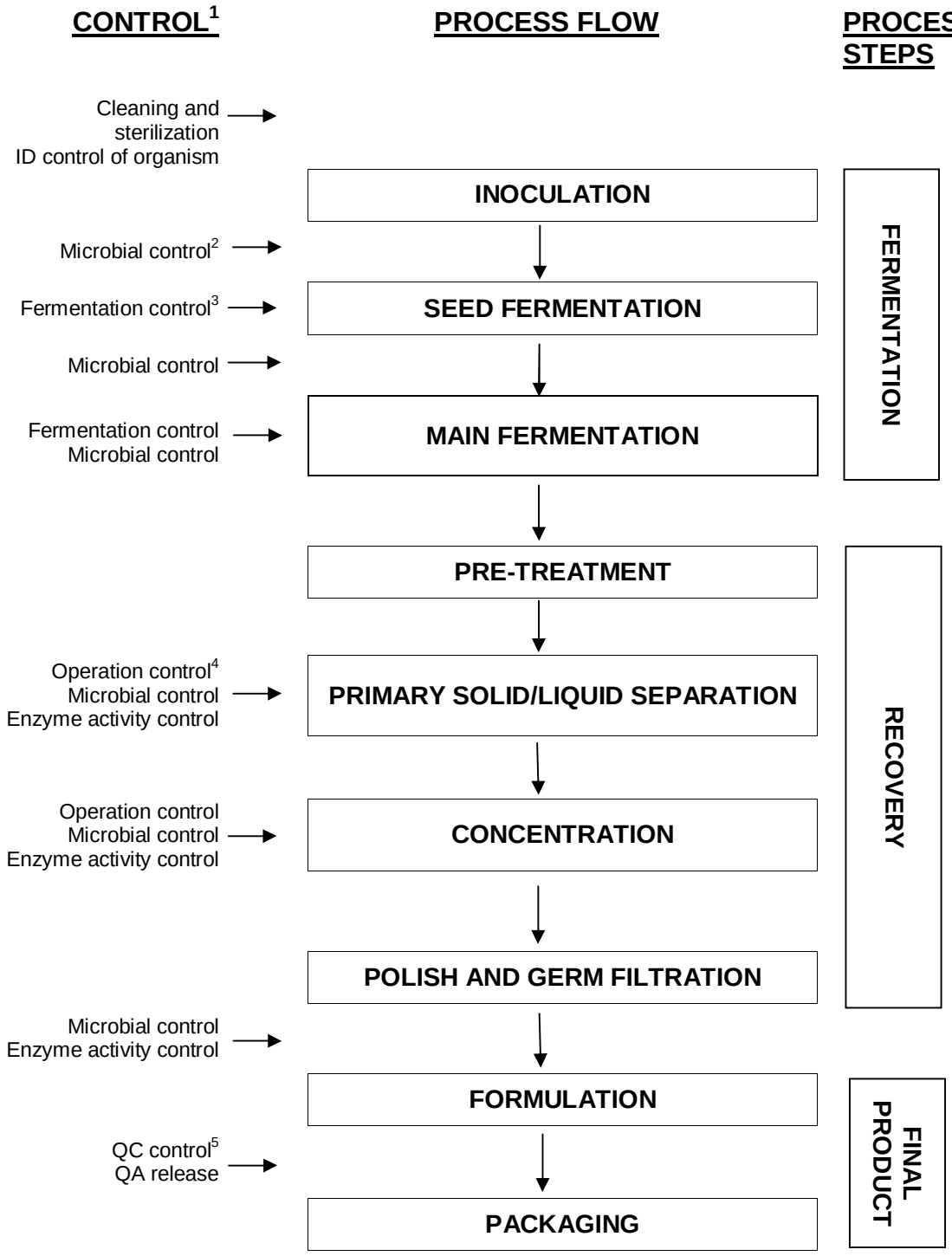
Batch	**P160010B**	**C160115D**	**C160116E**
Arsenic, As (mg/kg)	<0.5	<0.5	<0.5
Cadmium, Cd (mg/kg)	<0.05	<0.05	<0.05
Lead, Pb (mg/kg)	<0.05	<0.05	<0.05
Mercury, Hg (mg/kg)	<0.05	<0.05	<0.05

Heavy metals: ISO 17294-2:2003

Rajamäki 02.01.2018

Hanna-Riikka Pirttilahti
Quality Management Coordinator
Roal Oy

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.