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Chymosin and Phytase. Made by genetic engineering (No. 10 in a series of articles to promote a better understanding of the use of genetic engineering)

1. Chymosin, pure source of rennet provides unlimited availability to the cheese industry

The production process of cheese coagulation of milk proteins to 'curds' is achieved by the proteolytic activity of rennet added to milk. Rennet products obtained from suckling calves' stomachs have traditionally been used for this. However, for various reasons such as milk quota and fluctuating veal production, the number of suckling calves available for slaughter is declining. Consequently, there has been a corresponding reduction in the quantity of calves' stomachs available for use as a source of rennet. On the other hand, there is increasing demand for rennet as the world production of cheese is steadily increasing.

In the early eighties, Gist-brocades started research on making calves' rennet by fermentation of a genetically modified micro-organism. After an extensive evaluation of several possible hosts, it was decided to clone and express the DNA coding for calve stomach chymosin in an industrial strain of *Kluyveromyces lactis*. The natural habitat of *K. lactis* is milk and milk products. The yeast has been isolated from fermented milk products like kefir, koumiss, yoghurt, and from a variety of cheeses.

The yeast itself, when cultured on whey, was used in the 1950s and 1960s as an alimentary yeast with a high nutritional value in food and feed; in inactivated and dried form it was used in the 1960s and early 1970s as a health food and protein supplement. The micro-organism is known to be completely harmless and non-toxicogenic.

K. lactis has been used for many years by Gist-brocades for the production of the food enzyme lactase which has been affirmed GRAS status by the US FDA. GRAS status applies to ingredients generally regarded as safe to be used in food.

The industrial strain used for lactase production proved to be a suitable host to express the DNA coding for calf preprochymosin. With the aid of the yeast α-factor leader as a signal sequence the prochymosin is efficiently secreted into the medium. As *K. lactis* does not secrete appreciable amounts of endogenous proteins, the recovery of prochymosin can be very simple. The fermentation is followed by an acid step to autolyse prochymosin into active chymosin, the *K. lactis* biomass is subsequently removed by centrifugation or filtration and no further steps are required to obtain a chymosin preparation, brand name Maxiren®, with a much higher purity than traditional calves' rennet.

The chymosin in Maxiren® (Table 1) is absolutely identical to its natural counterpart in calves' rennet, both chemically and biologically. This is based on molecular size, amino acid sequence and composition, immunochemical behaviour, and biochemical characteristics.

Maxiren® has been commercially produced in the Gist-brocades enzyme plant at Seclin, France, since 1988. This 'Installation Classée' plant has

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also been authorised to use a genetically modified organism for production purposes. The plant has been awarded the ISO 9002 certificate as well as kosher certification. The product Maxiren® is a vegetarian product and is also kosher and halal approved. Maxiren® has been officially approved for sale to cheese manufacturers in the USA, Australia, a number of European countries, and countries in South America, Africa, and Asia.

With the availability of chymosin made by genetically modified *K. lactis*, the cheese producing industry is no longer dependent on the supplies of calves' stomach rennet. There is now an unlimited supply of cost-effective product of high and consistent purity, free of animal-source contaminants.

P.W.M. van Dijck
Regulatory Affairs Department
Royal Gist-brocades
P.O. Box 1
2600 MA Delft
The Netherlands
Tel.: +31 15 2799111
Fax: +31 15 2753482

2. Phytase enzyme product reduced the release of manure phosphate into the environment

Intensive farming can lead to environmental stress due to the production of large amounts of manure. In particular the nitrogen and phosphorus content of manure has, in certain areas of the world, deteriorated the quality of subsoil water used for making drinking water. Such 'overfertilization' has led to so-called eutrophication. The consequence of this has been excessive growth of algae, oxygen depletion in rivers, lakes and even seas, and harm to aquatic life.

Now, phosphorus is an essential element for the growth and viability of animals. In plant material phosphorus is abundantly available, bound in the form of phytin. The enzyme phytase present in nature in the stomachs of ruminants, enzymatically liberates the phosphates from the phytin molecule. However, this enzyme is not present in the stomachs of pigs and chicken. Consequently, phosphates have to be added to pig and chicken feed to ensure healthy and efficient growth of these animals.

Certain micro-organisms, e.g. *Aspergillus niger* produce the enzyme phytase. However, productivity was traditionally insufficient to make this an economically viable process. To achieve high expression levels of phytase a suitable host had to be selected. Research initiated at Gist-brocades in the mid-eighties led to the cloning of the DNA sequence coding for the phytase enzyme and to the development of proprietary PluGBug® technology allowing for the introduction of foreign genes (plugs) into safe industrial high-producing hosts (bugs) such as *A. niger*. *A. niger* is often used in the production of ingredients for foods, including several enzymes. As enzyme producer, Gist-brocades has extensive experience with this mould. An industrial strain of *A. niger* was used, producing huge amounts of the enzyme glucoamylase. So expression cassettes were developed

Table 1
Made by genetic engineering No. 10

Product	Chymosin	Phytase
Principal trade names	Maxiren®	Natuphos®
Principal uses	Manufacturing of cheese	Animal feed additive
Manufacturers	Royal Gist-brocades	Royal Gist-brocades
Donor organism	Calf stomach cells	*Aspergillus niger*
Host organism	*Kluyveromyces lactis*	*Aspergillus niger*
Advantages	Vegetarian product Approved for Kosher and Halal	Positive environmental effects Unlimited and cost-effective supply
	Cost-effective production Unlimited supply at consistent quality Free of animal-source contaminants	Consistent quality

Table 2
Summary of products published in the series *Made by Genetic Engineering*

Product	Lipase	Hepatitis B vaccine
Author/manufacturer	B. Diderichsen, Novo Nordisk, Denmark	P. Crooy, SmithKline Beecham Biologicals, Belgium
Made by Genetic Engineering No. 1, Biotech Forum Europe 8, 246–247, 1991		
Product	Human insulin	Human growth hormone
Author/manufacturer	E. Rasmussen, Novo Nordisk, Denmark	L. Fryklund, KabiPharmacia, Sweden
Made by Genetic Engineering No. 2, Biotech Forum Europe 9, 144–145, 1992		
Product	Protein G	Interferon alfa-2a
Author/manufacturer	R. Hjorth, Pharmacia LKB Biotechnology, Sweden	S. Ryser, F. Hoffmann–La Roche AG, Switzerland
Made by Genetic Engineering No. 3, Biotech Forum Europe 9, 641–642, 1992		
Product	AIDS test	α-Amylase
Author/manufacturer	E. Baumann, F. Hoffmann–La Roche AG, Switzerland	B. Diderichsen, Novo Nordisk, Denmark
Made by Genetic Engineering No. 4, J. Biotechnol. 38, 193–197, 1995		
Product	Erythropoietin	Interferon beta-1b
Author/manufacturer	C. Kionka, Boehringer Mannheim, Germany	T. Petri, Schering AG, Germany
Made by Genetic Engineering No. 5, J. Biotechnol. 43, 73–77, 1995		
Product	Interferon gamma	Rabies vaccine
Author/manufacturer	E. Falkner and I. Maurer-Fogy, Bender and Co GesmbH/Boehringer Ingelheim Vienna, Austria	J. Terré, G. Chappuis, M. Lombard and P. Desmettre, Rhone Mérieux, France
Made by Genetic Engineering No. 6, J. Biotechnol. 46, 155–159, 1996		
Product	Tissue plasminogen activator (rt-PA)	Granulocyte–macrophage colony-stimulating factor (GM-CSF)
Author/manufacturer	W. Werz and R.G. Werner, Dr. Karl Thomae GmbH/Boehringer Ingelheim Pharma, Germany	R. Till, Novartis/Schering Plough International, Switzerland
Made by Genetic Engineering No. 7, J. Biotechnol. 61, 157–161, 1998		
Product	Human coagulation factor VII	Folicle stimulating hormone (FSH)
Author/manufacturer	U. Hedner and T. Lund-Hansen, Novo Nordisk A/S, Denmark	J.C. Heikoop and W. Olijve, N.V. Organon, Oss, The Netherlands
Made by Genetic Engineering No. 8, J. Biotechnol. 61, 231–236, 1998		
Product	Alzheimer tau test	Detergent cellulase
Author/manufacturer	E. Vanmechelen and H. Vanderstichele, Innogenetics N.V., Belgium	B. Jones and W. Quax, Genencor International, The Netherlands
Made by Genetic Engineering No. 9, J. Biotechnol. 66, 229–233, 1998		
Product	Chymosin	Phytase
Author/manufacturer	P.W.M. van Dijck, Royal Gist-brocades, The Netherlands	P.W.M. van Dijck, Royal Gist-brocades, The Netherlands
Made by Genetic Engineering No. 10, J. Biotechnol. 67, 77–80, 1999		

based on the regulatory elements of the glucoamylase gene. The coding sequence of phytase was fused appropriately to the glucoamylase promoter sequence (P*glaA*) by recombinant DNA techniques in an expression cassette. Upon transformation, multiple copies of this cassette were randomly integrated into the genome of the *A. niger* strain.

This upgrading in productivity (by several orders of magnitude) made it possible to develop a production process for phytase that was cost-effective. The phytase enzyme is now produced

through fermentation in high quantities in the Gist-brocades enzyme production plant in Seclin, France.

After fermentation the production mould *A. niger* is killed and filtered out along with many small particles fond in the culturing fluid. The procedure removes the mould quantitatively from the broth. The resulting liquid, once concentrated, is ready for formulation. The product, brand name Natuphos® (Table 1), is supplied by our marketing partner BASF to the compound-feed industry in two forms, a liquid and a dry form. The dry product is formulated with wheat middlings as a carrier for the phytase enzyme. In the fluid formulation the concentrated phytase ultrafiltrate is mixed with sorbitol. The quality of both the production as well as the formulation is certified to ISO 9002.

Extensive feeding trials and data from several years of experience in the feed-industry have shown that the addition of extra phosphorus to feeds was made partially redundant and this in itself reduces the release of manure phosphate into the environment. By adding Natuphos® the total phosphorus level in the feed can be reduced by some 20%; the level emitted in manure is reduced by 25–30%.

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