GR

ORIGINAL SUBMISSION

GENENCOR

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December 20, 2007

Dr. Laura Tarantino, Director
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

Dear Dr. Tarantino:

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor, a Danisco Division, ("Danisco") has determined that its lipase enzyme preparation produced by *Hansenula polymorpha* expressing the gene encoding lipase enzyme from *Fusarium heterosporum* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. We are hereby submitting, in triplicate, a Generally Recognized As Safe ("GRAS") Notification, in accordance with proposed 21 C.F.R. § 170.36, informing FDA of the view of Danisco that the lipase enzyme preparation is GRAS, through scientific procedures, for use as an enzyme:

- To modify the endogenous lipids in bread dough and produce more polar lipids like lyso-phospholipids and lyso-galactolipids. The non-polar triglycerides in dough will also be modified during formation of mono- and diglycerides and free fatty acids. The properties of the endogenous lipids produced by addition of KLM1 contribute to improved dough stability and dough handling properties, and the polar lipids formed in the dough will produce a bread with improved volume and a homogenous crumb structure with homogenous pores.
- To modify the endogenous wheat lipids in noodle dough and produce more polar lipids. This in turn improves the quality of the finished noodle or pasta product giving a product which has lower cooking loss and improved eating quality.
- Used to modify phospholipids to lyso-phospholipids in egg yolk, which is used in cake manufacture and mayonnaise; and
- Used in oil degumming to hydrolyse phospholipids to lyso-phospholipids.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures (3 binders)

GENENCOR

Lipase Enzyme Preparation from *Hansenula polymorpha* Expressing the Lipase Gene from *Fusarium heterosporum* Is Generally Recognized As Safe For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

December 20, 2007

GENENCOR
A Danisco Division

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1. GENERAL INTRODUCTION

GRINDAMYL™ POWERBake 4100 and GRINDAMYL™ POWERBake 4200 are the Danisco trade names used for the lipase enzyme preparation produced by submerged fermentation of *Hansenula polymorpha* carrying the gene encoding lipase from *Fusarium heterosporum*, also called KLM1. DNA encoding the lipase protein was synthesized using the preferred codon usage for yeasts without changing the encoded amino acid sequence, and inserted into *H. polymorpha.*

The enzyme is intended to modify the endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids in dough for bread making to improve the quality of the baked products and in noodle or pasta products to improve eating quality and lower cooking loss; to modify egg yolk, which is used in cake manufacture; and to degum oils.

The enzyme is a lipase (EC 3.1.1.3) which hydrolyses ester bonds primarily in the 1- and 3-position of fatty acid in triglycerides. The enzyme also has activity towards sn-1 ester bonds in other lipid components including diacyl-phospholipids and diacyl-galactolipids.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its lipase enzyme preparation from a modified strain of *Hansenula polymorpha* expressing the lipase gene from *Fusarium heterosporum* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of a lipase enzyme preparation produced by a *H. polymorpha* host, which has been modified to express a gene encoding lipase from *Fusarium heterosporum*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for food use.[1,2] The safety of the production organism for this lipase *H polymorpha,* is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material. [3-8] The genetic modifications used to construct this production organism are well defined and are described in Section 2. Data showing this lipase to be substantially equivalent [5,7, 8-11] to naturally occurring lipase is provided in Section 3. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

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1.1 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.2 Common or Usual Name of Substance

Lipase enzyme preparation from *H polymorpha* expressing the gene encoding lipase enzyme from *F. heterosporum*

1.3 Applicable Conditions of Use

The lipase enzyme is intended to modify the endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids in dough for bread making to improve the quality of the baked products and in noodle or pasta products to improve eating quality and lower cooking loss; to modify egg yolk, which is used in cake manufacture; and to degum oils. The enzyme preparation is used at minimum levels to achieve the desired effect and according to requirements of normal production following current Good Manufacturing Practices. It is expected that bread, noodles, pasta, egg yolk in cakes, and degummed oils produced using this lipase will be consumed by the general population.

1.4 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.5 Availability of Information for FDA Review

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.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism of the lipase enzyme preparation is *H polymorpha* strain GICC03251. It is derived by recombinant DNA methods from a strain of *Hansenula polymorpha* modified to express a synthetic lipase gene identical to the lipase gene from *Fusarium heterosporum*, optimized for expression in yeast (see Section 2.2). According to the German gene technology legislation, *H polymorpha* is classified in the lowest risk group (Risk Group 1, no risk to human or environment), which applies to both research and production (Das Bundesministerium für Gesundheit, Zentralen Kommision für die Biologische Sicherheit, (http://www.rki.de/GENTEC/ZKBS/ORGLISTE/LISTE.HTML). It also meets the criteria for a safe production microorganism as described by Pariza and Johnson[1] and several expert groups. [2-8] It contains the synthetic lipase gene under the regulation of a native *H. polymorpha* promoter and terminator along with a selectable marker, *URA3* gene (encoding oritidine-5'-phosphate decarboxylase) from *S cerevisiae*
The inserted DNA was integrated into the host on a modified pBR322 plasmid which had been deleted for the genes coding for ampicillin resistance (*Apr*) and tetracycline resistance (*TCr*). The introduced DNA integrated into the Hansenula chromosome as tandem repeats.

2.2 Host Microorganism

The host microorganism used in the construction of the lipase production strain is a strain of Hansenula *polymorpha,* RB11 *H. polymorpha* is a yeast with a two-phase life cycle: reproduction by budding, and reproduction by creation of ascospores. *H. polymorpha* is able to reproduce by budding both as a haploid and as a diploid, but mainly as a haploid, as the diploid cells have a rather large tendency to sporulate.[12] The entire genome of *H. polymorpha* has been characterized.[13] The *H polymorpha* RB 11 strain genome consists of approximately 9.5 Mb and is organized as 6 chromosomes ranging in size from 0.9 to 2.2 Mb. *H. polymorpha* contains no extra chromosomal DNA and contains no known dormant genes.[14]

The families *Pichia* and *Hansenula* are closely related, and *H. polymorpha* has therefore been named in the past: *Pichia angusta, Hansenula angusta, Hansenula polymorpha,* and *Torulopsis methano-thermo.*[15] Some taxonomists find that the families *Pichia* and *Hansenula* should be joined in one family, and therefore *Hansenula polymorpha* has officially been named *Pichia angusta.*[16] Danisco obtained the parent host strain for the production strain from the German company Rhein Biotech GmbH (Düsseldorf, Germany), who uses the name *Hansenula polymorpha* This name is used in this document.

H. polymorpha was described for the first time in 1951 under the synonym *Hansenula angusta.*[17] When molecular biology evolved during the 1980's, *H. polymorpha* became very interesting as a host organism for production of heterologous proteins, as this species contains very strong promoters (*MOX,* methanol oxidase and *FMD,* formate dehydrogenase) and is able to secrete

large amounts of protein. Being a eucaryote, *H. polymorpha* is capable of glycosylating proteins.[18] However, unlike *S cerevisiae, H. polymorpha* rarely hyperglycosylates.[19]

H. polymorpha can be found naturally as an organism growing in putrescent plant material and soil rich in organic matter. The organism has also been isolated from maize flour, *Drosophila pseudobscura*,[15] putrid orange juice and milk from cows suffering from inflamed udders. *H. polymorpha* has been isolated in the USA, UK, Brazil, and South Africa (CBS, 2000).

In literature *H polymorpha* is described as a safe production organism, as this organism does not contain pyrogens, pathogens, or "viral inclusions".[20] According to the German gene technology legislation, *H. polymorpha* is classified in the lowest risk group (Risk Group 1, no risk to human or environment), which applies to both research and production (Das Bundesministerium für Gesundheit, Zentralen Kommision für die Biologische Sicherheit, (http://www.rki.de/GENTEC/ZKBS/ORGLISTE/LISTE.HTML).

Danisco has commercialized another enzyme product, hexose oxidase, using this host and vector system. The hexose oxidase has been approved by:

- JEFCA (see, Evaluation of certain food additives, Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 928, 2005),
- Australia/New Zealand (FSANZ Food Standards Application 475, http://www.foodstandards.gov.au/_srcfiles/A475%20Hexose%20oxidase%20FAR.pdf);
- Mexico (Diario Oficial, July 17, 2006, pg. 48);
- Denmark (private communication available on request);and
- A GRAS panel in the US (private communication available on request)

In these assessments, this *H. polymorpha* was determined to be a safe host for production of hexose oxidase. A paper documenting the safety evaluation of the hexose oxidase, including the safety of the host, was also published in 2003 by Cook and Thygesen.[40]

This lipase has also been approved in Mexico (by letter, not officially listed yet); in Australia/ New Zealand (FSANZ Food Standards Application 569, http://www.foodstandards.gov.au/_srcfiles/FAR_A569_Lipase_H_%20polymorphaL.pdf); Denmark (private communication available on request); and in the US by a GRAS panel (private communication available on request). In all these assessments, *H polymorpha* was determined to be a safe host for the production of lipase.

In addition, *H polymorpha* is used as the host for commercial production of hepatitis B vaccine. The commercial product AgR® produced by Laboratorio Pablo Casserá (LPC) is approved in Argentina[21] and the commercial product Hepavax-Gene® is approved for use by WHO (Appendix 1). Additionally phase II clinical trials for a therapeutic vaccine are being conducted in Europe.

Interferon alpha2a is produced in *H. polymorpha* and registered in Egypt (Rhein Minapharm) and insulin produced and registered in India (Wockhardt).

Two anticoagulants, saratin and hirudin, are expressed in *H. polymorpha* [22,23] Saratin is being tested in *in vitro* trials while hirudin is being tested in clinical trials in the form of PEGylated hirudin.

2.3 Lipase Expression Vector

The plasmid used in this construction was created from the *E coli* plasmid pBR322[38,39] by insertion of the *URA3* gene (encoding oritidine-5'-phosphate decarboxylase) from *S cerevisiae,* a native *H polymorpha* promoter and terminator, and the synthetic lipase gene from *F. heterosoporum* optimized for expression in yeasts. Furthermore, the genes coding for ampicillin resistance (*Apr*) and tetracycline resistance (*TCr*) in the original pBR322 have been removed.

The characteristics of the plasmid can be summarized as:

1. It is constructed from the *E coli* plasmid pBR322;
2. It contains the *S cerevisiae* URA3 gene encoding orotidine-5-phosphate decarboxylase as a selectable marker;
3. It contains the synthetic lipase gene, which encodes the lipase from *F heterosporum* optimized for expression in yeasts;
4. It contains a *Hansenula polymorpha* native promoter gene and terminator gene to control expression of the lipase;
5. It contains no genes conferring resistance to antibiotics;
6. It is non-conjugative and non-mobilizable;
7. It is designed to integrate into the chromosome of the host; and
8. The size is 6459 bp.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *H polymorpha* strain had been made.

2.4 Stability of the Introduced Genetic Sequences

Most probably, the plasmid was integrated as tandem repeats in the genome of *H polymorpha,* which theoretically makes the construction unstable as copies of plasmid might be able to recombine back out of the genome. In principle, the plasmid could therefore be transferred to other organisms. However, the frequency of this taking place is probably very low, as no selective advantage of taking up the plasmid is present, unless a potential recipient strain is orotidine-5'-phosphate decarboxylase negative.

Danisco has previously investigated the genetic stability of a recombinant *H. polymorpha* strain, which had been transformed with a plasmid which was almost identical to this plasmid except that it contains a gene encoding hexose oxidase instead of lipase.
The stability of the hexose oxidase strain was tested by inoculating each of five shake tubes (3 mL) with a single colony. After this, inoculation was carried out from shake tube to shake tube until a total of four times five shake tubes had been inoculated. The hexose oxidase activity of all shake tubes was measured, showing that the recovery of hexose oxidase from this strain does not change in the course of ca. 35 generations.

The organism was also grown in fed-batch fermentations (substrate added continuously to fermenting batch) in which the inoculum originated from the last step of the previous fed-batch fermentation. Thus passage of a high number of generation times was achieved, permitting detection of any in-stabilities in the organism. Three repeats were carried out under the correct process conditions, each with duration of 250 - 350 hours. Enzyme activity within 5-10% of the maximum was achieved in the three fermentations, indicating that in the described system the organism does not show any sign of instability.

The number of copies of the lipase gene in the organism is another indicator of the stability of the lipase organism, which has been studied. The number of lipase genes of three different fermentations (grown for different number of generations) was determined by Southern blot analysis, and this number was found to be stable, as no differences were observed within the uncertainty of the analysis. It seems obvious that a loss of lipase gene copies smaller than the accuracy of the analysis cannot be detected. The conclusion is that the production organism shows no significant loss of the lipase gene.

The results of the above mentioned stability studies are that a recombinant organism similar to the lipase production organism demonstrates constant expression level of its enzyme activity and the recombinant lipase strain itself shows a constant number of lipase gene copies. It is therefore concluded that the lipase production organism is genetically stable under the conditions used.

2.5 Antibiotic Resistance Gene

The DNA used for transforming the *H. polymorpha* host strain does not contain any antibiotic resistance genes.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by the IFBC[3] is satisfactorily addressed.

An antibiotic resistance gene was not used in this construction, therefore, there is none present in the enzyme preparation. The production strain does not carry any vectors. As no vector sequences are present in the final strain, the transfer frequency of the integrated expression cassettes is the same as for any other chromosomal sequence. The expression cassettes are completely derived from bacterial and synthetic DNA.

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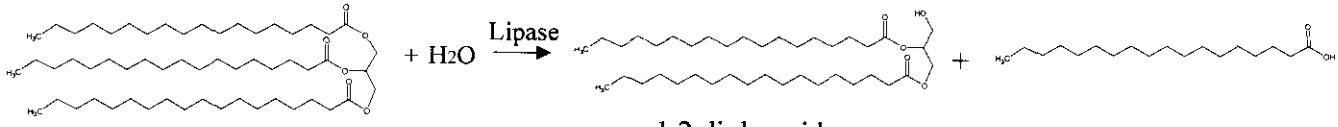
3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

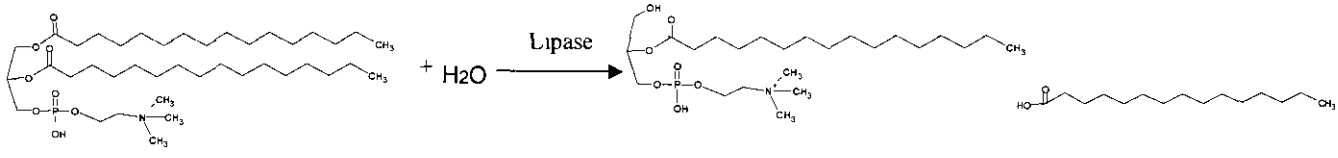
Key enzyme and protein chemical characteristics of the lipase are:

Classification: KLM1 hydrolyses ester bonds primarily in the 1- and 3-position of fatty acid in triglycerides.



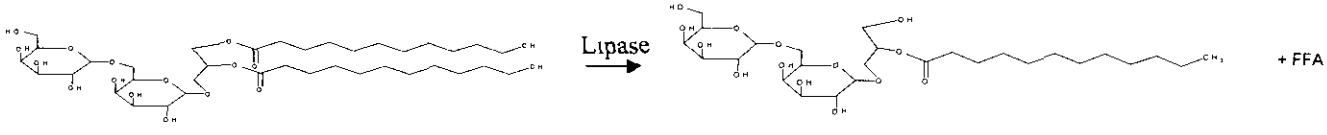
Triglyceride 1,2 diglyceride Fatty acid

The enzyme also has activity towards sn-1 ester bonds in other lipid components including diacyl-phospholipids, e.g.:



Phosphatidylcholine Lyso-phosphatidylcholine Fatty acid

and diacyl-galactolipids, e.g.:



Digalactosyl diglyceride Digalactosyl monoglyceride

IUB Nomenclature	triacylglycerol lipase
IUB Number:	3.1.1.3
CAS Number:	9001-62-1
EINECS Number:	232-619-9

Reaction catalyzed:	triacylglycerol + H_2O = diacylglycerol + a carboxylate
Molecular weight:	Approximately 30 kDa
Amino Acid Sequence:	The total nucleotide and amino acid sequences have been determined.

3.2 Amino Acid Sequence

The amino acid sequence of KLM1 lipase is identical to the amino acid sequence of native lipase. The nucleotide and amino acid sequence of the lipase is shown in Appendix 2.

3.3 Comparison to Other Lipases

The KLM1 lipase hydrolyzes triacylglycerols at the 1 and 3 positions (E.C. 3.1.1.3) (Appendix 3). This activity is identical to that of other GRAS approved lipases, for example, to the lipase enzyme preparation obtained from *Aspergillus oryzae* (*A. oryzae*) carrying a recombinant gene encoding a lipase from *Fusarium oxysporum* (*F. oxysporum*) of Novozymes (GRAS Notice No. GRN 000075). In addition to activity, KLM1 shares ~80% amino acid sequence similarity with that of the *F. oxysporum* lipase. Several lipases (animal derived and from *Rhizopus niveus*) are affirmed as GRAS (21 CFR §184. 1415 and 1420), the lipase from *Mucor miehei* (now known as *Rhizomucor miehei*) is approved for use as a food additive (21 CFR §173.140), and a lipase preparation produced by *A. oryzae* expressing a *Thermomyces lanuginosus* lipase is the subject of GRAS Notice No. 000043 (GRN 000043). These lipases all belong to the same classification as KLM1 (E.C.3.1.1.3).

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the KLM1 lipase which follows standard industry practice. [24-26] For a diagram of the manufacturing process, see Appendix 4. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with current FDA's Good Manufacturing Practices ("CGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for the lipase enzyme concentrate are standard ingredients used in the enzyme industry.[24-26] All the raw materials conform to the specifications of the Food Chemicals Codex, 5th edition, 2003 ("FCC"),[27] except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco uses a supplier quality program to qualify and approve

suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoams in the production process is ≤1.5%.

4.2 Fermentation Process

The lipase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of H. *polymorpha* described in Section 2. 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 conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

A new lyophilized stock culture vial of the *H polymorpha* production organism described in Section 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

The fermentation is declared as 'contaminated' if colony forming units of bacteria or fungi are present other than the production strain at CFU levels of $>10^3$/ml.

If a fermentation is determined to be contaminated, it will be rejected if deemed appropriate. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

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

4.3.1 Concentration process

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

1. Primary separation –centrifugation
2. Concentration – ultrafiltration
3. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation.

4.3.2 Formulation and standardization process

The product is dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier, or stabilized by e.g. glycerol to produce a liquid product.

4.3.3 Quality control of finished product

The final lipase preparation from *H polymorpha* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives ("JEFCA") in 2006 and the FCC. These specifications are set forth in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The lipase enzyme preparation has the following typical composition:

Dry Enzyme Product

Enzyme :~0.78% (w/w) active protein
71% (w/w) wheat starch
12% (w/w) NaCl
1% (w/w) vegetable oil
4% (w/w) trisodium citrate

Liquid Enzyme Product:

Enzyme: 0.74-0.78% (w/w) active protein
Preservatives 10% (w/w) NaCl
Water: ~80-90% (w/w)

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5.2 Specifications

The lipase enzyme preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC. See FCC (2003)[27] at page 149. In addition, the lipase enzyme preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA).[28]

The following specifications have been established for the lipase enzyme:

(See next page)

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Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Lipase (TIPU/g)	921W	Genencor/Danisco SOP E112, "Determination of phospholipase-activity by Konelab"	1500 TIPU/g
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	800V	ISO 4833 - "Microbiology - General guidance for the enumeration of micro-organisms - colony count technique at 30°C" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/g
Total Coliforms	810V	ISO 4832 - "General guidance for the enumeration of coliforms - colony count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/g
E coli	819V	ISO 7251 - Microbiology - "General Guidance for Enumeration of Presumptive *Escherichia coli* - Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Salmonella	832V	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition;	Negative/25g

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Property	Method Number	Reference Method	Specification
		1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	
Production strain	892V	Genencor/Danisco Method	Negative by test
Antibacterial Activity	899V	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Negative by test
OTHER ASSAYS			
Heavy Metals, as Pb	603W	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests Test solutions, and other reference materials, 1983, 2nd revision	Less than 30 mg/kg
Arsenic	603W-AS	Same as Heavy Metals as Lead	Less than 3 mg/kg
Cadmium	603W-CD	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Mercury	603W-HG	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Lead	603W-PB	Same as Heavy Metals as Lead	Less than 5 mg/kg
Mycotoxins	604W	Patterson & Roberts, Assoc. of Anal. Chem. (vol. 62, no. 6, 1979)	Negative by test

Heavy metals, lead, arsenic, cadmium, mercury, antibacterial activity, and mycotoxins are analyzed at regular intervals. Activity and microbial specifications are analyzed on every production batch of enzyme.

The lead, Coliforms and Salmonella specifications meet FCC and JEFCA requirements. The *E coli*, antibacterial activity and mycotoxin specifications meet JEFCA requirements and are not included in FCC (although FCC mentions mycotoxins, but has not established tolerances.) The

production microorganism specification is a Danisco specification and is not mentioned in FCC or JEFCA.

The lipase activity assay measures phospholipase cleavage of the fatty acids in both the 1 and 2 positions at pH 7.0 at 30 degrees C. The free fatty acids are measured via a commercially available kit that contains a coupled enzyme scheme. The rate of fatty acid generation is proportional to the to the phopholipase activity. The method is calibrated using a linear regression of standard dilutions prepared from a standard material.

6. APPLICATION

6.1 Mode of Action

As noted above, lipase hydrolyses ester bonds primarily in the 1- and 3-position of fatty acid in triglycerides. The enzyme also has activity towards sn-1 ester bonds in other lipid components including diacyl-phospholipids and diacyl-galactolipids.

The lipase enzyme preparations are intended to modify the endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids in dough for bread making to improve the quality of the baked products and in noodle or pasta products to improve eating quality and lower cooking loss; modify egg yolk, which is used in cake manufacture; and to degum oils.

6.2 Use Levels

KLM1 will be used in dough for bread making to improve the quality of the baked products. Wheat flour used for bread making typically contains 2% lipids. Half of these lipids are nonpolar lipids including triglyceride, diglyceride, sterol and sterolester. The other part of the lipids in wheat flour is polar lipids containing mainly galactolipids and phospholipids. KLM1 will be used in dough to modify these endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids. The non-polar triglycerides in dough will also be modified during formation of mono- and diglycerides and free fatty acids. The properties of the endogenous lipids produced by addition of KLM1 contribute to improved dough stability and dough handling properties, and the polar lipids formed in the dough will produce a bread with improved bread volume and a homogenous crumb structure with homogenous pores.

Noodles and pasta are normally produced by using the same raw material, wheat flour, which is used for manufacture of bread. KLM1 will in pasta or noodle dough also modify the endogenous wheat lipids and produce more polar lipids. This in turn improves the quality of the finished noodle or pasta product in terms of improved eating quality and a product which has lower cooking loss.

The use level of KLM1 in bakery and noodle applications is 100-4000 TIPU/kg flour.

KLM1 may be used to modify egg yolk, which is used in cake manufacture. The emulsification property of the egg yolk is improved because the phospholipids are modified.

The use level of KLM1 in egg for bakery applications is 250-1000 TIPU/kg bakery product.

Egg yolk is well known for use in the food industry because of its emulsification properties. The emulsification properties of egg yolk is explained by the fact that approx 30% of the egg yolk lipid are phospholipids. These emulsification properties are utilized in many food applications including mayonnaise, dressings, sauces and cakes. In some food applications the emulsification properties of egg yolk is however not sufficient to obtain a homogenous product without separation.
An example of such a product is mayonnaise, which during the production is pasteurised at high temperatures. This heat treatment will cause the product to separate.

KLM1 will be used to modify phospholipids to lyso-phospholipids in egg yolk. When enzyme modified egg yolk is used to produce mayonnaise, product separation can be avoided even at high pasteurisation temperatures.

The use level of KLM1 in eggs for mayonnaise production is 5000-20000 TIPU/kg egg yolk.

Crude soy bean oil contains 1-2% phospholipids, which during the refining process to produce edible oil are removed. This is done to improve the quality and taste of the oil. During the refining process, KLM1 will be used to hydrolyse phospholipids to lyso-phospholipids. Lyso-phospholipids are then washed out of the oil with small amounts of water (approx. 5%). By this washing process the enzyme is also removed from the edible oil.

The use level of KLM1 in oil refining is 200-2000 TIPU/kg crude oil.

6.3 Enzyme Residues in the Final Foods

As described in the sections on Enzyme characteristics and on Position specificity, KLM1 will release fatty acids from triglycerides, phospholipids and galactolipids. KLM1 is sensitive to heat and has a temperature optimum at 40°C (104°F). At temperatures above 40°C (104°F), the activity readily decreases and at 60°C (140°F) less than 10% of the optimum activity is retained.

The released free fatty acids and the remaining fraction of the lipids will be digested as any other lipid component in the digestive system. No effects on nutrients are foreseen.

The enzyme will be inactivated by heat during baking, cooking and/or pasteurization or will be removed with the water phase during the refining of oils.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

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. [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, are safe to consume.[3] Pariza and Foster[2] define a nontoxigenc 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. polymorpha* meets these criteria for nontoxigenicty and nonpathogenicity.

7.1.1 Safety of the host

The safety of the host was discussed in section 2.2.

7 1.2 Safety of the donor source

The donor organism for the lipase gene used in construction of the new microorganism *H polymorpha* GICC03278 was the mould *Fusarium heterosporum.* The donor organism *F heterosporum* is deposited with Centraalbureau voor Schimmelcultures, Holland, as CBS 782.83.

Even though only the lipase gene has its origin from *F heterosporum* and even though it is a synthetic lipase gene which was inserted in the host organism, the safety of the donor organism was assessed, as *F heterosporum* does not have a long history of safe use in the food industry.

7.1.2.1 *F heterosporum* and health in humans and animals

Fusarium species are plant pathogens and common in soil.[30] *F. heterosporum* is able to produce fusaric acid,[31] zearalenoles and zearalenones[32] as well as trichothecene mycotoxins.[33,34] The production of mycotoxins has resulted in intoxication of humans after consumption of contaminated rice.[35]

A review from 1999 on mycotoxin biosynthesis schematically explains the biosynthesis of trichothecene mycotoxins in *Fusaria.*[35] This biosynthesis clusters more genes in *Fusarium sporotrichioides,* and the function of seven genes was determined. None of these genes code for a lipase. It was not possible to find literature on the biosynthesis of fusaric acid; however, the chemical structure of fusaric acid does not point to a lipase being involved in the synthesis.

To investigate whether lipase or phospholipase is involved in the toxin production of *Fusaria,* a literature search was carried out in Medline (http://www.ncbi.nlm.nih.gov/PubMed/) searching for "lipase and toxin and fusarium" and "phospholipase and toxin and fusarium" (April 2005). No references indicate involvement of lipases or phospholipases in the toxin production of *Fusaria.*

Fusarium species are known to cause local infections in skin, nails and cornea, whereas reports are rare on infections deep in tissue.[30] In cases of *Fusarium* species causing systemic infections, e.g. to organs, the patients were typically weak from disease, or undergoing treatment for severe illness (aplasia, kidney transplant, disease in the immuno system, ("chronic granulomatous disease"), burns).[30,36] *Fusarium* species involved in infections in humans include: *F. solani, F. verticillioides, F. oxysporum. F. moniliforme, F. antophylum, F proliferatum,* and *F. chlamydosporum.* [30,36] As seen from the above-mentioned *Fusaria*, no incidents are reported of *F. heterosporum* causing systemic infections in humans.

It should also be mentioned that a lipase encoded by a lipase gene, which originates from *Fusarium oxysporum,* is commercially available for the food market (FDA, GRAS Notice No. GRN 000075, http://www.cfsan.fda.gov/~rdb/opa-g075.html).

7.1.2.2 The gene

The inserted gene which encodes the lipase is a synthetic gene, which encodes the same amino acid sequence as the native gene isolated from *Fusarium heterosporum* (CBS 782.83). To obtain optimal codon usage, the synthetic gene was constructed with codon usage optimised for yeast.

7.1.2.3 Design and construction of a synthetic lipase gene

The synthetic gene was developed as follows: A protein having lipase activity was isolated from *F heterosporum* (CBS 782.83). The protein was reduced with dithiothreitol and the cysteine residues were protected by carboxymethylation using iodoacetamide. The protein was then cleaved by trypsin and the fragmentation pattern of the tryptic peptides were examined by MALDI-TOF analysis. The peptides were separated by chromatography on a C_{18} - reverse-phase HPLC column, and the degree of purification was monitored by MALDI-TOF analysis. The amino acid sequence was determined by Edman degradation.

The amino acid sequences of the peptide fragments were ordered by alignment with an amino acid sequence of a Japanese strain of *F. heterosporum.*[37] The complete amino acid sequence thus obtained was back translated into a nucleic acid sequence to reveal all possible codons. For each codon, the codon most favorable for expression in *H. polymorpha* was chosen according to the codon preference table of genes expressed in *H*

polymorpha. Synthetic oligonucleotides, each about 100 nucleotides long, comprising the complete gene, were synthesized, and the gene was assembled by PCR.

7.1.3 Lipase Expression Vector

The plasmid was created from the *E. coli* plasmid pBR322[38,39] by insertion of the *URA3* gene (encoding oritidine-5'-phosphate decarboxylase) from *S. cerevisiae,* a native *H. polymorpha* promoter and terminator, and the synthetic lipase gene from *F. heterosoporum* optimized for expression in yeasts. Furthermore, the genes coding for ampicillin resistance (*Apr*) and tetracycline resistance (*TCr*) in the original pBR322 have been removed.

The characteristics of the plasmid can be summarized as:

1. It is constructed from the *E. coli* plasmid pBR322;
2. It contains the *S. cerevisiae* URA3 gene encoding orotidine-5-phosphate decarboxylase as a selectable marker;
3. It contains the synthetic lipase gene, which encodes the lipase from *F. heterosporum* optimized for expression in yeasts;
4. It contains a *H. polymorpha* native promoter gene and terminator gene to control expression of the lipase;
5. It contains no genes conferring resistance to antibiotics;
6. It is non-conjugative and non-mobilizable;
7. It is designed to integrate into the chromosome of the host; and
8. The size is 6459 bp.

7.2 Safety of Lipase

7.2.1 Allergenicity

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens. Below we describe briefly why ingestion of enzymes used as food processing aids is unlikely to elicit an allergic response after consumption.

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions.

Because exposure to enzymes is very low, even if they were potentially allergenic by the oral route, the likelihood of allergic sensitization of consumers to these proteins is virtually nil. In addition, the lipase is either removed from the food or denatured by heat prior to consumption.

7.2.2 Substantial Equivalence

The KLM1 lipase hyrdrolyzes triacylglycerols at the 1 and 3 positions (E.C. 3.1.1.3) (Appendix 3). This activity is identical to that of other GRAS approved lipases, for example, to the lipase enzyme preparation obtained from *Aspergillus oryzae* (*A. oryzae*) carrying a recombinant gene encoding a lipase from *Fusarium oxysporum* (*F. oxysporum*) of Novozymes (GRAS Notice No. GRN 000075). In addition to activity, KLM1 shares ~80% amino acid sequence similarity with that of the *F oxysporum* lipase. Several lipases (animal derived and from *Rhizopus niveus*) are affirmed as GRAS (21 CFR §184. 1415 and 1420), the lipase from *Mucor miehei* (now known as *Rhizomucor miehei*) is approved for use as a food additive (21 CFR §173.140), and a lipase preparation produced by *A oryzae* expressing a *Thermomyces lanuginosus* lipase is the subject of GRAS Notice No. 000043 (GRN 000043). These lipases all belong to the same classification as KLM1 (E.C.3.1.1.3).

7.3 Safety of the Manufacturing Process

The manufacturing process for the production of lipase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration, filtration and formulation, resulting in a liquid lipase enzyme preparation. In addition, a dry product is also produced; it is dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier. The process, described in Section 4, is conducted in accordance with FDA's cGMPs as set forth in 21 C.F.R. Part 110. The resultant products meet the general requirements for enzyme preparations of the FCC and WHO/JEFCA.

7.4 Safety Studies

To confirm the safety of KLM1 produced by *Hansenula polymorpha* the following tests were conducted:

Study	Test Item
Acute oral toxicity studies in the rat	KLM1, TOX1
Sub-chronic 13 week toxicity in the rat	KLM1, TOX2
Ames Mutagenicity studies	KLM1, TOX1
In vitro Chromosomal Aberration Study	KLM1, TOX1

7.4.1 Test item preparations

Test items for the studies, as indicated above, were prepared as follows:

7.4.1.1 KLM1, TOX1

KLM1, TOX1 is the freeze-dried preparation of the liquid material KLM1, TOX2 (see below) to obtain a highly concentrated powder. The freeze dried preparation is characterized by:

Enzyme activity	62,000 TIPU/g
Dry matter content	94.2%
Ashes	4.1%
%TOS (100 – (%ashes +(100 -%dry matter)))	90.1%
Protein content	6.2%

KLM1, TOX1 was stored frozen until use.

7.4.1.2 KLM1, TOX2

The KLM1 enzyme was produced by fermentation of the recombinant strain of *Hansenula polymorpha*GICC03251. The test item is a blend of two fermentations designated Bio00951 and Bio00953. After the fermentation process the individual fermentation broths were diluted 1:1.5 = broth:water, before centrifugation. The centrates were concentrated on a UF filter and finally sterile filtered. KLM1, TOX2 is characterized by:

Enzyme activity	5800 TIPU/ml
Dry matter content	6.88%
Ashes	0.19%
%TOS (100 – (%ashes +(100 -%dry matter)))	6.69%
Protein content	0.40%

KLM1, TOX2 was a brownish liquid and was stored frozen at –18°C until use.

Despite the frozen storage of KLM1, TOX2 and thawing of samples in the refrigerator before use, some instability problems were seen. At the time of manufacture of KLM1, TOX2 the enzyme activity was 5800 TIPU/ml and the test article was prepared just before the sub-chronic 13-week study was initiated. Samples of the dose solutions for the 13 week study were drawn at the beginning of the study, after half of the feeding period and at the end of the study. These samples were frozen again and kept frozen until

analysis after the study period. At this time the enzyme activity was markedly lower than at the beginning of the study.

The enzyme activities for the samples drawn at the three individual dates were similar. This was the case for all three dose levels. This indicates that there was an initial loss of activity between manufacture of sample and study start or a loss of activity during thawing and re-freezing of the samples. No matter at which time the loss occurred, it seems clear the rats got the same amount of enzyme activity throughout the study, even though it was at a lower level than intended. Loss of enzyme activity was more pronounced in the diluted samples (samples for the two lowest dose levels). The ratio between enzyme activity of the three dose levels in the 13 week study was intended to be 1:3:10. However, the new analysis shows that the ratio was most probably 1: 2.8:15.

Average enzyme activity for the three dose groups for all three days was 100, 280 and 1500 TIPU/ml respectively. As the samples had this activity at time of re-analysis after the study, at least these amounts of activity were also present in the samples at the time of dosage. Consequently these dose levels represent the minimum of activity that the rats received. The total organic solids (TOS) and total protein content in the KLM1, TOX2 preparation were not affected by the loss of enzyme activity. Therefore, dose levels in the sub-chronic study are expressed in terms of TOS.

7.4.2 Acute Oral Toxicity in the Rat

An acute oral toxicity study in the rat was performed with a preparation of KLM1, TOX1 according to the method recommended in OECD Guideline No. 420, "Acute Oral Toxicity – Fixed Dose Method," adopted by the Council on 17th December 2001, Paris and according to OECD (2001) Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. OECD Series on Testing and Assessment Number 33. ENV/JM/MONO(2001)6, 14 August 2001. The study was performed at SafetyCity, Finland between 11 October and 28 October 2004.

7.4.2.1 Test performance

The experiment was performed in Sprague Dawley: Hsd:SD:Tu Wistar rats. The test item, KLM1, was suspended in sterile water and administered orally by gavage to rats that had been fasted over-night. The objective of this study is to assess the acute toxicity of KLM1 when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purpose. The study was initiated with a sighting study using a single dose level of 1.33 g KLM1 test item/kg body weight using one female rat. The main study was performed in four additional female rats given a dose of 1.33 g KLM1 test item/kg bw.

Animals were observed individually after dosing and daily thereafter. At the end of the 14 day observation period the animals were weighed and sacrificed. A gross necropsy was performed and all macroscopic signs were recorded.

7.4.2.2 Results

No abnormal clinical signs were observed during the study and all animals survived in good condition during the whole experiment. The body weight gains were normal in all animals during the observation period.

7.4.2.3 Conclusion

Under the experimental conditions described in this report, it was found that the dose level tested (1.33 g test item/kg bw, 82000 units/kg bw equalling at least 2000 mg/kg bw of a product with a concentration of 40000 units/g), which is the highest required dose level according to OECD guideline no.420, did not produce mortality.

Based on this study, the oral LD50 of KLM1, TOX1 is > 2000 mg product/kg bw and the test item can be classified to the category 5/unclassified.

7.4.3 Subchronic toxicity – 13-week rat feeding study

A 13 week oral toxicity test in rats was performed on the sample designated KLM1, TOX2. The study was conducted in accordance with the methods recommended in OECD Guideline No. 408, "Repeated Dose 90-days Oral Toxicity Study in Rodents", September 1998.

The study was performed at Safety-City, Finland between 14 October 2004 and 21 January 2005.

7.4.3.1 Test Performance

The objective of this study is to investigate the potential of KLM1 to induce systemic toxicity after repeated daily oral administration to SPF Hsd:Sprague Dawley rats of both sexes for 91 consecutive days. Groups of 10 rats/sex each were gavaged daily with 0 (sterile water), 4, 12 or 40 mg total protein/kg body weight in a constant volume of 10 ml/kg body weight corresponding to 67, 201 or 669 mg TOS/kg bw/day, respectively.

The groups, dose levels and animal numbers were as follows:

	Treatment	Dose			Animal numbers	
		Units[1] /kg bw	mg TOS /kg bw	mg total protein /kg bw	Female	Male
	Control (vehicle)	0	0	0	1-10	11-20
	Low	5800	67	4	21-30	31-40
	Middle	17400	201	12	41-50	51-60
	High	58000	669	40	61-70	71-80

[1]Intended enzyme activity. See paragraph on test article preparations above for details on actual enzyme activity

All animals were observed daily for mortality and signs of morbidity. Animals of the same sex were pair-housed in transparent Polycarbonate Macrolon III cages with softwood sawdust as bedding and had access to water (via bottle) and feed *ad libitum*. All groups were housed under controlled temperature, humidity and lightning conditions. Body weight and feed consumption were recorded weekly. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Hematology was conducted on Day 0, 30, 60 and 90. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was conducted on selected organs from control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

7.4.3.2 Results

All animals survived in good condition during the whole study. No abnormal test item related clinical signs were observed during the study. No abnormal changes in the eyes were observed in ophthamological examinations. There were no changes in body weights and weight gains between test item treated animals and control animals. However, statistically significant differences between the test item treated group and the control group were observed in food and water consumption.

At necropsy, isolated incidences of macroscopic findings were noted (enlarged lymph nodes, hyperemia in small intestine, erosion of stomach mucus) but the occurrence was

not dose-related and, hence, could not be attributed to treatment. There are some statistically significant differences in the treated groups in some hematological values (single points for BRC, PLT and APTT). The total serum bilirubin was statistically significantly increased in groups III and IV at study termination. No treatment-related effects were noted in organ weights and urinalysis. Histological findings were minor and are considered to be of little biological significance.

7.4.3.3 Discussion

A dose related decrease in the food consumption was observed but did not result in body weight loss suggesting that the feed efficiency was unaffected. Therefore, the statistical differences are of no biological significance. The statistical decrease in water consumption is expected due to the direct relationship between water and feed consumption. Although several hematological values were statistically significantly increased in the treated groups, they were still within the historical control values. A dose related increase in the serum bilirubin in both female and male animals was observed but the increased value is still within historical control values and thus is considered as of questionable significance. Further, microscopic examinations of the liver and kidneys did not reveal any functional changes. The few incidences of microscopic findings were considered as incidental findings for animals of this strain and age.

7.4.3.4 Conclusion

Under the conditions of this study, there were no observed treatment related signs of clinical and systemic toxicity. A No Observed Adverse Effect Level (NOAEL) can be established at the highest dose tested, 40 mg total protein/kg bw/day or 669 mg TOS/kg bw/day.

7.4.4 Bacterial reverse mutagenicity test (Ames assay)

An Ames assay was performed on the sample designated KLM1, TOX1. The test was performed at BioTest Ltd., Czech Republic between 28 December 2004 and 3 March 2005. The test was conducted in accordance with the guideline recommended by OECD "Bacterial Reverse Mutation Test", No. 471, 1997.

7.4.4.1 Test performance

The objective of this assay is to assess the mutagenic potential of KLM1 in four strains of *Salmonella typhimurium* (TA 1535, TA 97, TA 98 and TA 100) and one strain of *Eschericia coli* (WP2uvrA). The *Salmonella* tester strains carry a mutation in the histidine operon, while the *Eschericia* strain is a tryptophan auxotrophic strain. The test material was tested in both the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix).

A dose range-finding study test was carried out first using strain *S. typhimurium* TA100 with and without metabolic activation. The appropriate five doses used in the main tests were deduced from levels of solubility or toxicity.

Two independent main tests were performed. In each test all five strains were used with and without metabolic activation, 3 plates per dose level. Positive and negative controls with and without metabolic activation were included in both tests.

In the dose range-finding study the bacterium was treated with doses at 10, 100, 500, 1000, 2500 and 5000 µg/plate.

In the main study the bacteria were treated with doses at 10, 100, 500, 2000 and 5000 µg/plate. The highest concentration tested (5000 µg/plate) was the highest required by the guidelines for materials of low toxicity.

Cultures of each strain were grown for 16 hours. Test tubes were set up with 0.5 mL of buffered physiological solution or S-9 mix, 0.1 mL of concentrated bacterial suspension, 100 µL of test solution and 2 ml 0.6% top agar at 45°C. After shaking the mixture was poured onto minimal glucose agar plates. The number of revertant colonies on each plate was counted after 72 hours incubation at 37°C.

7.4.4.2 Results

The negative and positive control values were acceptable.

In the dose range-finding study no cytotoxic effects of KLM1, TOX1 were observed for bacteria up to the dose of 5000 µg/plate with or without metabolic activation. The test item showed no precipitation or contamination at any agar plate.

In the main tests, bacterial strains were treated with 10-5000 µg/plate. In none of the tests were there significant increases in the number of induced revertant colonies in any strain at any dose in the tests both with and without metabolic activation.

7.4.5 Chromosomal Aberration Assay (In Vitro) with human peripheral lymphocytes

The objective of this assay is to investigate the potential of KLM1-TOX1 to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes) both in the presence and absence of metabolic activation. The test was performed at BioTest, Ltd, Czech Republic from 28 December 2004 to 16 May 2005. The test was performed in accordance with the guideline recommended by OECD "In Vitro Mammalian Chromosome Aberration Test", No. 473, (1997).

7.4.5.1 General description of the test system

KLM1 concentrate was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main tests. Five concentrations of KLM1 were used in the preliminary assay and at least 3 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index).

In both main tests, all cultures (with or without S-9 mix) were treated for 4 hours or 48 hours (corresponding to 2 cell cycles). Two hours prior to the end of the 4 or 48 hour periods, Colchicine was added to all cultures to arrest all cells at the metaphase-stage of mitosis. At the harvest time of 4 or 48 hours, all cultures were centrifuged and the supernatant discarded. The cell pellets were resuspended in a KCl solution, incubated for 10 minutes, centrifuged and the supernatant removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations. Two slides from each culture were prepared and slides were then coded. Two slides from each culture with 50 well-spread metaphases on each slide were examined under the microscope, and the number and type of the observed chromosomal aberrations were recorded. A measure of the clastogenic effect of the test substance was obtained by comparing the frequency of chromosomal aberrations observed in the test cultures and in the control cultures.

7.4.5.2 Performance of chromosomal analysis

In the dose range-finding study the mitotic index was calculated as the percentage of cells at metaphase (%MI). The test substance was determined to be toxic to the cell cultures if %MI for the test substance was lower than half of the %MI for the negative control sample.

In all tests slides for the test substance and slides for the negative and positive controls were examined for the presence of chromosomal aberrations. The aberrations were recorded as gaps, breaks or exchanges, and classified as chromatid- or chromosome-type in each case. The number of aberrant cells calculated as cells bearing chromatid- and chromosome breaks or exchanges or showing aneuploidy or polyploidy while cells bearing gaps do not contribute to the number of aberrant cells because gaps are of questionable biological significance.

7.4.5.3 Results

In the dose range-finding study, strong or medium toxic effects were observed at the 4 and 5 mg/ml doses, and slight toxicity was observed at 3 mg/ml in the tests with and without metabolic activation. Consequently the following three doses of KLM1 were chosen for performing the main mutagenicity test: 0.5, 1 and 2 mg/ml of culture.

In the first main study, cells were exposed to the test substance both with and without metabolic activation for 4 hours. Average values of % frequencies of aberrant cells did not increase to two times the control values at any dose. There were no concentration-related increases of aberrations.

In the second main study, the cells were exposed for 48 hours. There was no concentration-related increases of aberrations.

7.4.5.4 Conclusion

Under the test conditions used, KLM1 does not induce structural chromosome aberrations in cultured mammalian somatic cells. The test article is not a mutagen or clastogen.

7. 5. Overall Safety Assessment and Conclusions

7.5.1 Identification of the NOAEL

In a 90-day oral study with KLM1, there were no treatment-related effects in all parameters investigated from clinical observations to histopathologic examinations. Under the conditions of this experiment, the NOAEL (No Observed Adverse Effect Level) is established at 40 mg total protein/kg bw/day or 669 mg TOS/kg bw/day.

7.5.2 Human Exposure Assessment

KLM1 is used in bakery and noodles and the use level in both applications is 100-4000 TIPU/kg flour. KLM1 is also used as an emulsifying agent in egg yolk at the use level of 250-1000 TIPU/kg bakery products, in eggs for mayonnaise production at the level of 5000-20000 TIPU/kg egg yolk and in oil degumming at a use level of 2000 TIPU/kg oil.

GENENCOR

7.5.2.1 Estimated Daily Intake of KLM1

Data from the USDA Continuing Survey of Food Intake by Individuals, 1994-1996 was used to estimate intake of KLM1 in the US. Data from the Danish Food survey, 1995 [41] was used to estimate in-take of KLM1 in Denmark.

The estimated daily intake in both countries was calculated as follows:

US:

	Food intake		Max enzyme dose	Estimated daily enzyme intake	
	g/person/day	g/kg bw/day	Units/kg food	Units/kg bw/day	mg TOS/kg bw/day*
Bread	137	2 28	4000	9 12	0 41
Eggs via bakery products	-	-	1000	-	-
Eggs via mayonnaise	1	0 02	20000	0 4	0 02
Oils	50	0 83	2000	1 66	0 07
Total	-	-	-	11 18	0 50

*The enzyme preparation KLM1, TOX2 which was used for the 13 week toxicity study had an enzyme activity of at least 1500 TIPU/ml %TOS was 6 69% equaling 67 mg TOS/1500 TIPU

DK:

	Food intake		Max enzyme dose	Estimated daily enzyme intake	
	g/person/day	g/kg bw/day	Units/kg food	units/kg bw/day	mg TOS/kg bw/day*
Bread	182	3 03	4000	12 13	0 54
Eggs via bakery products	10	0 17	1000	0 17	0 01
Eggs via mayonnaise	1	0 02	20000	0 4	0 02
Oils	50	0 83	2000	1 66	0 07
Total	-	-	-	14 36	0 64

*The enzyme preparation KLM1, TOX2 which was used for the 13 week toxicity study had an enzyme activity of at least 1500 TIPU/ml %TOS was 6 69% equaling 67 mg TOS/1500 TIPU

The Danish intake numbers are higher than the US values so these will be used for further evaluation. It is estimated that the daily intake of KLM1 is 14.36 TIPU/kg bw/day or 0.64 mg TOS/kg bw/day. These estimated intakes represent a worst-case scenario as they are based on an assumption that KLM1 is still active in the foods and is not removed or inactivated by heat and also on the assumption that 100% of the foods on the market are treated with KLM1.

In reality, it is expected that residues of a processing aid in the final products would be negligible after processing.

Maximum Estimated Daily Intake of KLM1 = 0.64 mg TOS/kg bw

7.5.3 Margin of safety

The margin of safety is calculated as a ratio of the NOAEL from the 90-day oral study in rats and the human exposure level. Several scenarios are presented – the first one in which it is estimated that the intended enzyme activity was stable; the second one in which a decrease in enzyme content as described above is taken into account and the third one based on TOS.

No observed adverse effect level = Daily exposure	58,000TIPU/kg bw/day = 14.36 TIPU/kg bw/day	3830

No observed adverse effect level = Daily exposure	15,000TIPU/kg bw/day = 14.36 TIPU/kg bw/day	1044

No observed adverse effect level = Daily exposure	669 mg TOS/kg bw/day = 0.64 mg TOS/kg bw/day	1045

7.6 Conclusion

The safety of KLM1 as a food processing aid to modify the endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids in dough for bread making to improve the quality of the baked products and in noodle or pasta products to improve eating quality and lower cooking loss; to modify egg yolk, which is used in cake manufacture; and to degum oils is assessed in a battery of toxicology studies investigating its acute oral, genotoxic and systemic toxicity potential. KLM1 is not acutely toxic by ingestion. A battery of genotoxicity assays was conducted and under the conditions of these assays KLM1 is not a mutagen, not a clastogen, not an aneugen. Daily administration of KLM1 for 91 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 40.0 mg total protein/kg bw/day (equivalent to 669 mg TOS/kg bw/day).

Based on a worst-case scenario that a person is consuming KLM1 from the egg yolk and whole eggs for cakes and mayonnaise, in bread and noodles, and in degummed oils this NOAEL still offers more than a 1000X fold margin of safety.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

The safety of lipase as a food processing aid to modify the endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids in dough for bread making to improve the quality of the baked products and in noodle or pasta products to improve eating quality and lower cooking loss; to modify egg yolk, which is used in cake manufacture; and to degum oils is assessed using the published literature and the toxicology data generated on the enzyme product. A NOAEL is established at 40 mg total protein/kg bw/day or 669 mg TOS/kg bw/day. Even under a worst case scenario that a person is consuming KLM1 from the egg yolk and whole eggs for cakes and mayonnaise, in bread and noodles, and in degummed oils, this NOAEL still offers more than a 1000X fold margin of safety.

Based on the publicly available scientific data from the literature and additional supporting data generated by Danisco, the company has concluded that the use of lipase from *Hansenula polymorpha* strain GICC03251 in these food applications is safe and suitable, and is Generally Recognized as Safe (GRAS). The safety of KLM1 enzyme was assessed using the decision tree of Pariza and Johnson for assuring the safety of new microbially derived enzymes. The conclusion was that the enzyme is 'Accepted' as safe for its intended uses. The enzyme product has been approved in Denmark, Mexico, and Australia/New Zealand; the food safety authorities in these three locations also agreed that the product is safe for its intended uses. In addition, the safety determination including construction of the production organism, the production process and materials, and safety of the product were reviewed by Dr. Michael W. Pariza, Dr. Herbert Blumenthal and Dr. Joseph F. Borzelleca and they concurred with the company's conclusion that the product is GRAS (private communication available upon request). The panel was provided with:

- A search of the scientific literature conducted by Danisco via the MEDLINE, Chemtox and Chem Abstracts databases through April 2005;
- Estimated dietary exposure data;
- A detailed description of the genetically modified microorganism and the lipase gene; and
- Reports of the safety studies and relevant FAO/WHO JECFA review documents on other enzymes for food uses.

The panel independently critically evaluated these documents and other materials deemed relevant. The panel concluded that the lipase meeting the described specifications and produced in compliance with cGMP is GRAS by scientific procedures for used in foods as described by Danisco.

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10. LIST OF APPENDICES

Appendix 1 - Hepavax-Gene® approval by WHO

Appendix 2 - The nucleotide and amino acid sequence of the lipase

Appendix 3 – IUBMB definition of 3.1.1.3

Appendix 4 - Diagram of the manufacturing process

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Appendix 1 - Hepavax-Gene® approval by WHO

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Appendix 16

Enclosure 15

Rhein Biotech

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Dr. Claus L Johansen

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Dear Dr. Johansen,

referring to the document of WHO qualifying GCVC/KGCC , Korea (now GCVC member of the Rhein Biotech Group) as pre-qualified supplier of Hepatitis B Vaccine, trade name Hepavax-Gene, please be informed as follows

The antigen used in the formulation of the vaccine is produced via recombinant technology in yeast Hansenula polymorpha.

The vaccine was first registered in the Republic of Korea (South Korea) 1996 and subsequently in 35 countries.

Since the registration more than 150 Mio doses of the vaccine has been administrated over national and supranational vaccination programs proving the safety and efficacy of the vaccine

Sincerely Yours

Dr. Z.A. Janowicz
V-President R&D
Rhein Biotech Group

2

Appendix 2 - The nucleotide and amino acid sequence of the lipase

GENENCOR

14 April 2005 -
JB/HVT

Appendix 2 Nucleotide and amino acid sequence

Nucleotide sequence and corresponding amino acid sequence of the synthetic *F heterosporum* (CBS 782.83) lipase gene, which is fused with the α-signal sequence. The amino acid sequence is shown above the nucleotide sequence. Start and stop codes for translation are marked with double under-lining. An arrow head marks the position for the fusion between the α-signal sequence and the lipase gene. Horizontal arrows show the PCR primers, which are used to assemble the gene. Restriction sites for *Eco* RI and *Bam* HI are underlined.

```
            M  R  F  P   S  I  F   T  A  V   L  F  A  A   S  S  A   L  A  A   P  V  N     23
AATTCAAACG ATGAGATTTC CTTCAATTTT TACTGCAGTT TTATTCGCAG CATCCTCCGC ATTAGCTGCT CCAGTCAACA   80
                                                   alp
T  T  T  E   D  E  T   A  Q  I  P   A  E  A   V  I  G   Y  S  D  L   E  G  D   F  D  V     50
CTACAACAGA AGATGAAACG GCACAAATTC CGGCTGAAGC TGTCATCGGT TACTCAGATT TAGAAGGGGA TTTCGATGTT  160

 A  V  L  P   F  S  N   S  T  N   N  G  L  L   F  I  N   T  T  I   A  S  I  A   A  K  E    77
GCTGTTTTGC CATTTTCCAA CAGCACAAAT AACGGGTTAT TGTTTATAAA TACTACTATT GCCAGCATTG CTGCTAAAGA  240

  E  G  V   S  L  E  K   R  E  A   E  A  A   V  G  V  T   S  T  D   F  T  N   F  K  F    103
AGAAGGGGTA TCTCTCGAGA AAAGAGAGGC TGAAGCTGCC GTTGGAGTGA CCTCTACTGA CTTCACTAAC TTTAAGTTCT  320
                                                                   alp cbss
                                                   cbss.alp
Y  I  Q  H   G  A  A   A  Y  C  N   S  G  T   A  A  G   A  K  I  T   C  S  N   N  G  C    130
ACATTCAGCA TGGTGCTGCC GCATACTGTA ACTCCGGTAC CGCCGCAGGT GCAAAGATCA CTTGTTCGAA TAACGGTTGC  400

 P  T  I  E   S  N  G   V  T  V   V  A  S  F   T  G  S   K  T  G   I  G  G  Y   V  S  T    157
CCTACTATCG AGTCCAACGG CGTGACTGTG GTCGCCTCCT TCACTGGTTC GAAGACTGGC ATCGGCGGTT ACGTGTCCAC  480

  D  S  S   R  K  E  I   V  V  A   I  R  G   S  S  N  I   R  N  W   L  T  N   L  D  F    183
CGATAGCTCG AGAAAAGAGA TCGTGGTCGC AATCAGAGGT TCCAGCAACA TCCGGAATTG GCTGACTAAT CTTGACTTTG  560

D  Q  S  D   C  S  L   V  S  G  C   G  V  H   S  G  F   Q  N  A  W   A  E  I   S  A  Q    210
ACCAGTCCGA CTGTTCCCTT GTTTCGGGCT GTGGTGTTCA CTCCGGTTTC CAGAACGCTT GGGCCGAGAT CTCCGCACAG  640

 A  S  A  A   V  A  K   A  R  K   A  N  P  S   F  K  V   V  A  T   G  H  S  L   G  G  A    237
GCCTCGGCTG CCGTGGCAAA AGCTAGAAAG GCCAACCCAT CCTTCAAGGT TGTCGCCACT GGCCACTCGC TCGGCGGCGC  720

  V  A  T   L  S  A  A   N  L  R   A  A  G   T  P  V  D   I  Y  T   Y  G  A   P  R  V    263
TGTGGCGACC CTGTCCGCTG CCAACCTTCG AGCTGCAGGT ACTCCAGTCG ACATCTACAC TTATGGTGCA CCTAGAGTTG  800

G  N  A  A   L  S  A   F  I  S  N   Q  A  G   G  E  F   R  V  T  H   D  K  D   P  V  P    290
GCAACGCCGC ACTGTCTGCT TTCATCTCGA ACCAAGCAGG CGGTGAATTT AGAGTCACTC ACGACAAGGA CCCAGTGCCT  880

 R  L  P  P   L  I  F   G  Y  R   H  T  T  P   E  Y  W   L  S  G   G  G  G  D   K  V  D    317
CGGCTTCCAC CTCTGATCTT CGGTTACAGA CACACTACCC CAGAGTACTG GCTGTCAGGT GGCGGCGGAG ACAAGGTGGA  960

  Y  A  I   S  D  V  K   V  C  E   G  A  A   N  L  M  C   N  G  G   T  L  G   L  D  I    343
CTACGCAATC TCCGACGTGA AGGTCTGCGA GGGAGCCGCA AACCTCATGT GTAACGGCGG TACACTGGGA CTGGACATCG 1040

D  A  H  L   H  Y  F   Q  A  T  D   A  C  N   A  G  G   F  S  W  R   R  Y  R   S  A  K    370
ACGCACACTT GCACTACTTC CAGGCAACTG ATGCTTGCAA CGCCGGAGGT TTCTCCTGGA GAAGGTATAG GTCCGCAAAG 1120

 R  E  S  I   D  M  R   A  T  M   T  D  A  Q   L  E  A   K  L  N   S  Y  V  A   M  D  Q    397
AGAGAGTCCA TCGACATGAG AGCTACCATG ACAGACGCAC AGTTGGAGGC CAAGCTCAAC TCCTATGTTG CCATGGACCA 1200

  E  Y  V   K  T  H  Q   N  R  T                                                            407
GGAGTATGTC AAGACTCACC AAAACAGAAC ATAGGCCATG GAGAGAATTG GATCC                                1255
      cbss.t
```

3

Appendix 3 - IUBMB definition of 3.1.1.3

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Appendix 3
IUBMB Enzyme Nomenclature

EC 3.1.1.3

Accepted name: triacylglycerol lipase

Reaction: triacylglycerol + H_2O = diacylglycerol + a carboxylate

Other name(s): lipase; triglyceride lipase; tributyrase; butyrinase; glycerol ester hydrolase; tributyrinase; Tween hydrolase; steapsin; triacetinase; tributyrin esterase; Tweenase; amno N-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride lipase; liver lipase; hepatic monoacylglycerol acyltransferase

Systematic name: triacylglycerol acylhydrolase

Comments: The pancreatic enzyme acts only on an ester-water interface; the outer ester links are preferentially hydrolysed.

Links to other databases: BRENDA, EXPASY, GTD, KEGG, ERGO, PDB, CAS registry number: 9001-62-1

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[EC 3 1 1 3 created 1961]

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Appendix 4 - Diagram of the manufacturing process

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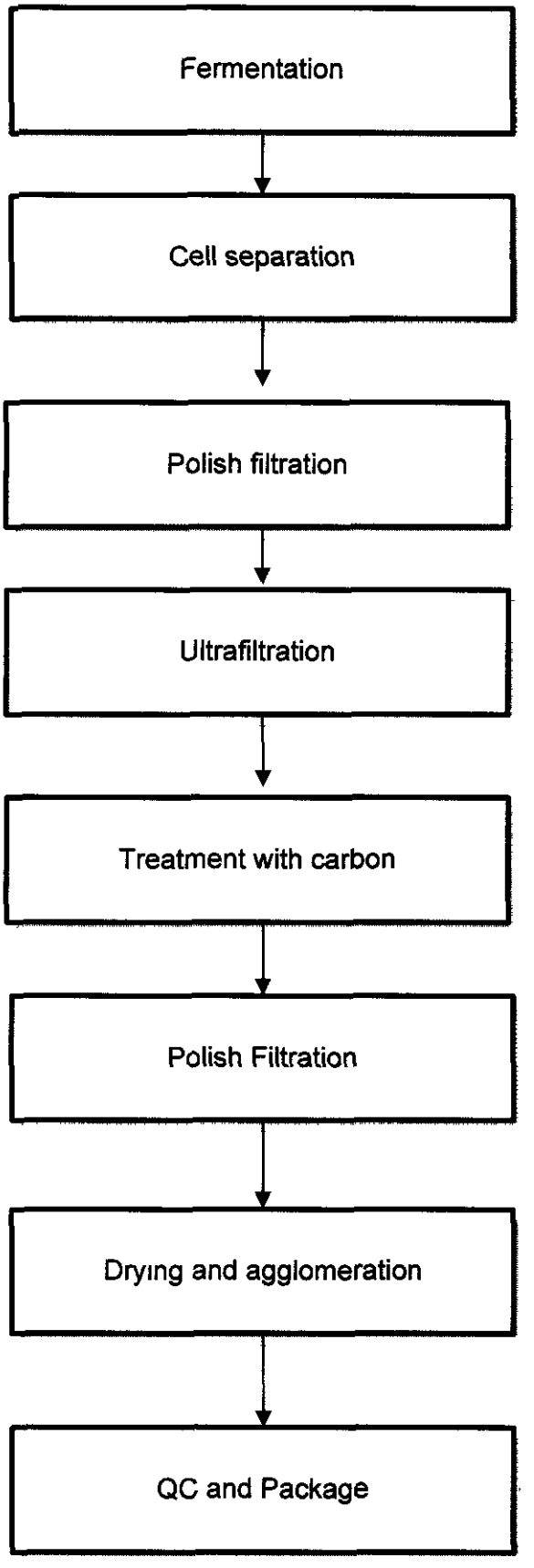
Fermentation
Cell separation
Polish filtration
Ultrafiltration
Treatment with carbon
Polish Filtration
Drying and agglomeration
QC and Package

SUBMISSION END