XYLANASES FROM *BACILLUS SUBTILIS* EXPRESSED IN *B. SUBTILIS*

CHEMICAL AND TECHNICAL ASSESSMENT (CTA)

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1 Summary

Xylanase is an enzyme that catalyzes the hydrolysis of 1,4-beta-D-xylosidic linkages in xylans that are constituents of hemicellulose, a structural component of plant cell walls. Arabinoxylans (also known as pentosans) are highly branched xylans that occur in wheat and rye flour. Several xylanases from fungal and bacterial sources are currently marketed for use in baking (Amfep; Pariza and Johnson, 2001). Xylanases described in this Chemical and Technical Assessment are derived from genetically engineered nonpathogenic and nontoxigenic strains of *Bacillus subtilis.* The information about these enzymes is provided in a dossier submitted to JECFA by the sponsor, Danisco, Inc. (Danisco, 2003).

The xylanase-encoding gene was isolated from the wild-type *B. subtilis* strain 168 and re-introduced into *B. subtilis* using recombinant DNA technology. Three recombinant production strains of *B. subtilis* were created by transformation of the *B.subtilis* host strain with an appropriate transformation vector. Two of these strains express xylanases BS1 and BS2 that are identical to the native (wild-type) *B. subtilis* xylanase A derived from strain 168. The third strain expresses xylanase BS3 that differs from the wild-type enzyme by two amino acids and is resistant to the xylanase inhibitor present in flour.

The host strain was developed from the well-characterized nonpathogenic and nontoxigenic *B. subtilis* wild-type strain 168. Three transformation vectors were constructed on the basis of the commonly used plasmid pUB110. The vector encoding xylanase BS1 contains two antibiotic resistance genes that confer resistance to kanamycin/neomycin and phleomycin. The vector encoding xylanase BS2 was genetically engineered to remove the antibiotic resistance genes. The vector encoding the modified xylanase BS3 is also devoid of the antibiotic resistance genes.

The xylanase production strains contain multiple copies of the respective transformation vectors. All of the DNA introduced into the xylanase production strains is well-characterized and is not expected to result in the production of any toxic or undesirable substances.

Each xylanase is produced by pure culture fermentation of the respective production strain. Xylanase is secreted into the fermentation medium from which it is subsequently recovered, concentrated, and formulated. The formulated xylanase is referred to below as the xylanase preparation. Two xylanase products, GRINDAMYL^{TM} H 640 that contains xylanase BS2 and GRINDAMYL POWERBake 900 that contains the modified xylanase BS3, are currently marketed. The sponsor has no plans to market xylanase BS1.

Xylanases naturally present in food and xylanases used in food processing as enzymes have not been reported to cause allergic reactions. By analogy, the *B. subtilis* xylanases discussed in this document are not likely to cause allergic reactions after ingestion of food containing the residues of these enzymes. The recombinant *B. subtilis* xylanases are unstable at temperatures above 50^{o} and are expected to be inactivated during baking or cooking.

2 Description

The xylanase preparation is an off-white non-dusting microgranulate or a brownish liquid. It is formulated with appropriate substances suitable for use in food such as starch and salt. The solid preparation can also be prepared in the form of tablets.

3 Manufacturing

3.1 Bacillus subtilis production strains

B. subtilis is a nonpathogenic and nontoxigenic bacterium that has been used as a source of enzymes used in food for many years. Examples of enzymes derived from *B. subtilis* include alpha-amylase, beta-glucanase, glutaminase, maltogenic amylase, protease, pullulanase, and xylanase (Pariza and Johnson, 2001). Some of these enzymes are derived from genetically engineered production strains. The natural properties of *B. subtilis* include sporulation, which occurs under nutritional deprivation, and production of extracellular proteases that can degrade the enzyme of interest. As both properties interfere with the production of enzymes, *B. subtilis* strains have been modified to eliminate sporulation and minimize the synthesis of extracellular proteases (Harwood, 1992). Other modifications may also be introduced, such as a requirement of a specific nutrient for growth.

The *B. subtilis* host strain used by the sponsor to produce the recombinant xylanases has the following characteristics:

- Is devoid of the chromosomal alpha-amylase gene
- Is asporogenic
- Produces low levels of extracellular proteases
- Requires thiamine for optimal growth
- Is unable to use nitrate as a nitrogen source

The stability of the asporogenic phenotype was confirmed by assessing the ability of the strain to form spores in repeatedly re-inoculated cultures incubated at 80^{o} for 20 minutes. No surviving cells were found. That means that the host strain is incapable of spore formation (and survival) under adverse conditions represented by the heat treatment procedure typically used by microbiologists to assess sporulation.

The host strain was transformed with three different vectors to create three production strains used as sources of xylanases BS1, BS2, and BS3. Each strain contains multiple copies of the vector per cell. Therefore, xylanase is produced at substantially higher levels than by the native *B. subtilis* strain that contains a single chromosomal xylanase gene. The vector is not integrated into the *B. subtilis* genome and is propagated as an extrachromosomal DNA.

3.2 Xylanase gene

The gene encoding xylanase was isolated from a well-characterized *B. subtilis* strain 168 widely used in research and industrial applications. This xylanase gene (known as xylanase A gene) was introduced into the *B. subtilis* host strain using two separate procedures that resulted in two production strains expressing xylanases BS1 and BS2. These xylanases are identical to each other and to the native xylanase A from strain 168. In addition, the xylanase gene was genetically modified and introduced into the *B. subtilis* host strain to obtain the production strain expressing xylanase BS3. The modified xylanase BS3 differs from the native xylanase by two amino acids and is resistant to inhibition by the xylanase inhibitor present in flour.

3.3 Transformation vectors and transformation of B. subtilis host strain

Three transformation vectors were constructed on the basis of a completely sequenced and extensively characterized plasmid pUB110. The pUB110 plasmid contains DNA sequences encoding proteins with the following functions:

- Kanamycin/neomycin resistance
- Phleomycin resistance
- Primary replication initiation
- Mobilization

The protein that provides resistance to kanamycin and neomycin is an enzyme that inactivates these antibiotics by phosphorylation. The phleomycin resistance protein binds to phleomycin and thereby neutralizes the effects of this antibiotic. The primary replication initiation protein (known as ORF alpha) is the DNA binding protein that initiates the copying of the plasmid. The mobilization protein (known as ORF beta) enables the transfer of pUB110 from one *Bacillus* strain to another with the participation of two other plasmids naturally present in *B. subtilis*.

To construct the xylanase transformation vectors the pUB110 plasmid was modified as described below in sections 3.3.1 – 3.3.3.

Transformation vector for xylanase BS1

The transformation vector for xylanase BS1 was constructed as follows: the BS1 xylanase expression cassette containing the *B. stearothermophilus* alpha amylase promoter and the *B. subtilis* xylanase A gene was inserted into the pUB110 plasmid by replacing a large portion of the mobilization gene. Therefore, the mobilization gene could not be expressed and the plasmid could not be tramsferred to other *B. subtilis* strains. Such modified vector was then introduced into the *B. subtilis* host strain by transformation. The transformed cells were selected on an antibiotic-containing medium. Although the antibiotic resistance proteins are expressed in the xylanase BS1 production strain, they remain within bacterial cells and are not carried over to the final xylanase preparation.

Transformation vector for xylanase BS2

The vector used to construct the production strain for xylanase BS2 was developed from the transformation vector carrying the xylanase BS1 gene by deleting substantial portions of the antibiotic resistance genes. Following this modification, the vector was transformed into the *B. subtilis* host strain in the same manner as the BS1 vector. As the BS2 vector does not express antibiotic resistance genes, the transformed cells were identified by their high production of xylanase.

Transformation vector for xylanase BS3

The transformation vector for xylanase BS3 was constructed using the same steps as in the construction of the transformation vector for xylanase BS2. However, instead of the xylanase BS2 gene, the modified BS3 gene was inserted. The vector does not express the antibiotic resistance genes.

4 Fermentation, recovery, and formulation

Xylanases BS1, BS2, and BS3 are produced by submerged fermentation of the respective production strains using a fermentation medium composed of food grade materials. The xylanases are secreted to the fermentation broth. After the fermentation has been completed, the broth is filtered on a rotary vacuum drum filter to remove the cells of the production strain. The clear liquid is subjected to ultrafiltration to concentrate and purify xylanase. The remaining microorganisms are removed by microfiltration. The enzyme concentrate is then either spray-dried on a suitable carrier, for example, a mixture of salt and starch, or formulated as a liquid preparation. The solid preparation can be converted into tablets. The xylanase activity is measured and, if necessary, adjusted according to the declared product activity. The final enzyme preparation is analyzed to verify its conformance to the product specifications.

5 Chemical characterization

5.1 Xylanase

Xylanase is described by the International Union of Biochemistry and Molecular Biology (IUBMB, Web Version) as follows:

Common name: endo-1,4-beta-xylanase

Reaction: Endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans

Other name(s): endo-(1→ 4)-beta-xylanase; (1→ 4)-beta-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; beta-1,4-xylanase; endo-1,4-xylanase; endo-beta-1,4-xylanase; endo-1,4-beta-D-xylanase; 1,4-beta-xylan xylanohydrolase; beta-xylanase; beta-1,4-xylan xylanohydrolase; endo-1,4-beta-xylanase; beta-D-xylanase

Systematic name: 1,4-beta-D-xylan xylanohydrolase

The Chemical Abstract Service Registry number (the CAS No.) of xylanase is 9025-57-4. Sometimes, xylanase is referred to as hemicellulase or pentosanase.

As noted in section 3.2., xylanases BS1, BS2, and the wild-type *B. subtilis* xylanase A are identical, i.e., have the same amino acid sequence. Xylanase BS3 is encoded by the gene that was subjected to site-specific mutagenesis in order to introduce two amino acid substitutions in the xylanase amino acid sequence. The purpose of these modifications was to reduce binding of the xylanase inhibitor to xylanase and prevent the inhibition of xylanase activity. Comparative studies on the effects of the xylanase inhibitor on the activity of five different microbial xylanases including the BS1 and BS3 xylanases showed that the BS3 xylanase was the only enzyme unaffected by the xylanase inhibitor.

The identity of the recombinant xylanases was confirmed by N-terminal sequencing and molecular weight determination. Molecular weight was determined by mass spectrometry for xylanase BS1 and by SDS-PAGE for xylanase BS3.

The xylanase activity is determined by measuring the rate of hydrolysis of the azurine-crosslinked wheat arabinoxylan substrate. Xylanase hydrolyses the substrate to water-soluble fragments with the concomitant change in color. The reaction is terminated after a designated time and the optical density (OD) of the reaction mixture is measured at 590 nm. Xylanase activity is calculated in xylanase units per gram of the enzyme preparation. One xylanase unit (XU) is defined as the amount of the enzyme that increases the OD_{590} at a rate of one OD per 10 minutes under standard conditions (pH=5.00; 40^{o}).

The xylanase activity can also be determined relative to the xylanase standard with known activity, as described in the dossier for xylanase (Danisco, 2003), and expressed either in total endo-xylanse units (TXU) or Danisco endo-xylanase units (DXU). The relationship between all these units is: 1XU = 5.43 TXU = 131 DXU. The specific activity of xylanase BS1 is 25000 TXU/mg enzyme protein and that of xylanase BS3 is 8400 TXU/mg enzyme protein.

Some enzymes manufactured for use in food were reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities. As other enzymes, xylanase may also cause occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the xylanase residues in food (mainly baked goods) seems remote. In 1998, the Association of Manufacturers of Fermentation Enzyme Products (currently Association of Manufacturers and Formulators of Enzyme Products) Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products. The Group concluded that there were no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers, and that the enzyme residues in food do not represent any unacceptable risk to consumers (Amfep).

Xylanases of fungal and bacterial origin (including *B. subtilis* wild-type xylanase) have been used in food for a number of years. We have no knowledge of any reports of allergic reactions to the residues of xylanase in food as well as to the residues of other enzymes used in food processing.

5.2 Xylanase preparation

The xylanase preparation may be sold as microgranulates, tablets, or as a liquid preparation. Both solid and liquid forms are formulated with appropriate ingredients such as stabilizers, preservatives and carriers, which are suitable for use in food. Two xylanase products are currently marketed: GRINDAMYL™ H 640 that contains xylanase BS2 and GRINDAMYL POWERBake 900 that contains the modified xylanase BS3. The products conform to the General Specifications and Considerations for Enzyme Preparations Used in Food processing prepared at the 57th JECFA and published in FNP 52, Addendum 9 (JECFA, 2001). The products also conform to the General and Additional Requirements set forth in the Food Chemicals Codex, 5th edition (FCC, 2003).

6 Functional use

Both fungal and bacterial xylanases degrade arabinoxylans present in flour into mono- and oligosaccharides. They hydrolyze the beta-(1,4)-xylosidic linkages in the arabinoxylan backbone. The *B. subtilis* xylanases BS2 and BS3 discussed in this CTA are used in bakery applications. The enzyme is added to the flour and remains active during the dough preparation and the leavening of the unbaked bread. Its use provides several benefits including:

- Increased tolerance towards variations in process parameters
- Reduced stickiness of the dough
- Increased volume of the final bread

The use of xylanase BS3 is advantageous in comparison to xylanse BS2 because the same technical effects can be accomplished at lower use levels (based on enzyme activity) due to resistance of the BS3 enzyme to the inhibition by the xylanase inhibitor. Typical use levels on a weight basis are 0.12-1.6 mg xylanase/kg flour (equivalent to 3000-40000 TXU/ kg flour) for xylanase BS2 and 0.06-1.6 mg/kg flour (equivalent to 500-13300 TXU/kg flour) for xylanase BS3.

7 Reactions and fate in food

Xylanases BS2 and BS3 are sensitive to heat and would be inactivated during baking or cooking. Experiments performed with xylanase BS3 showed that the enzyme is active at temperatures below 50^{o}. Above 50^{o}, the enzyme is rapidly inactivated. Therefore, no active enzyme would be present in the finished food.

8. References

Amfep. Association of Manufacturers and Formulators of Enzyme Products. http://www.amfep.org

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