BIOCATALYSTS
exceeding enzyme expectations

May 16th, 2020

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition,
Food and Drug Administration,
5100 Paint Branch Parkway, College Park, MD 20740.

Subject: GRAS notice for Porcine Phospholipase A2 enzyme produced by a genetically modified strain of *Yarrowia lipolytica*

Dear Sir or Madam:

Pursuant to proposed 21 C.F.R § 170.30, Biocatalysts Limited is providing in electronic media format (determined to be free of computer viruses), and based on scientific procedures, a generally recognized as safe (GRAS) notification for Phospholipase A2 enzyme produced by a genetically modified strain of *Yarrowia lipolytica* expressing the gene encoding Porcine Phospholipase A2 for use in egg yolk processing, i.e. as a processing aid in the manufacture of sauces and dressings such as mayonnaise, at a recommended dose of 28 – 279 ml Phospholipase A2/tonne egg yolk.

The Phospholipase A2 enzyme preparation described herein when used as described above and in the attached GRAS notice is exempt from the premarket approval requirements applicable to food additives set forth in Section 409 of the Food, Drug, and Cosmetic Act and corresponding regulations.

Please contact the undersigned by telephone or email if you have any questions or additional information is required.

Dr Andrew Ellis
Technical and Compliance Director (Biocatalysts Limited)
Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
E-mail: customerservices@biocats.com

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000940	May 19, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person: Dr Andrew Ellis		Position or Title: Technical and Compliance Director	
Organization *(if applicable)*: Biocatalysts Limited			
Mailing Address *(number and street)*: Cefn Coed, Parc Nantgarw			
City: Cardiff	State or Province: Wales	Zip Code/Postal Code: CF15 7QQ	Country: United Kingdom
Telephone Number: 0044 1443 843 712	Fax Number:	E-Mail Address: customerservices@biocats.com	

1b. Agent or Attorney *(if applicable)*

Name of Contact Person:		Position or Title:	
Organization *(if applicable)*:			
Mailing Address *(number and street)*:			
City:	State or Province:	Zip Code/Postal Code:	Country:
Telephone Number:	Fax Number:	E-Mail Address:	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Porcine Phospholipase A2 produced by a genetically modified strain of Yarrowia lipolytica

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Phospholipase A2 catalyses the hydrolysis of natural phospholipids present in foodstuffs, resulting in the formation of lyso-phospholipids with emulsifying properties. Phospholipase A2 is used as a processing aid in the manufacture of enzyme modified egg yolk. Phospholipase A2 will be added in an amount between 28 - 279 ml Phospholipase A2 concentrate/tonne egg-yolk which is equivalent to 3.4 - 33.9 g TOS/tonne egg yolk.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

1. The undersigned is informing FDA that Biocatalysts Limited
(name of notifier)

has concluded that the intended use*(s)* of Porcine Phospholipase A2 produced by a genetically modified strain of Yarrowia lipolytica
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Biocatalysts Limited agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

Cefn Coed, Parc Nantgarw, CF15 7QQ, Cardiff, Wales (United Kingdom)
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Andrew Ellis Digitally signed by Andrew Ellis Date: 2020.05.19 14:40:51 +01'00'	Dr Andrew Ellis, Technical and Compliance Director	11/01/2019

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	CoverLetterPLA2FromYarrowiaLipolytica_May2020.pdf	Administrative
	GRASNoticePLA2FromYarrowiaLipolytica_May2020.pdf	Submission
	Form3667-GRASNoticePLA2FromYarrowiaLipolytica_2020.pdf	Incoming Correspondence/Submission Form

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

GRAS notification for Porcine Phospholipase A2 enzyme produced by a genetically modified strain of *Yarrowia lipolytica*

Biocatalysts Limited

May 16th, 2020

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

GRAS notification for Porcine Phospholipase A2 enzyme produced by a genetically modified strain of *Yarrowia lipolytica*

Table of Contents

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

1. Part 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

1.1 §170.225(c)(1) – Submission of GRAS Notice:

Pursuant to 21 C.F.R. Part 170, subpart E, Biocatalysts Limited is hereby submitting a GRAS (Generally Recognised as Safe) notice and claims that the use of Porcine Phospholipase A2 enzyme manufactured by submerged fermentation of a selected, pure culture of a genetically modified strain of *Yarrowia lipolytica*, as described in Section 2.3 below, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because we have determined that such uses, as described in Section 6 below, are Generally Recognised as Safe (GRAS).

1.2 §170.225(c)(2) – The name and address of the notifier:

Biocatalyst Limited
Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom

1.3 §170.225(c)(3) – Appropriately descriptive term:

Porcine Phospholipase A2 enzyme produced by a genetically modified strain of *Yarrowia lipolytica*.

1.4 §170.225(b) – Trade secret or confidential:

This notification does not contain confidential information.

1.5 §170.225(c)(4) – Intended conditions of use:

Phospholipase A2 can be used for production of bread, baked goods, egg-yolk based sauces and dressings and vegetable oil degumming. Phospholipase A2 is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. There are no maximum limits set, just suggested dosages. The "general" population is the target population for the consumption of foodstuffs produced with Phospholipase A2.

1.6 §170.225(c)(5) – Statutory basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures.

1.7 §170.225(c)(6) – Premarket approval:

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

1.8 §170.225(c)(7) – Availability of information:

This notification package provides a summary of the information which supports our GRAS conclusion of the notified substance. Complete data and information that are the basis for this GRAS conclusion is available to the Food and Drug Administration for review and copying at reasonable times (during customary business hours) at Biocatalysts Limited or will be sent to FDA upon request (electronic format or on paper).

1.9 §170.225(c)(8) – FOIA (Freedom of Information Act):

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

1.10 §170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favourable and unfavourable information, known to Biocatalysts Limited and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

Signed,

[redacted]

Andrew Ellis
Technical and Compliance Director
Biocatalysts Limited

16th May 2020

Date

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2. Part 2 §170.230 – IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 Identity of the notified substance:

The subject of this notification is porcine Phospholipase A2 enzyme produced by a genetically modified strain of *Yarrowia lipolytica*.

Key enzyme and protein chemical characteristics of Phospholipase A2 are given below:

Enzyme Commission Number:	EC 3.1.1.4
Systematic name:	Phosphatidylcholine 2-acylhydrolase
Other names:	Phospholipase A2; Lecithinase A; Phosphatidase; Phosphatidolipase; Phospholipase A
Class:	Hydrolases; Acting on ester bonds; Carboxylic-ester hydrolases
CAS number:	9001-84-7

2.2 Identity of the source

2.2.1 Donor Organism

The sequence of Phospholipase A2 was derived from *Sus scrofa* (pig):

Kingdom: Animalia
Phylum: Chordata
Division: Ascomycota
Sub-phylum: Vertebrata
Class: Mammalia
Order: Artiodactyla
Family: Suidae
Genus: *Sus*
Species: *scrofa*

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2.2.1.1 Amino Acid Sequence of the Enzyme

The sequence of *Sus scrofa* Phospholipase A2 was optimized for expression in *Yarrowia lipolytica*.

ATGCAGGAGGGTATCTCTTCTCGAGCCCTGTGGCAGTTCCGATCTATGATCAAGTGCGCTATCCCCGGCTCTCACCC
TCTGATGGACTTCAACAACTACGGCTGCTACTGCGGCCTCGGCGGCTCTGGCACCCCTGTGGACGAGCTGGACCGA
TGCTGCGAGACTCACGACAACTGCTACCGAGATGCCAAGAACCTGGACTCTTGCAAGTTCCTGGTGGACAACCCCTA
CACCGAGTCTTACTCTTACTCCTGCTCTAACACCGAGATCACCTGTAACTCTAAGAACAACGCCTGCGAGGCCTTCAT
CTGCAACTGCGACCGAAACGCCGCCATCTGCTTCTCTAAGGCTCCCTACAACAAGGAACACAAGAACCTCGACACCA
AGAAGTACTGCTAA

This nucleotide sequence encodes the Phospholipase A2 amino acid sequence:

MQEGISSRALWQFRSMIKCAIPGSHPLMDFNNYGCYCGLGGSGTPVDELDRCCETHDNCYRDAKNLDSCKFLVDNPYTE
SYSYSCSNTEITCNSKNNACEAFICNCDRNAAICFSKAPYNKEHKNLDTKKYC

The sequence map is detailed below:

Phospholipase A2 consists of 132 amino acids with the pro-peptide, and 124 amino acids result in the mature sequence of the enzyme. The molecular weight of Phospholipase A2 is 15kDa.

2.2.1.2 Sequence Comparison to Other Phospholipase A2 Enzymes

Comparison of the synthetic phospholipase A2 expressed in *Yarrowia lipolytica* to *Sus scrofa* (wild pig) phospholipase A2 showed that the sequences of both enzymes were 100% identical.

Biocatalysts Ltd. Phospholipase A2 sequence was 88% identical to the sequence of *Orcinus orca* (killer whale). The sequence of *Sus scrofa* Phospholipase A2 was optimized for expression in *Yarrowia lipolytica*.

2.2.2 Production Organism

The host organism used for production of Phospholipase A2 is *Yarrowia lipolytica*.

The taxonomic position of the recipient organism was first established by van der Walt and von Arx (1980), with the following microbiological taxonomy:

Kingdom: Fungi
Sub-kingdom: Dikaryota
Division: Ascomycota
Sub-division: Saccharomycotina
Class: Saccharomycetes
Order: Saccharomycetales
Family: Dipodascaceae

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Genus: *Yarrowia*
Species: *Yarrowia lipolytica*

Yarrowia lipolytica is a common host used for expression of a large variety of biotechnology products.

The host organism was obtained from an established research laboratory in the Institut National de la Recherche Agronomique (INRA), France. The strain is auxotrophic for uracil and defective for the secretion of an extracellular alkaline protease and three lipases. These strain modifications are highly likely to render it unable to compete in the environment against adapted wild-type strains.

2.2.3 Construction of the Production Strain

The original strain was obtained from the INRA collection. The parent strain of *Yarrowia lipolytica* has been modified to over-express the genes responsible for the production of Phospholipase A2.

The Phospholipase A2 gene originates from *Sus scrofa* but has been codon optimised for maximum expression in the yeast. The gene was synthesized at GeneArt (Germany).

The production strain *Yarrowia lipolytica* carries the *Sus scrofa* PLA2 gene sequence inserted in its genome.

The yeast *Yarrowia lipolytica* is not known to harbour genes encoding for toxins or otherwise harmful sequences so it is not expected that targeted introduction of DNA sequences will lead to an increased risk because of unintended pleiotropic effects.

2.2.4 Antibiotic resistance gene [Confidential]

No antibiotic resistance markers are inserted into *Yarrowia lipolytica*.

2.2.5 Stability of the Introduced Genetic Sequences

Biocatalysts Ltd. follows a robust internal procedure to ensure the continued availability of viable, stable cultures for the production of enzymes.

Biocatalysts Ltd. maintains a master cell bank (MCB) of multiple vials of the host organism carrying the production strain harbouring the Phospholipase A2 gene stored at -80 °C. The MCB is preserved in 15% glycerol and aseptically aliquoted in pre-labelled cryovials to ensure traceability. The vials are promptly snap-frozen in absolute ethanol chilled with dry ice to minimise cell damage. The vials are quickly stored at -80 °C to ensure preservation and genetic stability.

In addition, a working cell bank (WCB) is maintained at the production facility. The WCB is prepared from the MCB under aseptic conditions to ensure the absence of contamination, following the procedure described above. Each new batch of WCB is checked for identity, viability and microbial purity. Providing all these parameters are correct, the strain is tested for production capacity at laboratory scale. If the productivity and the product quality meet the required standards, the new WCB is accepted for production runs.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2.2.6 Absence of Production Organism in the Product

The downstream process following the fermentation includes several unit operations to prevent presence of production organism in the final product. This is ensured through both process design and testing.

Cell debris is removed during the cell separation step. The filtration steps further ensure that no production organism is present in the final product.

The ability of these processing steps to remove the production organism from the enzyme preparation has been verified by testing the final product.

2.2.7 Absence of Transferable rDNA Sequences in the Product

Three batches of Phospholipase A2 samples have been analysed for the presence of residual *Yarrowia lipolytica* DNA. Both the qualitative and quantitative analyses suggest the absence of residual DNA, as no DNA is detected in the experimental conditions followed at Biocatalysts Ltd (Section 7.4).

2.2.8 Absence of Antibiotic Resistance Gene in the Product

As noted above, the transformed DNA does not contain any antibiotic resistance genes and therefore no antibiotic resistance markers are present in the strain.

2.3 Method of manufacture

2.3.1 Overview

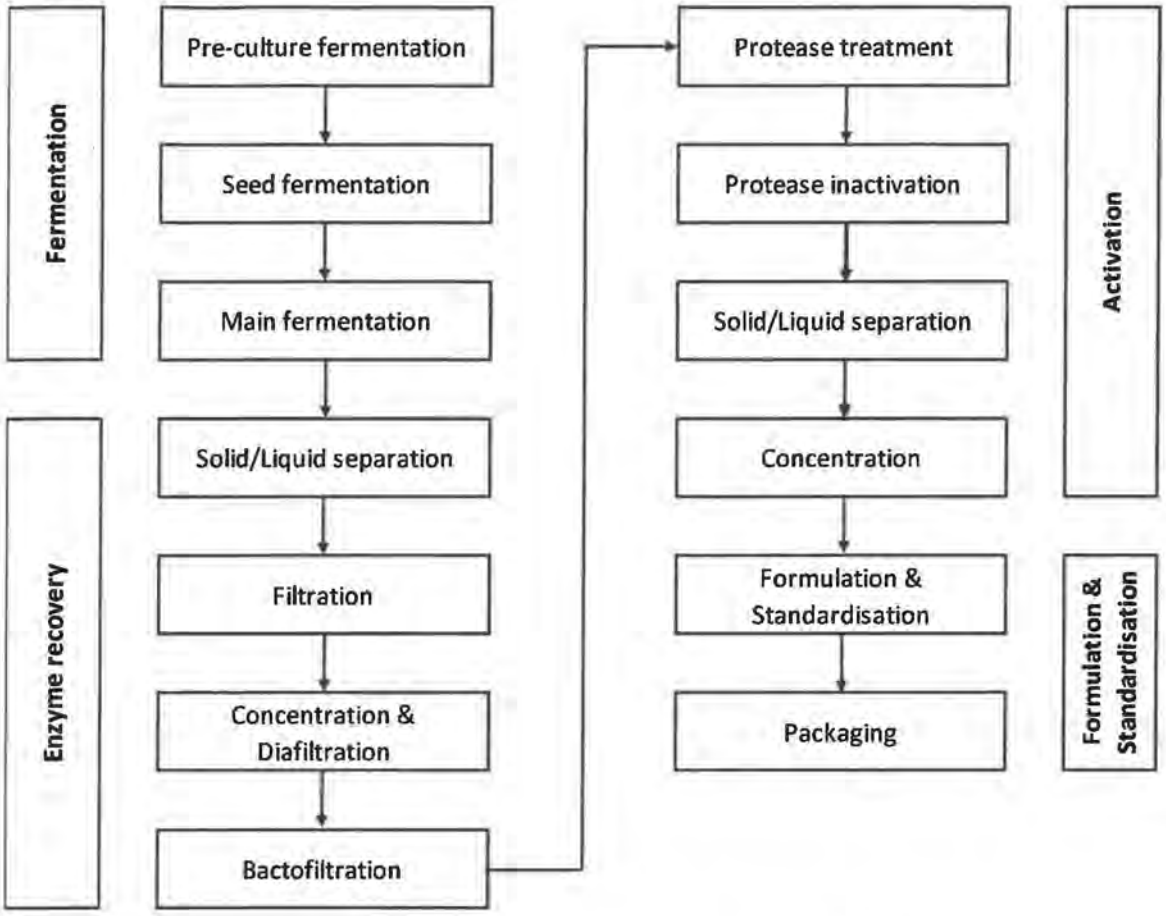
Phospholipase A2 is produced in accordance with current Good Manufacturing Practices for Food (cGMPs) and following the principles of Hazard Analysis of Critical Control Points (HACCP) for food production (Section 7.7). The enzyme manufacturing factory has the following certifications:

- FSMA accredited
- BSI:IS0 9001:2015
- BSI:ISO 14001:2015
- OHSAS 18001:2007
- FSSC 22000 4.1
- Local Trading Standards

Phospholipase A2 is manufactured under controlled fed-batch submerged fermentation of a selected, pure culture of *Yarrowia lipolytica*. The production process is split into four stages: fermentation, recovery (downstream processing) of the enzyme, activation of the enzyme and formulation/standardisation.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

PHOSPHOLIPASE A2 FLOWCHART



Flowchart 1: Phospholipase A2 manufacturing process at Biocatalysts Ltd.

2.3.2 Raw Materials

Biocatalysts Ltd. uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The raw materials used in the manufacture of Phospholipase A2 are of a grade suitable for the intended use and meet defined quality and safety standards set by the Food Safety and Quality Assurance Team at Biocatalysts Ltd. The raw materials used for the formulation of the product are food grade.

The materials used in the fermentation process include:

- Water
- A carbon source
- A nitrogen source
- Vitamins
- Salts and minerals
- pH adjustment agents
- Foam control agents

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

The antifoam agent used in the fermentation is used in accordance with the cGMP per the FDA correspondence to Enzyme Technical Association submission dated September 11, 2003. The maximum use level of the antifoam in the production process is < 0.15%.

The materials used during the recovery and activation include:

- Water
- Buffer salts
- Filter aids
- pH adjustment agents
- Food grade protease

The materials used during the formulation and standardisation include:

- Water
- Glycerol
- Sodium chloride
- Potassium sorbate

2.3.3 Fermentation Process

The main fermentation steps include the pre-culture fermentation, the seed fermentation and the main fermentation. During the fermentation process samples are taken before inoculations and at regular intervals during cultivation and harvest to test for purity. Should evidence of contamination exist, the batch is rejected.

2.3.3.1 Pre-culture fermentation

A pure culture of *Yarrowia lipolytica* harbouring the Phospholipase A2 gene is aseptically transferred to flasks containing sterile growth media and incubated under controlled conditions to ensure healthy growth of the biomass.

2.3.3.2 Seed fermentation

The pre-culture is aseptically transferred to the seed fermenter containing sterile media and is let to grow at controlled pH and temperature conditions.

2.3.3.3 Main fermentation

When an acceptable concentration of biomass is achieved in the seed fermenter, this is used as inoculum and aseptically transferred to the main fermenter containing sterile media. The fermentation runs as a normal submerged fed-batch fermentation where sterile feed media is added into the fermenter using a feed-rate regime tailored to maximise productivity of the enzyme. Environmental factors such as pH, temperature, aeration and agitation are constantly controlled.

During the main fermentation, the enzyme production rate is monitored, and the fermentation is stopped when no significant increase in production yield is observed.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2.3.4 Recovery Process

During fermentation, Phospholipase A2 is excreted by the producing microorganism into the fermentation medium. The recovery process is a multi-step operation including solid/liquid separation of the cell debris, filtration, concentration, diafiltration and bactofiltration carried out to recover the enzyme from the fermentation media and remove the production organism.

During the recovery process, the temperature, pH and turbidity of the solution are controlled to maximise protein recovery and minimise microbial growth.

2.3.4.1 Solid/Liquid separation

Separation of the Phospholipase A2 enzyme containing fermentation medium from the solids takes place at a defined pH and temperature using continuous centrifugation.

2.3.4.2 Filtration

At the end of the centrifugation step the centrate containing the Phospholipase A2 is filtered to remove fine particles.

2.3.4.3 Concentration and Diafiltration

The liquid containing Phospholipase A2 is concentrated to the desired enzyme activity. Diafiltration is then used as a method of removing permeable molecules (low molecular weight impurities, such as fermentation ingredient traces) from the solution while maximising protein recovery during the purification of the enzyme.

A further polish filtration may be required at this point to remove insoluble substances in order to facilitate the final bactofiltration step.

2.3.4.4 Bactofiltration

During bactofiltration the production organism is removed from the Phospholipase A2 containing solution. This step is also beneficial to minimise microbial presence in the final concentrated enzyme liquid.

2.3.5 Activation Process

Phospholipase A2 enzyme produced in the fermentation is in inactive form and must be cleaved by a trypsin-like protease to become functional. This is achieved by following a multi-step process that involves protease treatment, protease inactivation, solid/liquid separation and concentration.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2.3.5.1 Protease treatment

The pH and temperature of the Phospholipase A2 containing concentrated filtrate is adjusted to the optimum conditions for the protease incubation treatment.

2.3.5.2 Protease inactivation

After activation of the Phospholipase A2 enzyme, the temperature conditions of the solution are adjusted to inactivate the protease enzyme.

2.3.5.3 Solid/liquid separation

A clarification step is included in order to separate Phospholipase A2 from the insoluble solids.

2.3.5.4 Concentration

Phospholipase A2 is concentrated to the desired enzyme activity.

2.3.5.5 Bactofiltration

Phospholipase A2 is bactofiltered again to ensure that the microbial load is reduced to a minimum prior to its formulation into the final product.

2.3.6 Formulation and Standardisation Process

The commercial product of Phospholipase A2 as manufactured by Biocatalysts Ltd is named Lipomod™ 833L. The starting material for Lipomod™ 833L is the bactofiltered concentrate Phospholipase A2 post activation (known as "Phospholipase A2 concentrate"].

The Phospholipase A2 concentrate is standardised using water, glycerol and sodium chloride. The last two help reduce the water activity level to aid with the increase of stability of the enzyme product. In addition, the commercial product contains potassium sorbate which is used as preservative.

Phospholipase A2 concentrate and product Lipomod 833L are tested by the Quality Control Department for all quality related aspects. Providing the enzyme preparation passes all tests, the batch is released by Quality Control and packed in labelled, food grade containers before storage.

2.3.7 Quality Control of Finished Product

Lipomod™ 833L containing Phospholipase A2 concentrate complies with the JECFA/FAO/WHO and FCC recommended specifications for enzymes preparations used in food processing.

The specification for Lipomod™ 833L is analysed for the specifications given in Section 2.4.2.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2.4 Composition and Specifications

2.4.1 Enzyme activity

The main activity of the enzyme preparation is phospholipase A2 (EC 3.1.1.4).

PLA2 is a lipolytic enzyme that catalyses the hydrolysis of the sn-2 ester bond into a variety of different phospholipids. The reaction catalysed can be described as follows:

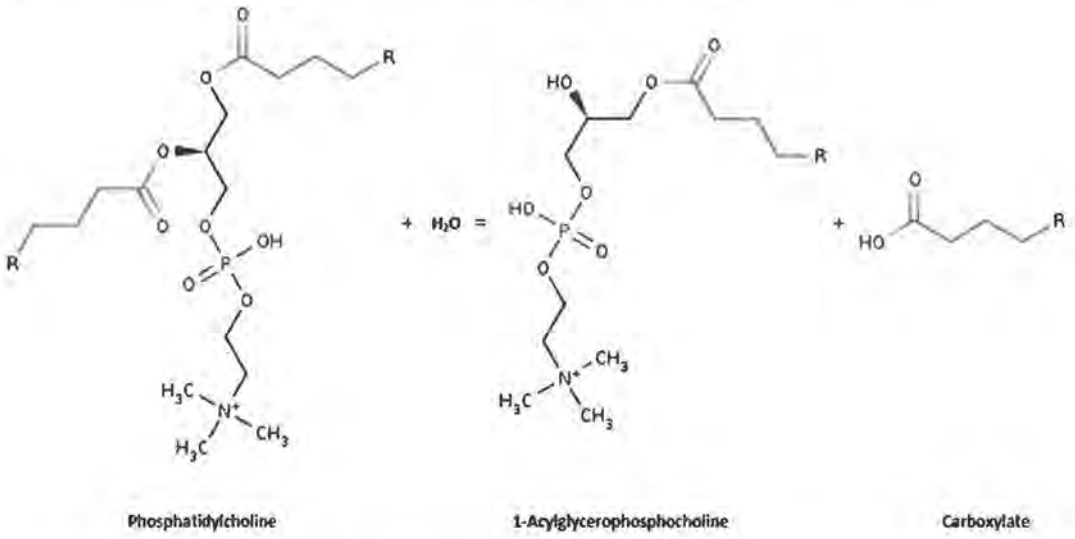


Figure 2: Enzymatic reaction of Phospholipase A2

The enzyme assay method to analyse the phospholipase A2 activity in the enzyme is company specific and can be found in Section 7.5. This assay has been developed and validated by Biocatalysts Ltd. to ensure that is reproducible and fit for purpose.

During production of Phospholipase A2, *Yarrowia lipolytica* also produces other enzymes that it requires for the breakdown of nutrients and other cell material. Although phospholipase A2 is produced in excess, the enzyme preparation could contain other minor enzymes activities such as protease.

The enzyme assay method to analyse the protease side activity in the enzyme is company specific and can be found in Section 7.6. This assay has been developed and validated by Biocatalysts Ltd. to ensure that is reproducible and fit for purpose.

2.4.2 Finished Product Specification

Specifications for the finished product Lipomod™ 833L meet the requirements of the Food Chemicals Codex (10th Edition) and Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006) monographs for enzyme preparations used in food processing (Section 7.1). Biocatalysts Ltd. has additionally included limits for total viable count, which is known as of concern to the food industry, and additional heavy metals specifications.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

The table below identifies the specifications for Lipomod™ 833L and the analysis results of different batches of Phospholipase A2.

Item	Lipomod™ 833L	Phospholipase A2 concentrate				Analysis method
	Specification (target)	Pilot Scale #22510	Pilot Scale #22921	Pilot Scale #23502	Commercial Scale #7006019	
Phospholipase A2 activity (U/ml)	>10,000 U/ml	25,593 U/ml	36,494 U/ml	36,352 U/ml	40,952 U/ml	Biocatalysts Ltd. validated internal method
Lead (mg/kg)	<5 mg/kg	0.055 mg/kg	<0.05 mg/kg	0.203 mg/kg	0.07 mg/kg	ISO 17294 (EPA 200.8)
Total viable count (cfu/g)	<50,000 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	BS EN ISO 4833-1:2013
Total Coliforms (cfu/g)	<30 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	BS EN ISO 4832:2006 at 37C
Salmonella (in 25g)	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	BS EN ISO 6579:2002
***Escherichia coli* (in 25g)**	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	ISO 16649-3:2015
Yeast and moulds (cfu/g)	<100 cfu/g	< 20 cfu/g	< 20 cfu/g	< 20 cfu/g	Yeast = 80 cfu/g Mould <20 cfu/g	BS ISO 21527-1:2008
Antimicrobial activity (in preparation)	Absent in preparation	Not tested	Not tested	Absent	Absent	FAO/JECFA Monograph 1

Table 1: Specifications for Lipomod™ 833L and analysis results of different batches of Phospholipase A2.

The Phospholipase A2 concentrate and Lipomod™ 833L may contain low concentrations of harmless substances derived from the microorganism and the fermentation medium. These may include polypeptides, proteins, carbohydrates and salts as shown in the nutritional analysis results of the food enzyme.

Parameter	Phospholipase A2 concentrate			
	Pilot Scale #22921	Pilot Scale #23502	Commercial Scale #7006019	Average

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Moisture (g/100g)	88.2	87.5	86.3	87.3
Total fat (g/100g)	<0.5	<0.5	<0.5	<0.5
Crude protein (g/100g)	7.0	6.8	7.2	7.0
Ash (g/100g)	<0.5	<0.5	<0.5	<0.5
Carbohydrates (g/100g)	4.6	5.4	6.3	5.4

Table 2: Nutritional analysis results of different batches of Phospholipase A2.

The Total Organic Solids (TOS) values of 3 different batches of Phospholipase A2 concentrate were as follows:

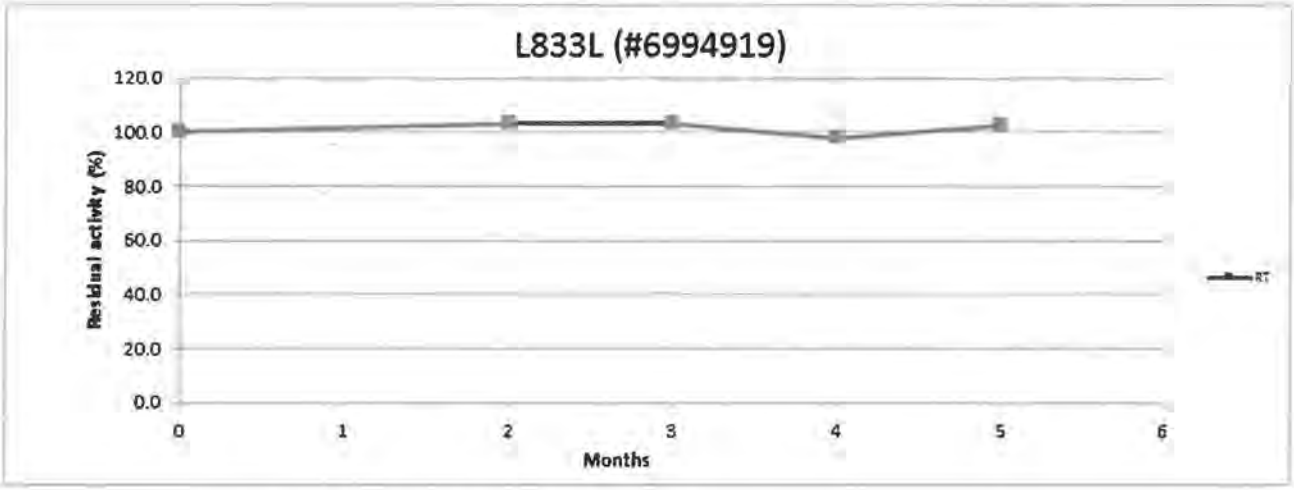
Parameter	**Phospholipase A2 concentrate**			
	Pilot Scale #22921	**Pilot Scale #23502**	**Commercial Scale #7006019**	**Average**
Phospholipase A2 activity (U/ml)	36,494	36,352	40,952	37,932.7
Protein (%)	7.0	6.8	7.2	7.0
Ash (%)	<0.5	<0.5	<0.5	<0.5
Water (%)	88.2	87.5	86.3	87.3
Total organic solids (TOS)[a] (%)	11.3	12.0	13.2	12.2
Phospholipase A2 activity / mg TOS (U/mg TOS)	323.0	302.9	310.2	311.8

Table 3: Calculated TOS of different batches of Phospholipase A2 concentrate.

2.4.3 Stability of the Notified Substance

To assess the stability of Lipomod™ 833L, one batch (#23502) of the enzyme preparation was stored at 8°C and 20°C and tested for phospholipase activity at different time points. Stability trials are still on-going but 5.5 month data shows that the Lipomod™ 833L retains 100% activity when stored at 20°C as shown in Graph 1.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com



Graph 1: Lipomod™ 833L stability data when stored at 20°C.

Phospholipase A2 activity profile has been characterised and pH and temperature activity data can be found in Section 7.2.

2.5 Physical or Technical Effect

2.5.1 Mode of Action

Phospholipases are classified as hydrolases which are a group of enzymes that hydrolyse various bonds. Phospholipase A2 catalyses the hydrolysis of natural phospholipids present in foodstuffs, resulting in the formation of lyso-phospholipids. Lyso-phospholipids are surface-active agents with emulsifying properties and can mimic the effects of chemical emulsifiers.

After hydrolysis, no substrate is left for the enzyme Phospholipase A2 to act upon. As a result, it is not functional in the foodstuff and can be regarded as a processing aid.

2.5.2 Application

Egg yolk is a complex oil water emulsion composed of 50% water, 32% lipids and 16% protein. Approximately 28% of the lipids are phospholipids, of which approximately 80% is phoshatidylcholine, 12% is phosphatidylethanolamine with other phospholipids such as sphingomyelin and lyso-phosphatidylcholine. The surface-active properties of these phospholipids can act a little like soap in stabilising oil water emulsions.

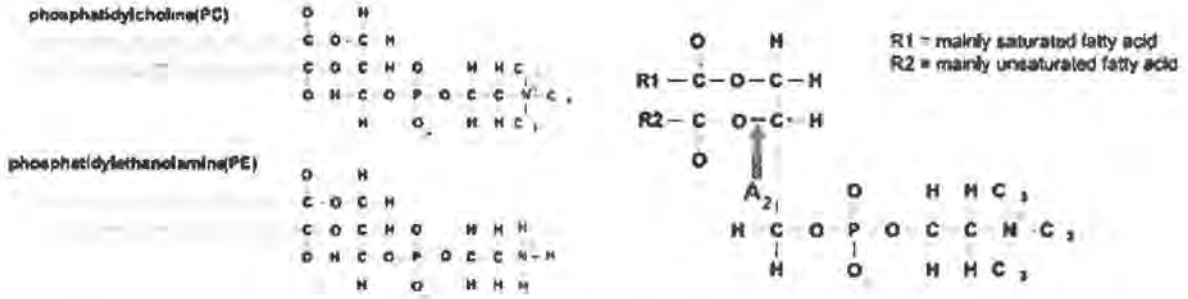
Enzyme-modified yolk greatly improves emulsification and gelation properties so that less modified yolk is required to produce the same viscosity as normal yolk in foods such as mayonnaise and salad dressings. Another key benefit to using enzyme-modified yolk is that the mayonnaise is more heat stable and can now be pasteurised without separating, resulting in increased microbial safety and a longer shelf-life.

2.5.2.1 Sauces and dressings

Egg-yolk is used in mayonnaise, sauces and dressings because of its emulsifying properties due to the presence of naturally occurring phospholipids.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Phospholipase A2 cuts at the Sn-2 position on the glycerol backbone to produce new molecules with different and superior emulsifying properties.



Structure of main phospholipids in egg yolk Phospholipase A2 specificity

Figure 3: Phospholipase A2 mode of action.

Biocatalysts Ltd. conducted laboratory tests on the efficiency of Phospholipase A2 in the hydrolysis of egg-yolk phospholipids. Test results demonstrated the positive effect of phospholipase A2 treated egg-yolk in the production of mayonnaise. These tests are described in Section 7.8.

2.5.3 Use levels

Enzyme preparations are generally used in *quantum satis* ("Q.S."); at the minimum level necessary to achieve the desired effect and according to requirements for normal production following GMP.

The dosage applied by the food manufacturer depends on the substrate type and quality, the addition of other ingredients into the foodstuff, enzyme incubation time and pH and temperature during the enzymatic reaction.

2.5.3.1 Sauces and Dressings

The recommended dosage of Phospholipase A2 is 28 – 279 ml Phospholipase A2 concentrate (equivalent to 100 – 1,000 ml of Lipomod 833L) per tonne of egg yolk (3.4 – 33.9 g TOS per tonne egg yolk) (Section 7.3). The use levels are not considered to be self-limiting to achieve the required technological benefits.

As a guide, whole egg or a 65 - 80 % w/v aqueous solution of egg yolk can be prepared. It is often advisable to add salt to prevent microbial growth during the process. The enzyme is stimulated by the presence of calcium. There is usually sufficient calcium present in egg products but in some cases, addition of extra calcium may increase the efficiency of the reaction. No pH adjustment is required. The reaction takes 2 – 4 hours at 40 – 60°C with gentle mixing. To prevent damage to the egg, some processors prefer to incubate the reaction at lower temperatures (25°C) for longer periods (overnight).

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2.5.4 Enzyme residues in the final food

2.5.4.1 Sauces and Dressings

Phospholipase A2 hydrolyses the phospholipids naturally present in egg-yolk. After hydrolysis, the substrate (the phospholipids) for the enzyme is depleted and the enzyme remains inactive in the egg-yolk like any other protein.

Ingredient	Concentration (%)
Sunflower oil	80.0
Water	7.5
Pasteurised egg yolk	6.0
Vinegar (4 - 4.5% acetic acid)	4.0
Sugar	1.0
Salt	1.0
Mustard	0.5

Table 4: Example of a standard mayonnaise recipe.

Based on the information given in Section 2.5.3.1 and the standard composition of mayonnaise, as described above, the TOS per tonne of mayonnaise can be calculated.

Foodstuff	Phospholipase A2 concentrate use in food ingredient	Amount of ingredient in final food	Residual amount of Phospholipase A2 concentrate in final food (in mg TOS)
Sauces and dressings; i.e. mayonnaise	3.4 – 33.9 g TOS/tonne egg yolk	6.0 g egg yolk/100g mayonnaise	203.4 – 2034.2 mg TOS/tonne mayonnaise

Table 5: Phospholipase A2 calculation of TOS in foodstuffs.

Phospholipase A2, like any other enzyme, performs its technological function during food processing. The reasons why enzymes do not typically exert enzymatic activity in the final food could be due to a combination of various factors, depending on the application and the process conditions used by food producers, such as:

- the enzyme protein must be in its 'native' (non-denatured) form,
- the substrate must still be present,
- the enzyme must be free to be able to reach the substrate,
- conditions like pH, temperature and water content must be favourable for the enzyme.

Failing to meet the conditions above, the enzyme will not be active in the final food product.

At the end of the egg-yolk hydrolysis process using Phospholipase A2, no substrate is left for the enzyme to act upon. In addition, during pasteurisation of the mayonnaise, the enzyme would be denatured by the heat treatment applied.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

As a result, it can be concluded that it is unlikely that Phospholipase A2 will be functional in the foodstuff and can be regarded as a processing aid.

2.5.5 Possible Effects on Nutrients

The catalytic activity of the enzyme preparation is very specific towards the hydrolysis of the sn-2 ester bond between a fatty acid and glycerol in phospholipids. Like the substrate and the enzyme, these reaction products are also natural constituents in various organisms from bacteria to mammals. As a result, phosphatides and fatty acids are quite abundant in the human diet.

Consequently, it is not expected that the reaction products obtained by the use of Phospholipase A2 will lead to a new or unintended effect on other constituents or nutrients present in foodstuffs and adverse effects on nutrients are not to be expected.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

3. Part 1 §170.325 – DIETARY EXPOSURE

3.1 Description of the Population Expected to Consume the Substance

As described in Section 6.2, Phospholipase A2 has a long history of safe use. This enzyme is ubiquitous in nature and is naturally present in animal and plant cells. It has been isolated from a number of food sources (including wheat flour) and animals such as pig. It is also a constituent of the digestive pancreatic juice of humans (de Haas et al. (1968); Rossiter (1968); Johnson and McDermott (1974)).

Similar Phospholipase A2 preparations from microbial sources such as *Aspergillus niger*, *Trichoderma Reesei* and from *Streptomyces violaceruber* have already been the subject of a GRAS notification and animal derived Phospholipase A2 has been affirmed a GRAS substance.

The products of the enzymatic reaction carried out by Phospholipase A2 (lyso-phospholipids and free fatty acids) play important roles in a number of metabolic processes in all organisms, from bacteria to mammals. As a result, lyso-phospholipids and free fatty acids are quite abundant in the human diet. Therefore, there is no basis to believe that conversion of phospholipids to lyso-phospholipids and free fatty acids will have a significant effect, if any, on processed foods or on the human body.

Since Phospholipase A2 produced in genetically modified *Yarrowia lipolytica* strain is a protein composed of natural amino acids and present in the final dressings and sauces at such low levels, it is expected it will be digested in the human gastrointestinal tract just as any other food protein/enzyme. In addition, because Phospholipase A2 reaction products are also naturally formed in the human body and so far the consumption of phospholipases worldwide has not led to any adverse events or allergic reactions, there is no basis to believe Phospholipase A2 can cause unfavourable reactions in humans and that the consumer population will not be affected by its presence in food.

3.2 Estimates of Human Consumption

The average yearly consumption of mayonnaise is estimated at 7g/person/day (g/p/d) and mayonnaise consumption by the 90th percentile consumer was 14g/p/d. in the U.S. (Pao, E.M. (1982)). 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 Phospholipase A2 concentrate contains 311.8 U per mg TOS and that all TOS remain in the mayonnaise.
b) It is assumed that all mayonnaise is produced using Biocatalysts Ltd Phospholipase A2 concentrate as a processing aid during the production process, and that it is used at the highest recommended dosage of 28 – 279 ml per tonne egg yolk.
c) The daily maximum consumption of the mayonnaise is 30 g/p/d.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Foodstuff	Residual amount of inactive Phospholipase A2 concentrate in final food (mg TOS/tonne mayonnaise)	Mayonnaise intake level (g food/person/day)	Estimated daily intake of inactive Phospholipase A2 concentrate (kU/kg body weight/day)*1	Estimated daily intake of inactive Phospholipase A2 concentrate (mg TOS/kg body weight/day)*1
Mayonnaise	203.4 – 2034.2 mg TOS/tonne mayonnaise	30	0.00003 – 0.00032 kU/kg body weight/day	0.00010 – 0.00102 mg TOS/kg body weight/day

*1 calculated for a person of 60 kg

Table 6: Inactive Phospholipase A2 calculation of TOS in foodstuffs.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

4. Part 4 §170.240 – SELF-LIMITING LEVELS OF USE

This part is not applicable to this notified substance, see Section 2.5.3.1 for further details regarding use levels.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

5. Part 5 §170.245 – EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This part is not applicable to this notified substance.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

6. Part 6 §170.250 – NARRATIVE OF THE CONCLUSION OF GRAS STATUS

6.1 Safety of the Production Organism: *Yarrowia lipolytica*

Yarrowia lipolytica is a dimorphic ascomycetous yeast that is readily isolated from various food products. The species was originally classified as a *Candida lipolytica* until the perfect form (sexual stage) was identified in the late sixties by Wickerham at the Northern Regional Research Laboratory of the USDA at Peoria (Barth and Gaillardin (1997)). Then it was reclassified first as *Endomycopsis lipolytica*, then as *Saccharomycopsis lipolytica* (Wickerham L.J. (1970)) and finally as *Yarrowia lipolytica* (Yarrod (1972)).

Yarrowia lipolytica is one of the more intensively studied yeast species. Jean-Marc Nicaud (2012) published a history of *Yarrowia lipolytica* research, including specific physiological, metabolic and genomic characteristics. Barth and Gaillardin (1997) also provided a comprehensive review on the available data on the physiology, cell biology, genetics and molecular biology of *Yarrowia lipolytica*. The environmental and industrial applications of *Yarrowia lipolytica* have been reviewed most recently by Bankar et al. (2009), food-related applications were described by Smita S. Zinjarde (2014) and a safety assessment review was published by Groenewald et al (2014).

Yarrowia lipolytica is generally regarded as a biosafety class 1 microorganism. This biosafety class encompasses microorganisms which are not known to cause disease in healthy adult humans (Lelieveld et al. (1996)). The safety issues of *Yarrowia lipolytica* were thoroughly evaluated and this yeast was labelled as a "safe-to-use" organism (Groenewald et al. (2014)). The aspects regarding the safety of the yeast are evident because (i) it is inherently associated with dairy, poultry, and meat products, (ii) yeast biomass is a safe nutritional supplement, (iii) it is consumed as food and feed, and (iv) food-grade additives have been obtained from this yeast (Zinjarde (2014)).

Yarrowia lipolytica can naturally be found in a number of foods such as yoghurts, kefir and in various types of cheese (e.g. cheddar cheese, Stilton Blue cheese, Armada cheese, Reblochon cheese, Italian-style cheeses, Rokpol). Even though not added deliberately to cheese, *Yarrowia lipolytica* has been reported to be among the common yeast species therein (Roostita and Fleet (1996); Welthagen and Viljoen (1998); Larpin et al., (2006); Monnet et al., (2010); Larpin-Laborde et al. (2011)).

There is a long history of *Yarrowia lipolytica* use the industry for food and feed. In the 1950s, *Yarrowia lipolytica* was used by British Petroleum Co. (BP) to produce single cell protein (SCP) for animal feeding (Groenewald et al. (2013)). Citric acid production using *Yarrowia lipolytica* has been granted "Generally Regarded as Safe" (GRAS) status by the US FDA (21 CFR 173.165). *Yarrowia lipolytica* has obtained GRAS status for the production of eicosapentaenoic acid (EPA)-rich triglyceride oil (GRN000355), erythritol. (GRN000382), rebaudioside A (GRN000632) and steviol glycosides consisting primarily of rebaudioside M (GRN 000759).

In addition, *Yarrowia lipolytica* is included in the 2012 update of the "authoritative list of microorganisms with a documented use in food", originally established in a joint project between the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (Bourdichon et al. (2012)).

More recently, in January 2019, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) concluded that given the qualified presumption of safety status for production purposes of *Yarrowia lipolytica* granted by the EFSA Panel on Biological Hazards (BIOHAZ) (Ricci et al. (2018)) and the fact that the production process of a yeast biomass as novel food did not raise safety concerns, no toxicological studies were needed for the safety assessment of the novel food and it was therefore deemed safe under the proposed conditions of use (Turck et al. (2019)). The applicant proposed to use the novel food, *Yarrowia lipolytica* yeast biomass, as a food supplement in the form of capsules,

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

tablets or powder and the target population for the novel food as food was the general population above 3 years of age.

Yarrowia lipolytica is regarded as non-pathogenic and non-toxigenic (Holzschu et al. (1979)) but has been associated with disease. A review of the safety of *Yarrowia lipolytica* concluded that the species causes rare opportunistic infections in severely immunocompromised or otherwise seriously ill people with other underlying diseases or conditions. However, those infections can be treated effectively with the use of regular antifungal drugs, and in some cases, disappeared spontaneously. The occasional occurrence of opportunistic infections of *Yarrowia lipolytica* in immunocompromised and catheterized patients does not differ from other microorganisms with a history of safe use, such as *Saccharomyces cerevisiae* (Groenewald et al. (2013)). In addition, strain specific differences were observed in the *Yarrowia lipolytica* ability to stimulate the formation of biogenic amines. However, the concentrations of biogenic amines associated with the use of *Yarrowia lipolytica* in cheese ripening (up to 120 mg/kg) were stated not to give any reason for health concerns (Wyder et al. (1999)).

If the production organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (Pariza and Johnson (2001)), as shown in the examples above. Pariza and Foster (1983) define a non-toxigenic 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 non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances".

Yarrowia lipolytica is a safe strain for production of food ingredients, as reported in the literature available in the public domain. The modifications performed by Biocatalysts Ltd. did not introduce antibiotic production or resistance genes into the production organism; neither did they introduce any toxin-production genes. The modifications inserted the Phospholipase A2 gene of *Sus scrofa*, which has a history of safe use.

The safety of the Phospholipase A2 enzyme preparation produced using *Yarrowia lipolytica* has been evaluated using the decision tree scheme of Pariza and Johnson (2001). These authors report that the safety of the production microorganism is the prime consideration when assessing the safety of an enzyme preparation intended for use in food.

The decision tree did not reveal concerns and since the aforementioned characteristics of the production organism are safe, Biocatalysts Ltd. concludes that the use of the genetically modified *Yarrowia lipolytica* presents no known safety concerns.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Question	Answer	Outcome
1. Is the strain genetically modified?	Yes. The *Yarrowia lipolytica* production strain was genetically modified to express Phospholipase A2 from *Sus scrofa*.	(If yes, go to question 2)
2. Modification by recDNA?	Yes. The production strain was modified using recombinant DNA techniques.	(If yes, go to question 3a)
3a. Expressed product history of safe use?	Yes. *Yarrowia lipolytica* has been modified to produce the enzyme Phospholipase A2. The safety of Phospholipase A2 is described in the current GRAS document.	(If yes, go to question 3c)
3c. Test article free of transferable antibiotic resistance gene DNA?	Yes. As described in the GRAS document no antibiotic resistance gene is present in the production strain *Yarrowia lipolytica*.	(If yes, go to question 3e)
3e. All introduced DNA well characterized and safe?	Yes. The plasmid containing the Phospholipase A2 enzyme was sequenced and is well characterised.	(If yes, go to question 4)
4. Introduced DNA randomly integrated?	No. Copies of the PLA2 gene are present in the *Yarrowia lipolytica* genome were inserted into pre-defined locus. The yeast *Yarrowia lipolytica* is not known to harbour genes encoding for toxins or otherwise harmful sequences so it is not expected that targeted introduction of DNA sequences will lead to an increased risk because of unintended pleiotropic effects.	(If no, go to question 6.)
6. *Production strain from safe lineage?*	Yes. The strain of *Yarrowia lipolytica* used is from a safe lineage.	**(If YES: The test article is accepted)**

Table 7: Analysis based on the Decision Tree of MW Pariza and EA Johnson (2001).

6.2 Safety of the Phospholipase A2

Phospholipases are classified as hydrolases which are a group of enzymes that hydrolyse various bonds. Phospholipase A2 catalyses the hydrolysis of the sn-2 fatty acyl bond of phospholipids to liberate free fatty acids and lysophospholipids. The products of the reaction do not have toxic properties and are readily metabolised by the human body.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Phospholipase A2 is found in animal tissues including pig (de Haas et al. (1968)), rat (Arnesjö et al. (1967)), ox (Dutilh et al. (1975)), sheep (Dutilh et al. (1975)), ostrich (Ben Bacha et al. (2007)) and horse pancreas (Evenberg et al. (1977)). It has also been found in human pancreatic juice (Grataroli et al (1982)). In addition, Phospholipase A2 genes have been identified in plants (Lee et al (2005)).

Animal derived lipases have been affirmed as GRAS substances (21 CFR§184.1415). These lipases are derived from edible forestomach tissue of calves, kids, or lambs, or from animal pancreatic tissue.

Animal and microbial derived Phospholipase A2 is currently used as a processing aid to improve the emulsifying capabilities of naturally present or added phospholipids (primarily lecithins) to improve the desired characteristics of the foods [21 CFR§184.1063]. Furthermore, Phospholipase A2 enzymes, have been on the market for decades and have been approved for use in food on the basis of safety documentation in countries such as USA, Canada, Mexico, Brazil, France, Australia and New Zealand, Japan or China.

Examples of the use of phospholipases in food industry can be found in the production of edible oils, dairy, and baking products or emulsifying agents. Thus, phospholipases are incorporated in processes such as the degumming of vegetables oils during refinement for removing undesirable compounds, the manufacture of cheese for yield increasing, or the production of bread as bakery improvers for reducing the inclusion of emulsifying compounds or manufacture of sauces and dressings with improved properties (Ramrakhiani and Chand (2011)).

Phospholipase A2 enzyme produced by *Yarrowia lipolytica* is equivalent to the Phospholipase A2 enzyme derived from pig pancreas, an enzyme which already has a history of safe use.

The safety of the Phospholipase A2 was assessed using the Pariza and Johnson (2001) decision tree (Table 7).

Based on the information above, Biocatalysts Ltd. concludes that Phospholipase A2 enzyme has a history of safe use in food. In spite of this, no information about the use of the notified substance in food prior to 1958 has been found in literature.

6.2.1 Allergenicity

Enzymes are proteins and as such, they have the potential to cause allergic responses in sensitive individuals.

Industrial enzymes are typically used as processing aids, thus in very small amount during food processing. They are generally not functional in the final food because they are removed and/or denatured; therefore, resulting in a low exposure to the food consumers. As a result, in spite of the vast variety of applications of enzyme in food industry and their long history of use, there have been no confirmed reports of allergies in consumers caused by ingestion of enzymes used in food processing (Pariza and Foster (1983)).

The absence of allergenicity caused by the use of food enzymes has also been reviewed by AMFEP's Working Group on Consumer Allergy Risk from Enzyme Residues in Food (Section 7.9) and concluded there are no scientific indications that the small amounts of enzymes in foods can sensitise or induce allergy reactions in consumers.

Nevertheless, an evaluation of the potential to cause allergy is conducted for every new enzyme developed. To evaluate the potential allergenicity of phospholipase A2 enzyme from *Sus scrofa*, the sequence comparison with known allergenic proteins was done using three dedicated servers available on internet as follows:

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

- The AllergenOnline tool is recommended by the Protein Allergenicity Technical Committee of ILSI-HESI (International Life Science Institute-Health and Environmental Sciences Institute).
- SDAP (Structural Database of Allergenic Proteins)
- Algpred (Prediction of Allergenic Proteins and Mapping of IgE Epitopes

Server	Search	Number of matches	Allergen alignment
Allergen Online	Match for full-length alignments by FASTA	9 Hits	Allergen See below
	80 amino acid sliding window search	0 Hits	Non-Allergen
	Match for 8 consecutive amino acid sequence	0 Hits	Non-Allergen
SDAP	Match for full-length alignments by FASTA	30 Hits	Allergen See Below
	80 amino acid sliding window search	53 Hits	Allergen See Below
	Match for 8 consecutive amino acid sequence	0 Hits	Non-Allergen
	Match for 6 consecutive amino acid sequence	2 Hits	Allergen See Below
AlgPred	Mapping of IgE epitope	0 Hits	Non-Allergen
	MAST Results	0 Hits	Non-Allergen
	Prediction by SVM method based on amino acid composition	Score threshold -0.4 Positive Prediction Value = 64.55% Negative Prediction Value = 86.61%	Allergen
	Prediction by SVM method based on dipeptide composition	Score threshold -0.4 Positive Prediction Value = 63.10% Negative Prediction Value = 85.56%	Allergen
	BLAST results of ARPS	0 Hits	Non-Allergen
	Hybrid approach (SVMc + IgE epitope + ARPs BLAST + MAST)	N/A	Allergen

Table 8: Summary of servers used and number of matches.

No similarities found for:

- 80 amino acid sliding window search (Allergen Online)
- Match for 8 consecutive amino acid sequence (Allergen Online & SDAP)
- Mapping of IgE epitope (AlgPred)
- MAST scan (AlgPred)
- BLAST search against 2890 allergen-representative peptides (ARPs) (AlgPred)

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Significant hits were identified with:

- Match for full-length alignments by FASTA (Allergen Online& SDAP) total of 39 hits.
- Match for 6 consecutive amino acid sequence (SDAP)

Significant BLAST hits, but with no contiguous stretches of more than 4 amino acids include:

- Phospholipase A2 (Bee)
- Serum Albumin (Dog)
- Agglutinin isolectin A (Wheat)
- Cereal trypsin/alpha-amylase inhibitor; CM16 protein (Wheat)
- Salivary antigen 1; FS-I (Cat flea)
- Thaumatin-like protein (various plants)
- Serine protease inhibitor (Nematode)
- Non-specific lipid transfer protein type 1 (French bean)

Significant BLAST hits, with one contiguous 6 amino acid sequence include:

- vitellogenin (Chicken)

The AlgPred Hybrid approach is generally taken to provide a clear overview result and says that the PLA2 is an allergen. Only one contiguous 6 amino acid sequence match was identified (Chicken vitellogenin), the majority of other hits are statistically significant with the default search parameters but probably do not represent significant allergens. Although PLA2 has significant matches to Phospholipase A2 from insect venom, it is unlikely, due to the diversified functionality of these enzymes that this represents an allergen potential in PLA2 similar to bee venom, for example.

The lack of hits to known IgE epitopes is important since Allergen-specific immunoglobulin E (IgE) antibodies play a pivotal role in the development of food allergy.

Based on the analysis described above and the literature review carried out, Biocatalysts Ltd. concludes that it is improbable that Phospholipase A2 from *Sus scrofa* is a significant allergen.

6.3 Safety on the Manufacturing Process

The manufacturing process used to make Phospholipase A2 enzyme employs a pure culture submerged fermentation of the *Yarrowia lipolytica* production strain. Good Manufacturing Practices are used throughout the process which utilizes generally accepted, published methods for manufacture, purification and formulation of microbial enzymes. The fermentation process of microbial food enzymes and the recovery process are substantially equivalent across the world.

All raw materials used as processing aids in the fermentation and recovery processes are standard materials used in the enzyme industry and of a grade suitable for use in the intended use.

The final Phospholipase A2 enzyme preparation meets the general and additional requirements set for enzyme preparations as outlined in Food Chemicals Codex and by JECFA.

6.4 Safety Summary

On the basis of the evaluation above, including a review of the published literature and history of safe use of Phospholipase A2 and *Yarrowia lipolytica*, the limited and well defined nature of the genetic

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

modifications as described in Section 2 and the low TOS present in the end foodstuffs as described in Section 3, it is concluded that Phospholipase A2 produced by submerged fermentation of the genetically modified microorganism *Yarrowia lipolytica* for the proposed uses in food can be safely manufactured and used as a processing aid.

6.5 Conclusion

The regulatory framework for determining if a substance can be considered generally recognised as safe (GRAS) in accordance with sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (the Act) states that:

a) FDA's implementing regulations in 21 CFR 170.3 and 21 CFR 170.30, the use of a food substance may be GRAS either through scientific procedures or, for a substance used in food before 1958, through experience based on common use in food Under 21 CFR 170.30(b), general recognition of safety through scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of the substance as a food additive. General recognition of safety through scientific procedures is based upon the application of generally available and accepted scientific data, information, or methods, which ordinarily are published, as well as the application of scientific principles, and may be corroborated by the application of unpublished scientific data, information, or methods.
b) Under 21 CFR 170.30(c) and 170.3(f), general recognition of safety through experience based on common use in foods requires a substantial history of consumption for food use by a significant number of consumers.

This criterion has been applied in this GRAS notification, and as discussed above, Biocatalysts Ltd. has concluded that Porcine Phospholipase A2 enzyme produced by the genetically modified strain of *Yarrowia lipolytica* is GRAS via scientific procedures for use as a processing aid in the production of sauces and dressings. All documentation provided in the GRAS determination is publicly available and generally known, and therefore meet the "general recognition" standard under the FFDCA.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7. Part 7 §170.255 – LIST OF SUPPORTING DATA AND INFORMATION

This section contains a list of all the data and literature discussed in this dossier to provide a basis that the notified substance is safe under the conditions of its intended use.

7.1. Technical Product Specification

BIOCATALYSTS
exceeding enzyme expectations

Technical Product Specification
Lipomod™ 833L (L833L)

Product Specification

Test	Specification
Phospholipase A2 activity	>10,000 U/ml
Lead	<5mg/kg
Arsenic	<3mg/kg
Cadmium	<0.5mg/kg
Mercury	<0.5mg/kg
Total viable count	<50,000cfu/g
Coliforms	<30cfu/g
Yeasts and Moulds	<100cfu/g
E.coli	Absent in 25g
Salmonella	Absent in 25g
Antibiotic	Absent

Material complies with the JECFA/FAO/WHO and FCC recommended specifications for enzymes used in food processing.

Activity Unit Definition
Phospholipase A2 - One unit of enzyme activity is defined as that amount of enzyme that causes the release of one micromole of free fatty acid per minute at pH 8.0 and 40°C.

Ingredient List

Component	Approx. %*
Enzyme	2.0%
Water	57.9%
Glycerol	30.0%
Sodium chloride	10.0%
Potassium sorbate	0.1%

*Values are representative and may vary from batch to batch.

Nutritional Composition

Component	per 100g (calculated)*
Carbohydrates	Not currently available
of which sugars	
Fat	
Protein	
Moisture	
Calories	

*Values are representative and may vary from batch to batch.

Product Type/Origin

Test/Characteristics	
Colour*	Brown
Form	Liquid
Activity	Phospholipase A2
Origin	Microbial
Production organism	*Yarrowia* sp.

*Colour of product may vary from batch to batch. Colour is not an indication of enzyme activity.

Allergens

Allergen Group	Present in product
Cereals containing gluten	No[1]
Crustaceans and products thereof	No
Egg and products thereof	No
Fish and products thereof	No
Peanuts and products thereof	No
Soybeans and products thereof	No
Milk and products thereof (including lactose)	No
Nuts and products thereof	No
Celery and products thereof	No
Mustard and products thereof	No
Sesame seeds and products thereof	No
Sulphur dioxide and/ or sulphites at concentrations of more than 10mg/kg or 10mg/L (litre) in terms of the total SO_2	No
Lupin and products thereof	No
Molluscs and products thereof	No

1. If No: Gluten free i.e. <20ppm (EU Regulation 41/2009)

GMM/GMOs
This product is not a GMO and does not contain GMOs. This product does therefore not require labelling as GMO on food labels.[1,2]

1. Regulation EC 1829/2003 Article 12
2. http://ec.europa.eu/food/food/biotechnology/reports_studies/docs/COM_2006_626_en.pdf

Visit our website for further relevant & current information www.biocatalysts.com

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

BIOCATALYSTS
exceeding enzyme expectations

Technical Product Specification
Lipomod™ 833L (L833L)

Storage
Best before date: Detailed on batch specific certificate of analysis and on container label.

The majority of products are made to order and delivered with >90% of shelf-life. At a guaranteed minimum the material is delivered with >50% of the product shelf-life remaining. Shelf-life can potentially be extended with an activity retest. May incur additional cost.

Recommended storage conditions: 0 - 8°C to achieve the documented shelf-life.

Regulatory Status
Enzyme legislation is in place in various countries, please contact Biocatalysts for information on country specific regulations.

Kosher/Halal Status
This product is Kosher and Halal certified. For our Kosher/Halal certificate please contact our Customer Services Department.

Biocatalysts' manufacturing facilities are Kosher and Halal certified.

Packaging
The contact packaging used for all food grade enzymes is food grade. All packaging is recyclable.

Integrity of packaging must be maintained. Store in a dry environment out of direct sunlight.

Quality & Food Safety
Biocatalysts operates a preventative risk-based Food Safety System that ensures the environment and processes are designed to produce safe products every time. FSSC22000 and FSMA compliant.

Compliance - The Company's integrated management system encompasses Quality, Food Safety, Health and Safety and GMP.

Certification – Biocatalysts is certified to ISO9001, ISO14001, OHSAS 18001 and FSSC22000.

Certificates are available on request from the Customer Services Department.

Health & Safety
Always read the Material Safety Datasheet (MSDS) before use and retain. If you are in any doubt about recommended product handling and safety, please contact Biocatalysts before use. Generally, when using enzymes avoid contact with the skin and eyes and do not breathe dusts or aerosols containing them. MSDSs are available in other languages. Please contact Customer Services.

Contact Us: Please send any enquiries regarding the above information to customerservices@biocats.com.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

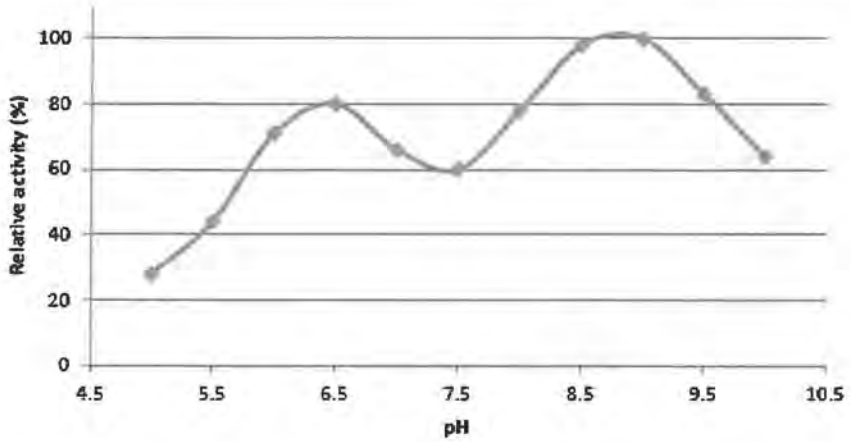
7.2 Activity Profiles

BIOCATALYSTS
exceeding enzyme expectations

Biocatalysts Ltd
Cefn Coed, Parc Nantgarw, Cardiff
CF15 7QQ Wales UK
Tel: 01443 843712
email: sales@biocats.com
www.biocatalysts.com

Activity Profile - Lipomod™ 833L

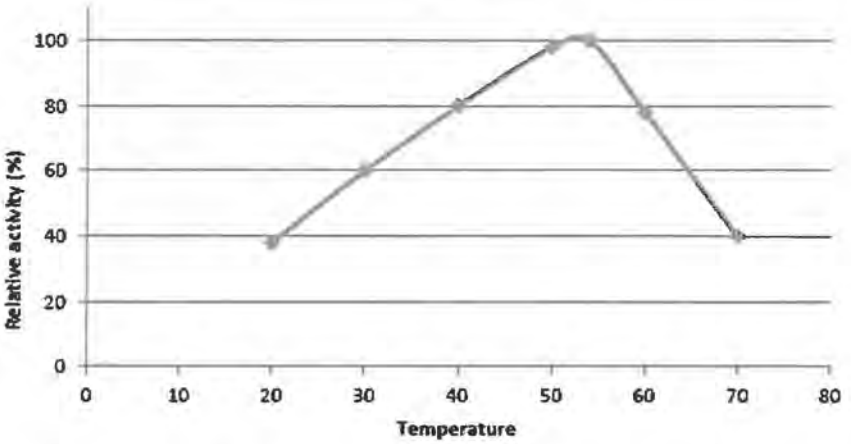
pH Profile



The pH profile of Lipomod™ 833L was created using a standard assay procedure.

The activity-pH profile for Lipomod™ 833L might be slightly different depending on the substrate and conditions used during the application.

Temperature Profile



The temperature profile of Lipomod™ 833L was created using a standard assay procedure.

The activity-temperature profile for Lipomod™ 833L might be slightly different depending on the substrate and conditions used during the application.

Visit our website for further relevant & current information www.biocatalysts.com

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.3 Datasheet

BIOCATALYSTS
exceeding enzyme expectations

Lipomod™ 833L (L833L)

Features/Benefits

- Microbial phospholipase A2 for the production of lyso-lecithin emulsifiers
- Enzyme modified yolk provides increased viscosity and heat stability in mayonnaise manufacture
- Kosher, Halal and vegetarian.

Biocatalysts supply enzymes that modify the functionality of egg and can therefore be used as an aid to egg processing. During commercial processing eggs are separated into egg white or egg yolk products and the effect of enzymes on this process is covered in depth by our Technical Bulletin titled "The use of Enzymes in Egg Processing."

Egg yolks have extremely useful emulsifying and gelation properties due to the presence of various lipid and protein types. Phospholipids can be modified by the action of Lipomod™ 833L to produce lyso-lecithin with superior emulsifying properties especially useful in mayonnaise manufacture.

Lipomod™ 833L is a microbial phospholipase A2. It does not contain any animal products and is halal and kosher certified.

Specification

Activity	10,000 U/ml minimum
Biological Source	*Microbial*
Form	Brown liquid
Optimum pH Range	5.0 – 9.0
Optimum Temperature Range	40 - 50°C

Application & Dose

Lipomod™ 833L can be used to improve the emulsifying properties of egg yolk, whole egg or purified lecithin. Egg yolk or whole egg should be warmed to between 40 - 60°C. No pH adjustment is required if pH of egg yolk is between pH 5.0 and pH 8.0. L833L should be added at 200 – 1000 mL per ton of egg yolk. Ensure the enzyme is evenly distributed throughout the mixture with gentle mixing. The reaction takes 2-4 hours to reach completion at 50°C.

Health & Safety

Always read the Material Safety Datasheet (MSDS) before use and retain. If you are in any doubt about recommended product handling and safety, please contact Biocatalysts before use. Generally, when using enzymes avoid contact with the skin and eyes and do not breathe dusts or aerosols containing them. MSDSs are available in other languages. Please contact Customer Services.

Storage

Liquids: Activity will remain within specification for at least 6 months from the date of manufacture when stored at 0-5°C.

Allergens

None present.

Food Status

Material complies with the JECFA/FAO/WHO and FCC recommended specifications for enzymes used in food processing.

GM Status

This product is not a GMO and does not contain GMOs. This product does therefore not require labelling as GMO on food labels.

Quality & Food Safety

Biocatalysts operates a preventative risk-based Food Safety System that ensures the environment and processes are designed to produce safe products every time. FSSC22000 and FSMA compliant.

Compliance - The Company's integrated management system encompasses Quality, Food Safety, Health and Safety and GMP.

Certificates are available on request from the Customer Services Department.

Availability

Liquids: Available in 1, 5 or 25kg packs.

Visit our website for further relevant & current information www.biocatalysts.com

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.4 DNA quantification in Phospholipase A2

1. EXECUTIVE SUMMARY

PLA2 samples from Biocatalysts Ltd. production batches 23502, 22921, 7006019 have been analysed for the presence of residual *Yarrowia lipolytica* DNA. Both the qualitative analysis (such as agarose gel electrophoresis before/after DNaseI or RNaseA treatment) and the quantitative analysis (such as qPCR with primers annealing a housekeeping gene from *Yarrowia lipolytica*) indicate the absence of residual DNA. It should be noted that in the neat samples and their dilutions up to 1:20, there are qPCR inhibition effects which might affect the detection of DNA in the samples at these very concentrated levels. These inhibition effects clearly disappear when samples are diluted between 20- and 320-fold.

2. OBJECTIVES

Qualitative and quantitative analysis of residual *Yarrowia lipolytica* DNA content in PLA2 batches 23502, 22921, 7006019.

3. METHODS

- **Samples preparation**

Samples were diluted with sterile water at a concentration of 20 mg/mL and used for all the analysis described in this report.

- **Agarose gel electrophoresis**

10 µL of each sample (20 mg/mL) were loaded on a 1% agarose gel, with addition of 2 µL 6X Loading dye (SYBRSafe®, Cat: S33102, Invitrogen)

- **DNase/RNase treatment**

5 µL of each sample were incubated for 1h at 37C in the presence of 1 µL DNaseI (2,000 units/mL, M0303L, NEB) or 1 mL RNaseA (10 mg/mL, Cat: 12091021, ThermoFisher), in a 10 µL reaction mix containing DNaseI/RNaseA buffer. After incubation, 2 µL of 2X loading dye (SYBR®Safe®, Cat: S33102, Invitrogen) were added, and samples were loaded on 1% agarose gel. As a negative control, each sample was incubated in the presence of buffer only.

- **qPCR analysis**

qPCR experiments were performed using a set of primers (Table 1) annealing the *Yarrowia lipolyca* housekeeping gene Actin 1 (*GenBank: AJ250347.1*). qPCR was run by means of an Agilent® qPCR AriaMx system, with a set up following reported (Table 2).
A standard curve was created using genomic DNA extracted from *Yarrowia lipolytica* strain Y1212 within the range 20 ng/µL – 0.000125 ng/µL).
Samples were properly diluted, and each dilution was analysed by qPCR with or without the addition of doping DNA, such as 5 ng of gDNA from Y1212 (Table 3). All the experiments have been performed in triplicate.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Table 1: Primers set

Primer ACTYI-F1	CGAAAGGATCTCTACGGAAAC
Primer ACTY-R1	GCGGTGATCTTGACCTTGAT

Tm= 65C

Table 2: qPCR method

Segment	Plateau	Temperature	Duration
Amplification	1	95	00:00:10
Amplification	2	65	00:00:30
Amplification	3	72	00:01:00
Melt	1	95	00:00:30
Melt	2	65	00:00:30
Melt	3	95	00:00:30

Table 3: qPCR reaction mix

Q5® polymerase (Cat: M0491L, NEB)	0.2
Q5® buffer	4
Primer ACTYI-F1	1
Primer ACTY-R1	1
EvaGreen® Dye (Cat:31000, Biotum)	1
dNTP (Cat: 18427013, Thermofisher)	0.4
H2O	7.4

When needed: 1µl of 5 ng/µl Y1212 gDNA were added

4. RESULTS

- **Agarose gel electrophoresis and DNAseI/RNaseA treatment**

The following data show that the brown pigment of the samples (typically produced during *Yarrowia lipolytica* fermentations) is responsible for the smear visible on the agarose gel (Figure 1). In fact, when the treatment with either DNaseI or RNaseA is performed (Figure2A – 2B), no differences can be observed (differently from the case of a positive control sample (Figure 3) which clearly suggests the action of DNaseI and RNaseA on gDNA and RNA, respectively), suggesting that the fluorescence is not due to the presence of DNA.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Figure 1: 1% Agarose gel electrophoresis

Figure 2A: DNaseI/RNaseA treatment on PLA2 samples

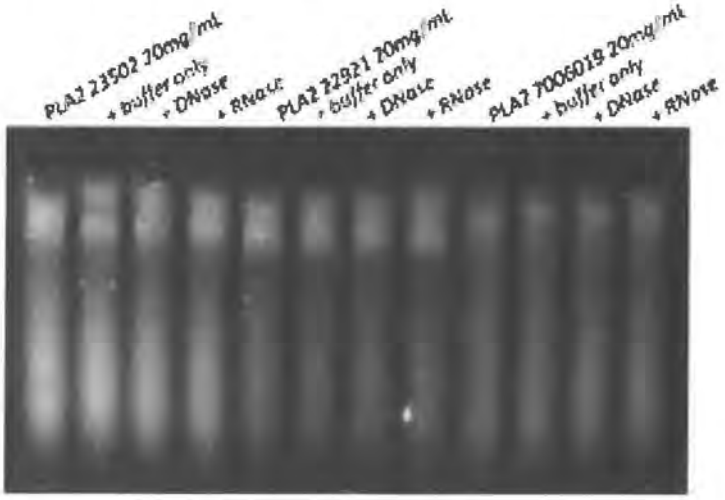


Figure 2B: DNaseI/RNaseA treatment on a positive control

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

- **qPCR analysis of doped/not doped samples**

qPCR results (Table 4) demonstrate for all the samples the occourrence of inhibition effects on the DNA amplification. In fact, when the doping occours (i.e. addition of 5ng gDNA to the sample), the external DNA can be detected only starting from a 1:20 dilution.
At the dilutions where inhibition effects do not occour (from 20- to 320-fold), no DNA can be detected other than the 5 ng added. In accordance with the DNaseI/RNaseI treatment results, this suggests that residual *Y. lipolytica* DNA is either absent in PLA2 samples or below the detection limit for the experimental conditions here reported.

Table 4: qPCR results

	Sample	Equiv. ng DNA in reaction mix		Sample	Equiv. ng DNA		Sample	Equiv. ng DNA in reaction mix
PLA2 batch 23502	1	n.d.	PLA2 batch 22921	1	nd	PLA2 batch 7006019	1	nd
	1:5	n.d.		1:5	nd		1:5	nd
	1:10	n.d.		1:10	nd		1:10	nd
	1:20	n.d.		1:20	nd		1:20	nd
	1:40	n.d.		1:40	nd		1:40	nd
	1:80	n.d.		1:80	nd		1:80	nd
	1:160	n.d.		1:160	nd		1:160	nd
	1:320	n.d.		1:320	nd		1:320	nd
	1 + 5ng gDNA	n.d.		1 + 5ng gDNA	nd		1 + 5ng gDNA	nd
	1:5 + 5ng gDNA	n.d.		1:5 + 5ng gDNA	nd		1:5 + 5ng gDNA	nd
	1:10 + 5ng gDNA	n.d.		1:10 + 5ng gDNA	nd		1:10 + 5ng gDNA	nd
	1:20 + 5ng gDNA	2.52 ± 0.89		1:20 + 5ng gDNA	3.72 ± 0.42		1:20 + 5ng gDNA	2.04 ± 1.39
	1:40 + 5ng gDNA	4.18 ± 1.19		1:40 + 5ng gDNA	4.73 ± 0.26		1:40 + 5ng gDNA	4.18 ± 0.47
	1:80 + 5ng gDNA	4.87 ± 0.03		1:80 + 5ng gDNA	5.14 ± 0.14		1:80 + 5ng gDNA	4.59 ± 0.07
	1:160 + 5ng gDNA	4.76 ± 0.35		1:160 + 5ng gDNA	5.48 ± 0.31		1:160 + 5ng gDNA	4.82 ± 0.41
	1:320 + 5ng gDNA	5.37 ± 0.26		1:320 + 5ng gDNA	5.33 ± 0.30		1:320 + 5ng gDNA	4.82 ± 0.35

5. CONCLUSIONS AND RECOMMENDATIONS FOR NEXT STEPS

Phospholipase A2 samples from Biocatalysts Ltd. production batches 23502, 22921, 7006019 have been analysed for the presence of residual *Yarrowia lipolytica* DNA. Both the qualitative and quantitative analyses indicate the absence of residual DNA, as no DNA is detected in the experimental conditions followed at Biocatalysts Ltd.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.5 Phospholipase A2 Activity Assay

Assay Procedure *Auto-titrator assay for the analysis of PHOSPHOLIPASE A2*

This is based on the release of fatty acids from the substrate lecithin by a Phospholipase A_2 enzyme. The fatty acids subsequently released are continuously titrated at pH 8.0 using 0.01M NaOH. The activity of the enzyme is determined by the amount of 0.01M NaOH required to keep the pH within these limits over a 5 minute period.

ASSAY CONDITIONS

pH	8.0
Temperature	40 °C
Substrate	Lecithin Soy Bean
Incubation time	5 minutes

UNIT DEFINITION One unit of enzyme activity is defined as the amount of enzyme that causes the release of 1 micromole of free fatty acid per minute at 40°C and pH 8.0.

EQUIPMENT

Stirring Water bath set at 40°C
Blender
Thermometer
Stirring bars
Multi pipette
Gilson p5000 pipette
Gilson p100 pipette
Mitsubishi Automatic Titrator (Model GT-100)

All equipment should be calibrated to the requirements set out in the appropriate EOP, according to the Biocatalysts ISO9001 Manual.

BIOCATALYSTS
exceeding enzyme

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

REAGENTS

Reagent	CAS No	Item Code	Supplier
Sodium Deoxycholate	302-95-4	27836.135	VWR
Calcium Chloride Dihydrate	10035-04-8	223506-500G	Sigma
L-Phosphatidylcholine, Soybean type IV-S	8002-43-5	P5638	Sigma
1M HCl	7647-01-0	32050.602	VWR
0.01M NaOH	1310-73-2	1.60309.4000	Merck

Water should be RG grade unless otherwise specified. Record lot numbers and quantities used for all reagents. Make sure timings are exact.

1. **0.016M Sodium Deoxycholate** – prepare fresh daily

   Dissolve 0.67g sodium deoxycholate *(HARMFUL)* in approximately 80ml of water. Make up to 100ml in a volumetric flask.

2. **0.32M Calcium Chloride** - prepare fresh daily in a universal container

   Dissolve 0.47g calcium chloride dihydrate ($CaCl_2.2H_2O$) *(IRRITANT)* in 10ml of water – use a 5ml pipette.

3. **Lecithin Substrate** – prepare fresh daily. L-Phosphatidylcholine, Soybean type IV-S (Sigma P5638) check batch number.

   For the analysis of 20 samples (including blanks), weigh out 10g of soybean lecithin in a 500ml glass beaker.

   Add 200ml of water and stir for 20 minutes on speed setting 2.

   Slowly add 10ml $CaCl_2$ *(IRRITANT)* solution in a drop wise fashion. Stir for exactly 5 minutes on speed setting 2.

   Add 100ml sodium deoxycholate *(HARMFUL)* solution. Stir for 20 minutes on speed setting 2.

   Make up to 500ml with water in a volumetric flask. Make sure to rinse the beaker.

   Blend for exactly 1 minute on full power using the grey glass blender with the rubber lid on the 'smoothie' setting.

   Dispense a 25ml aliquot using a 5ml pipette and place in the water bath at 40°C to stir (on speed 300) for exactly 30 minutes before commencing the assay.

4. **0.001M HCl** - stable for 1 year if stored at 15 - 25°C. Make fresh daily.

   Dilute a stock solution of 1M HCl *(CORROSIVE)* to give a working dilution of 0.001M (1/1000).

5. **0.01M NaOH** – stable for 1 year if stored at 15 - 25°C.

   Bought in from Sigma/Merck *(CORROSIVE)*.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

6. **Enzyme Dilutions** – prepare fresh daily

Liquid and solid enzymes should be first inverted to distribute the sample and weighed in the analytical balance (+/-0.001g), add this weight to the sheet so that an exact dilution can be calculated. The enzyme sample should be diluted in 0.001M HCL (4) to a concentration which when assayed, gives a test-blank titre of 1.5-2.5mls (this is the linear range of the assay).

For a liquid sample:

$$\text{dilution} = \text{expected activity (u/ml)}$$

For a solid at 1mg/ml:

$$\text{dilution} = \frac{\text{expected activity (u/g)}}{1000}$$

PROCEDURE

Switch on the auto-titrator and carry out the following checks before commencing analysis:

Place pH 7 buffer solution under the electrodes. The pH should read between pH 7.1 and pH 6.9. If the displayed pH is outside these limits a calibration is required as described below:

1. Calibration of pH electrodes

(1) Press **[Option]**.
(2) Press the **[down arrow]** twice to select Hardware Set-up and Press **[OK]**.
(3) Press **[OK]** to enter the pH calibration section.
(4) Select the **Point 2 (Manual)** option with the **[down arrow]** and Press **[OK]**.
(5) Enter 7 into the 1st standard point and press **[Enter]**.
(6) Rinse electrode with water and place the pH 7 buffer under the electrode. Change buffers each month.
(7) Leave for 10 minutes and press **[Enter]**.
(8) Enter 10 into the 2nd standard point and press **[Enter]**.
(9) Rinse electrode with water and place the pH 10 buffer under the electrode.
(10) Leave for 10 minutes and press **[Enter]**.
(11) Press **[OK]** to accept the calibration.
(12) Press **[Cancel]** repeatedly to return to the Start screen.
(13) Place buffer pH 7 and pH 10 under the electrodes again to recheck the calibration. The pH should read between 7.1 and 6.9 for pH 7 and between 10.1 and 9.9 for pH 10. Keep record of the pH reading for each buffer.

2. Priming the Glass titration nozzle

(1) Press **[Buret]** and then **[Mode]**.
(2) Select *Manual operation* using the **[down arrow]** and press **[Enter]**
(3) Press the **[right arrow]** to rotate the valve to the nozzle.
(4) Press the **[up arrow]** continuously to purge the system.
(5) Press **[Escape]**, wait while the unit initialises.
(6) Press **[Mode]** to bring the burette back online. Make sure it is back online, **[Mode]** may have to be pressed twice.
(7) Press **[Cancel]** to return to the Start screen.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

3. Changing / Refilling the Titrant (if required)

(1) Prime the burette (as above) until the 0.01M NaOH titrant bottle and vessel are empty
(2) Replace / Refill the 0.01M NaOH titrant bottle.
(3) Press [Escape] and wait while the vessel fills with 0.01M NaOH.
(4) Prime the burette again (as above).
(5) Press [Escape].
(6) Once the vessel is full, prime the burette a further 3 times.
(7) Press [Mode] to bring the burette back online.
(8) [Press [Cancel] to return to the Start screen.

4a. Loading the correct software files

(1) Press [Method] followed by the [down arrow] to access the Titration files.
(2) Press [Enter] and the use the arrow keys to scroll through the files.
(3) Press [OK] to enter the relevant files. This assay requires File 9, which should be set up with the parameters shown below:

File 9 – Sigma PLA2

Reag : NaOH M=0.01 E=1
Mode : stat
Detect : pH sens
Brt : 1
Speed : 400 ul/s
V max : 50.0ml
P_e : 7.7 pH
P_e : 8 pH
V_{up} : 50 ul
Cont : 2 sec
W tint : 0.1 min
Gain : 5
P-cyc : 0.1 min
T_{max} : 5.1min

Adjust the P_e and P_e (if necessary) using the arrows keys and keypad. Press [OK] and then [Cancel] to save the changes and return to the Start screen.

4b. Loading the correct software files (continued)

(1) Press [Method] followed by [Enter]
(2) Use the arrow keys to select the PLA2 file
(3) Press [OK] to enter the file.

The sample list will come up as below. An asterisk in the size column indicates that results are stored for those samples (they cannot be re-run). To remove these samples, navigate to the size column above the samples you wish to remove, enter 0 and press [Enter]. This will remove the asterisk and therefore these sample numbers can now be re-used.

No	Sample	Func	Size	Unit	P	FN	S
1				ml		9	
2				ml		9	
3				ml		9	
4				ml		9	

Press [OK] followed by [Cancel] to return to the start screen. The instrument is now ready to commence the assay.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

ANALYSIS

(1) Prepare enzyme samples at required dilutions in 0.001M HCl. Keep samples on ice.
(2) The probes and titrator nozzle are positioned, using a clamp, in the water bath so that the pH and reference electrodes are submerged in the substrate.
(3) Manually adjust the pH to 8 (+/-0.01 pH units) using 0.01M NaOH. This usually requires 6-8ml of NaOH. 6ml can be added in 2ml aliquots to bring the pH close to 8. A Pasteur pipette should then be used after this. Approximately 20 drops from the Pasteur pipette equates to 1ml. Keep record of how much NaOH is required. If it is greater than 9ml then do not use this sample. Be very careful when the pH is close to 8 and make sure the pH is between 7.992-8.008 before commencing the assay.
(4) Add 2ml of the enzyme sample to the substrate and press [Start]. Titrant will be added appropriately to maintain the pH at 8. Record the titre to calculate the activity of the enzyme, when reading the titre read the second value with a 1 before it on the titrator
(5) The blank, standard and positive control should be run before any samples – see below.

A blank must be carried out at the start and end of analysis by adding 2 ml of 0.001M HCl to the substrate instead of your enzyme sample (the average of the 2 results is used in the calculation), the titre for the blank should be 0.3-0.4.

The standard should be filtered before use and should be assayed at a 1/10000 dilution, this should be achieved by completing two 0.5ml in 50ml dilutions. The titre should be around 1.8-2.2.

A positive control using (L699L) should also be run at a 1/10000 dilution.

Run repeats for each sample. The duplicate runs should be ±10% of each other.

For each run record the sample name, dilution, titre, initial pH reading when the sample was added and the approximate volume of NaOH required to begin the assay at pH 8. See assay sheet.

When not in use the probes should be kept in pH 7 buffer solution.

Calculation Theory

Number of moles of titrant used = $\frac{V \times M}{1000}$ (divide by 1000 as titrant in mls and molarity in and molarity in litres).

To convert to micro-moles

$$= \frac{VxM}{1000} \times 1{,}000{,}000 = V \times M \times 1000$$

$$U/ml = \frac{V \times 1000 \times M \times d}{v \times t}$$

$$= V \times d$$

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

$$U/g = \frac{V \times 1000 \times M \times d}{[E] \times v \times t} \times 1000$$

$$= \frac{V \times d}{[E]} \times 1000$$

Where V = volume of 0.01M NaOH added (test titre-average blank titre)
t = time of assay (5)
M= Molarity of NaOH (0.01)
v = Volume of enzyme sample (2)
[E] = concentration of enzyme in mg/ml
1000 = to convert from u/mg to u/g
d = dilution

A FACTOR IS THEN INTRODUCED TO BRING THE ACTIVITY IN LINE WITH BIOCATALYSTS UNITS, BASED ON THE ACTIVITY CALCULATED ABOVE OF A STANDARD. THIS WILL CHANGE FOR EACH NEW CONTAINER OF L-PHOSPHATIDYLCHOLINE. SOYBEAN TYPE IV-S (SIGMA P5368) OPENED.

TO ACHIEVE THIS FACTOR A VALIDATION EXERCISE MUST BE CARRIED OUT ON A STANDARD PRODUCT FOR EACH CONTAINER OF SUBSTRATE.

HEALTH AND SAFETY ISSUES

1. Ensure you have read and understood the COSSH Assessments for all reagents used in this assay (found on the COSSH database)
2. Wear a mask when weighing out enzyme powders, or use the extractor fan on the weigh safe.
3. Laboratory glasses should be worn at all times.
4. Ensure all Hazard cards are filled out in detail and any appropriate hazard labels used. *(Refer to notes in italics throughout the procedure)*

Related Documents

Number	Type	Title
<NO DATA>	<NO DATA>	<NO DATA>

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.6 Caseinase Activity Assay

CONFIDENTIAL

Procedure For *Casein Protease Assay*

The rate of generation of peptides from the substrate is a measure of the catalytic activity of the protease being tested. The peptides released during the assay are separated from the substrate protein using trichloroacetic acid (TCA) and the TCA-soluble peptides are then measured by the method of Folin and Ciocalteu.

ASSAY CONDITIONS

pH	7.5
Temperature	37 °C
Substrate	2% Hammarsten casein
Incubation time	10 minutes

UNIT DEFINITION

One unit of protease activity is defined as that amount of enzyme which will liberate one micromole of tyrosine equivalents per minute at pH 7.5 and 37°C.

EQUIPMENT

Waterbath set to 37°C
Spectrophotometer set to 578nm
Whatman No1 filter papers
P5000, P1000, P200 pipettes
pH meter
Bench top centrifuge
RG water should be used

All equipment should be calibrated to the requirements set out in the appropriate EOP, according to the Biocatalysts ISO9001 Manual.

exceeding expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

CONFIDENTIAL

Procedure For *Casein Protease Assay*

Reagent	Supplier/ Supplier Code	CAS No.
Sodium Hydroxide pellets	S8045	1310-73-2
Potassium dihydrogen orthophosphate	VWR 26936.293	7778-77-0
di-potassium phosphate-3-hydrate	VWR 103495H	16788-57-1
Hydrochloric Acid (Convol)	VWR 32050.602	7647-01-0
Hammarsten Casein Solution	VWR 440203H	9000-71-9
Tyrosine	Sigma T3754	60-18-4
Trichloroacetic acid	Sigma T4885-500G	76-03-9
Folin and Ciocalteu's phenol reagent	Sigma F9252-500ml	

REAGENTS

1M Sodium Hydroxide – Stable for 1 year when stored at room temperature
Dissolve 40g sodium hydroxide pellets *(Sodium Hydroxide is a CORROSIVE chemical)* in 800ml water and make up to 1 litre.

1M Phosphate Buffer, pH 7.5 - Stable for 6 months when stored at room temperature
Add 26.3g potassium dihydrogen orthophosphate and 183.9g di-potassium phosphate-3-hydrate to 900ml RG water.

Check pH is 7.5. Adjust accordingly if required. Make up to 1L in a volumetric flask.

1M Hydrochloric Acid - Stable for 1 year when stored at room temperature
Empty the contents of a 1M 'Convol' vial into a volumetric flask and make up to the volume specified *(Hydrochloric Acid is a CORROSIVE chemical)*

Hammarsten Casein Solution (2% w/v) – Prepare fresh daily
Suspend 2g casein in 10ml water by manually mixing with a stirrer. Add approximately 3ml of sodium hydroxide and stir until a partially clear solution is

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Procedure For *Casein Protease Assay*

produced. Add 50ml water and 10ml 1M phosphate buffer. Stir using the magnetic stirrer until dissolved (approximately 30mins). Adjust pH to 7.5 slowly using 1M HCl with constant stirring to ensure that all the casein remains in solution. Make up to a final volume of 100ml with RG water.

0.05M Hydrochloric Acid - Stable for 6 months when stored at room temperature
Dilute 5ml 1M HCl with 95ml water.

Standard Tyrosine Solution (100mM) - Stable for 6 months stored at room temperature
Dissolve 1.812g L-Tyrosine in 100ml 1M HCl (3). Dilute 1 in 20 with RG water prior to assay.

Enzyme Samples – Prepare fresh daily
Liquid and solid enzymes should be first inverted to distribute the sample and weighed in the analytical balance (+/-0.001g).
Dilute with water immediately prior to assay to a concentration which gives an OD change of 0.1 to 0.4.

0.3M Trichloroacetic Acid (TCA) - Stable for 1 year when stored at room temperature
Dissolve 24.5g TCA *(TCA is a very CORROSIVE chemical)* in 400ml water and make up to 500ml in a volumetric flask.

0.5M Sodium Hydroxide - Stable for 1 year when stored at room temperature
Dilute 1M NaOH 1 in 2 in water.

Folin Ciocalteu's Phenol Reagent – Prepare fresh daily
Perform a 1 in 4 dilution of Folin reagent with RG water. *(Folin Reagent is a TOXIC chemical)*

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Procedure For *Casein Protease Assay*

PROCEDURE

Time / mins	Reagent	Tube Sample 1	Tube Sample 2	Tube Sample Blank	Tube Tyrosine Standard 1	Tube Tyrosine Standard 2	Tube Colour blank
0	Casein substrate	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml	-
5	Enzyme	0.2ml	0.2ml	-	-	-	-
	Tyrosine	-	-	-	0.2ml	0.2ml	-
	0.05M HCl	-	-	0.2ml	-	-	-
15	0.3M TCA	5ml	5ml	5ml	5ml	5ml	-
	Enzyme	-	-	0.2ml	-	-	-
	0.05M HCl	0.2ml	0.2ml	-	0.2ml	0.2ml	-
	Mix, and filter through Whatman No1 filter paper. In plastic test-tubes, add the following:						
20	Filtrate	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml	-
	Water	-	-	-	-	-	2.5ml
	0.5M NaOH	5ml	5ml	5ml	5ml	5ml	5ml
	Folin	1.5ml	1.5ml	1.5ml	1.5ml	1.5ml	1.5ml
	Mix and centrifuge for 10 minutes at 4000rpm and 4°C.						
30	Read the absorbance of the supernatant at 578nm against the colour blank.						

NOTES

1. During substrate preparation ensure casein has dissolved properly and none has precipitated following the pH change.
2. After addition of TCA to the tubes to end the reaction, make sure the tubes are mixed thoroughly before filtering them.
3. Add filtrate (2.5ml) to centrifuge tubes followed by the sodium hydroxide and phenol reagent as stated in the protocol. If completed in any other order the assay will not work.

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Procedure For *Casein Protease Assay*

CALCULATION THEORY

$$U/g = \frac{\text{Average OD sample} - \text{OD sample blank}}{\text{Average OD standard}} \times \frac{500}{C}$$

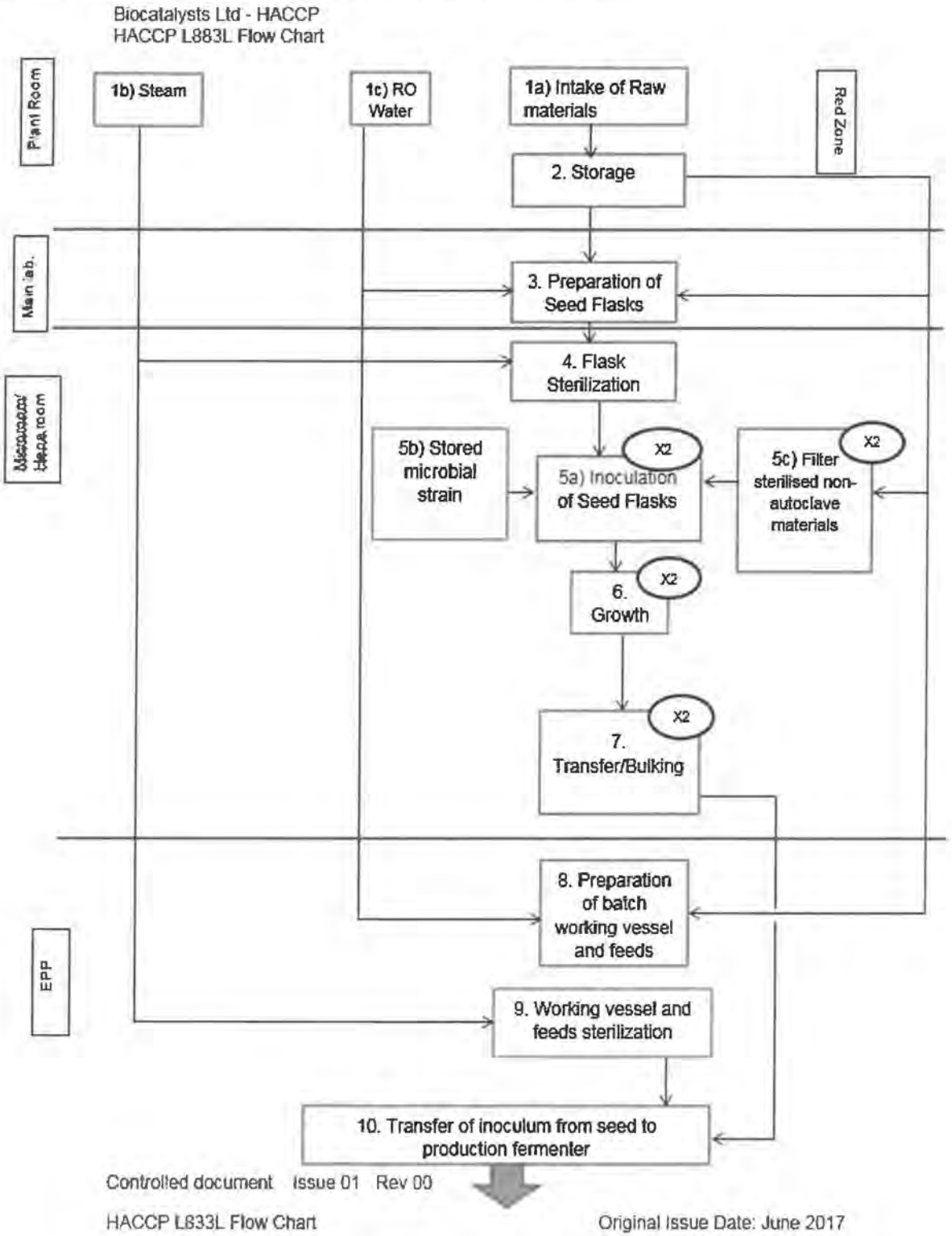
Where C = concentration of enzyme (mg/ml).

HEALTH AND SAFETY ISSUES

1. Ensure you have read and understood the COSHH Assessments for all reagents used in this assay.
2. Wear a mask when weighing out enzyme powders or use the extractor fan on the weigh safe.
3. Safety glasses should be worn at all times.
4. Ensure all Hazard cards are filled out in detail and any appropriate hazard labels used.
   (Refer to notes in italics throughout the procedure)
5. Empty contents of any tubes or cuvettes used at the spectrophotometer into a waste container and clean after each assay.

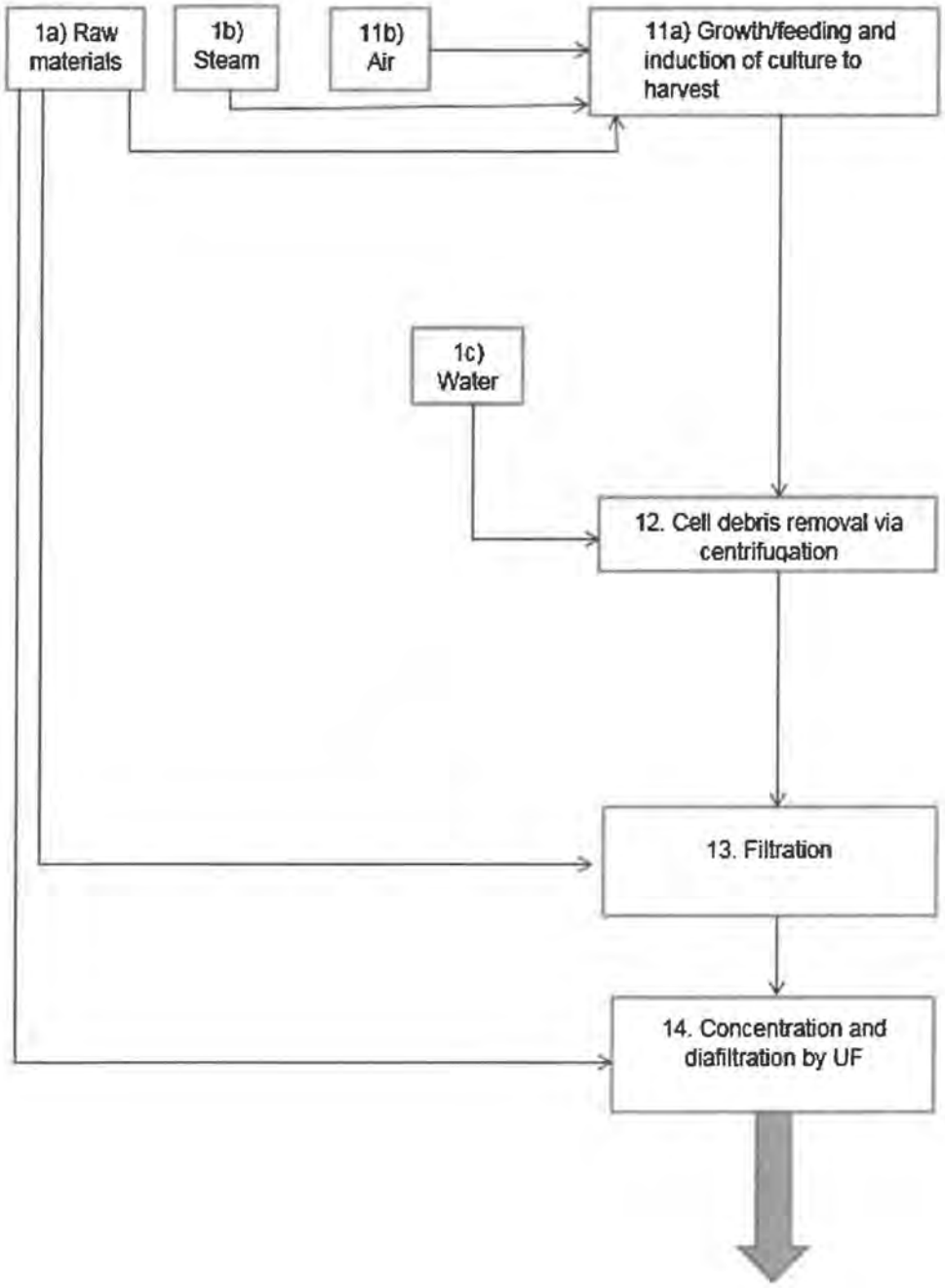
Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.7 HACCP Flow Chart (Confidential)



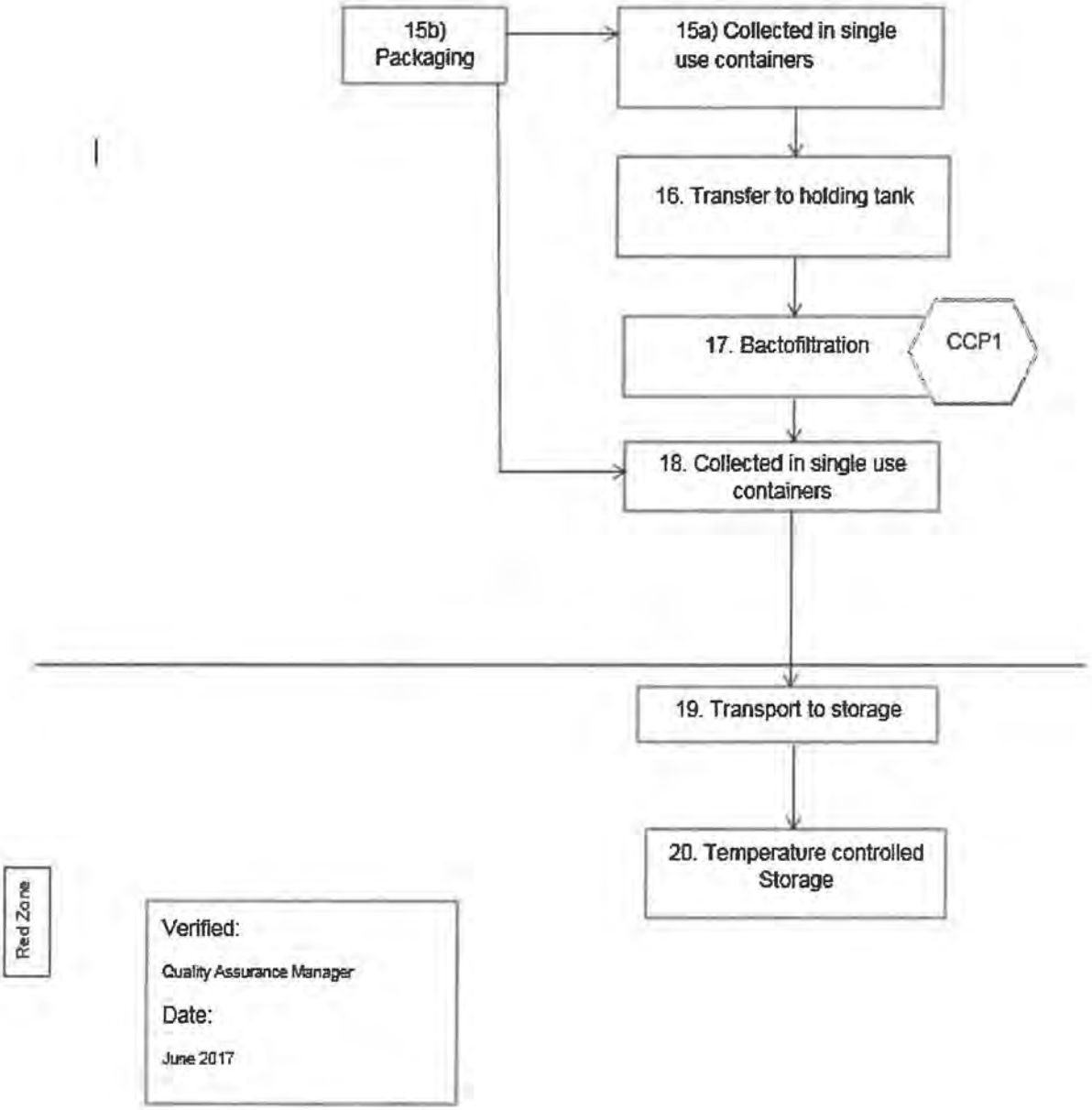
Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Biocatalysts Ltd - HACCP
HACCP L883L Flow Chart



Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Biocatalysts Ltd - HACCP
HACCP L883L Flow Chart



Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.8 Application trials

Introduction to L833L

- L833L Kosher / Halal microbial phospholipase A2
- Production not reliant on supply of animal raw materials
- Production organism *Yarrowia lipolytica*
- Dosage 100 - 1000ml/ ton of egg yolk, no pH adjustment required
- Incubate 2-4 hours at 40-60°C with gentle mixing

L833L Specification	
Activity	10,000U/mL minimum
Form	Liquid (colour brown)
Optimum pH Range	5.0 – 9.0
Optimum Temperature Range	40 – 50°C
Storage	0 - 8°C

BIOCATALYSTS
exceeding enzyme expectations

External Evaluations

An external evaluation was conducted by Campden BRI

- Evaluate and compare the functionality of mayonnaise manufactured with egg yolk modified by L833L and a competitor enzyme
- Both enzymes added to egg yolk at 2 dosage levels
  - 250ml per ton of egg yolk
  - 400ml per ton of egg yolk
- All batches of egg yolk incubated at 50°C for 4 hrs
- At 4 hrs enzyme activity stopped by decreasing temperature to 10°C

BIOCATALYSTS
exceeding enzyme expectations

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Mayonnaise Manufactured for External Evaluations

Mayonnaise produced by a 2-stage process

1. Pre-mixed in a pilot scale Silverson high shear mixer
2. Crude emulsion then passed through a colloid mill creating a finer emulsion

Mayonnaise Formulation Used:-

Sunflower oil	80%
Water	7.5%
Pasteurised egg yolk	6%
Vinegar (4-4.5% acetic acid)	4%
Sugar	1%
Salt	1%
Mustard	0.5%

BIOCATALYSTS
exceeding enzyme expectations

External Evaluations Testing using L833L and Competitor Enzyme

Phospholipids hydrolysis study

- Measured at different time points using pH meter

Viscosity study

- Viscoelastic properties of mayonnaise measured using rheometer
- Shear rate range used 0.1 – 100 1/s
- Tested in duplicate at 20°C

Firmness study

- Firmness of mayonnaise measured using texture analyser
- 25mm diameter cylinder and probe set at 10g force
- Tested in triplicate

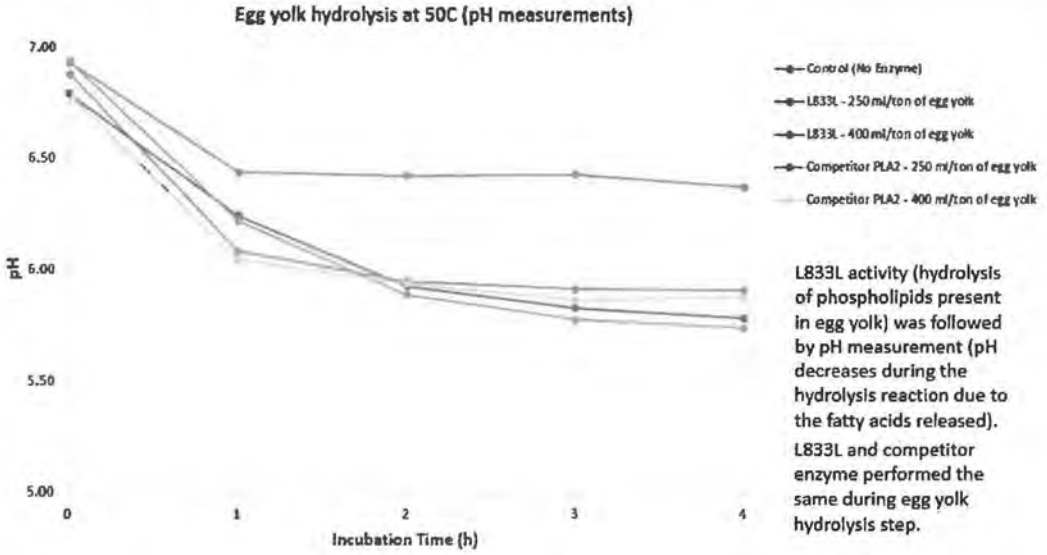
Firmness stability study

- Visual observation of samples at two different time points

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Phospholipids hydrolysis study

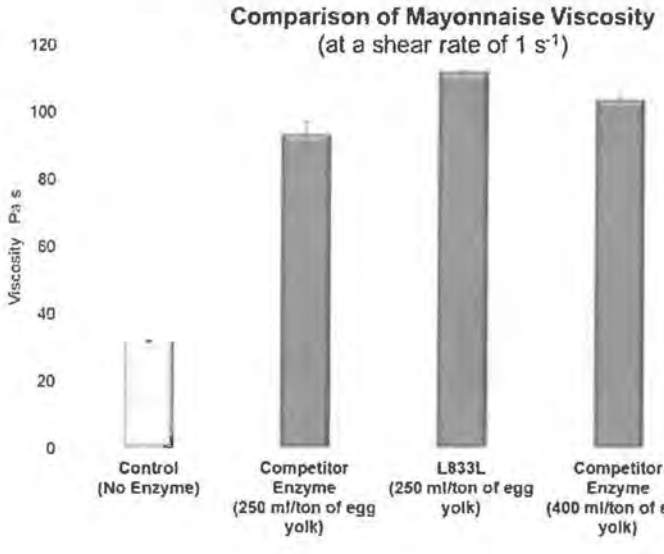


L833L activity (hydrolysis of phospholipids present in egg yolk) was followed by pH measurement (pH decreases during the hydrolysis reaction due to the fatty acids released).

L833L and competitor enzyme performed the same during egg yolk hydrolysis step.

BIOCATALYSTS
exceeding enzyme expectations

Viscosity Study

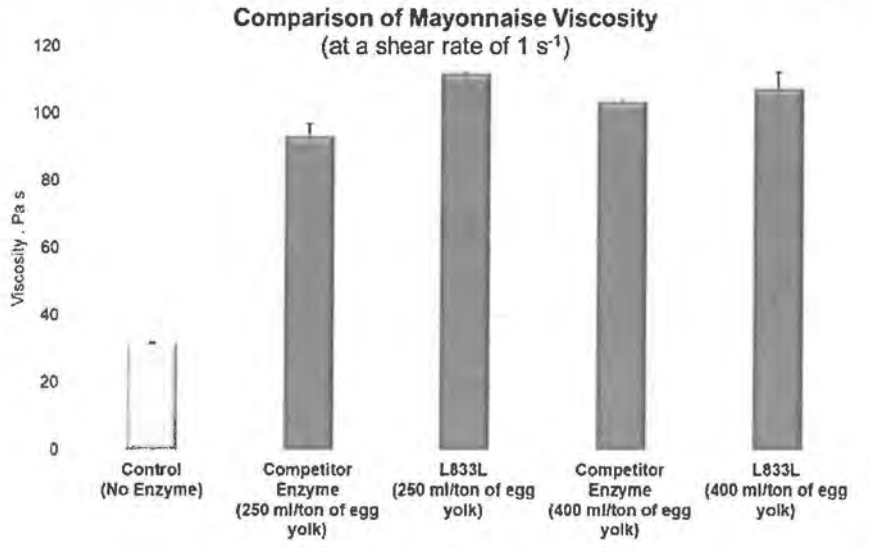


L833L and competitor enzyme performed the same in improving mayonnaise viscosity.

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Viscosity Study



L833L and competitor enzyme performed the same in improving mayonnaise viscosity.

Firmness Stability Study

Time = 0 minutes

L833L and competitor enzyme performed the same in improving mayonnaise firmness stability.

Time = 60 minutes

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.9 Working Group on Consumer Allergy Risk from Enzyme Residues in Food

WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

AMFEP	*Members*
Thierry Dauvrin	Frimond
Gert Groot	Gist-brocades
Karl-Heinz Maurer	HenkelCognis
David de Rijke	Quest International
Henning Ryssov- Nielsen	Danisco Ingredients
Merete Simonsen	Novo Nordisk
Torben B. Sorensen (chairman)	TBS Safety Consulting ApS

Copenhagen. August 1998

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Contents

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

1.0. Introduction

Since the late 80'ies, and particularly since 1992 it has been repeatedly claimed that enzyme residues in foods may represent a hazard to consumers in the form of allergies, and that a certain percentage of the population are at risk of having allergicy reactions to enzymes in bread and other foods.

In particular it has been claimed that consumers were at risk of developing severe allergy symptoms caused by α-amylase. The public was somewhat alarmed and there have been complaints, questions and other reactions of concern to bakers and other suppliers.

The media's interest was based on results from a study by Schata[1], published only as a 1/2-page abstract which does not allow for scientific evaluation.

However the issue was effectively raised within the public, and industry had no data with which to make a response.

Since 1992, the issue of allergy risk in consumers have emerged from time to time on television in the TV and the printed media. The general issue as it has emerged over these years is that there is a concern in the public that enzymes are unsafe, and as far as the bakers and the flour improvers are concerned, require and request data to oppose the allegations.

An additional concern is the possible cross reaction between enzymes produced by fermentation of certain moulds which may be related to common moulds. In theory, a person with a preexisting allergy to *Aspergillus sp.* might react to enzymes from e.g. *Aspergillus niger or A. oryzae.*

2.0 Background

2.1 General

In the public mind there is some confusion about the frequency of allergy, and in particular on food allergy. However, in the scientific community there seem to be consensus of the following:

- The frequency of common allergy (all allergies included) is 20 - 30%, in most populations around the world. The figure is increasing. Part of the increase may be due to higher awareness and improved diagnostic methods, however, a true increase cannot be ruled out.
- The frequency of occupational allergy in bakers is 8 - 27%. About 30 - 35%, of the bakers with occupational allergy to flour have an additional respiratory allergy to α-amylase and/or other baking enzymes.
- There is a reasonably good documentation of the frequency of food allergy in the general population at 1 - 2%. However, the frequency of perceived food allergy allergy in the general population is 12 - 16%
- Food allergy does not differ from inhalation allergies with regard to the biological mechanisms taking place in the immune system. Any 'true' allergy is based on allergy antibodies (IgE). Allergy antibodies are produced by the white blood cells called lymphocytes after the allergen has been introduced to these cells by inhalation or by ingestion. This process is called 'sensitisation'.
- Sensitisation then, is merely the event of the body recognising the foreign aller genic protein and reacting to it by producing allergy antibodies specifically recognising the particular allergen.
- Sensitisation is not a disease.
- It only becomes an allergic disease if the person develop symptoms related to exposure to the particular allergen.
- Not all sensitised people exhibit symptoms of allergy have allergy-symptoms.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

2.2 *Occupational respiratory allergy*

allergy caused by inhalation of airborne particles of proteins, incl. Enzymes

Fungal enzymes, bacterial enzymes and extracted plant and animal enzymes are equally capable of inducing respiratory allergy - Papain and Bromelain[2-4], Trypsin[5], protease's from the skin yeast Candida albicans[6], from bacteria/ subtilisins[7,8], fungal amylases[9,10], bacterial amylases[11], fungal hemicellulases[12], lipases[13], xylanases and cellulases14,15 are all examples of industrial enzymes known to induce allergic sensitisation and respiratory occupational allergy. This is a feature characterised by highly purified enzyme protein products rather than the origin or the methods of production.

They all share the structural and biological properties that may cause sensitisation when inhaled.

The classical food allergens are also capable of inducing respiratory allergy when they are brought into a dust- or aerosol form and inhaled. Soya[16], eggs[17,18], milk[19] and fish[20] are just examples. Soya may be one of the best described examples of epidemic inhalation allergy to an allergen also well recognised as a food allergen[21].

3.0. Food allergy

3.1. Allergy caused by ingestion of proteins in foods

Eight percent of children under 3 years of age are allergic to food[22]. In, and in this age group, milk, egg, fish and soya are examples of common allergens. Many of these allergies disappear with age, but food allergy is seen also in older children and in adults. The overall frequency of verified food allergy is 1 - 2% of the population[22-25].

Food allergy is the adverse reaction to food characterised by allergic sensitisation to food proteins and elicitation of symptoms by ingestion of the same food proteins.

Symptoms

The symptoms of food allergy are gastrointestinal with vomiting and diarrhoea, sometimes accompanied by urticaria, asthma or hay-fever. Generalised very severe reactions occur in rare cases.

Many food allergies are very mild, with symptoms of itching and burning sensation in the mouth. This is also a feature of most of the well known cross-reactions between common inhalation allergens and foods. An example can be found in patients with a birch pollen allergy who also react to e.g. fresh apples, without having a specific allergy to apples. Another well known cross reaction is that of latex and bananas. There are a number of such cross reactions between common pollen allergens and certain foods.

Types of food allergens

Examples of 'true food allergens' are proteins in milk, egg, soya, wheat, fish, nuts and, peanuts and a few more. There are others, but only about 10 food allergens account for more than 95% of severe cases. However the list of food allergens is extremely long and a large number of food allergens only give rise to allergy in sporadic cases.

The common features of food allergens are largely shared by those of respiratory allergens. However, foods are very often treated by cooking and other physico-chemical means that may destroy part of the protein structure and thereby its allergenic properties.

Properties of food allergens

The molecular weights of allergens are typically in the range of 10 -70 (90) kDa.

They have a number of 'epitopes', i.e. sequences of 8 - 16 amino acids. These are the structural 'units' which can be identified by the immune system and lead to production of specific IgE (sensitisation). In the sensitised individual the specific IgE readily recognises the epitopes on the par-ticular protein, resulting in allergy symptoms. Some of these epitopes are described in literature26-28.

Food allergens are stable to digestion and most also to heating by cooking, and in most cases, food allergens can represent a very large proportion of the food itself Enzymes are not well described with regard to neither their fate after ingestion nor their allergenic properties after cooking.

The TNO Institute performed a study[33] on native α-amylase from *Aspergillus oryzae* in a gastrointestinal model simulating the physiological events in the stomach.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

The results indicate that about 92%, of the epitopes of the α-amylase are destroyed and about 8%, of the epitopes on the α-amylase are intact at the delivery from the stomach to the duodenum.

However, it can be expected that the proteolytic pancreatic enzymes will reduce even further, the remaining 7 – 8%, of the α-amylase during the passage through the duodenum.

Doses at which food allergy occurs

The doses and other conditions necessary to sensitise an individual are not well known. It is believed that the sensitising doses must be considerably higher than doses required for elicitation of symptoms in patients already sensitised. There are many examples of sensitised people reacting to trace amounts of allergens in the food - some of them with fatal outcomes.

It is therefore understandable that there is some focus on hidden allergens like traces of milk, nuts and peanuts in other foods.

Steinman[29] wrote a leading article in the August 1996 issue of J. Allergy Clin. Immunol. regarding hidden allergens in food. It is representative of the concern in the medical profession and in the public. He suggested a number of preventive measures including labelling in clear language. His article does not mention enzymes.

Food produced by GMO's

Genetically Modified Organisms (GMO's), and enzymes produced by GMO's have raised concern in general and also specifically for enzymes used in food processing.

Scientists in the fields of gene technology[30-33] and allergy seem to agree that gene technology and the results thereof expressed in foods should not cause concern with regard to allergy risk. However, gene technology does bring about new proteins, and it is important to be aware that some of these new proteins may be allergenic.

Genetically modified proteins may, or may not share allergenic properties with traditional allergens. This would relate to the nature of the protein as it does in all other circumstances, and there are no examples of involuntary (or voluntary) changes of allergenicity of proteins in food.

A possibility may be that in the future, gene technology may be used as a tool to produce less allergenic proteins. This might be a future example of voluntary change of allergenicity.

Enzymes produced by GMO's have been on the market in some countries for many years. Enzyme producers have not experienced any difference in allergenicity of these enzymes as compared to traditional extracted or fermented enzymes. They appear to have the same sensitising potential as are capable of sensitising exposed employees at the same rate as traditional enzymes.

3.2. Epidemiology of Food Allergy

In a survey of 5000 households in the USA carried out in 1989, 1992 and again in 1993[25] it was found that 13.9 -16.2% of the households reported at least one member to be allergic to foods.

A study of food allergy in a random sample of 1483 adults in Holland[23] showed that 12.4% reported allergy to foods, but by controlled tests only 2.4% could be confirmed by Double Blind Placebo Controlled Food Challenge (DBPCFC).

In Spain, 3034 patients from the outpatient allergy clinics at two hospitals were tested for food allergy[24]. The patients were tested by skin prick, RAST and open food challenge. They found 0.98% positive to one or more foods.

When looking at food additives, the same pattern emerges. In a survey of a population sample in the UK, 7% claimed to have reactions to food additives. Double blind challenge tests could verify only 0.01 - 0.23% to be true reactions to food additives[34].

The frequencies of confirmed food allergy in different countries in Europe and the USA are quite uniform at 1 - 2.5% of the populations.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

A number of explanations to the discrepancy of perception and verified cases has been offered. There are indications that the public attribute a number of conditions to 'something in the food' and consider themselves allergic without ever having it tested.

A certain number of perceived food allergy may be induced by members of the medical profession, conducting less efficiently controlled test programs. In some cases, patients are declared food allergic solely based on skin prick tests -which may well over-diagnose food-reactions. High focus on food allergy in the media combined with personal and psychological conditions may also play a role. Actually some specialists in food allergy consider the psychological disorders the most important differential- diagnosis from food allergy.

A diagnosis must rest upon a combination of a medical history and objective tests to confirm or reject the tentative diagnosis. In the field of food-related allergies, the diagnostic test systems have been difficult to establish. However, the Double Blind Placebo Controlled Food Challenge (DBPCFC)[35,36], is the method of choice to confirm or reject indications of food allergy that may derive from the patient's perception and in many cases also from skin prick testing.

The experience from food allergy centres is that objective test programs to confirm or reject a suspected 'food allergy', requires skin- and blood tests and up to 6 placebo controlled challenges to be reliable.

Therefore a diagnosis of food-related allergy, based solely on medical history and a skin prick test is not good clinical practice and must be regarded un-ethical

3.3. Enzymes in food

In theory, enzyme sensitisation and allergy symptoms may be induced by direct ingestion of consumer products containing enzyme residues may occur

The tendency in recent years to focus on allergy and food allergy in particular may explain part of the marked discrepancy between the public perception of allergy to food - and the relatively few cases that can be verified in controlled clinical tests.

Papain is relatively widely used as a meat tenderiser, often supplied in a powder form to apply to the meat before cooking.

In 1983 Mansfield and co-workers[37] published a case story of a person who had allergicy symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients[38] with allergy of which 5 had a positive skin prick test to Papain.

The 5 papain positive were subjected to oral challenge with papain and all had positive reactions to the challenge.

Unfortunately, the challenge was only single blinded, and there is no report of occupational exposure or the use of powdered meat tenderisers that may have caused respiratory sensitisation.

In one other case story by Binkley[39], described below in the section 3.6.2, it can't be totally excluded that sensitisation took place by ingestion of a food product containing relatively high amounts of industrial produced enzymes.

A recent review by Wüthrichl[40] of enzymes in food concluded that orally ingested enzymes are not potent allergens and that sensitisation to ingested enzymes is rare as is also the case of reactions to bread in bakers with occupational allergy to enzymes.

The member companies of AMFEP have not registered, experienced or heard of consumers that have become sensitised to enzymes or enzyme residues in consumer products by ingestion.

It has not been possible to verify the claims in the media of such cases, and they seem as yet un-substantiated as examples of enzyme allergies in consumers. The patients presented and the symptoms and tests described are not documented, merely describing sensations and feelings,however presented as facts.

A large proportion of adverse reactions to food must be ascribed to digestive disorders such as intolerance to for example gluten and lactose, which are not allergic reactions.

3.4. The Theory of cross reactions

people sensitised with common moulds might react to enzymes produced in related moulds

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

The theory that people with allergy to common moulds which are related to those used for the fermentation of enzymes might react to enzyme residues in food was one of Schata's[1] claims and was given relatively high coverage in the media.

The theory could not be readily rejected as cross-reactions are relatively common in allergy. A number of food allergy reactions are merely cross reactions than caused by primary sensitisation.

The most commonly used moulds for fermenting enzymes are *Aspergillus oryzae* or *A. niger*.

According to the theory, people with allergy to *Aspergillus*-moulds would be a high risk population. *Aspergillus* allergy occurs in less than 0.5%, of the population.

A study by Cullinan[41] was conducted with the objective of testing if patients with a well-documented allergy to the widely distributed common mould *Aspergillus fumigatus* reacted upon the ingestion of bread prepared with enzymes of *Aspergillus* origin. The study was a double blind placebo controlled food challenge study on 17 *Aspergillus* allergic people.

The 17 test persons all had allergy antibodies to *Aspergillus fumigatus*, but in addition, 6 also reacted at the skin prick test to the enzymes produced in *A. oryzae* or *A. niger*.

Each patient was challenged with bread baked with the 2 enzymes in standard doses and with placebo bread baked without enzymes. Allergy symptoms and a number of general physiological parameters were monitored before, during and for 24 hours after the challenge.

No allergicy reactions were seen upon ingestion of enzyme containing bread as compared to placebo bread.

This study clearly demonstrates that patients who must be considered at the highest risk for cross reactions to baking enzymes do not react with clinical symptoms when they eat enzyme containing bread containing enzymes.

It is a general experience that once a person is sensitised, even very small amounts of the allergen can elicit allergy symptoms.

In the case of baking enzymes it seems well documented that even patients with severe asthma caused by *Aspergillus fumigatus* did not react to the baking enzymes produced in *A. oryzae* and *A. niger*.

3.5. *Food related reactions in occupationally sensitised people*

The situation of possible reactions to enzymes in bread in patients with occupational allergy to enzymes

There are a few papers describing cases of allergy symptoms elicited by the ingestion of enzymes in people who have occupational allergy to enzymes:

Kanny & Moneret-Vautrin..[42] and Baur & Czuppon[43] each describes one patient who since late childhood, has had asthma and occupational asthma with allergy to flour and enzymes for several years. Both patients were tested for elicitation of symptoms by ingestion of bread baked with and without enzymes. Kanny & Moneret-Vautrin's patient was tested in a blinded design, Baur's patient in an open, non-controlled programme. In both cases the result was elicitation of respiratory symptoms after challenge with bread baked with enzymes. Baur's patient also had a slight reaction to bread without enzymes, however not as pronounced as the reaction after the enzyme containing bread.

Losada et al[44] investigated occupational allergy to α-amylase in a pharmaceutical plant and found a number of employees sensitised to α-amylase. None reported reactions related to ingestion of bread. Five patients, all positive to α-amylase were given oral doses of native α-amylase in doses up to 10 mg.

At this dosage, one of the 5 test persons reacted with respiratory- and generalised allergy symptoms. Four did not react.

Baur et al[45] described the possible background for consumer sensitisation to α-amylases in bread. 138 subjects, of which 98 were allergic, and 11 bakers with occupational allergy were tested. The bakers reacted to α-amylase as may be expected. None of the atopics and none of the control persons reacted to skin prick test with α-amylase. Two atopics had weak RAST to native α-amylase and one reacted also to heated ce-amylase. Reactions to other related compounds, for example *Aspergillus* was not tested.

Tarlo and co-workers[46] reported results of testing for papain allergy in 330 allergy patients. - Seven had positive RAST and Skin prick test but none of them had any gastrointestinal or other allergic symptoms to papain.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

The elicitation of gastrointestinal symptoms upon respiratory sensitisation is also reported for flours. One example is reported by Vidal et al[47] and describes a man with occupational asthma after exposure to flours and other grain dusts. He was sensitised to barley, and experienced gastrointestinal reaction upon ingestion of foods and beverages made from barley.

Enzyme producers and other companies handling concentrated enzymes do see cases of employees being sensitised to baking enzymes. These would be the people at the highest risk of reacting to enzyme residues in bread.

However, none of the members of AMFEP had any reports of sensitised employees who had experienced allergy symptoms in connection to ingestion of bread, and there are no reports of α-amylase sensitised employees avoiding bread.

Cases of people with occupational allergy to flours and food-related reactions to ingestion of flours/bread do occur. One case report describes a person with asthma to barley dust and also with reaction to beverages and foods produced from barley.

The conclusion from these reports of people with pre-existing occup. allergy to α-amylase is:

- Allergic reactions after ingestion of enzyme containing foods are described in 3 individuals.
- The 3 cases are people with definite occupational respiratory allergy to flour and an additional sensitisation to α-amylase. It means they are most probably sensitised by inhalation of flour dust and enzyme dust and not by eating bread or other foods with enzyme residues in it.

3.6 The consumption of enzymes for medical purposes and as digestive aids:

Many people around the world eat enzymes for medical purposes or for convenience as digestive aids.

In many countries enzymes are used routinely as digestive aids by healthy people. The number of people in the world, frequently eating enzyme preparations must be counted in millions.

A number of diseases require the daily addition of enzyme preparation to the food to compensate the patient's insufficient production of digestive enzymes.

3.6.1. Medical uses:

Medical use of enzyme preparations are subject to clinical trials, the results of which are normally reported to the health authorities, and such adverse effects are described in the pharmacopoeia/registry of drugs.

Patients with chronic pancreatitis suffer from insufficient production of digestive enzymes from the pancreas. They are dependent on daily intake of enzymes, some of these produced from *Aspergillus* and other moulds, some extracted from animal glands. The doses of these enzymes are in the order of gram's a day. - we have not been able to identify published documentation of allergy to enzymes in these patients, and the drug registry's does not even mention allergy as an adverse effect.

Proteolytic enzymes and mixtures of different enzymes are commonly used for treatment of a number of physical lesions and also for a number of more special conditions[48-50].

The enzymes are administered in the form of tablets with mixtures of enzymes and in doses of 6 to 600 mg per day, in some cases several times more.

We have not been able to find any evidence of sensitisation or allergy symptoms caused by the ingestion of enzymes from these enzyme preparations. One example is the use of enzymes given as tablets for the treatment of non-articular rheumatism. Uffelmann[51] describes a double blind study of 424 patients, of which 211 received enzyme treatment. The daily doses of the mixed enzyme preparations was 240 mg Lipase, 240 mg Amylase, 1,44 g Papain, 1.08 g Bromelain and 2.4 g Pancreatin,. This dosage was given for 8 weeks and no serious adverse effects and no allergy reactions were reported.

Patients with Cystic Fibrosis suffer a hereditary disease characterised by severe lung symptoms and insufficient production of digestive pancreatic enzymes. They too are dependent of daily intake of grain-doses of enzymes. - There are a few reports of parents

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

and hospital staff who have become sensitised by inhalation of dust from these enzyme preparations[52-54]. This of course might also happen to the Cystic fibrosis patients when they handle the enzyme preparations themselves. However no cases of enzyme allergy in Cystic Fibrosis patients have been described. but there are reports of allergy to common food allergens[55].

An informal telephone survey on unpublished cases of enzyme allergy to European Cystic fibrosis Centres, resulted in only one possible case. The patient was a boy who reacted with vomiting after administration of the enzyme preparation containing amylase. protease and lipase. - The enzyme treatment had been stopped because of suspected allergy to the enzymes. However, testing for specific allergy antibodies by Maxisorp RAST[56] did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed[57]

3.6.2. Digestive aids one possible case of allergy to digestive aid enzymes

In some cultures the use of digestive enzymes after large meals is very common. Enzymes for this purpose are 'over the counter' (OTC) drugs. We have found no studies of possible allergy to enzymes in these populations. That may be irrelevant if no-one ever thought of the possibility that enzymes might be the cause of allergicy symptoms had not been considered. - However, with millions of people using enzymes frequently, some cases of adverse effects in the form of allergic symptoms would be expected to emerge and be described in the literature. In most patients with allergic reactions, symptoms would appear immediately or very shortly after the intake.

Binkley[58], described a case of allergic reaction to ingested lactase. This patient had a respiratory allergy with positive skin prick test reaction to *Aspergillus sp.*

He had had two incidents with allergic reactions in the form of swelling and burning s tion of Lactaid tablets. The lactase was produced from fermentation of Aspergillus oryzae. Skin prick test with extracts of Lactase tablets gave a very strong positive reaction. He had not taken Lactaid tablets previous to the first experience of symptoms, but he had taken milk products containing lactase from *Saccharomyces fragilis* and from *Kluyveromyces lactis*. Although highly unlikely, it may be speculated if these may cross react with Lactaid. In this case it seems unlikely that sensitisation was caused by the Lactaid tablets as the symptoms appeared the first time he ever took Lactaid. It could be a 'cross reaction' based on sensitisation to yeast-produced lactase and symptoms elicited by the ingestion of Lactaid. Another possibility may be a cross reaction from his pre-existing *Aspergillus sp.* allergy.

This case may be regarded a possible but not verified case of oral sensitisation to enzymes in food.

A few other consumers haves claimed allergy to these OTC drugs but thorough testing has not verified allergy to enzymes in any of these cases.

With the background of the very high awareness of food related allergy in the populations, the widespread use of digestive aid and medical uses of enzymes should have attracted interest if allergy to ingested enzymes were of importance. However, up to now, only the single case mentioned above have been described.

To evaluate the risk of sensitisation from ingestion of enzymes and eventually experience of symptoms, we are aware of only the one case that may have become sensitised by ingestion.

This has to be related to the total number of people world-wide who ingest enzymes for short periods of time as part of a medical treatment, and to those who are dependent of daily intake of high amounts of digestive enzymes.

4.0. Conclusion

The working group has studied the available literature on these subjects and come to the conclusion that from a scientific point of view there is no indication that enzyme residues in bread or in other foods may represent an unacceptable risk for consumers.

Lack of scientific data is not evidence of lack of risk, and the working group realises that evidence of 'no risk' is extremely difficult or impossible to generate.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

The group wish to stress that a 'zero-risk' can never be proved by science, and it must be anticipated that even an extremely low risk (e.g. 1 in 50 or 100 millions) of verified allergy to enzymes in food may well be perceived as a significant and unacceptable risk by the public in which more than 10% believe they are allergic to food.

Scientific data are of high value as the credible background for promotion to the public, to trade organisations and individual customers and for an ongoing dialogue with opinion leaders and consumer organisations.

It is the opinion of the group that many cases of perceived allergy to enzymes may be attributed to insufficient diagnostic procedures employed by members of the medical profession.

A minimum requirement for establishing a diagnosis of food related enzyme allergy should be a well conducted DBPCFC.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

5.0. Bibliography

1. Schata M. Allergische reaktionen durch alpha-amylase in backmitteln. Allergologie 1992;57:s0(Abstract).

2. Baur X. Studies on the specificity of human IgE-antibodies to the plant proteases papain and bromelain. Clin Allergy 1979;9:451-457.

3. Wuthrich B. [Proteolytic enzymes: potential allergens for the skin and respiratory tract?] Proteolytische Enzyme: Potente Allergene fur Haut- und Respirationstrakt? Hautarzt 1985;36:123125.

4. Gailhofer G, Wilders Truschnig M, Smolle J, Ludvan M. Asthma caused by bromelain: an occupational allergy. Clin Allergy 1988; 18:445-450.

5. Colten HR, Polakoff PL, Weinstein SF, Strieder DJ. Immediate hypersensitivity to hog trypsin resulting from industrial exposure. N Engl J Med 1975;292:1050-1053.

6. Akiyama K, Shida T, Yasueda H, Mita H, Yamamoto T, Yamaguchi H. Atopic asthma caused by Candida albicans acid protease: case reports. Allergy 1994;49:778-78 1.

7. Flood DF, Blofeld RE, Bruce CF, Hewitt JI, Juniper CP, Roberts DM. Lung function, atopy, specific hypersensitivity, and smoking of workers in the enzyme detergent industry over I I years. Br J Ind Med 1985;42:43-50.

8. Pepys J, Mitchell J, Hawkins R, Maio JL. A longitudinal study of possible allergy to enzyme detergents. Clin Allergy 1985; 15:101-115.

9. Alvarez MJ, Tabar AI, Quirce S, Olaguibel JM, Lizaso MT, Echechipia S, Rodriguez A, Garcia BE. Diversity of allergens causing occupational asthma among cereal workers as demonstrated by exposure procedures. Clinical and Experimental Allergy 1996;26:147-153.

10. De Zotti R, Larese F, Bovenzi M, Negro C, Molinari S. Allergic airway disease in Italian bakers and pastry makers. Occup Environ Med 1994;51:548-552.

11. Bernstein DI, Bernstein IL, Gaines WG, Jr., Stauder T, Wilson ER. Characterization of skin prick testing responses for detecting sensitization to detergent enzymes at extreme dilutions: inability of the RAST to detect lightly sensitized individuals. J Allergy Clin Immunol 1994;94:498-507.

12. Baur X, Weiss W, Sauer W, Fruhmarm G, Kimin KW, Ulmer WT, Mezger VA, Woitowitz HJ, Steurich FK. [Baking ingredients as a contributory cause of baker's asthma] Backmittel als Mitursache des Backerasthmas. Dtsch Med Wochenschr 1988; 113:1275-1278.

13. Bossert J, Fuchs E, Wahl R, Maasch HJ. Occupation sensitization by inhalation of enzymes diaphorase and lipase. Allergologie 1988;11:179-181.

14. Losada E, Hinojosa M, Moneo I, Dominguez J, Diez Gomez ML, Ibanez MD. Occupational asthma caused by cellulase. J Allergy Clin Immunol 1986;77:635-639.

15. Tarvainen K, Kanerva L, Tupasela O, Grenquist Norden B, Jolanki R, Estlander T, Keskinen H., Allergy from cellulase and xylanase enzymes. Clin Exp Allergy 1991;21:609-615.

16. Aceves M, Grimalt JO, Sunyer J, Anto JM, Reed CE. Identification of soybean dust as an epidemic asthma agent in urban areas by molecular marker and RAST analysis of aerosols. J Allergy Clin Immunol 1991;88:124-134.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

17. Oehling A, Garcia B, Santos F, Cordoba H, Dieguez I, Fernandez M, Sanz ML. Food allergy as a cause of rhinitis and/or asthma. J Investig Allergol Clin Immunol 1992;2:78-83.

18. Valero A, Lluch M, Amat P, Serra E, Malet A. Occupational egg allergy in confectionary workers. AllergyNet 1996;51:588-592.

19. Bemaola G, Echechipia S, Urrutia I, Fernandez E, Audicana M, Fernandez de Corres L. Occupational asthma and rhinoconjunctivitis from inhalation of dried cow's milk caused by sensitization to alpha-lactalbumin. Allergy 1994;49:189-191.

20. Droszcz W, Kowalski J, Piotrowska B, Pawlowicz A, Pietruszewska E. Allergy to fish in fish meal factory workers. Int Arch Occup Environ Health 1981;49:13-19.

21. Ferrer A, Torres A, Roca J, Sunyer J, Anto JM, Rodriguez Roisin R. Characteristics of patients with soybean dust-induced acute severe asthma requiring mechanical ventilation [published erratum appears in Eur Respir J 1990 Jul;3(7):846]. Eur Respir J 1990;3:429-433.

22. Burks AW, Sampson H. Food allergies in children. Curr Probl Pediatr 1993;23:230-252.

23. Jansen JJ, Kardinaal AF, Huijbers G, Vlieg Boerstra BJ, Martens BP, Ockhuizen T Prevalence of food allergy and intolerance in the adult Dutch population. J Allergy Clin Immunol 1994;93:446-456.

24. Joral A, Villas F, Garmendia J, Villareal 0. Adverse reaction to food in addults. J Invest Allergol Clin Immunol 1995;5:47-49.

25. Altman DR. Public perception of food allergy. J Allergy Clin Immunol 1996;97:124-151.

26. O'Neil C, Helbling AA, Lehrer SB. Allergic reactions to fish. Clin Rev Allergy 1993;11:183-200.

27. Shimojo N, Katsuki T, Coligan JE, Nishimura Y, Sasazuki T, Tsunoo H, Sakamaki T, Kohno Y, Niimi H. Identification of the disease-related T cell epitope of ovalbumin and epitope-targeted T cell inactivation in egg allergy. Int Arch Allergy Immunol 1994;105:155-161.

28. Mole LE, Goodfriend L, Lapkoff CB, Kehoe JM, Capra JD. 'Me amino acid sequence of ragweed pollen allergen Ra5. Biochemistry 1975;14:1216-1220.

29. Steinman HA. Hidden allergens in foods. Journal of Allergy and Clinical Immunology 1996;98:241-250.

30. Lehrer SB, Homer WE, Reese G. Why are some proteins allergenic? - Implications for Biotechnology. Cri Rev in Food Science and Nutr 1996;36:553-564.

31. Metcalfe DD, Fuchs R, Townsend R, Sampson H, Taylor S, Fordham J. Allergenicity of foods produced by genetic modification. 1995;(Abstract).

32. Metcalfe DD. Public perception of food-allergy problems is high, experts say. Food Chemical News 1994;April 25:45-46.

33. Fuchs R, Astwood JD. Allergenicity Assessment of Foods Derived from Genetically Modified Plants. Food Technology 1996;February:83-88.

34. Young E, Stoneham M, Petruckevitch A, Barton J, Rona R. A population study of food intolerance. The Lancet 1994;343:1127-1130.

35. Bock SA, Sampson HA, Atkins FM, Zeiger RS, Lehrer S, Sachs M, Bush RK, Metcalfe DD. Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. J Allergy Clin Immunol 1988;82:986-997.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

36. Ortolani C, Pastorello EA, Ansaloni R, Incorvaia C, Ispano M, Pravettoni V, Rotondo F, Scibilia J, Vighi G. Study of nutritional factors in food allergies and food intolerances. 1997;EUR 16893 en:1-196.(Abstract).

37. Mansfield LE, Bowers CH. Systemic reaction to papain in a nonoccupational setting. J Allergy Clin Immunol 1983;71:371-374.

38. Mansfield LE, Ting S, Haverly RW, Yoo TJ. The incidence and clinical implications of hypersensitivity to papain in an allergic population, confirmed by blinded oral challenge. Ann Allergy 1985;55:541-543.

39. Binkley KE. Allergy to supplemental lactase enzyme. J Allergy Clin Inummol 1996;97:1414-1416.

40. Wuthrich B. Enzyme als ingestive Allergene. Allergie für die Praxis 1996;4:74-91.

41. Cullinan P, Cook A, Jones M, Cannon J, Fitzgerald B, Newman Taylor AJ. Clinical responses to ingested fungal alpha-amylase and hemicellulase in persons sensitized to Aspergillus fumigatus? Allergy Eur J Allergy Clin Immunol 1997;52:346-349.

42. Kanny G, Moneret Vautrin DA. alpha-Amylase contained in bread can induce food allergy. J Allergy Clin Immunol 1995;95:132-133.

43. Baur X, Czuppon AB. Allergic reaction after eating alpha-amylase (Asp o 2)-containing bread. A case report. Allergy 1995;50:85-87.

44. Losada E, Hinojosa M, Quirce S, Sanchez Cano M, Moneo I. Occupational asthma caused by alpha-amylase inhalation: clinical and immunologic findings and bronchial response patterns. J Allergy Clin Immunol 1992;89:118-125.

45. Baur X, Sander I, Jansen A, Czuppon AB. [Are amylases11 in bakery products and flour potential food allergens?] Sind Amylasen von Backlaitteln und Backmehl relevante Nahrungsmittelallergene? Schweiz Med Wochenschr 1994;124:846-851.

46. Tarlo SM, Shaikh W, Bell B, Cuff M, Davies GM, Dolovich J, Hargreave FE. Papain-induced allergic reactions. Clin Allergy 1978;8:207-215.

47. Vidal C, Gonzalez Quintela A. Food-induced and occupational asthma due to barley flour. Ann Allergy Asthma Immunol 1995;75:121-124.

48. Stauder G, Pollinger W, Fruth C. Systemic enzyme therapy. A review of the new clinical studies. Allgemeinmedizin 1990;19:188-191.

49. Rahn HD, Kilic M. The effectiveness of a hydrolytic enzyme in traumatology. The results of two prospective, randomized, double-blind studies. Allgemeinmedizin 1990;19:183-187.

50. Ransberger K, Stauder G. Use of catabolic enzymes for controlling the acquired immune deficiency syndrome (AIDS) and its precursors (LAS, ARC). 1991;5,002,766:1-8.(Abstract).

51. Uffelmann K, Vogler W, Fruth C. The use of a hydrolytic enzyme in extra-articular rheumatism. Allgemeinmedizin 1990;19:151-153.

52. Moss RB. Drug allergy in cystic fibrosis. Clin Rev Allergy 1991;9:211-229.

53. Sakula A. Bronchial asthma due to allergy to pancreatic extract: a hazard in the treatment of cystic fibrosis. Br J Dis Chest 1977;71:295-299.

54. Lipkin GW, Vickers DW. Allergy in cystic fibrosis nurses to pancreatic extract. Lancet 1987;Feb 14:

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

55. Lucarelli S, et al. Food allergy in cystic fibrosis. Minerva Paediatr 1994;46:543-548.

56. Poulsen LK et al. Maxisorp PAST Afflergy 1989.44:173-180.

57. Dab I. - personal communication. Not published.

58. TNO Nutrition and Food Research Institute. Stability of fungal and bacterial (-amylase in gastric compartment of TIM. 1998, in prep.

BIOCATALYSTS
exceeding enzyme expectations

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

AMFEP, Av. De Roodebeck, 30, B-1030 Bruxelles
Tel. +32 2 743 8730 /1 Fax. +32 2 736 8175

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

7.10. List of Appendices

1. Arnesjö, B., Barrowman, J., Borgström, B. The zymogen of phospholipase A2 in rat pancreatic juice. Acta Chem Scand; 1967; 21(10): 2897-2900.
2. Bankar A.V., Kumar A.R., Zinjarde. S.S.; Environmental and industrial applications of *Yarrowia lipolytica*; Applied Microbiology and Biotechnology; 2009; 84(5):847-65.
3. Barth, G. and Gaillardin, C.; Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*; FEMS Microbiology Reviews; 1997, 19(4): 219–237.
4. Ben Bacha, A., Gargouri, Y., Bezzine, S., Mosbah, H., Mejdoub, H. Ostrich pancreatic phospholipase A2: Purification and biochemical characterization; Journal of Chromatography B Analyt Technol biomed Life Sci; 2007; 857(1):108–114.
5. Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Ter Schure, E., van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., Hansen, E.B. Food fermentations: microorganisms with technological beneficial use; Int J Food Microbiol; 2012; 154(3):87–97.
6. Dauvrin, T., Groot, G., Maurer, K-H., de Rijke, D., Ryssov-Nielsen, H., Simonsen, M., Sorensen, T.B. AMFEP's Working Group on Consumer Allergy Risk from Enzyme Residues in Food (1998).
7. de Haas G.H., Postema N.M., Nieuwenhuizen W., van Deenen L.L. Purification and properties of phospholipase A from porcine pancreas; Biochim Biophys Acta; 1968; 159(1):103-17.
8. Dutilh, C.E., van Doren, P.J., Verheul, E.E., de Haas, G.H. Isolation and Properties of Prophosholipase A2 from Ox and Sheep Pancreas; European Journal of Biochemistry; 1975; 53(1):91-97.
9. Evenberg, A., Meyer, H., Verheij, H.M., de Haas, G.H. Isolation and properties of prophospholipase A2 and phospholipase A2 from horse pancreas and horse pancreatic juice. Biochim Biophys Acta; 1977; 491(1):265-74.
10. Grataroli, R., de Caro, A., Guy, O., Amic, J., Figarella, C. Studies on prophospholipase A2 and its enzyme from human pancreatic juice. Catalytic properties and sequence of the N-terminal region. Eur J Biochem; 1982; 122(1):111-7.
11. Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., van Dijck, P.W.M., Wyss, M. *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential; Crit Rev Microbiol; 2014; 40(3):187–206.
12. Groenewald, M., Boekhout, T., Neuveglise, C., Gaillardin, C., van Dijk, P.W.M., Wyss, M. *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential; Critical Reviews in Microbiology; 2013; 40:187–206.
13. Holzschu, D.L., Chandler, F.W. Ajello, L., Ahearn, D.G. Evaluation of industrial yeasts for pathogenicity; Sabouraudia; 1979; 17(1):71–78.
14. Jean-Marc Nicaud; *Yarrowia lipolytica*; Yeast; 2012; 29: 409–418.
15. Johnson AG, McDermott SJ. Lysolecithin: a factor in the pathogenesis of gastric ulceration? Gut; 1974; 15(9):710-3.
16. Larpin-Laborde, S., Imran, M., Bonaïti, C., Bora, N., Gelsomino, R., Goerges, S., Irlinger, F., Goodfellow, M., Ward, A.C., Vancanneyt, M., Swings, J., Scherer, S., Gueguen, M., Desmasures, N. Surface microbial consortia from Livarot, a French smear-ripened cheese; Canadian Journal of Microbiology; 2011; 57(8):651–660.
17. Larpin-Laborde, S., Imran, M., Bonaïti, C., Bora, N., Gelsomino, R., Goerges, S., Irlinger, F., Goodfellow, M., Ward, A.C., Vancanneyt, M., Swings, J., Scherer, S., Gueguen, M. and

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

Desmasures, N. Surface microbial consortia from Livarot, a French smear-ripened cheese; Canadian Journal of Microbiology; 2011; 57(8):651–660.

18. Lee, H.Y., Bahn, S.C., Shin, J.S., Hwang, I., Back, K., Doelling, J.H., Ryu, S.B. Multiple forms of secretory phospholipase A2 in plants; Progress in Lipid Research; 2005; 44(1):52-67.
19. Lelieveld, H.L., Boon, B., Bennett, A., Brunius, G., Cantley, M., Chmiel, A., Collins, C.H., Crooy, P., Doblhoff-Dier, O., Economidis, I., Elmqvist, A., Frontali-Botti, C., Havenaar, R., Haymerle, H., Käppeli, O., Leaver, G., Lex, M., Lund, S., Mahler, J.L., Marris, R., Martinez, J.L., Mosgaard, C., Normand-Plessier, C., Romantschuk, M., Werner, R.G. (1996). Safe biotechnology. 7. Classification of microorganisms on the basis of hazard. Working party "Safety in Biotechnology" of the european federation biotechnology. Appl Microbiol Biotechnol.; 1996; 45:723–9.
20. Monnet, C., Bleicher, A., Neuhaus, K., Sarthou, A.S., Leclercq-Perlat, M.N., Irlinger, F. Assessment of the antilisterial activity of microfloras from the surface of smear-ripened cheeses; Food Microbiology; 2010; 27(2):302–310.
21. Pao, E.M., Fleming, K.H., Guenther, P.M., Mickle S.J. Consumer Nutrition Center, Human Nutrition Information Service, USDA. Home Economics Research Report Number 44; 1982.
22. Pariza, M.W. and Foster, E.M. Determining the Safety of Enzymes Used in Food Processing; Journal of Food Protection; 1983; 46(5):453-468.
23. Pariza, M.W and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century; Regulatory Toxicology and Pharmacology; 2001; 33(2):173–186.
24. Ramrakhiani, L. and Chand, S. Recent progress on phospholipases: different sources, assay methods, industrial potential and pathogenicity; Appl Biochem Biotechnol; 2011; 164(7):991-1022.
25. Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Salvador, P., Girones, R., Koutsoumanis, K., Lindqvist, R., Nørrung, B., Robertson, L., Ru, G., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter, N., Threlfall, J., Wahlstr€om, H., Sandro Cocconcelli, P., Peixe, L., Prieto, M., Querol, A., Evaristo, J., Sundh, I., Vlak, J., Barizzone, F., Correia, S., Herman, L. EFSA Panel on Biological Hazards (BIOHAZ). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 8: suitability of taxonomic units notified to EFSA until March 2018; 2018.
26. Roostita, R. and Fleet, G.H. The occurrence and growth of yeasts in Camembert and blue-veined cheeses; International Journal of Food Microbiology; 1996; 28:393–404.
27. Rossiter, R.J., Metabolism of Phosphatides, Metabolic Pathways; Greenberg, D.M., Ed; Vol II; 1968; Academic Press: New York; 69-115.
28. Smita S. Zinjarde; Food-related applications of *Yarrowia lipolytica*; Food Chemistry; 2014; 152(1):1-10.
29. Turck, D., Castenmiller, J., de Henauw, S., Hirsch-Ernst, K-I., Kearney, J., Maciuk, A., Mangelsdorf, I., McArdle, H-J., Naska, A., Pelaez, C., Pentieva, K., Siani, A., Thies, F., Tsabouri, S., Vinceti, M., Cubadda, F., Engel, K-H., Frenzel, T., Heinonen, M., Marchelli, R., Neuhäuser-Berthold, M., Pöting, A., Poulsen, M., Sanz, Y., Schlatter, J.R., van Loveren, H., Ackerl, R., Knutsen, H-K. EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA). Safety of *Yarrowia lipolytica* yeast biomass as a novel food pursuant to Regulation (EU) 2015/2283; 2019.
30. van der Walt, J.P. and von Arx, J.A. The yeast genus Yarrowia gen. nov. Antonie van Leeuwenhoek; 1980; 46(6):517–521.
31. Welthagen, J.J. and Viljoen, B.C. Yeast profile in Gouda cheese during processing and ripening; International Journal of Food Microbiology; 1998; 41(3):185–194.

Cefn Coed, Parc Nantgarw
Cardiff, CF15 7QQ
United Kingdom
Tel: +44(0)1443 843712
Email: customerservices@biocats.com
Website: www.biocatalysts.com

32. Wickerham, L.J., Kurtzman, C.P. Herman, A.I. Sexual reproduction in *Candida lipolytica*; Science, 1970; 167; 1141.
33. Wyder, M-T., Bachmann, H.P., Puhan, Z. Role of selected yeasts in cheese ripening: an evaluation in foil wrapped raclette cheese; Lebensm-Wiss Technol; 1999; 32:333–43.
34. Yarrow D. Four new combinations in yeasts; Antonie van Leeuwenhoek; 1972; 38(3):357–360.
35. Zinjarde, S.S. Food-related applications of *Yarrowia lipolytica*; Food Chem; 2014; 152(0):1–10.