PULLULANASE FROM *BACILLUS DERAMIFICANS* EXPRESSED IN *BACILLUS LICHENIFORMIS*

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the pullulanase enzyme preparation submitted to JECFA by Genencor, a Danisco Division (Genencor),[1] in a dossier dated 29 November 2010 (Genencor, 2010). This document also discusses published information relevant to pullulanase, the *Bacillus licheniformis* production organism, and the *Bacillus deramificans* organism that is the source for the pullulanase gene.

Pullulanase is an enzyme that catalyses the hydrolysis of the (1,6-α-D) glucosidic linkages in liquefied starch to produce linear oligosaccharides. It is used as a processing aid in the manufacture of starch hydrolysates (maltodextrins, maltose, and glucose) and high fructose corn syrup (HFCS), and in the production of beer and potable alcohol.

Pullulanase is manufactured by pure culture fermentation of a genetically modified strain of *B. licheniformis* containing the pullulanase gene from *B. deramificans*. *B. licheniformis* is a Gram-positive bacterium that is widely distributed in nature and is considered to be nonpathogenic and nontoxigenic. *B. licheniformis* has a long history of use in the production of enzymes used in food processing, including enzymes from genetically engineered strains of the organism.

The gene encoding pullulanase was cloned from *B. deramificans*, a Gram-positive, aerobic, spore-forming bacterium that was originally isolated from a soil sample from Canada. The bacterium was classified as a Risk Group 1 microorganism by German authorities.

Prior to the introduction of the pullulanase gene, the *B. licheniformis* host strain was genetically modified through deletion of sporulation capability, amylase activity, and chloramphenicol acetyl transferase activity. The modified host strain was then transformed with an amplifiable DNA cassette containing the pullulanase gene from *B. deramificans* and the chloramphenicol acetyltransferase (*cat*) *gene* from *B. licheniformis*. The *cat* gene was used as a selectable marker in the transformation of the host strain. The transformed host strain was further modified by deletion of genes that encode two major endogenous proteases. The strain was subsequently subjected to a gene amplification procedure to increase the number of pullulanase gene copies. The final production strain was designated as BMP139.

Pullulanase is secreted into the fermentation broth and is subsequently purified and concentrated. The final product is formulated and standardized to a desired activity. The pullulanase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

Pullulanase is not known to be allergenic when used in food processing. Nevertheless, the potential for the enzyme to be a food allergen was examined by comparing the pullulanase amino acid sequence with the sequences of known allergens in the Structural Database of Allergenic Proteins (SDAP) using internationally accepted search criteria. No meaningful homology with food allergens was detected by this analysis. The amino acid sequence of pullulanase was also

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analyzed for hydrophilicity because two short amino acid sequences were homologous to the sequences of two non-food allergens. Since neither of the matching amino acid sequences is strongly hydrophilic they are not likely to be located on the surface of the intact pullulanase, and are thus unlikely to serve as IgE binding sites.

2. Description

Amber liquid.

3. Method of manufacture

3.1. B. licheniformis

B. licheniformis is a Gram-positive, spore-forming bacterium that is widely distributed in the environment and occurs as a contaminant in raw agricultural products. Additionally, spores from the microorganism may be found in processed or dried foods. *B. licheniformis* has been classified as a Risk Group 1 microorganism (i.e., not associated with disease in healthy adult humans) by the U.S. National Institutes of Health (NIH, 2011). It has also been granted a Qualified Presumption of Safety status by the European Food Safety Authority (EFSA). The qualification for Gram-positive sporulating bacteria such as *B. licheniformis* has been recently updated to "absence of toxigenic potential" (EFSA, 2010).

B. licheniformis has been described in the literature as a safe source of food-processing enzymes and has been used to produce these enzymes for nearly 25 years (De Boer et al., 1994; Pedersen et al., 2002; Olempska-Beer et al., 2006). In 2003, JECFA evaluated α-amylase from *B. licheniformis* and established an acceptable daily intake of "not specified" (FAO/WHO, 2003a). In 1983, the U.S. Food and Drug Administration (FDA) affirmed mixed carbohydrase and protease from *B. licheniformis* as generally recognized as safe (GRAS; 21 CFR 184.1027). In recent years, FDA has reviewed several GRAS notices for enzyme preparations derived from *B. licheniformis*, and has issued "no questions" letters in response to each of these notices. One notice, designated GRN 000072, discussed data and information supporting the safe use in food of the pullulanase enzyme preparation from *B. licheniformis* expressing the pullulanase gene from *B. deramificans*. This pullulanase enzyme preparation was originally described in GRAS affirmation petition GRP 5G0415, which was submitted to FDA in 1995 by Solvay Enzymes, Inc. (Solvay). In 1996, Solvay transferred the rights and interests in GRP 5G0415 to Genencor International Inc., and at Genencor's request, the petition was converted to GRN 000072. It should be noted that the genetic procedures that Solvay used to construct the pullulanase-producing *B. licheniformis* strain differ from those described in Genencor's dossier, which is the subject of this Chemical and Technical Assessment.

3.2. B. licheniformis production strain

The pullulanase-producing *B. licheniformis* strain was derived from *B. licheniformis* strain Bra7. Bra7 was developed from a wild-type parent strain using classical genetic improvement methods. To construct the production strain, Bra7 was modified by deleting the genes encoding amylase, chloramphenicol acetyl transferase (CAT), and the *spo*IIAC gene responsible for sporulation. The resulting strain, designated as BML612, was then transformed with a plasmid carrying: 1) an amplifiable DNA cassette containing the *cat* gene; 2) the Bra7 amylase promoter region, and 3) a signal peptide fused to the mature *B. deramificans* pullulanase gene and terminal sequences of the *cat* gene to allow integration of the cassette at the *cat* locus and subsequent amplification of the pullulanase gene. The plasmid used to introduce the

pullulanase expression cassette is not present as such in the final strain because the vector sequences have been excised by homologous recombination, leaving only the amplifiable pullulanase expression cassette including the *cat* gene. A transformant that was resistant to chloramphenicol and contained the pullulanase gene was designated as BMP61 and selected for further development. To increase pullulanase production, BMP61 was modified by deletion of genes encoding two endogenous proteases and was subsequently subjected to a gene amplification procedure to increase the number of pullulanase gene copies. The final production strain, designated BMP139 was tested to show that it contains genes encoding pullulanase and the endogenous *B. licheniformis* chloramphenicol acetyl transferase and that it does not contain plasmid DNA. The strain was also shown to be stable after culturing for 61 generations.

3.3. B. deramificans donor strain

The source of the pullulanase gene was *B. deramificans* wild-type strain TD89.117D from Solvay. The strain was isolated from a Canadian soil sample and was deposited in the Belgian Coordinated Collections of Microorganisms (LMG Culture Collection, Universiteit Gent, Lab. Voor Microbiolgie. K. L. Ledrganckstraat 35, B-9000 Gent, Belgium) as LMG P-13056. The strain was classified as a Risk Group 1 microorganism by German authorities (Zentralen Kommision för die Biologische Sicherheit (ZKBS)). Solvay used TD89.117D as the source of the pullulanase gene for the production of the pullulanase enzyme preparation that was the subject of GRN 000072. The pullulanase enzyme preparation described in GRN 000072 was evaluated for safety as reported by Modderman and Foley (1995).

3.4. Fermentation, recovery, and formulation

Pullulanase is produced by submerged, aerobic, pure culture fermentation of the genetically modified *B. licheniformis* strain. The fermentation medium consists of food-grade raw materials that provide nutrients (carbohydrates, proteins, minerals, and vitamins) and compounds used for pH control. The fermentation process is conducted under sterile conditions to prevent contamination with foreign microorganisms. Pullulanase is secreted into the fermentation broth and when its concentration is maximal, the fermentation broth is treated with lysozyme to inactivate the production organism and facilitate its removal. Cell debris is separated from the broth by filtration, centrifugation, or a combination of both processes. The liquid containing pullulanase is concentrated by ultrafiltration. The enzyme concentrate is subsequently formulated with dextrose, sodium benzoate, and potassium sorbate to achieve the desired pullulanase activity and stability.

During the secretion of pullulanase into the fermentation broth, pullulanase is cleaved in two distinct ways, resulting in the formation of pullulanase with different molecular weights. Two major bands that appear on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels have molecular weights of approximately 105,000 and 95,000. The catalytic activity of these forms appears to be equivalent.

4. Characterization

4.1. Pullulanase

Pullulanase catalyses the hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin, and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen. The Chemical Abstract Service Registry Number (CAS No.) of pullulanase is 9075-68-7. Pullulanase

is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB, online edition) as follows:

Accepted name: pullulanase

Other name(s): limit dextrinase (erroneous); amylopectin 6-glucanohydrolase; bacterial debranching enzyme; debranching enzyme; α-dextrin endo-1,6-α-glucosidase; R-enzyme; pullulan α-1,6-glucanohydrolase

Reaction: hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen

Systematic name: pullulan 6-α-glucanohydrolase

EC No.: 3.2.1.41

The activity of pullulanase is measured relative to the pullulanase standard using insoluble Red Pullulan as a substrate. The substrate is hydrolysed and solubilized by the action of pullulanase. The insoluble residue is precipitated and removed by centrifugation and the absorbance of the supernatant is measured at 510 nm. Color intensity is proportional to pullulanase activity, which is expressed in Acid Stable Pullulanase Units (ASPU). One ASPU is defined as the amount of pullulanase that liberates reducing sugars equivalent to 0.45 µmole of glucose per minute from pullulan at pH 5.0 and a temperature of 40°.

Pullulanase has been evaluated for potential allergenicity using bioinformatics criteria recommended by the Codex Alimentarius Commission (FAO/WHO, 2003b). An amino acid sequence homology search between pullulanase and known allergens listed in the SDAP database (http://fermi.utmb.edu/SDAP/index.html) was conducted. A primary search for 80-amino acid stretches with greater than 35% sequence identity yielded no matches. A secondary search for exact matches of short amino acid fragments that could serve as potential linear IgE binding sites yielded two seven-amino acid matches with non-food allergens from the German cockroach and the mold *Penicillium citrinum*. No homology was found for sequence fragments of eight contiguous amino acids. Due to insufficient data on allergenic epitopes, the seven-amino acid fragments matching allergens from the German cockroach and *P. citrinum* could not be confirmed as IgE binding sites, and may be random matches.

To further explore the allergenic potential of the short contiguous amino acid stretches, the amino acid sequence of pullulanase was analyzed for hydrophilicity with ExPaSy ProtScale software (http://www.expasy.org/cgi-bin/protscale.pl) using the Hopp and Wood hydrophilicity algorithm (Hopp and Woods, 1981). The match with the German cockroach allergen was found to be mildly hydrophilic, while the match with the *P. citrinum* allergen was strongly hydrophobic. Since neither of the matching amino acid sequences is strongly hydrophilic they are not likely to be located on the surface of the intact pullulanase, and are thus unlikely to serve as IgE binding sites.

4.2. Pullulanase enzyme preparation

The pullulanase enzyme preparation is marketed as a liquid product under the trade name OPTIMAX L - 1000. The product is standardized to 1000-1260 ASPU/g. The composition of OPTIMAX L - 1000 is variable. As an example, the composition of one batch of the pullulanase enzyme preparation is provided below:

Total Organic Solids (TOS)	4.4%
Dextrose	33.0%
Sodium benzoate	0.34%
Potassium sorbate	0.13%
Water	62.1%

The TOS content is calculated according to the following equation:

TOS (%) = 100 – (A + W + D)

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients (NAS/NRC, 1981; FAO/WHO, 2006). TOS consists of the enzyme of interest and residues of organic materials, such as proteins, peptides, and carbohydrates, derived from the production organism and the manufacturing process.

The pullulanase enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006). It does not contain significant levels of secondary enzyme activities and is free from the production strain and transformable DNA.

5. Functional uses

The pullulanase enzyme preparation is used as a processing aid in the manufacture of corn syrups, high fructose corn syrup (HFCS), potable alcohol, and beer. In the production of sweeteners from starch, pullulanase is used in conjunction with glucoamylase to saccharify starch after its gelatinization with α-amylase. The action of these enzymes allows nearly complete starch hydrolysis to monomeric glucose (>95.5%). The pullulanase enzyme preparation is typically used at levels ranging between 0.1-0.5 litre per metric ton of starch on a dry weight basis.

In a typical brewing application, the recommended dosage of the pullulanase enzyme preparation is 0.5-2.0 kg per metric ton of grist and up to 2 g/100 litres during fermentation. In the production of potable alcohol, the pullulanase enzyme preparation is used at a rate of 0.2-0.3 kg per metric ton of grist during saccharification and at 0.15 kg per metric ton of grist during fermentation.

6. Reactions and fate in food

The production of corn sweeteners, beer, and potable alcohol includes steps that would inactivate and/or remove pullulanase. Thus, the carry-over of the active enzyme to food is expected to be negligible.

7. References

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