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An application to amend the Australia New Zealand Food Standards Code with a Triacylglycerol lipase preparation produced by a genetically modified *Trichoderma reesei*

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Appendix 13 – CCI version

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I. Table of contents

Elements in Appendix 13 that are to be treated as confidential commercial information (CCI) are marked in highlighted text in this CCI version (the corresponding text will be marked as [redacted] in the non-CCI version).

II. The host/receipient organism

The recipient strain used for the genetic modifications in constructing RF10625 was ***Trichoderma reesei*** **strain [redacted], a genetically modified derivative of [redacted] mutant strain.**

a. Taxonomy

Trichoderma reesei is a hypercellulolytic fungus which was found on deteriorating military fabrics such as tents and clothing.

Taxonomic studies have shown that the species *Trichoderma reesei* consists only of a single isolate QM6a and its derivatives (Nevalainen et al. 1994).

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

T. reesei can be identified by PCR-fingerprinting assay and sequence analyses of the nuclear ribosomal DNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene (Kuhls et al. 1996).

Synonyms[1]**:** T*richoderma reesei* is the species name given to the anamorphic form (the form which reproduces asexually) of the fungus whose teleomorphic form (the form which reproduces sexually) is now understood to be *Hypocrea jecorina* (Kuhls et al. 1996; Seidl et al. 2008*)*.

Trichoderma reesei was formerly known as *Trichoderma longibrachiatum*. Therefore, it is of relevance to note that enzymes have been approved that are produced by *T. reesei* under the name of *T. longibrachiatum*[2].

The *T. reesei* parental strain [redacted] was characterized by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands as *Trichoderma reesei*. It was identified based on the

[1] *Reference: Mycobank taxonomic database (see*: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic).

[2] *see*: http://amfep.drupalgardens.com/sites/amfep.drupalgardens.com/files/Amfep-List-of-Commercial-Enzymes.pdf

sequences of Internal Transcribed Spacer 1 and 2 and the 5.8S gene and Translation Elongation Factor 1α. *T. reesei* [redacted] was deposited as a CBS culture (safe deposit) as CBS 114041.

The classification of [redacted] (also referred to as [redacted][3]) as *Trichoderma reesei* has been confirmed by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands (Appendix #13.1).

Therefore the recipient can be described as followed:

Genus:	*Trichoderma*
Species:	*Trichoderma reesei*
Subspecies (if appropriate):	not applicable
Generic name of the strain:	[redacted]
Previous or other name(s) (if applicable):	none
Commercial name:	Not applicable. The organism is not sold as such.

Synonyms[4]: *Trichoderma reesei* is the species name given to the anamorphic form (the form which reproduces asexually) of the fungus whose telemorphic form (the form which reproduces sexually) is now understood to be *Hypocrea jecorina*.

Trichoderma reesei was formerly known as *Trichoderma longibrachiatum*.

b. Construction of the host

The *Trichoderma reesei* host strain is a classical mutant derived from *T. reesei* QM6a.

Trichoderma reesei [redacted] was developed from the wild type strain QM6a by conventional mutagenesis.

The genetically modified strain [redacted] used as a recipient in construction of RF10625 derives from [redacted]. For a summary of the genetic construction steps from [redacted] see Appendix #13.2.

[3] *Both numbers are indifferently referring to the same strain, depending on ROAL or AB Enzymes reference system. For easy reading, the RF reference number will be use most of the time throughout the dossier.*

[4] Reference: Mycobank taxonomic database (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic).

III. Origin and donor of vector and inserts

a. The enzyme gene

Origin

The *Fusarium oxysporum* lipase gene for lipase protein overproduced by RF10625 was designed and synthesized using the preferred codon usage for *Trichoderma reesei*. A codon-optimized *Fusarium oxysporum* lipase [redacted] encoding sequence was designed based on the *Hypocrea jecorina* preferred codon usage (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=51453) and synthesized by Eurofins (Germany). The lipase DNA sequence, with ATG (Met) codon at position +1 comprises an open reading frame of [redacted]. The codon-optimized lipase sequence expressed in *T. reesei* showed 86% identity to the original lipase sequence from *Fusarium oxysporum*. **The amino acid sequence remained unchanged.**

Allergenicity

In order to specifically evaluate the risk that the lipase enzyme would 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 a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 14, January 20, 2014), which contains the amino acid sequences of known and putative allergenic proteins.

The resulting alignments of the full-length lipase protein sequence to any allergenic proteins in the allergen database showed no sequences with E () <1.000000. In addition, the lipase protein sequence

showed no matches of greater than 35% to the known allergens when searching for 80 amino acid alignments and no perfect match when searching for a stretch of eight amino acids.

See Appendix #13.3 for further information.

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 lipase produced by *Trichoderma reesei* RF10625 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

b. Vector

The *Fusarium oxysporum* lipase [redacted] encoding sequence was designed and synthesized using the preferred codon usage for *Trichoderma reesei*. The lipase gene contains an *Avr*II site four nucleotides upstream of the ATG and a *Pac*I site downstream of the stop-codon. The lipase gene was cloned into the pCR4-TOPO vector, resulting in the plasmid Lip-FO.

The plasmid Lip-FO was digested with *Avr*II and *Pac*I. The *Avr*II-*Pac*I fragment containing the lipase gene was ligated into the *Spe*I and *Pac*I sites of the plasmid pAB140SP (Appendix #13.4) resulting in the plasmid pAB140SP-LipFO. In this plasmid the *Fusarium oxysporum* lipase gene was placed under the control of the *Trichoderma reesei* [redacted] promoter and [redacted]-terminator.

The plasmid pAB140SP-LipFO was characterized by restriction with endonucleases and the construct was confirmed by DNA sequencing. The expression plasmid pAB140SP-LipFO is shown in more detail in Appendix #13.5.

The description of the plasmid Lip-FO used in the construction of the plasmid pAB140SP-LipFO is included in Appendix #13.6.

The plasmid pAB140SP-LipFO was digested with *Not*I and the expression cassette containing the lipase gene was isolated (Appendix #13.7). The purified expression cassette was used for transformation of *T. reesei* [redacted].

c. Promoter

The lipase is expressed under the strong [redacted] promoter.

IV. Introduced genetic sequence

Standard molecular biology methods were used in the construction of the expression plasmid. The expression cassette fragment used in fungal transformation does not contain any vector derived sequences as it is isolated from the expression plasmid by restriction digestion and purification from an agarose gel. It is free from any harmful sequences and contains the following genetic materials:

- ***Fusarium oxysporum lipase gene:*** The lipase gene was synthesized using the preferred codon usage for *Trichoderma reesei*. [redacted]. The sequence of the lipase gene and the deduced amino acid sequence of the encoded protein are included Appendix #14.8. For the construction of the expression vector, the gene is fused at its 5´-end to the [redacted] promoter. This promoter is strong and is used to drive *lipase* expression, to obtain high yields of lipase enzyme.

- **Linker**: synthetic DNA sequence contained *Pac*I and *Bam*HI restriction sites

- [redacted] **terminator:** To ensure termination of transcription the native [redacted] terminator is used.

- **Linker:** synthetic DNA sequence with *Stu*I restriction site

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

Of the above genetic materials, the lipase gene and *Aspergillus nidulans amdS* gene are not naturally present in the host genome.

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

V. Construction of the recombinant production organism

The transformation of *T. reesei* host strain with the expression cassettes was performed as described by Penttilä et al. (1987) with the modifications described in Karhunen et al. (1993).

Southern blot analyses were performed to the genome of the production strain RF10625. Results indicated that [redacted] of the expression cassettes were integrated in the genome of strain RF10625 (Appendix #13.9).

VI. Description of the production organism

a. Identity and taxonomy

The transformed production strain containing the lipase gene is ***Trichoderma reesei* strain RF10625** which is deposited in the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS 134213.

The taxonomic classification of the *T. reesei* is: *Hypocreaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota*, Fungi, according to Index *Fungorum* database. See section II.a. for more taxonomy details.

b. Stability of the genetic traits in the GMM

T. reesei strains are widely used in biotechnological processes because of their known stability.

The production strain RF10625 is stable in terms of genetic traits. The genetic materials in the expression cassettes have been integrated as part of the genome and are as stable as any natural gene. The integrated genetic materials are not acting as mobilisable elements and they do not contain mobilisable elements.

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 RF10625 strain (see Appendix #13.10). The results revealed that the lipase gene stays genetically stable in *T. reesei* genome over necessary time that is needed for industrial fermentation process of the RF10625 production strain.

Additionally, the stability is also followed as equal production of the lipase in a number of fermentation batches performed for the *T.reesei* strain RF10625. The activity measurements from parallel successful fermentations showed that the productivity of the RF10625 strain remains unchanged.

c. Mobilisation and transfer capacity

The inserted DNA does not include any mobile genetic element. Additionally, it should be highlighted that *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome (Kubicek et al. 2011; Martinez et al. 2008). This results to low risk of transfer of genetic material.

d. Presence of acquired antimicrobial resistance genes

The review article by Nevalainen et al. (1994) reveals that some 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 absent of antibiotic activities (Hjortkjaer et al. 1986; Coenen et al. 1995). The absence of antibiotic activities, according to the specifications recommended by JECFA was also confirmed.

Additionally, no genes have been introduced during the genetic construction that encode antimicrobial resistance.

VII. Information on any Significant Similarity between the Amino Acid Sequence of the Enzyme and that of Known Protein Toxins

A study was conducted to assess the toxicity of the *Fusarium oxysporum* lipase using bioinformatics tools.

A homology search was performed from the non-redundant protein sequences database (nr) using the BLAST-P (protein – protein BLAST) program, v. 2.6.1+ (Altschul *et al*., 1997; http://blast.ncbi.nlm.nih.gov/). The amino acid sequence of the lipase ([redacted]) was used as the query sequence in the searches. For the purpose of toxicity analysis, additional search criterion was used to limit the search to sequences that are related to toxins. The word "toxin" was given as the Entrez Query.

For detailed methods and results, see Appendix #13.11.

According to the results obtained from the searches performed it can be concluded that the lipase protein does not shown significant homology to any protein sequence identified or known to be a toxin.

VIII. Appendices

13.1. Centraalbureau voor Schimmelcultures (CBS) of host strain - Confidential

13.2. [redacted]

13.3 [redacted]

13.4 [redacted]

13.5 [redacted]

13.6 [redacted]

13.7 [redacted]

13.8 [redacted]

13.9. [redacted]

13.10) [redacted]

13.11. [redacted]

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