

# ALPHA-AMYLASE FROM BACILLUS LICHENIFORMIS CONTAINING A GENETICALLY ENGINEERED ALPHA- AMYLASE GENE FROM B. LICHENIFORMIS (THERMOSTABLE)

Chemical and Technical Assessment (CTA)

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## 1 Summary

The  $\alpha$ -amylase described in this document is a genetically engineered enzyme that is thermo-stable and active at a relatively low pH and low calcium concentration. These characteristics make the enzyme particularly suitable for use in starch hydrolysis conducted at high temperatures, for example, for starch liquefaction in the production of nutritive sweeteners from starch. The genetically engineered  $\alpha$ -amylase is manufactured by pure culture fermentation of the nonpathogenic and nontoxigenic *Bacillus licheniformis* production strain. The enzyme is subsequently purified, concentrated, and formulated with appropriate substances. The formulated  $\alpha$ -amylase is referred to below as the  $\alpha$ -amylase preparation.

The  $\alpha$ -amylase preparation is currently marketed under a trade name Liquozyme X. The manufacturer of these products, Novozymes A/S, submitted a dossier to JECFA containing detailed information about the  $\alpha$ -amylase and  $\alpha$ -amylase preparation (Novozymes A/S, 2002). This Chemical and Technical Assessment is based on Novozyme's dossier.

The  $\alpha$ -amylase was developed from another thermo-stable  $\alpha$ -amylase (Termamyl LC) by changing four amino acids in the polypeptide chain. These modifications were accomplished by introducing appropriate mutations into the DNA sequence encoding the Termamyl LC  $\alpha$ -amylase. The engineered gene, designated as the LE399  $\alpha$ -amylase gene, was introduced into the host strain SJ5550.

The host strain was developed from a parent strain DN2717, a derivative of a natural *B. licheniformis* isolate. The DN2717 strain was genetically engineered to inactivate the following native genes: the *apr* gene encoding the "Alkalase" protease; the *amyL* gene encoding the "Termamyl"  $\alpha$ -amylase; the *xyl* gene encoding xylose isomerase; and the *gnt* gene encoding gluconate permease. Three copies of the LE399  $\alpha$ -amylase gene were successively inserted in place of the inactivated *amyL*, *xyl*, and *gnt* genes. In a separate step, the gene encoding C-component protease was deleted. The resulting strain was designated as MOL2083 and used as a production strain.

The DNA introduced into the production strain is well characterized and does not contain genes that would result in the production of toxic or undesirable substances. The LE399  $\alpha$ -amylase gene is stably integrated into the *B. licheniformis* chromosome. Therefore, the probability of its transfer to other microorganisms is low. The production strain does not contain genes encoding antibiotic-inactivating proteins. Thus, neither antibiotic-inactivating proteins, nor DNA encoding such proteins would be present in the  $\alpha$ -amylase enzyme preparation.

The LE399  $\alpha$ -amylase was assessed for potential allergenicity. Its amino acid sequence was compared to the amino acid sequences of known allergens listed in publicly-available protein databases. No immunologically significant sequence homology was detected. This result suggests that the LE399  $\alpha$ -amylase is not likely to cause allergic reactions in individuals consuming food manufactured with the use of the LE399  $\alpha$ -amylase. In addition, the LE399  $\alpha$ -amylase is functionally equivalent to other  $\alpha$ -amylases used in food as well as  $\alpha$ -amylases naturally present in food. Alpha-amylases present in food have not been reported to cause allergy.

The  $\alpha$ -amylase preparation is intended for use in starch liquefaction in the production of sweetener syrups, as well as in the production of alcoholic beverages and beer. The LE399  $\alpha$ -amylase is not expected to be carried over to syrups. The absence of the  $\alpha$ -amylase protein in the final (purified) syrup was experimentally confirmed. The LE399  $\alpha$ -amylase would not be carried over to alcoholic beverages because ethanol is removed from the fermentation mash by distillation. In brewing beer, the LE399  $\alpha$ -amylase is active in the mash. It would be subsequently inactivated during wort boiling and removed during beer purification. In conclusion, no residues of the LE399  $\alpha$ -amylase are expected to be present in food processed with the use of the LE399  $\alpha$ -amylase preparation.

## 2 Description

The  $\alpha$ -amylase preparation is a brown liquid with a density of about 1.25 g/ml. It contains active  $\alpha$ -amylase and other components that stabilize the enzyme and prevent microbial growth.

## 3 Manufacturing

### 3.1 Construction of the host strain

A *Bacillus licheniformis* strain SJ1707 was used to develop the host strain for the genetically engineered  $\alpha$ -amylase LE399 gene.

The SJ1707 strain was developed from a parent strain DN2717 that is a sporulation-deficient derivative of a natural isolate. The DN2717 strain naturally produces a subtilisin-type protease, known as “Alkalase” and an  $\alpha$ -amylase, known as “Termamyl.” The strain has been used in the production of enzymes since 1972. The strain has also been used to develop a number of amylase production strains through genetic engineering.

The DN2717 strain was genetically modified to inactivate genes coding for “Alkalase” protease and “Termamyl”  $\alpha$ -amylase. The “Alkalase” gene (*apr*) was inactivated by inserting a chloramphenicol resistance gene, *cat*. The  $\alpha$ -amylase gene (*amyL*) was replaced with a fusion gene consisting of the  $\alpha$ -amylase signal peptide sequence and the *Thermoanaerobacter* cyclodextrin glucanotransferase (CGTase) coding sequence (*cgtA*). Subsequently, the chloramphenicol resistance gene, *cat*, was removed by replacing it with an “Alkalase” gene inactivated by an internal deletion.

The resulting strain SJ1707 is CGTase positive, “Termamyl”  $\alpha$ -amylase negative, “Alkalase” protease negative, and chloramphenicol sensitive. The SJ1707 strain was subsequently modified by introduction of deletions in the *xyl* gene encoding xylose isomerase and the *gnt* gene encoding gluconate permease. The inactivation of these genes resulted in the loss of the ability to utilize xylose and gluconate as carbon sources. The modified strain containing these deletions (designated as SJ5550) requires either xylose or gluconate for growth in the absence of other carbon sources. The SJ5550 was used as a host for the LE399  $\alpha$ -amylase gene.

### 3.2 Construction of the $\alpha$ -amylase gene

The LE399  $\alpha$ -amylase is an enzyme with improved specificity and specific activity as well as stability at low pH and low calcium concentration at high temperatures in comparison with other  $\alpha$ -amylases.

Alpha-amylases used in food processing require calcium ions for stability. In industrial applications, such as starch processing, up to 40 mg/kg calcium is added when wild-type (naturally-occurring)  $\alpha$ -amylases are used. Calcium must be subsequently removed to avoid fouling of evaporators and inactivation of glucose isomerase used in the production of fructose syrup. This is done by ion exchange and results in a large volume of wastewater high in salts.

The starting gene for the development of the LE399  $\alpha$ -amylase was the modified *B. licheniformis*  $\alpha$ -amylase gene used in the production of Novozyme’s product Termamyl LC (Novo Nordisk, 1999). The Termamyl LC gene was constructed by combining selected DNA sequences from the *Bacillus amyloliquefaciens*  $\alpha$ -amylase gene and the wild-type *B. licheniformis*  $\alpha$ -amylase gene and by introducing mutations (via site directed mutagenesis) that resulted in five amino acid substitutions. The Termamyl LC gene was modified by introduction of additional mutations. The mutated gene was designated as the

LE399  $\alpha$ -amylase gene. The introduced mutations resulted in substitutions at four additional amino acid positions as compared with the Termamyl LC  $\alpha$ -amylase. These changes improved performance of the LE399 enzyme and increased its stability at low pH and low calcium concentration at high temperatures.

The LE399  $\alpha$ -amylase coding sequence is associated with the *amyL* promoter (variant P<sub>4199</sub>) and *amyL* terminator both derived from the *B. licheniformis*  $\alpha$ -amylase (*amyL*) gene. Only well-known bacterial vectors were used in the construction of the LE399 gene.

The modified LE399 *B. licheniformis*  $\alpha$ -amylase amino acid sequence has been compared with the amino acid sequences of other naturally occurring *B. licheniformis*  $\alpha$ -amylases. The homology was found to be within the range of natural sequence variation of *B. licheniformis*  $\alpha$ -amylases.

### 3.3 Construction and characterization of the production strain

To construct the  $\alpha$ -amylase production strain, three copies of the LE399  $\alpha$ -amylase gene were successively introduced into the chromosome of the SJ5550 host strain using conjugative plasmids carrying the LE399 gene flanked by either *xyl*, *gnt*, or *amyL* *B. licheniformis* chromosomal DNA sequences. As expected, the integration occurred at the *xyl*, *gnt*, and *amyL* loci by homologous recombination. In a separate step, the gene encoding C-component protease was replaced by a deletion and thereby inactivated. The plasmids used for insertion of the LE399  $\alpha$ -amylase gene and for inactivation of the C-component protease gene were constructed from known bacterial plasmids. The details of plasmid construction are complex and are not described here.

The production strain, designated as MOL2083, is “Alkalase” protease negative and C-component protease negative. It contains three copies of the modified *B. licheniformis*  $\alpha$ -amylase gene (LE399 gene). The identity and location of the three inserted copies of the LE399 gene were verified by PCR (Polymerase Chain Reaction) amplification followed by DNA sequencing of the amplified fragments. In addition, the presence and configuration of the inserted copies were determined by Southern blot analysis. The inserted copies of the LE399 gene are stably integrated into the host strain chromosome and are, therefore, poorly mobilizable for genetic transfer to other microorganisms. The production strain does not contain antibiotic resistance genes or any other selectable marker genes.

### 3.4 Fermentation, recovery and formulation

#### *Fermentation*

The LE399  $\alpha$ -amylase is produced by submerged fed-batch pure culture fermentation of the modified *Bacillus licheniformis* strain MOL2083. The raw materials used in the fermentation include: corn steep liquor; potato protein; soybean meal or soy grits; glucose or sucrose; ammonium sulfate; sodium and potassium phosphates; citric acid; and calcium carbonate. For pH control, the following substances may be used: ammonia; sodium carbonate; sodium and potassium hydroxide; and phosphoric acid. If necessary, defoaming agents are used. Examples of suitable defoamers include:

Polyoxyethylene-polyoxypropylene copolymer (Pluronic PE 6100 or L61)

Polypropylene glycol (Pluriol 2000)

Polyalcoxyether (Clerol 3003)

Each batch of the fermentation process is initiated with the stock culture of the production strain. 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) contaminating microorganisms are found in two or more samples by microscopy or 2) contaminating microorganisms are observed on 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. During fermentation,  $\alpha$ -amylase is secreted to the fermentation broth and its activity is periodically measured.

### Recovery

After the fermentation has been completed, the fermentation broth is processed to purify and concentrate  $\alpha$ -amylase. Raw materials used in the recovery process include flocculants such as:

Calcium chloride

Aluminates (sodium aluminate or poly(aluminum hydroxy) chloride)

Anionic polyacrylamide (Superfloc A-130)

Cationic polyamine (Superfloc C-521)

Dimethyldiallylammonium chloride polymer (Cat-Floc L)

Other substances used in enzyme recovery include pH-controlling substances such as acetic acid, phosphoric acid, and sodium and potassium hydroxide. Defoamers, such those listed in section 3.4.1, are used only if necessary. Diatomaceous earth is used as a filter aid.

The recovery process consists of the following steps: primary separation; concentration; and pre- and germ filtration. Before primary separation, the fermentation broth is diluted with water. Calcium chloride and sodium aluminate are added to precipitate carbohydrate impurities. Subsequently, the pH is adjusted and flocculating agents are added. The cell mass and other flocculated solids are separated from the broth using well-known techniques such as centrifugation, pre-coat drum filtration, or fluid pressure filtration.

The filtrate is subsequently subjected to ultrafiltration or evaporation. Ultrafiltration is used to remove low molecular weight impurities and to increase the  $\alpha$ -amylase activity/dry matter ratio. Evaporation is used to increase the enzyme 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 fermentation. Pre-filtration is included when needed.

### Formulation

In order to stabilize the enzyme and prevent microbial degradation, the liquid concentrate is blended with water, sucrose or glucose, methionine, and sodium chloride. The  $\alpha$ -amylase activity is adjusted according to the declared product activity. The final enzyme preparation is analyzed to verify its conformance to the product specifications.

## 4 Chemical Characterization

### 4.1 Alpha-amylase

Alpha-amylase is described by the International Union of Biochemistry and Molecular Biology (IUBMB, 1992) as follows:

EC 3.2.1.1.  $\alpha$ -amylase

Reaction: Endohydrolysis of 1,4- $\alpha$ -D-glucosidic linkages in polysaccharides containing three or more 1,4- $\alpha$ -linked D-glucose units

Other name: Glycogenase

Systematic name: 1,4- $\alpha$ -D-Glucan glucanohydrolase

Comments: Acts on starch, glycogen and related polysaccharides and oligosaccharides in a random manner; reducing groups are liberated in the  $\alpha$ -configuration

The Chemical Abstract Service Registry number (the CAS No.) of  $\alpha$ -amylase is 9000-90-2.

The LE399  $\alpha$ -amylase is functionally equivalent to other  $\alpha$ -amylases. However, it distinguishes itself from other  $\alpha$ -amylases with improved performance and increased stability at high temperatures, low pH, and low concentration of calcium ions. Because of these characteristics, the LE399  $\alpha$ -amylase is particularly suitable for starch hydrolysis conducted at high temperatures, for example, in the production of glucose or fructose syrups. In these processes, the LE399  $\alpha$ -amylase is used in the liquefaction of starch, a process that is conducted initially at temperatures of 105-110°C, pH 5.1, and a calcium concentration of 5 mg/kg starch. The LE399  $\alpha$ -amylase was designed to avoid bonds near  $\alpha(1\rightarrow6)$  branch points in amylopectin.

When such bonds are hydrolyzed, the branch points are not recognized by pullulanase during saccharification of the liquefied starch. As a result, starch hydrolysis is incomplete.

The LE399  $\alpha$ -amylase activity is determined relative to an  $\alpha$ -amylase standard with known activity and is expressed in Kilo Novo  $\alpha$ -amylase Units (KNU(T)). One KNU(T) is the amount of  $\alpha$ -amylase which, under standard conditions (pH 7.1; 37°) dextrinizes 5.26 g starch dry substance (Merck Amylum soluble No. 9947275 or equivalent) per hour. In practice, the  $\alpha$ -amylase activity is measured using ethylidene-G<sub>7</sub>-PNP as a substrate. Ethylidene-G<sub>7</sub>-PNP is 4,6-ethylidene(G<sub>7</sub>)-*p*-nitrophenyl(G<sub>1</sub>)- $\alpha$ ,D-maltoheptaoside. The compound is hydrolyzed by the LE399  $\alpha$ -amylase to G<sub>2</sub>-PNP and G<sub>3</sub>-PNP where G means glucose and PNP means *p*-nitrophenol. G<sub>2</sub>-PNP and G<sub>3</sub>-PNP are subsequently hydrolyzed by  $\alpha$ -glucosidase, which is added to the reaction mixture, to glucose and *p*-phenol. The latter compound is measured spectrophotometrically at 409 nm under standard reaction conditions. One KNU(T) corresponds to the amount of  $\alpha$ -amylase that hydrolyzes 672 micromoles of ethylidene-G<sub>7</sub>PNP per minute under standard conditions (pH 7.1; 37°). The quantification limit of the method is approximately 0.3 KNU(T)/g.

Although  $\alpha$ -amylases are present in food and are not known to be allergenic when ingested, Novozymes assessed potential allergenicity of the LE399  $\alpha$ -amylase by comparing its amino acid sequence to the amino acid sequences of known allergens listed in the SWALL database. 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 amino acid sequences. Novozymes detected only one matching seven amino acid sequence and four matching six amino acid sequences. These results suggest that, like other  $\alpha$ -amylases, the LE399  $\alpha$ -amylase is not likely to cause allergic reactions.

## 4.2 Alpha-amylase preparation

The  $\alpha$ -amylase preparation is currently available as Liquozyme X. The product contains 8% Total Organic Solids (TOS)<sup>1</sup>. Other ingredients include water, sucrose and/or glucose, sodium chloride, and the amino acid methionine. A declared activity of Liquozyme X is 200 KNU(T)/g.

The  $\alpha$ -amylase preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing prepared at the 57<sup>th</sup> JECFA (2001) and published in FNP 52 (Addendum 9) as well as to the General and Additional Requirements set forth in the Food Chemicals Codex, 3<sup>rd</sup> supplement to the 4<sup>th</sup> edition (2001).

## 5 Functional Use

The  $\alpha$ -amylase preparation is used in starch liquefaction that is the initial step in starch processing for production of starch-based sweeteners. The LE399  $\alpha$ -amylase catalyzes the hydrolysis of amylose and amylopectin to soluble dextrans and oligosaccharides. In subsequent production steps, the dextrans and oligosaccharides are hydrolyzed to glucose by other enzymes. Glucose syrup is either used as a final product or is converted to fructose syrup through enzymatic isomerisation of glucose to fructose.

The  $\alpha$ -amylase preparation is also used for starch liquefaction in the production of alcohol and in brewing beer. It is also used in the production of sugar to break down the starch present in sugar cane juice. Reducing starch content facilitates the filtration of the juice.

## 6 Reactions and Fate in Food

The LE399  $\alpha$ -amylase is an enzyme that performs the same enzymatic function in food processing as other  $\alpha$ -amylases. It catalyzes the hydrolysis of starch components, amylose and amylopectin, to dextrans and oligosaccharides. In the production of sweeteners, the hydrolysis is continued to completion using other enzymes. The final hydrolysis product is glucose. Glucose can be subsequently isomerised to fructose. The final food products are either glucose or fructose syrups or the corresponding crystalline products.

The LE399  $\alpha$ -amylase is likely to be inactivated by the pH reduction at the end of starch liquefaction. The residues of the enzyme are removed during subsequent stages of syrup purification such as clarification,

<sup>1</sup> Total Organic Solids 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.

filtration, and ion exchange or carbon treatment. This has been confirmed by immunochemical analysis using ELISA (Enzyme-Linked Immunosorbent Assay). No  $\alpha$ -amylase protein was found in the final syrup at the detection limit of 1.4 ng/g (Novozymes A/S, 2001).

In the production of alcoholic beverages, ethanol is separated from the fermented mash by distillation. The LE399  $\alpha$ -amylase is inactivated during this process and remains in the mash. In beer production, the enzyme is inactivated during wort boiling and removed during subsequent purification steps.

In summary, no residues of the LE399  $\alpha$ -amylase are expected to be present in foods processed with the use of the LE399  $\alpha$ -amylase preparation.

## 7 References

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