LACCASE FROM *MYCELIOPHTHORA THERMOPHILA* EXPRESSED IN *ASPERGILLUS ORYZAE*

Chemical and Technical Assessment (Cta)

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1 Summary

Laccase is an enzyme that catalyzes the oxidation of phenolic compounds such as ortho- and para-diphenols to their corresponding quinones with the concomitant reduction of oxygen to water. The laccase described in this document is manufactured by pure culture fermentation of a genetically modified nonpathogenic and nontoxigenic strain of *Aspergillus oryzae* that contains the laccase gene derived from *Myceliophthora thermophila*. This production strain was developed from a nonpathogenic *A. oryzae* strain IFO 4177 (also known as A 1560) using recombinant DNA techniques and traditional mutagenesis. During fermentation, laccase is secreted to the fermentation broth. It is subsequently purified, concentrated, and formulated with appropriate substances. The formulated laccase, referred to below as the laccase preparation, is marketed for use in brewing beer to prevent the formation of off-flavor compounds, such as trans-2-nonenal. Laccase scavenges oxygen which otherwise would react with fatty acids, amino acids, proteins, and alcohols to form off-flavor precursors.

The laccase preparation is marketed under a trade name "Flavourstar." The manufacturer of this product, Novozymes A/S, submitted a dossier to JECFA containing detailed information about laccase and laccase preparation (Novozymes A/S, 2002). This Chemical and Technical Assessment is based on Novozymes' information as well as published information relevant to the source microorganism, A. *oryzae.*

The *A. oryzae* production strain, designated as Mt, was developed by transformation of the *A. oryzae* host strain How B711 (derived from the A 1560 strain) with two plasmids pRaMB17.WT and pToC90. The pRaMB17.WT plasmid contains the laccase gene from a thermophilic fungus *M. thermophila* that occurs in decaying manure and other organic matter. The laccase gene is linked to the DNA regulatory sequences, promoter and terminator. The pRAMB17.WT plasmid also contains the ampicillin resistance gene *bla* and other well-characterized DNA sequences. The pToC90 plasmid contains the acetamidase gene *amdS* that makes it possible for *A. oryzae* to metabolize acetamide in the absence of other sources of carbon or nitrogen. The pToC90 plasmid also contains the *bla* gene.

After incubation of the host strain with the plasmids, the transformed cells were identified by their ability to produce laccase and grow on acetamide as a sole nitrogen source. One colony was selected and subjected to chemical mutagenesis and screening for high yield of laccase. A transformant producing adequately high level of laccase was selected for use as a laccase production strain.

The DNA introduced into the production strain was well characterized using known molecular biology methods. It does not contain any sequences that would result in the production of toxic substances. It is stably integrated into the *A. oryzae* genomic DNA. Therefore, the probability of its transfer to other microorganisms is very low. Although the introduced DNA contains the *bla* gene, the gene is not expressed because it is under the control of a bacterial promoter that is not functional in *A. oryzae*. Thus, the laccase preparation does not contain the *bla* gene product, i.e., the enzyme beta-lactamase that hydrolyses and inactivates ampicillin. The laccase preparation was tested for the presence of the *bla* gene. No *bla* DNA was detected.

The laccase enzyme was assessed for potential allergenicity. The amino acid sequence of the *M. thermophila* laccase was compared to the amino acid sequences of allergens listed in publicly available protein data bases. No immunologically significant sequence homology was detected. In addition, laccases commonly occur in food and there have been no reports in the literature about allergic reactions to these enzymes. Therefore, the *M. thermophila* laccase is not likely to cause allergic reactions in individuals consuming beer (or other foods) manufactured with the use of laccase.

The laccase preparation is used in brewing beer during mashing of malt, barley, or other starch sources. It is subsequently inactivated during wort boiling and removed during subsequent stages of beer production.

2 Description

The laccase preparation is a brown liquid containing active laccase. It is formulated with appropriate substances that stabilize the enzyme and prevent microbial growth.

3 Manufacturing

3.1 Aspergillus oryzae

A. oryzae is a filamentous fungus that occurs in soil mainly in Japan and China. It has been used in Asia in the production of soy sauce, miso and sake for over 2000 years. At the beginning of the 20th century, *A. oryzae* was used as a source of the first enzyme (α-amylase) produced on an industrial scale. (Nielsen *et al*., 1994). More recently, *A. oryzae* has been used as the source of several enzymes used in food processing, including α-amylase, lipase, glucoamylase, lactase, pectin esterase, protease, and xylanase (Pariza and Johnson, 2001). Although *A. oryzae* is generally considered to be nonpathogenic and nontoxigenic, some strains are capable of producing low levels of one or more of the mycotoxins, beta-nitropropionic acid, kojic acid, and cyclopiazonic acid (Barbesgaard *et al.*, 1992).

3.2 A. oryzae host and production strains

The production strain for the laccase enzyme is the *A. oryzae* strain Mt. The strain contains the gene encoding laccase from *Myceliophthora thermophila*. *M. thermophila* is a thermophilic fungus that occurs in decaying manure, silage, wood chips, etc., in Europe and North America.

The production strain was developed by transformation of the *A. oryzae* host strain How B711 with two plasmids, pRaMB17 and pToC90. The transformed strain, designated as How B711/RaMB17.WT, was subsequently subjected to classical mutagenesis with nitrosoguanidine followed by screening to select for high yield of *M. thermophila* laccase. The selected high-yielding strain (Mt) is the source of the laccase enzyme.

The host strain How B711 is a derivative of a well-know industrial production strain *A. oryzae* (Ahlburg) Cohn that was originally obtained from the Institute for Fermentation in Osaka, Japan. The strain is known as either IFO 4177 or A1560. The host strain How B711 was obtained from *A. oryzae* A1560 by site-specific gene disruption of three endogenous TAKA amylase genes. The How B711 strain does not express TAKA amylase.

3.3 Plasmids used in the transformation of the host strain

Two plasmids, pRaMB17.WT and pToC90, were used in the transformation of the host strain How B711. The pRaMB17.WT plasmid is the laccase expression vector. It contains the laccase gene from *M. thermophila.* The pRMB17.WT plasmid also contains DNA sequences necessary for the proper expression of laccase in *A.* oryzae and for plasmid replication in the intermediate host, *Escherichia coli*. These sequences include: the TAKA-amylase promoter from *A. oryzae*; the transcription termination sequence from the *Aspergillus niger* amyloglucosidase gene; a fragment of the well-known *E. coli* vector pUC18 that contains the *E. coli* replication origin and the ampicillin resistance gene (*bla*); and four synthetic polylinker sequences interspersed between the functional DNA sequences.

The pToC90 plasmid contains the selectable marker gene *amdS* from *Aspergillus nidulans* encoding acetamidase that makes it possible for *A. oryzae* to metabolize acetamide. Thus, the *A.* oryzae cells

containing acetamidase can be selected when provided with acetamide as a sole carbon or nitrogen source. The pToC90 plasmid also contains the following DNA sequences: the promoter and terminator of the *amdS* gene from *A. nidulans* and a fragment of the *E. coli* pUC19 vector that carries the *E. coli* origin of replication and the *bla* gene.

3.4 Transformation, selection, and mutagenesis

Both plasmids, pRaMB17.WT and pToC90, were incubated with protoplasts (cells from which the cell wall has been removed) of the host strain How B711. The transformed cells were identified by growing on a medium containing acetamide as a sole nitrogen source and screened for expression of laccase. A single colony expressing laccase (transformant HowB711/RaMB17.WT) was selected for further development.

The production strain, Mt, was obtained from the HowB711/RaMB17.WT transformant by mutagenesis with nitrosoguanidine and screening for high yield of the *M. thermophila* laccase.

3.5 Characterization of the production strain

The production strain contains recombinant DNA derived from plasmids pRaMB17.WT and pToC90. The introduced DNA is well characterized and does not contain any sequences that would result in the production of toxic substances. The presence and configuration of the introduced DNA was assessed by Southern blot analysis. The transformed DNA is stably integrated into the *A.* oryzae chromosomal DNA and, as such, is poorly mobilizable for transfer to other organisms. The ampicillin resistance gene that was introduced into the production strain is not expressed because it is under the control of a bacterial promoter that is non-functional in *A. oryzae*. The production organism complies with the OECD (Organization for Economic Cooperation and Development) criteria for Good Industrial Large Scale Practice (GILSP) for microorganisms (OECD, 1992).

3.6 Fermentation, recovery, and formulation

Fermentation

Laccase is produced by submerged fed-batch pure culture fermentation of the genetically modified *A. oryzae* production strain Mt. All materials used during fermentation are standard ingredients used in the enzyme industry, such as sucrose, glucose, maltose, starch hydrolysates, potato protein, soybean meal, corn steep liquor, yeast extract, citric acid, ammonia, urea, salts (e.g., Na_2HPO_4, KH_2PO_4, K2HPO4, K_2SO_4, $(NH_4)_2SO_4$, $MgSO_4$), trace minerals (e.g., $ZnSO_4$, $NiCl_2$, $FeSO_4$, $MnSO_4$), and alkali and acids for pH adjustments (e.g., NaOH, H_3PO_4). Copper sulfate ($CuSO_4$) is added to the fermentation medium because laccase requires copper for activity. Defoaming agents, such as polypropylene glycol (P2000) or polyoxyethylene-polyoxypropylene copolymer (Pluronic PE 6100 or L61), are used if necessary.

Each batch of the fermentation process is initiated with a stock culture of the production microorganism. Before use, the stock culture is tested for identity, absence of contaminating microorganisms, and enzyme-generating ability. The stock culture is initially grown in a seed fermenter and is subsequently transferred to the main fermenter. Samples are taken from both the seed fermenter and the main fermenter at regular intervals and tested for microbial contamination by microscopy and by plating on a nutrient agar followed by a 24-48 h incubation period. The fermentation is considered "contaminated" if 1) infection is observed in two or more samples by microscopy or 2) infection is observed in two successive agar plates plated within at least six hours of each other. Any contaminated fermentation is rejected. The parameters of the fermentation process such as temperature, pH, agitation, aeration, and pressure, are strictly controlled. Laccase is secreted into the fermentation broth and its activity is periodically measured.

Recovery

The enzyme recovery process begins immediately after the fermentation has been completed. Raw materials used in the recovery process include flocculants (e.g., Superfloc A 130 or a similar product), diatomaceous earth, acids and bases for pH adjustment, and defoamers if necessary. The recovery process consists of the following steps: primary separation; concentration; and pre- and germ filtration.

The primary separation is conducted at well-defined pH and temperature ranges. The cell mass and other solids are separated from the broth using either pre-coat drum filtration or centrifugation. Diatomaceous earth is used as a filter aid. The filtrate is subsequently subjected to ultrafiltration and/or evaporation. Ultrafiltration is used to remove low molecular weight impurities and to increase the laccase activity/dry matter ratio. Evaporation is used to increase the activity while maintaining the activity/dry matter ratio. The pH and temperature are controlled during concentration. The concentrated enzyme is subjected to germ filtration to remove the residual production strain and any other microbial contaminants as well as insoluble particles derived from the fermentation. The pre-filtration step is included before germ filtration when needed.

Formulation

To ensure product stability, the liquid concentrate is blended with water, sorbitol, glucose, glycine, sodium lactate, potassium sorbate, and sodium benzoate. The laccase activity is adjusted according to the product specification.

4 Chemical Characterization

4.1 Laccase

Laccase from *M. thermophila* belongs to the family of laccases characterized by the International Union of Biochemistry and Molecular Biology (IUBMB, 1992) as follows:

EC 1.10.3.2	Laccase
Reaction:	4 Benzendiol + O_2 = 4 benzosemiquinone + 2 H_2O
Other name:	Urishiol oxidase
Systematic name:	Benzendiol:oxygen oxidoreductase
Comments:	A group of multi-copper proteins of low specificity acting on both o- and p-quinols, and often acting also on aminophenols and phenylenediamine. The semiqunone may react further either enzymatically or non-enzymatically

Laccase is also known as p-diphenol oxidase. The Chemical Abstract Service Registry number (the CAS No.) of laccase is 80498-15-3.

Laccases have broad substrate specificity and catalyze oxidation of a wide range of diphenols and other substrates with the concomitant reduction of oxygen to water. Peroxides are not produced in the reaction. Laccases require copper for activity. They commonly occur in fruits and vegetables such as potatoes, apples, bananas, peaches, and other foods of plant origin. There is no known information on allergenicity caused by laccases present in food.

M. thermophila laccase is a single polypeptide with three internal disulfide bonds and four copper atoms. Both the nucleotide sequence of the laccase gene and the amino acid sequence of laccase have been determined. The molecular mass of laccase is 85 kDa. The enzyme is active at temperatures up to 70^0C. Laccase activity is measured using siringaldazine as a substrate. Siringaldazine is oxidized in the presence of atmospheric oxygen to tetramethoxy-azo bis(methylene) quinone that is measured spectrophotometrically at 530 nm. Laccase activity is expressed in Laccase Myceliophthora Units (LAMU). One LAMU is defined as the amount of enzyme that under standard conditions (pH 7.5; 30^0C) oxidizes 1 micromole syringaldazine per minute.

Although laccases commonly occur in food and are not known to be allergenic, Novozymes compared the amino acid sequence of *M. thermophila* laccase to the amino acid sequences of known allergens listed in the SWALL (SWISSPROT and TrEMBL) and GenBank databases. According to published reports (Fuchs *et al*., 1996; Metcalfe *et al*., 1996), an immunologically significant sequence similarity requires a match of at least eight contiguous amino acid residues. Novozymes has not detected any matching eight or seven amino acids sequences. Novozymes detected only two matching six amino acid sequences identified in the SWALL database and none in the GeneBank database. These results suggest that, like other laccases present in food, the *M. thermophila* laccase is not likely to cause allergic reactions.

4.2 Laccase preparation

The laccase preparation is marketed under the trade name "Flavourstar." The product contains approximately 5.3% Total Organic Solids (TOS).[1] Other ingredients include water, sorbitol, glucose, glycine, sodium lactate, potassium sorbate, and sodium benzoate. The declared activity of Flavourstar is 1000 LAMU/g.

As noted in section **3.1**, certain strains of *A. oryzae* are capable of producing low levels of one or more of the mycotoxins, beta-nitropropionic acid, kojic acid, and cyclopiazonic acid (Barbesgaard *et al.*, 1992). The toxicity of these mycotoxins is considered to be low to moderate (Burdock and Flamm, 2000). To conform to JECFA's General Specifications for Enzyme Preparations (JECFA 2001), Novozymes tested representative batches of the concentrated laccase for the presence of beta-nitropropionic acid, kojic acid, and cyclopiazonic acid. Neither mycotoxin was detected at the detection limits of 6 mg/kg for beta-nitropropionic acid and kojic acid and 10 mg/kg for cyclopiazonic acid.

To address the question regarding DNA encoding antibiotic resistance (see JECFA 2001, "Other Considerations"), Novozymes assessed the laccase preparation for the presence of the ampicillin resistance gene (*bla*) using the dot blot assay and a radiolabeled plasmid pUC19 as a probe. The pUC19 plasmid carries the *bla* gene. No DNA was detected in the laccase preparation at the detection limit of 0.01-0.02 ng DNA (pUC19) per gram. Novozymes has not assessed the laccase preparation for the presence of the *bla* gene product, the enzyme beta-lactamase that hydrolyzes and inactivates ampicillin, because the enzyme is not expressed in the Mt production strain.

he laccase preparation is used in the production of beer to prevent the formation of off-flavor compounds. The general process for beer production involves four basic stages:

1. Mashing of malt/barley/other starch sources with fermentable sugars for production of wort
2. Separation of the wort from insoluble material
3. Boiling of the wort to sterilize it before fermentation, typically for 1.5-2 hours at temperatures 101^0C -105^0C initially without pressure and later under slight pressure.
4. Fermentation

The laccase preparation is added to the mash to scavenge dissolved oxygen and prevent the formation of off-flavor precursors. The enzyme catalyzes the oxidation of phenolic substances present in the mash with concomitant reduction of oxygen to water. Precursors to off-flavor compounds are formed during mashing as a result of oxidation of fatty acids (e.g., linoleic acid), amino acids, proteins, and alcohols by atmospheric oxygen dissolved in the mash. The oxidation reactions are likely to be catalyzed by various oxidases and a lipoxygenase present in the mash. The conversion of the off-flavor precursors to off-flavor compounds occurs during subsequent stages of beer production or even during storage. Trans-2-nonenal has been identified as one of the major off-flavor compounds. It results in a papery taste and has a taste threshold of 0.06-0.11 micrograms/kg. The use of laccase effectively reduces the level of trans-2-nonenal in beer. A typical dosage of the laccase preparation is up to 0.35 kg per ton of malt or barley equivalent to up to 350 thousand LAMU per ton of malt or barley. One ton of malt or barley yields approximately six thousand liters of beer.

5 Reactions and Fate in Foods

Laccase is active only during mashing of malt and barley. It catalyzes the oxidation of phenolic substances present in the mash. The oxidized phenolics undergo polymerization and are removed during later stages of brewing. Laccase itself is denatured and inactivated during wort boiling. Barley and malt have their own endogenous laccases. These laccases are also denatured during wort boiling. Thus, no active laccase and no unusual reaction products would be present in beer processed with laccase.

[1] TOS is defined as the sum of the organic compounds present in the enzyme preparation and is calculated according to the following formula: TOS (%) = 100 – A – W – D, where A = % ash; W = % water; and D = % diluents, stabilizers, or carriers.

6 References

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