APPENDIX A

Product Data Sheet

novozymes

Alcalase® 2.4 L FG

Valid from 01-Oct-2004

Product Characteristics:

Enzyme Class	Protease (Subtilisin)
Declared activity	2,4 AU-A/g
Colour	Brown Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.
Physical form	Liquid
Stabiliser	Glycerol
Production organism	Bacillus licheniformis Produced by submerged fermentation of a micro organism. The enzyme protein is separated and purified from the production organism.

Product Specification:

	Lower Limit	Upper Limit	Unit
Protease Units AU-A	2.4		/g
Total Viable Count	-	50000	/g
Coliform Bacteria	-	30	/g
Enteropathogenic E.Coli		None Detected	/25g.
Salmonella		None Detected	/25g.

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before	When stored as recommended, the product is best used within 6 months from date of delivery.
Storage temperature	0-10°C (32°F-50°F)
Storage Conditions	In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.

novozymes

Enzymatic Hydrolysis of Proteins Using Novozymes Proteases

Background

Enzymatic hydrolysis of proteins is a way to improve the properties of proteins. The properties of a protein hydrolysate are determined by the degree of hydrolysis (see below) and by the structure of the peptides produced. These in turn are dependent on the nature of the protein and the specificity of the enzyme used, as well as on the hydrolysis conditions, particularly pH and temperature.

Degradation of a protein renders it more soluble. Other functional properties, such as emulsifying ability, foaming capacity, viscosity, gelatinization and water absorption capacity are also affected by hydrolysis.

The taste of a protein is affected by hydrolysis. This goes both for the flavour of the protein and for bitterness (please find more on taste development below).

The nutritional value of a protein is normally maintained or increased by enzymatic hydrolysis, which is carried out under mild reaction conditions. The protein is simply broken down into smaller units: peptides and amino acids.

Enzymes

The following food-grade proteases are recommended for the hydrolysis of proteins:

Endoproteases:	Alcalase® Food Grade Alcalase® AF 2.4 L Neutrase®, Protamex®
Exopeptidase/endoprotease complex:	Flavourzyme®,

Mode of Action

Endoproteases work by cleaving peptide bonds in the interior of polypeptide chains, whereas exopeptidases cleave off amino acids one at a time from the end of polypeptide chains. Figure 1 shows how the different proteases will hydrolyze different bonds in a protein.

Application

When preparing protein hydrolysates, the typical protein contains 8-12%, and the typical enzyme dosage with endoproteases is 0.5-2% based on the weight of protein. The typical enzyme dosage with Flavourzyme is 10,000-25,000 LAPU/kg protein, corresponding to 20-50 kg of Flavourzyme 500 L or MG per ton of protein.

The ratio between enzyme and substrate determines the speed of hydrolysis, which is highest initially and decreases with time. The hydrolysis eventually comes to a halt when there are no more peptide bonds available for the enzyme. The maximum attainable degree of hydrolysis depends on the nature of the protein and the specificity of the enzyme.

Degree of Hydrolysis

The degree of hydrolysis (%DH), defined as the percentage of peptide bonds cleaved, is a key parameter which characterizes a protein hydrolysate:

%DH= (Number of peptide bonds cleaved/total number of peptide bonds) x100%

The %DH can be measured by various methods, e.g.:

- Increase in osmolality measured as freezing point depression
- Determination of free amino groups, e.g. by using the OPA method
- Titration in a pH-stat of the free amino groups generated
- Formol titration

Descriptions of these methods are available on request.

When insoluble protein is solubilized by proteolysis, this can be followed conveniently by measuring the dry matter content in the soluble phase, e.g. by measurement of °Brix.

Taste

The flavour (meaty, vegetable, etc.) generally increases in intensity as the protein is broken down, eventually into small peptides and amino acids.

Degradation of proteins can result in the formation of bitter peptides. Some bitterness will usually be present at intermediate degrees of hydrolysis. Bitterness is believed to be caused by the presence of peptides of a certain size with terminal hydrophobic amino acids, see for instance Figure 1.

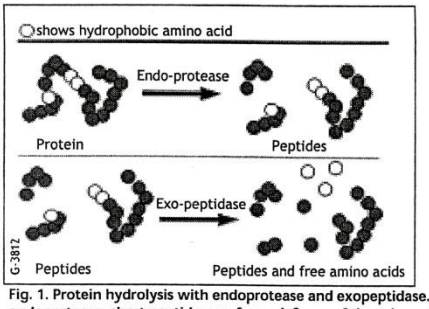

Fig. 1. Protein hydrolysis with endoprotease and exopeptidase. After hydrolysis with endoprotease, short peptides are formed. Some of these have terminal hydrophobic amino acids and are thus bitter peptides. Further hydrolysis with exopeptidases breaks down these bitter peptides.

The tendency to bitterness therefore depends not only on the degree of hydrolysis but also on the structure of the peptides produced. For example, casein will, when hydrolyzed with Alcalase, start to get bitter even at a degree of hydrolysis of 1%. The tendency to bitterness is reduced substantially when hydrolyzing casein with Protamex, and further reduced when using Flavourzyme.

Enzyme Hydrolysis

Optimal working conditions for the individual enzymes are summarized in Table 1, which also lists a number of characteristics of the protein hydrolysates produced by the enzymes.

	Optimum pH	Optimum Temp. in °C	Optimum Temp. in °F	Max. %DH
Alcalase	8	50-60	122-140	15-25
Alcalase AF 2.4 L	8	50-60	122-140	15-25
Neutrase	7	40-50	104-122	10-15
Protamex	7-8	50	122	10-20
Flavourzyme	5.5-7.5	50-55	122-131	~60

Table 1: Characteristics of Novozymes proteases and the protein hydrolysates produced by these.

The progress of hydrolysis with different Novozymes proteases is illustrated in Figure 2.

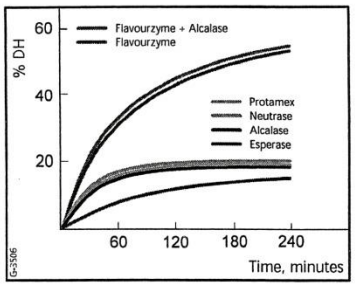

Fig. 2. Hydrolysis of casein using different proteases

The choice of enzyme for a given application depends on the substrate and the desired properties of the final hydrolysate. More detailed Application Sheets are available for several specific applications, and we would be pleased to help you identify the optimal enzyme(s) for your particular application.

Termination of the enzyme reaction

In order to control the functional properties of the hydrolysate it may be important to stop the enzyme reaction at a closely defined %DH value. All the proteases can be irreversibly inactivated by heat treatment. Table 2 suggests treatment times for inactivation at a given pH and temperature.

However, inactivation by heat treatment is very much dependent on the substrate (substrate concentration, pH, etc.). Thus, the documentation for efficient elimination of protease must be based on actual analysis for the detection of residual activity.

Protease	pH	Temp. °C	Temp. °F	Time, min.
Alcalase	4	50	122	30
	8	85	185	10
Alcalase AF 2.4 L	4	50	122	30
	8	85	185	10
Flavourzyme	6-8	90	194	10
Neutrase	4	50	122	30
	7	80	176	5
Protamex	4	50	122	30
	8	85	185	10

Table 2

The proteases Alcalase, Neutrase and Protamex are all inactive at pH 4 or below. The reaction can, therefore, be stopped instantaneously by the addition of a convenient acid, e.g. hydrochloric, phosphoric, malic, lactic or acetic acid.

See the Analytical Method for further information.

APPENDIX B

Product Data Sheet

novozymes®

Celluclast® 1.5 L

Valid from 30-May-2005

Product Characteristics:

Enzyme Class	Cellulase
Declared activity	700 EGU/g
Colour	Brown Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.
Physical form	Liquid
Approximate Density (g/ml)	1.20
Stabiliser	Sodium chloride Sorbitol
Preservatives	Potassium sorbate
Production organism	Trichoderma reesei Produced by submerged fermentation of a micro organism. The enzyme protein is separated and purified from the production organism.

Product Specification:

	Lower Limit	Upper Limit	Unit
Cellulase Units EGU	700		/g
Total Viable Count	-	50000	/g
Coliform Bacteria	-	30	/g
Enteropathogenic E.Coli		None Detected	/25g.
Salmonella		None Detected	/25g.

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Product Sheet

novozymes®

Celluclast® 1.5 L FG

Description

Celluclast 1.5 L FG is a liquid cellulase preparation produced by submerged fermentation of a selected strain of the fungus *Trichoderma reesei*. The enzyme catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers. The relative amounts of reaction products formed depend on the reaction conditions. Celluclast has a pronounced viscosity-reducing effect on soluble cellulosic substrates.

Product Properties

Appearance

Celluclast 1.5 L FG is a brown liquid with a density of approximately 1.2 g/ml.

Activity

Celluclast 1.5 L FG.....700 EGU/g.

EGU = Endo-Glucanase Units.
See the Analytical Method for further information.

Solubility

The active enzyme components of Celluclast are readily soluble in water at all concentrations which occur in normal usage. Turbidity which may occur in the enzyme preparation has no influence on the volumetric activity or handling characteristics of the product.

Food-grade status

Celluclast 1.5 L FG complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC), supplemented with maximum limits of 5 x 10^4/g for total viable count and 10^2/g for moulds.

Packaging

See the standard Packaging List for more packaging information.

Application

Celluclast 1.5 L FG can be used whenever the aim is the breakdown of cellulosic material for the production of fermentable sugars, reduction of viscosity or increase in extraction yield of valuable products of plant origin.

Production of fermentable sugars from cellulosic material

The main reaction products of cellulose material hydrolysis with Celluclast are cellobiose and glucose. Cellobiose is not a fermentable sugar. Therefore, when maximum convertion to fermentable sugar is desired, we recommend the use of a cellobiase such as Novozym® 188 in combination with Celluclast or Cellubrix® L.
For initial trials with industrial substrates, the following dosages of the products are recommended (% w/w based on the cellulose content):

Celluclast 1.5 L FG... .....1%
Novozym 188.....0.2%
Cellubrix L 1%

The optimal enzyme dosages depend on the reaction conditions, such as pH, temperature and substrate concentration, and the above dosages may be changed when the process is optimized.

Reduction of viscosity and increase in extraction yield of vegetable products

For initial trials aiming at a reduction of viscosity or increase in extraction yield, a dosage of 0.1% w/w (based on raw material dry matter) of Celluclast 1.5 L FG is recommended. Depending on the circumstances, it may be possible to reduce the dosage substantially.

Reaction Parameters

Figures 1 and 2 illustrate the activity of Celluclast at different pH values and temperatures, using CMC as substrate. The heat and pH stability of the enzyme in aqueous solutions can be seen from Figures 3 and 4. For practical applications, the optimum conditions are about 50-60 °C and a pH of 4.5-6.0.

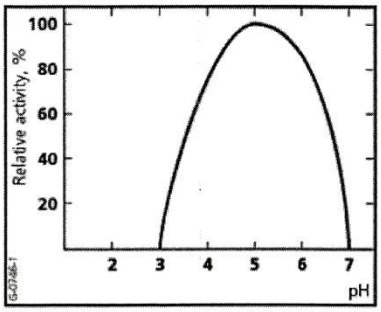

Fig. 1. Influence of pH on the activity of Celluclast.

Concentration of enzyme:	0.009 EGU/ml
Temperature:	50 °C (122 °F)
Reaction time:	20 minutes

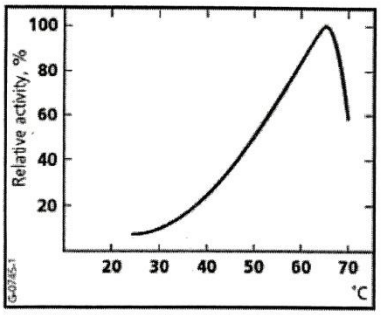

Fig. 2. Influence of temperature on the activity of Celluclast.

Concentration of enzyme:	0.009 EGU/ml
pH:	4.8
Reaction time:	20 minutes

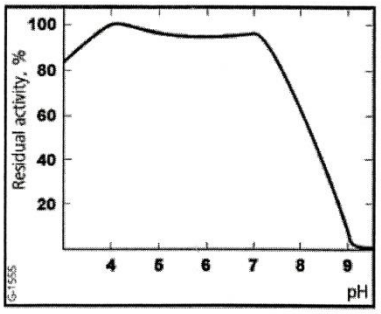

Fig. 3. Influence of pH on the stability of Celluclast.

Concentration of enzyme:	0.9 EGU/ml
Temperature:	25 °C (77 °F)
Incubation time	16 hours
Buffer system:	McIlvaine

novozymes®

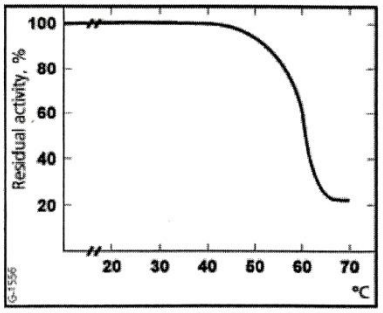

Fig. 4. Influence of pH on the activity of Celluclast.

Concentration of enzyme:	0.9 EGU/ml
pH:	4.8
Reaction time:	20 minutes

Safety

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. This product has been developed to resist mechanical effects. However, excessive mechanical wear and tear or crushing may create dust.

All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes.

Handling Precautions

Celluclast 1.5 L FG is non-flammable, completely miscible with water and safe when used according to directions. Observe standard handling precautions to avoid direct contact with the product or inhalation of dust from the dried product. In case of accidental spillage and contact with the skin or eyes, rinse promptly with water.

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

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LIST OF PUBLICATIONS

1. Hazrati Wazir, Cheng, S. F., Ong, A. S. H., & Chuah, C. H. (2012). Enzymatic Assisted Aqueous Extraction and Recovery of *Jatropha curcas* Oil. Journal of Oleo Science. [Submitted]

2. Hazrati Wazir, Cheng, S. F., & Chuah, C. H. (2011). Aqueous Enzymatic Extraction and Recovery of *Jatropha curcas.* Poster Presented at *9th Euro Fed Lipid Congress 2011*. 18-21 September, Rotterdam, The Netherlands.

3. Hazrati Wazir, Cheng, S.F., & Chuah, C.H. (2010). Effect of Enzyme Selection for Aqueous Extraction of *Jatropha curcas* Oil. Poster presented at *Oil and Fats International Congress 2010*. 20-22 October 2010, Kuala Lumpur Convention Centre, Kuala Lumpur.

4. Hazrati Wazir, Cheng, S.F., & Chuah, C.H. (2010). Enzymatic Assisted Aqueous Extraction of *Jatropha curcas* oil. Oral presentation at *16th Malaysian Chemical Congress 2010 (16th MCC)*, 12-14 October 2010, Putra World Trade Centre, Kuala Lumpur.

5. Hazrati Wazir, Cheng, S.F. and Chuah, C.H. (2010). Effect of Enzyme Selection for Aqueous Extraction of *Jatropha curcas* Oil. Poster presented at *Bilateral Symposium "Emerging Trends in Chemistry" between University of Malaya and University of Hyderabad.* 26-28 October 2010, Department of Chemistry, Faculty of Science, University of Malaya. Kuala Lumpur.

6. Hazrati Wazir, Cheng, S.F. and Chuah, C.H. (2009). A Comparative Study of Chemical and Enzymatic Extraction of *Jatropha curcas* Oil. Poster presented at *The 5th Mathematics and Physical Sciences Graduate Congress (5th MPSGC)*, 7-9 December 2009, Chulalongkorn University, University of Malaya and National University of Singapore.