GR

ORIGINAL SUBMISSION

novozymes®

REC'D MAR 3 2006

March 1, 2006

Linda S. Kahl, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Kahl,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' mixed beta-glucanase, xylanase enzyme preparation produced by *Humicola insolens*. The enzyme preparation is intended for use in the brewing industry.

Please contact me by direct telephone at 919 494-3151, direct fax at 919 494-3420 or email at lobg@novozymes.com if you have any questions or require additional information.

Sincerely,

Lori Gregg
Regulatory Specialist

Enclosures (3 binders)

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel· 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

February 28, 2006

novozymes®

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R.§ 170.36 (c)(1) Novozymes North America Inc. hereby claims that mixed beta-glucanase, xylanase preparations produced by submerged fermentation of *Humicola insolens* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Mixed beta-glucanase, xylanase enzyme preparation from *Humicola insolens.*

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

The enzyme preparation is intended for use in the brewing industry as a processing aid. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed §170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

28 February 2006
Date

John Carroll
Director, Regulatory Affairs

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

File No. 2006-12946-01

novozymes®

REC'D MAR 3 2006

Mixed beta-glucanase, xylanase enzyme preparation, produced by a strain of *Humicola insolens*

Lori Gregg, Regulatory Affairs, Novozymes North America Inc., USA
Birger Rostgård Jensen, Regulatory Affairs, Novozymes A/S, Denmark

March 2006

novozymes®

TABLE OF CONTENTS

		PAGE
1.	**GENERAL INTRODUCTION**	3
2.	**PRODUCTION MICROORGANISM**	3
2.1	Production Strain	3
2.2	Absence of the Production Organism in Product	4
3.	**MANUFACTURING PROCESS**	4
3.1	Raw Materials	4
3.2	Fermentation Process	5
3.3	Recovery Process	5
3.4	Quality Control of Finished Product	6
4.	**ENZYME IDENTITY**	6
5.	**COMPOSITION AND SPECIFICATIONS**	**7**
5.1	Quantitative Composition	7
5.2	Specifications	7
6.	**APPLICATION**	**8**
6.1	Mode of Action	8
6.2	Use Levels	9
6.3	Enzyme Residues in the Final Food	9
7.	**SAFETY EVALUATION**	**9**
7.1	Safety of the Production Strain	9
7.2	Safety of the β-glucanase and Xylanase Enzymes	10
7.3	Safety of the Manufacturing Process	11
7.4	Safety Studies	11
7.5	Estimates of Human Consumption and Safety Margin	12
7.6	Results and Conclusions	13
8.	**LIST OF APPENDICES**	**14**
9.	**LIST OF REFERENCES**	**15**

novozymes®

1. GENERAL INTRODUCTION

The subject of this notification is a mixed beta-glucanase and xylanase enzyme preparation (Novozymes A/S trade name Ultraflo® L) produced by submerged fermentation of a non-pathogenic and nontoxigenic strain of *Humicola insolens*. Ultraflo L is a multicomponent enzyme preparation that contains β-glucanase and xylanase as the main activities (EC 3.2.1.6 and EC 3.2.1.8). The enzyme preparation is to be used in the brewing industry as a processing aid. The enzyme hydrolyzes polysaccharide gums which may cause trouble in the brewing process.

The information provided in the following sections is the basis for our determination of general recognition of safety of the mixed β-glucanase, xylanase enzyme preparation produced by *H. insolens*. The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food [1,2]. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices (cGMP), is safe to consume[3]. Pariza and Foster (1983) define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *H. insolens* meets these criteria for nontoxigenicity and nonpathogenicity. The production organism for this enzyme preparation, *H. insolens*, is discussed in Sections 2 and 7.

Our safety evaluation in Section 7 includes a discussion of the safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production microorganism, *H. insolens,* (Cooney and Emerson 1964) belongs to the dematiaceous mitosporic fungi (hyphomycetes) having dark colored conidia. It is a thermophilic fungus commonly found in soil growing as a saprophyte on dead plant tissues typically in compost. It grows very well at temperatures between 35 and 50°C with an optimum at about 40°C[4,5].

No reports have been found connecting *Humicola insolens* (= *Scytalidium thermophilum*) with infectious diseases or mycotoxicosis in humans or animals. Hyphomycetes, like the *Humicola/Scytalidium* complex, are generally not regarded as human pathogens. They are adapted to a living as saprophytes and

novozymes®

with very few exceptions they have no inherent ability to invade and cause disease in man or animals[6].

We have found no reports in the literature describing isolates within the *Humicola insolens/Scytalidium thermophilum* complex as producers of any known mycotoxins.

Based on the literature it can be concluded that *Humicola insolens (= Scytalidium thermophilum)* does not present any risk to humans.

The production strain used to make the mixed β-glucanase, xylanase enzyme preparation has not been modified using recombinant DNA techniques.

2.2 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product, and as such, the production organism does not end up in the food.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the enzyme preparation derived from *H. insolens*, which follows standard industry practices[7-9]. The quality management system used in the manufacturing process for the enzyme complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with current good manufacturing practices (cGMP).

3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the enzyme concentrate are standard ingredients used in the enzyme industry[7-9]. The raw materials conform to *Food Chemicals Codex* (FCC)[10] specifications except those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The antifoams used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the enzyme preparation is less than 1%.

novozymes®

3.2 Fermentation Process

The mixed β-glucanase, xylanase enzyme preparation is manufactured by submerged fed-batch pure culture fermentation of a *H. insolens* strain. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and to confirm strain identity.

3.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *H. insolens,* described in Section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

3.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermentor and the main fermentor before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions is fulfilled:

1. Infection is observed in 2 or more samples by microscopy

2. Infection is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

3.3 Recovery Process

The recovery process is a multi-step operation, which starts immediately after the fermentation process and consists of both the purification and the formulation processes.

novozymes®

3.3.1 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Pretreatment - pH adjustment
2. Primary Separation - vacuum drum filtration
3. Concentration - ultrafiltration and/or evaporation
4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
5. Preservation and Stabilization- In order to prevent microbial degradation stabilizers are added immediately after the germ filtration. The enzyme concentrate is stabilized by addition of sorbitol, glycerol, and potassium sorbate.
6. Final concentration- If the concentration is too low to reach the target yield for the final product, further concentration may be carried out by evaporation and/or ultrafiltration.

3.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

4. ENZYME IDENTITY

The enzyme preparation contains the two main activities: β-glucanase and Xylanase and several side activities, e.g.: Cellulase, Hemicellulase, and Pentosanase.

Key enzyme and protein chemical characterizations of the enzyme are:

Classification:	Carbohydrases (generic name)
IUB nomenclature:	Endo-1,4-β-glucanase and Endo-1,4-β-xylanase
IUB No.:	3.2.1.6 and 3.2.1.8
CAS number:	62213-14-3 and 9025-57-4
Trade name:	Ultraflo™ L

The Product Data Sheet for Ultraflo L is enclosed as Appendix 1.

novozymes®

5. COMPOSITION AND SPECIFICATIONS

This enzyme preparation is presently available in a formula for use in brewing applications.

5.1 Quantitative Composition

Ultraflo® L has the following typical composition:

Enzyme solids (TOS [1])	approx.	5 %
Water	approx.	40 %
Glycerol	approx.	28 %
Sorbitol	approx.	27 %
Potassium sorbate		0.2 %

5.2 Specifications

The β-glucanase and xylanase enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in *Food Chemicals Codex*[10]. In addition, the β-glucanase and xylanase preparation conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications[11].

The following Novozymes' specifications have been established for the enzyme preparation:

Enzyme activity	according to declaration
Heavy metals	not more than 30 ppm
Lead	not more than 5 ppm
Arsenic	not more than 3 ppm
Total viable count/g	not more than 5×10^4
Total coliforms/g	not more than 30
Enteropathogenic *E. coli*/25 g	negative by test
Salmonella/25 g	negative by test
Antibiotic activity	negative by test
Production organism	negative by test
Mycotoxins	negative by test

[1] TOS = Total Organic Solids, defined as: 100% - water - ash - diluents

novozymes®

Heavy metals, lead, arsenic, antibiotic activity, and mycotoxins are analyzed at regular intervals.

Ultraflo L is standardized to have a typical activity of 45 FBG/g. In addition the enzyme preparation will contain approximately 470 FXU/g.

The definition of FBG (Fungal Beta Glucanase) is as follows:

1 β-glucanase unit (BG) is the amount of enzyme, which under standard conditions (pH 5.0 and 30.0°C), liberates glucose or other reducing carbohydrates with a reduction power corresponding to 1 µmol glucose per minute.

Principle:

β-glucanase reacts with beta-glucans under formation of glucose and other reducing carbohydrates which are determined according to the Somogyi-Nelson method [J. Biol. Chem. 153, p.375 (1944)].

The definition of FXU (Farbe Xylanase Unit) is as follows:

The FXU activity is determined relative to the activity of a reference enzyme standard. The activity of the reference standard, Humicola insolens xylanase batch no. 17-1194, is defined to have an enzymatic activity of 3550 FXU/g at pH 6.0 and 50.0°C in 30 min reaction time of colour release from remazol-xylan substrate.

Principle:

A xylanase sample is incubated with remazol-xylan substrate. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant is proportional to the endoxylanase activity. The activity is determined relative to an enzyme standard. The reaction conditions are pH = 6.0, temperature 50°C, and reaction time 30 min.

6. APPLICATION

6.1 Mode of Action

The most important active principles in the enzyme preparation are beta-glucanase and xylanase. The product is standardized according to the β-

novozymes®

glucanase activity, characterized by IUB "Enzyme Nomenclature 1992" as EC 3.2.1.6, (systematic name 1,3-(1,3;1,4) -β-D-glucan 3 (4) -glucanohydrolase). The preparation is mainly used in beer-brewing in the mashing process to ensure efficient breakdown of β-glucans, pentosans, and other gums. This reduces the viscosity of the solution and thereby increases the filtration rate of both wort and beer, and haze is avoided.

The enzyme is normally inactivated and removed during the industrial production process.

6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP.

The recommended dosage is 200 g of the enzyme preparation per ton of total raw materials. However, the optimal dosage depends on the composition of the raw materials and the specific process parameters.

6.3 Enzyme Residues in the Final Food

The enzyme preparation is added at mashing in, and the enzyme activities will be completely inactivated during wort boiling.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

This strain of *Humicola insolens* applied by Novozymes for production of cellulolytic and hemicellulolytic enzymes is derived from a natural isolate deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM 1800). This strain lineage has been used by Novozymes as an enzyme producer for many years without any safety problems.

Several toxicological tests have been undertaken to evaluate the safety of an enzyme product produced by this strain. In their paper "SAFETY EVALUATION OF ALKALINE CELLULASE", Fd. Chem. Toxic. Vol. 29, No. 11, pp. 781-785, 1991, R.J. Greenough, D.J. Everett and M. Stavnsbjerg conclude that *Humicola insolens* can be regarded as a non-toxigenic and a non-pathogenic strain. The paper is enclosed in Appendix 2.

The test substances described in that paper were declared in now obsolete cellulase activity units. In the table below the conversion to the currently used cellulase activity unit EGU has been done.

Test batch	CMCU/g	EGU/g	TOS%	EGU/TOS
PPC 1317	817	292	70.4	4.1
PPC 2809	3200	1143	69.1	16.5
PPC 5569	-	82.1	5.1	16.1

The product was investigated for general toxicity in mice and rats by a variety of routes, effects on reproduction, inhalation toxicity, mutagenic potential, irritation and skin sensitization. No significant adverse effects were observed in any of these studies. These results indicate that the production strain does not produce any harmful substances under production conditions.

The pathogenicity of the *Humicola insolens* production strain was also assessed in mice and rabbits by intraperitoneal and subcutaneous administration, respectively. From the results obtained in these investigations the authors concluded that no notable pathogenicity was observed and the production strain could be regarded as non-toxigenic and non-pathogenic.

The batch PPC 5569 and the safety studies performed using that batch are described in Section 7.4.

Based on these results and the information obtained from the literature (summarized in section 2) we consider this strain lineage of Humicola insolens, used by Novozymes for production of enzymes, to be safe for the production of food grade enzymes.

7.2 Safety of the β-Glucanase and Xylanase Enzymes

Enzyme proteins themselves do not generally raise safety concerns[2,12,13]. As indicated in section 4, the subject enzyme of this notification is a β-glucanase, IUB EC 3.2.1.6, which causes the endohydrolysis of 1,3- or 1,4-linkages in β-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3; and a xylanase, IUB EC 3.2.1.8, which causes endohydrolysis of 1,4-β-D-xylosidic linkages in xylans. Enzymes including β-glucanases and xylanases have a long history of safe use as processing aids in the food industry[1,2].

This mixed β-glucanase, xylanase enzyme preparation produced by *H. insolens* has been reviewed by JECFA at the 61st JECFA (2003). An ADI "not specified was established.

novozymes®

Ultraflo has been approved for use in brewing and alcohol in Canada since 1997.

The mixed beta-glucanase and xylanase preparation is also marketed for use in animal feed in the US and the EU. Beta-glucanase from *H. insolens* is included in Table 30.1 in Section 30 of the Official Publication of AAFCO.

Before a provisional permission to market a feed-additive within EU is granted, the product in question must be evaluated by the Scientific Committee on Animal Nutrition (SCAN).

The SCAN concluded in it's "Opinion of the Scientific Committee on Animal Nutrition on the use of certain enzymes in animal feedingstuffs (updated 16 October 2002) Annex, Part A." that:
"...on the basis of the information provided and knowing of no adverse reports elsewhere that the following enzyme preparations are safe for use as feed additives when used according to the manufacturers instructions and with the animal categories specified. The Committee also concludes that these enzyme preparations pose no risk to the wider environment, to those handling the preparations, or to individuals of any age consuming products derived from animals fed the enzyme-treated feed."

The mixed beta-glucanase and xylanase preparation from *Humicola insolens* is included in the Annex, Part A.

The opinion can be found at:
http://europa.eu.int/comm/food/fs/sc/scan/out96_en.pdf .

7.3 Safety of the Manufacturing Process

The enzyme preparation meets purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex*[10]. As described in Section 3, the preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes[7-9].

7.4 Safety Studies

This section describes the studies and analyses performed to evaluate the safety of the use of the Ultraflo L enzyme preparation.

novozymes®

7.4.1 Description of Test Material

For the safety studies, a liquid enzyme concentrate was prepared according to the description given in section 3, omitting stabilization and standardization.

7.4.2 Studies

The following studies were performed:

- 13 weeks oral toxicity study in rats
- Test for mutagenic activity (Ames Test)
- Human lymphocyte cytogenetic assay

The main conclusions of the safety studies can be summarized as follows:

The administration of the mixed beta-glucanase and xylanase enzyme preparation, PPC 5569 at 10.0 ml/kg/day for 13 weeks (equivalent to 0.51 g TOS/kg/day) caused no signs of toxicity. This dosage is therefore considered to be the no-observed-adverse-effect level (NOAEL) in this study. The enzyme preparation has shown no mutagenic activity in either Ames' test or the human lymphocyte test.

A summary of the safety studies is enclosed in Appendix 3.

7.5 Estimates of Human Consumption and Safety Margin

As stated in section 6.3 the enzyme activities are heat inactivated during the wort boiling process and mostly removed during filtration and further processing of the beer. However, in order to illustrate a "worst case" situation, the following calculation is made assuming that all enzyme activity is retained in the final beer.

Ultraflo L has a typical activity of 45 FBG/g and an approximate content of 5.1% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

In the US, in 2001 the per capita consumption of beer was 21.6 gallons[14] (81.76 liters).

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions:

a) The calculation is made assuming that all TOS remains in the final beer. Ultraflo L contains 5.1 % TOS.

novozymes®

b) It is assumed that all beers are produced using Ultraflo L as a processing aid, used at the recommended dosage.

The recommended dosage of Ultraflo L is 200 g per ton of barley and/or malt.

200 g of Ultraflo L contains 200g x 5.1% = 10.2 g TOS.

In a standard brewing procedure 1 ton of barley and/or malt will result in approximately 6000 liters of beer.

1 liter of beer will then contain 10.2 g TOS/6000 L = 1.7 mg TOS

Based on the US per capita consumption in 2001, the daily intake per person of Ultraflo L corresponds to:
1.7 (mg TOS/L) x 81.76 (L/year)/365 (days/year) = 0.38 mg TOS per day.

For an average person weighing 60 kg this corresponds to 0.0063 mg TOS/ kg body weight per day.

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption.

The NOAEL dose level in the 13 weeks oral toxicity study in rats was 10 ml/kgbw/day corresponding to 510 mg TOS/kgbw/day.

The estimated human consumption is 0.0063 mg TOS/kgbw/day

The safety margin can thus be calculated to be: 510/0.0063 = 80952 ~ **8×10^4**

7.6 Results and Conclusion

The results of the tests described in Section 7.4.2 show that the enzyme preparation does not exhibit any mutagenic activity or toxic effect under the conditions of each specific test. On the basis of the safety evaluations contained in Sections 7.1-7.5, a review of the literature, and the history of safe use of enzymes, the Ultraflo L enzyme preparation can be safely manufactured and used as a processing aid in the brewing industry as well as in other food or non-food applications.

novozymes®

8. LIST OF APPENDICES

1. Novozymes Product Sheet for Ultraflo® L (Brewing 2001-00468-02)

2. Greenough, R.J. and Everett, D.J.. 1991. Safety evaluation of Alkaline Cellulase. Food Chem. Toxic. Vol. 29, No. 11, pp.781-785.

3. Summary of Toxicity Data – Ultraflo (File no. 2002-01621-01)

novozymes®

9. REFERENCES

1. Pariza, M.W. and Johnson, E.A. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Reg. Toxicol. Pharm. 33:173-186, 2001.

2. Pariza, M.W. and Foster, E.M. Determining the safety of enzymes used in food processing. J. Food Protection, 46:5:453-468, 1983.

3. IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. Regulatory Toxicology and Pharmacology 12:S1-S196. 1990.

4. Ellis D.H. and Griffiths D.A.. The Fine Structure Of Conidial Development In The Genus Torula, Part 4 Torula-Thermophila. Canadian Journal of Microbiology Vol. 22(8), 1102-1112, 1976.

5. Ellis, D.H.. Ultrastructure of Thermophilic Fungi v. Conidial ontogony in Humicola Grisea var. thermopidea and H.insolens. Transactions of the British Mycological Society 78(1) 129-139, 1982.

6. Smith,J.M.B. . Opportunistic Mycoses of man and other Animals. C.A.B. International Institute, 1989.

7. Enzyme Applications in Encyclopedia of Chemical Technology, 4th ed., Ed. Kroschwitz, J.I., Volume 9, pp. 567-620, 1994.

8. Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K.. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Vol. I, Eds. Peppler, H.J. and Perlman, D., Chapter 9, pp.282-309, 1979.

9. Aunstrup, K.. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp. 28-68, 1979.

10. National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemicals Codex, Food Chemicals Codex, 5th Edition, The National Academies Press, Washington, D.C., p. 151, 2003.

novozymes®

11. JECFA (Joint FAO/WHO Expert Committee on Food Additives), General Specifications and Considerations for Enzyme Preparations Used in Food Processing, Compendium of Food Additive Specifications, FAO FNP (Food and Nutrition Paper) 52, Add.9, FAO, Rome, 2001.

12. Food and Drug Administration. Lipase Enzyme Preparation From *Rhizopus niveus*: Affirmation of GRAS status as a Direct Food Ingredient. Fed. Regist. 63:24416-24419, 1998.

13. Food and Drug Administration. Statement of Policy: Foods Derived From New Plant Varieties. Fed. Regist. 57:22984-23005, 1992.

14. USDA. 2003 ERS/USDA Data: Food consumption (per capita) data system. US Department of Agriculture. http://www.ers.usda.gov/data/foodconsumption/spreadsheets and select alcoholic beverages. Last accessed: 1/12/06.

Appendix 1

Product Sheet

novozymes®

Ultraflo® L

Description

Ultraflo L is a heat-stable multi-active beta-glucanase preparation produced by a selected strain of *Humicola insolens*. The most important side activities of Ultraflo L are cellulase, xylanase, pentosanase and arabanase.

Product Properties

Appearance

Ultraflo L is a brown liquid preparation with a density of approx. 1.2 g/ml.

Activity

Ultraflo L is standardized to contain 45 FBG/g (Fungal Beta Glucanase units)and contains a range of side activities as mentioned in the introduction. See the analytical method for further information.

Other Characteristics

Ultraflo L is specifically developed for the mashing process.

BEST ORIGINAL COPY

Food-grade status

Ultraflo L complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC), supplemented with maximum limits of 5 x 10^4/g for total viable count and 10^2/g for moulds.

Packaging

See the standard Packaging List for more packaging information.

Application

Ultraflo L is used for beer-brewing in the mashing process to ensure efficient breakdown of beta-glucans, pentosans and other gums. Two important reasons for poor lautering are high beta-glucan levels in the wort and lauter tun overload. High beta-glucan levels also give poor beer filtration. Reduction in load and/or the addition of extra beta-glucanases at mashing-in are therefore usually effective in increasing the lauter tun and beer filter capacity. Recent research work has shown that filtration

rates can be significantly influenced by other polysaccharides than beta-glucans, in particular pentosans, which are polysaccharides containing a backbone of xylan to which side chains of single arabinose units are attached. Therefore, there has been an increasing interest in beta-glucanase preparations with pentosanase and other gum-hydrolyzing activities.

Reaction Parameters

Activity and Stability

Ultraflo L remains active during the major part of mashing. Figure 1 shows that even at 75°C (167°F) Ultraflo L has a significant effect. Therefore, beta-glucan and other polysaccharides which are extracted towards the end of the mashing process will be effectively hydrolyzed. On the other hand, wort boiling will quickly and completely inactivate Ultraflo L, so that no active enzyme will be present during fermentation or in the final beer.

pH

The different enzymes in Ultraflo L have optimal activities and stabilities at typical mashing pH values.

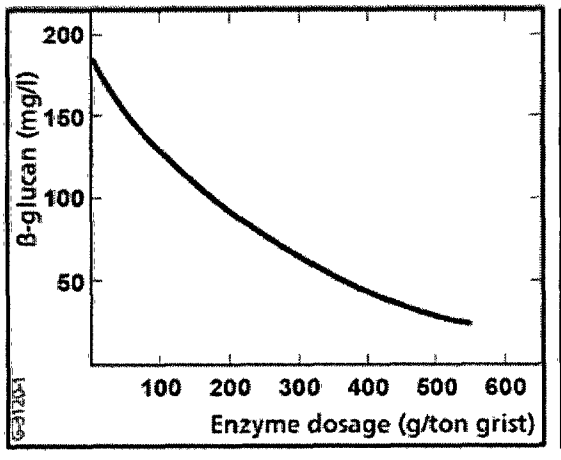

Fig. 1. Influence of Ultraflo on wort beta-glucan.
Substrate: Sweet wort, 10.1% Plato
Temperature: 75°C (167°F)
ncubation time: 30 minutes

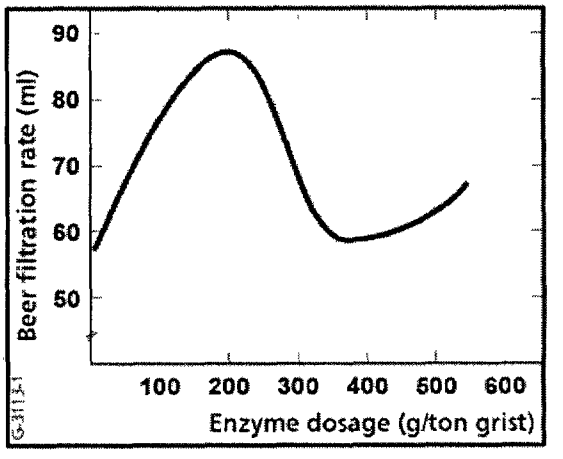

Fig. 2. Effect of Ultraflo on beer filtration rate.
Grist: 80% malt + 20% barley

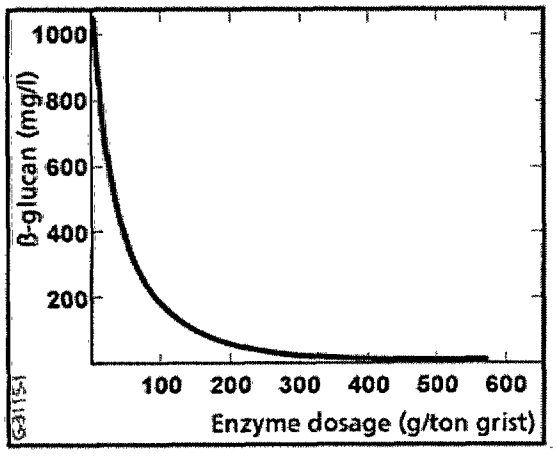

Fig. 3. Effect of Ultraflo on wort beta-glucan.
Grist: 80% malt + 20% barley

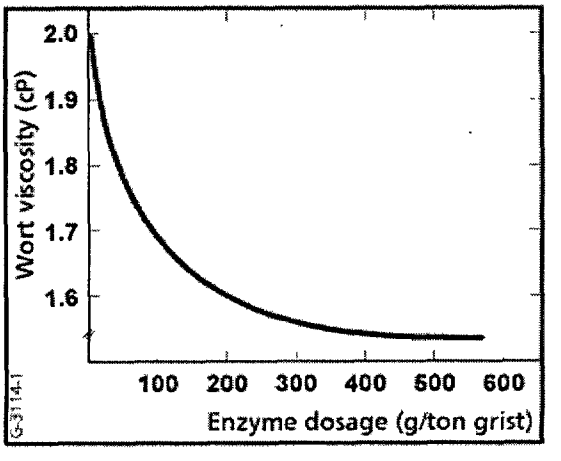

Fig. 4. Effect of Ultraflo on wort viscosity.
Grist: 80% malt + 20% barley

Dosage

For initial trials we recommend a dosage of 200 g Ultraflo L per ton of total raw materials, added at mashing-in.

The optimal dosage, however, depends on the raw materials composition and the specific process parameters and should be determined by testing different dosages at the brewery.

Application example

Filtration trials were conducted in laboratory scale for predicting the beer filtration.

Increasing dosages of Ultraflo L were added at mashing-in, and a standard mashing programme was applied to a grist composition of 80% malt and 20% barley. The beta-glucan content and the viscosity were measured in the filtered wort.

After the wort boiling, pH was adjusted and alcohol added to give the wort beer-like properties (modified Esser test), and the modified wort was filtered.

In the trials, an Ultraflo L dosage of 200 g per ton of total raw materials gave the highest beer filtration rate (Figure 2), whereas the beta-glucan level (Figure 3) and viscosity (Figure 4) continued to fall with higher dosages. This indicates that the beta-glucan level and the viscosity are not the only factors influencing beer filtration rates.

Safety

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. This product may create easily inhaled aerosols if splashed or vigorously stirred. Spilled product may dry out and create dust.

Spilled material should be flushed away with water. Avoid splashing. Left-over material may dry out and create dust. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes.

A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.

Storage

Enzymes gradually lose activity over time depending on storage temperature and humidity. It is recommended to store the product under cool and dry conditions in closed containers at 0-10°C (32-50°F) (e.g. in the hop storage room). Extended storage and/or adverse conditions incuding higher temperature or high humidity, may lead to a higher dosage requirement. Further information on product stablility is available on request.

Laws, regulations and third party rights may prevent customers from importing, processing, applying and/or reselling certain products in a given manner. It is the responsibility of the customers that their specific use of products from Novozymes does not infringe relevant laws and regulations and, furthermore, does not infringe patents or other third party rights.

The contents of this document are subject to change without further notice.

novozymes®

Appendix 2

Pages 000025 - 000029 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 3

Toxicology
Novozymes A/S

Date : 2002-01-24
File : 2002-01621-01
Ref.: PBjP

SUMMARY OF TOXICITY DATA

Ultraflo

CONTENTS

PAGE

1. INTRODUCTION 2

2. GENERAL TOXICITY 2

   2.1 13-weeks subchronic oral toxicity in rats 2
   2.2 Conclusion of general toxicity data 3

3. MUTAGENICITY 3

   3.1 Gene mutation test - Ames test 3
   3.2 In vitro cytogenetic test - cultured human lymphocytes 3
   3.3 Conclusion of mutagenicity data 4

LAST PAGE 4

Author :
Peder Bjarne Pedersen

Issued by :
Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

1. INTRODUCTION

In order to registrate Ultraflo investigations concerning the general toxicity and mutagenicity were undertaken to evaluate the safety of this. The documentation has been elaborated according to the Guidelines for the presentation of data on food enzymes as expressed by the Scientific Committee for Food, Commission of the European Communities (Twenty-seventh series, 1991).

The studies were carries out at TNO Nutrition and Food Research Institute, Zeist, The Netherlands, Corning Hazleton, Harrogate, North Yorkshire, England and Novo Nordisk A/S, Bagsværd, Denmark. All studies were carried out in accordance with current guidelines of OECD and EEC, and with the principles of Good Laboratory Practice (GLP), according to OECD principles of GLP, May 1981.
The results of these studies are summarised in this paper.

Ultraflo is a liquid enzyme concentrate. It is a multi-component enzyme preparation consisting of is a fungal xylanase (EC 3.2.1.8), an endo-cellulase, (EC 3.2.1.4) and a beta-glucanase (EC 3.2.1.6). It is produced by a strain of the fungus *Humicola insolens* by submerged fermentation and recovered by purification/concentration of the fermented culture broth. The production strain has been developed from the wild type by traditionally microbiological methods.

Ultraflo, Batch No. PPC 5569 has been used as test article in all the toxicological investigations referred to in this paper. This batch contains 5,7 % dry matters and 5.1 % "Total Organic Solids" (TOS), calculated as % dry matter - % ash. The enzyme activities are measured to 644 FXU/g (Fungal Xylanase Unit), 82.1 EGU/g (Endo-Cellulase) and 45 FBG/g (Fungal Beta-Glucanase).

2. GENERAL TOXICITY

2.1 13-Weeks Subchronic Oral Toxicity in Rat

TNO Nutrition and Food Research Report No.: V96.898. NN Study No.: 966020.
Sub-chronic (13week) oral toxicity study with Pentopan/Biofeed Plus in rat

The study was performed in accordance with the OECD guideline 408, 1981 and the requirements of the Annex to European Communities Council Directive 88/302/EEC.

Groups of ten male and ten female Wistar rats received Ultraflo at dosages of 1, 3.3 or 10.0 ml/kg/day (equivalent to 0.05, 0.17 or 0.51 g TOS/kg/day) by daily oral (gavage) administration for 13 weeks. A similarly constituted group received the vehicle and served to generate contemporaneous control data.

There were no treatment-related signs and no animals died. Weight gains, food intake and food conversion efficiencies were unaffected by treatment. There were no treatment-related ocular lesions. There were no haematological or biochemical findings related to treatment.

Analysis of organ weights and the macroscopic examinations did not identify any treatment-related change.

The histopathological examinations did not identify any histological alterations related to treatment.

2.2 Conclusion of general toxicity data

The administration of Ultraflo, PPC 5569 at 10.0 ml/kg/day for 13 weeks (equivalent to 0.51 g TOS/kg/day) caused no signs of toxicity. This dosage is therefore considered to be the no-observed-adverse-effect level (NOAEL) in this study.

The present data do not lead to a classification in accordance to the EU-criteria.

3. MUTAGENICITY

3.1 Gen mutation test - Ames test

Novo Nordisk A/S Report No.: QR-9615135. NN Study No.: 968084. Pentopan/Biofeed Plus (Batch No. PPC 5569): Testing for Mutagenic Activity with Salmonella typhimurium TA98, TA1537, TA100 and TA1535 in a Treat and Plate Assay.

Ultraflo was examined for mutagenic activity in four histidine-dependent auxotrophs of *Salmonella typhimurium*, strains TA98, TA100, TA1535 and TA1537, using a treat-and-plate assay. The procedures used complied in principle with OECD Guideline for Testing of Chemicals No.471 (1983) and the requirements of the Annex to European Commission Directive 92/69/EEC.

Cultures of each bacterial strain were exposed to a range of six concentrations of Ultraflo (highest dose: 10 mg/ml), in phosphate-buffered nutrient broth for three hours. After incubation, the exposed cells were separated from the test material by centrifugation prior to plating. The study was conducted in the presence and absence of an activating system derived from rat liver (S-9 mix). All tests included diluents (purified water) controls with and without S-9 mix.

Each test, in each strain, was conducted on two separate occasions. In both these experiments bacteria were exposed to 6 doses separated with bi-sections and with 10 mg/mL as the highest dose level applied.

Marked increases in the number of revertant colonies were induced by the known mutagens 2-nitrofluorene, 2-aminoanthracene, 9-aminoacridine and MNNG when examined under similar conditions as the test substance, confirming the sensitivity of the test.

No increases in reversion to prototrophy were obtained with any of the four bacterial strains at the Ultraflo concentrations tested, either in the presence or absence of S-9 mix.

3.2 In vitro cytogenetic test - cultured human lymphocytes

Corning Hazleton. Report No.: CHE 665/139. NN Study No.: 966022.
Pentopan/Biofeed Plus: Induction of chromosome aberrations in cultured human peripheral blood lymphocytes.

The effects on chromosomal structure of exposure to Ultraflo were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, 1983) and the requirements of the EEC Annex V, Test B 10 (1993).

Two independent experiments were conducted with and without the inclusion of a rat liver-derived metabolic activation system (S-9 mix). Without S-9 mix cells were exposed continuously for 20 hour and in the second experiment a delayed sampling 44 hour sampling time was included. With S-9 mix exposure was limited to three hours and cells were harvested 17 and 41 hours later respectively.

The test article dose levels for chromosome analysis were selected by evaluating the effect of Ultraflo on mitotic index. Chromosome aberrations were scored by

examination of 100 metaphases per culture, and the frequencies of cells with one or more aberrations were calculated both including and excluding gap-type aberrations.

In the first experiment chromosome aberrations were analysed at three consecutive dose levels. The highest concentration chosen for analysis, 5000 µg/mL, induced 59% and 24% mitotic inhibition in the absence and presence of S-9 respectively.
In the second experiment 5000 µg/mL induced 37% and 4% mitotic inhibition in the absence and presence of S-9 respectively. The effect of this single concentration only, (without and with S-9) was investigated at the delayed sampling time.

Treatment of cultures with Ultraflo in the absence and presence of S-9 resulted in frequencies of cells with aberrations which were similar to and not significantly different from those seen in concurrent negative controls. Number of aberrant cells in all treated cultures fell within historical negative control ranges.

It was concluded that Ultraflo did not induce chromosome aberrations in cultured human blood lymphocytes when tested to 5000 µg/mL in the presence and absence of S-9.

3.3 Conclusion of mutagenicity data

The present results show that Ultraflo does not induce gene mutations in *Salmonella typhimurium* or chromosome aberration test in cultured human lymphocytes under the conditions employed in these tests.

000034

SUBMISSION END