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UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

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ENZYME PRODUCTS DEVELOPMENT

DP/CPR/88/001/11-53

THE PEOPLE'S REPUBLIC OF CHINA

Technical report: Downstream processing and marketing*

Prepared for the Government of the People's Republic of China
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of P. Bouchez, Consultant in downstream processing and marketing

Backstopping Officer: Z. Csizer, Chemical Industries Branch

United Nations Industrial Development Organization
Vienna

* This document has not been edited.

V.93-84847

ABSTRACT

Title of the project:
Improvement of technology for the production and development of enzymes at Wuxi Enzyme factory

Number of the project: DP/CPR/88/001

Duties:
- Technical examination of the enzymes downstream processing.
- Preparation of a detailed work plan, plan of action with budget estimate, schedule and standard of performance for the period 1997-2000.
- Preparation of a strategic plan to identify partners, enter negotiations in the view of a joint-venture as to consolidate Wuxi's Enzyme Factory position in the Chinese market and to develop worldwide export potential.

Duration: Three weeks, two of which on site and two days in Vienna.

Mission scheduling:

Departure from Paris: Feb. 26 th
Departure from Wuxi: March 10 th (no flights available on March 11 th for Shanghai-HK)
Arrival in Paris: March 12 th.

Work on site:

The first week was devoted to the visit of the three main workshops which are downstream processing the main marketed enzymes.

At the same time the present expert was concerned by raising all the necessary questions on financial and commercial aspects of this business in order to be able to write a presentation document for the companies which might be interested by a venture.

However, a few days later, the present expert was given a negative answer to the queries. It appeared that WEF had recently signed a memorandum of agreement with an unnamed company. WEF had already many good contacts with several companies among which were cited FINN SUGAR, NOVO NORDISK, MILES, GIST BROCADES and AMANO.

It is thought that some of these companies were more interested in a mean of marketing their own products in China than by selling WEF products worldwide. They also did not agree on the amount of money to input in the joint-venture, arguing for a negative balance in hard currency.

The future joint-venture was presented to the present expert as WUXI SYNDER BIO-PRODUCTS Co LTD.

There has been a change in the scope of work since Mr. HUO told the expert about the recently signed memorandum of agreement. The expert was asked to give a survey of the enzyme world market with an emphasis given to the use of enzymes as reagents, a sector for which they have

regular contacts with a polish company. On this particular chapter, a short lecture was given to the WEF scientists.

Follow-up:

Concerning the possible short-term improvements, which are discussed further in this report and which concern particularly the filtration problems, the present expert has already met with frame filter clothes manufacturers and will get soon a set of samples which could be tested on site. At the request of WEF people the present expert could bring them in a size adapted for two industrial frames and bring also some laboratory equipment to test other supports for ultra filtration. All these tests could be conducted in a period of maximum ten days.

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DOWNSTREAM PROCESSING

A - GLUCO-AMYLASE SOLID FORM

This is the main product processed at WEF. It is usually produced in the 60 m^3 fermenters at their 70% level. Therefore, the downstream workshop has to handle about 45 m^3 of broth. There are 2x20 m^3 storage tanks and 2x20 m^3 salting-out tanks. After 8 hours of salting-out, the product is filtered through 6x60 m^2 filter plate units.

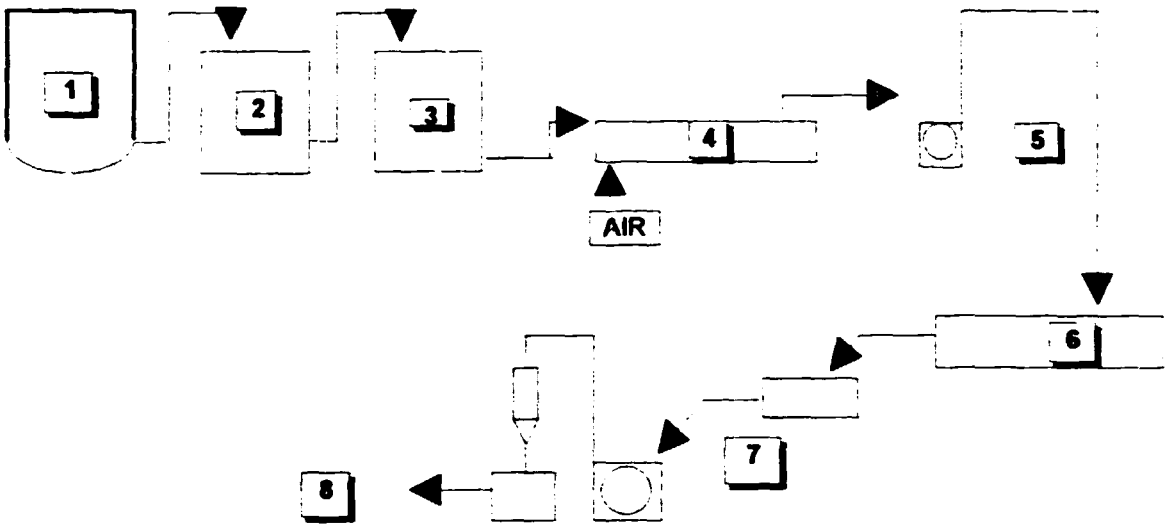
A 10 hours filtration cycle includes filtration (4 hr), air blowing (4 hr) and de-caking (2 hr). At a given time 4 filters are on filtration and 2 on air blowing. Filter cake is about 500-600 kg per unit and averages a 60 % dry matter content. De-caking is a manual operation as is the further handling to pre-grinding. During the following hot air pneumatic transport to the upper floor the product loss in humidity is around 3%. It is then handled manually to a set of 4 fluid bed dryers of 1 m^2 of surface area. Inlet air is admitted at 90°C and leaves at 40°C. One drying batch is lasting 20 minutes.

The dryers are emptied manually and the product is ground and sieved. At this stage, the activity of the product which may range from 80000 to 90000 U/g is standardized to a commercial level of 50000 U/g by adding sodium phosphate.

This workshop has 48 people on 3 shifts.

PRODUCTION SCHEME: GLUCOAMYLASE

SOLID FORM



LEGEND		EQUIPMENT number	vol./surf.	unit	handling
1	FERMENTER	1	20	m3	pump
2	STORAGE TANK	2	20	m3	pump
3	SALTING OUT TANK	2	20	m3	pump
4	FILTRATION	6	60	m2	pump
5	PRE-GRINDING AND HOT AIR TRANSPORT	1			manual
6	FLUID-BED DRYING	4	1	m2	manual
7	SIEVING AND GRINDING	1			manual
8	BAGGING				manual

BATCH TREATMENT UNIT			14 m3		
OPERATIONS	Unit	Amount	activity	10000	U/ml
SALTING OUT			total	1.40E+11	
ammonium sulfate	50%		dm	10%	
	kg	7000			
total volume	m3	21			
waiting time	hr	8			
FILTRATION					
surface area	m2	120			
duration	hr	4			
flow rate	l/sqm*hr	44			
air blowing	°C	ambient			
duration	hr	4			
decaking	hr	2			
cake dry matter	%	60	1389	kg dry	
cake weight	kg	2315	activity	90000	
PRE-GRINDING			total	1.25E+11	
increase in D.M.	%	3	yield	89%	
DRYING					
air inlet	°C	90			
air outlet	°C	40			
evaporation capacity	kg/h*m2	100			
water to evaporate	kg	926			
duration	hr	5			
Na-Phosphate	kg	611			
DRY ENZYME	kg	2000	activity	50000	
Total time	hr	23	total	1.05E+11	
Equivalence			yield	75%	
WEF U/g		50000			
NOVO AMG		167			

B - GLUCO-AMYLASE LIQUID FORM

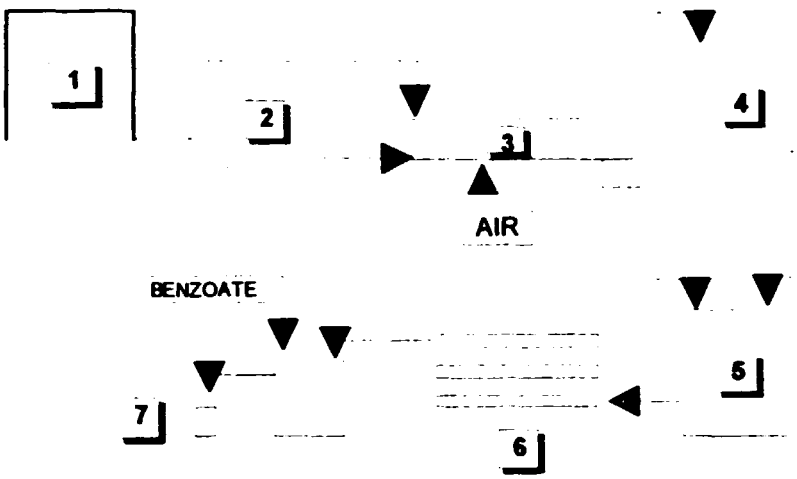
This enzyme is prepared in 20 m^3 fermenters. The resulting 15 m^3 are directly filtered with 3 to 5% diatomaceous earth as a filter aid. This step is carried on 7x60 m^2 filters in 4 to 5 hours followed by air blowing. Liquid is received on the floor in a concrete basement and pumped to a storage tank.

Ultra filtration on a COV 50000 organic membranes (120 m^2) is performed batchwise via a stainless steel recirculation tank with a 5 fold volume reduction. At this stage, 1% sodium benzoate is added for stabilization and the product is packed with a commercial activity of 50000 U/ml.

This workshop is run with 22 workers on 3 shifts.

PRODUCTION SCHEME: GLUCOAMYLASE

LIQUID FORM



LEGEND		EQUIPMENT number	vol./surf.	unit	handling
1	FERMENTATION TANK	1	20	m3	pump
2	FILTER AID TANK	1		m3	pump
3	FILTRATION	7	60	m2	pump
4	RECEIVING TANK	1	15	m3	pump
5	RECIRCULATION TANK	1			pump
6	ULTRAFILTRATION	1	120	m2	pump
7	BAGGING				manual

	BATCH UNIT		15 m3		
OPERATIONS	Unit	Amount	Activity	10000	U/ml
FILTRATION			Total	1.5E+11	
diatomaceous earth	4%	600	kg		
surface area	m2	420			
duration	hr	4			
flow rate	l/sqm*hr	9			
air blowing	°C	ambient			
duration	hr	4			
ULTRAFILTRATION	COV	50000			
surface area	m2	120			
input	m3	12			
volume reduction		5			
output	m3	2.4			
mean filtration rate	l/sqm*h	5			
duration	hr	4			
LIQUID ENZYME	l	2400	Activity	50000	U/ml
Total time	**hr**	**8**	total	1.2E+11	
Stabilisation	benzoate	1%	yield	80%	

C - α- AMYLASE SOLID FORM

Because of extensive foaming, only 12 m^3 of broth can be fermented in a 20 m^3 fermenter. This volume is withdrawn to a flocculation tank in which 2% NaH_2PO_4 and 2% $CaCl_2$ are added. After 30 minutes, the suspension is filtered during 4 to 5 hours on 2x60 m^2 of the 6 available filter units, while 2 others are on air blowing and 2 on de-caking.

Ultra filtration is performed batchwise for 5 hours with a membrane set of COV 30000 and 2-fold volume reduction.

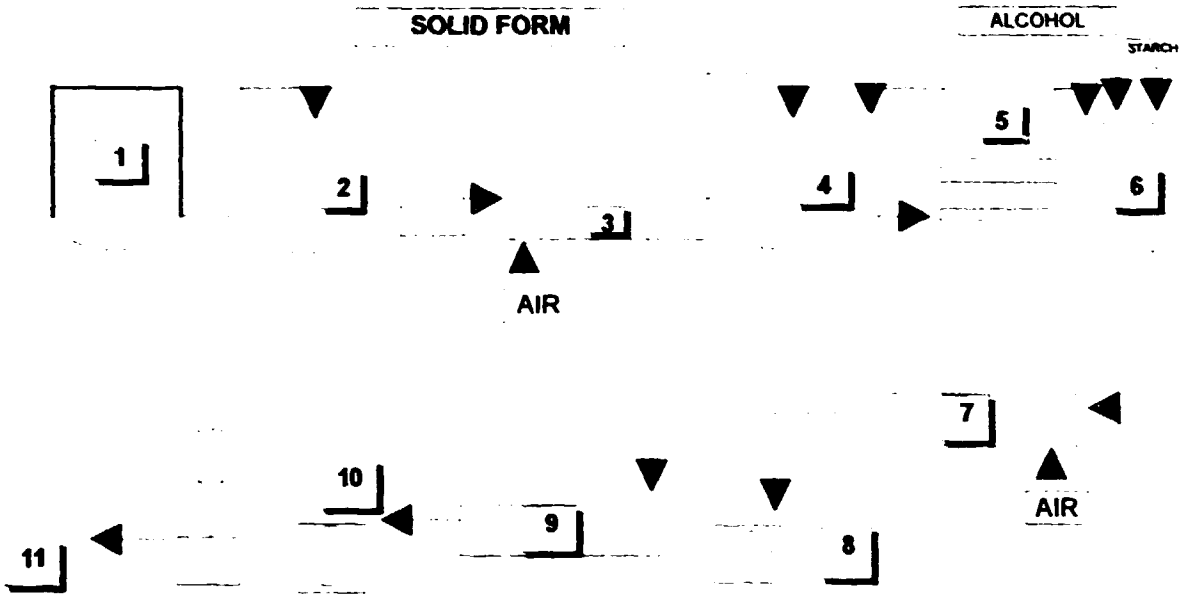
Distilled 95° alcohol is then poured in the storage tank to get a final 60° content and, after allowing 30 minutes for precipitation, an average 2% starch is added as a standardizing agent.

The precipitate is the filtered on 2x60 m^2 filters during 3 to 4 hours. Following a 4 hours air blowing, the 60% dry matter cake is pre-ground and air transported to the fourth floor to be manually introduced in a continuous 2 m^2 fluid bed dryer with 100 and 60°C air inlet and outlet temperatures, respectively. The residence time of the product is 40 minutes, the layer thickness being around 4 cm.

The finely ground and sieved 10000 U/g enzyme is manually bagged in 2 kg plastic bags.

This workshop is run with 41 people on 3 shifts.

PRODUCTION SCHEME: alpha-AMYLASE



LEGEND		EQUIPMENT number	vol./surf.	unit	handling
1	FERMENTATION TANK	1	20	m3	pump
2	COAGULATION TANK	1	25	m3	pump
3	FILTRATION	2	60	m2	pump
4	RECIRCULATION TANK	1			pump
5	ULTRA-FILTRATION	1			pump
6	COAGULATION TANK	1			pump
7	FILTRATION	2	60	m2	pump
8	PRE-GRINDING and HOT AIR TRANSPORT	1			manual
9	FLUID-BED DRYING	1	2.5	m2	manual
10	GRINDING	1			manual
11	BAGGING				manual

	BATCH UNIT		12 m3		
OPERATIONS	Unit	Amount	Activity	300	U/ml
COAGULATION			Total	3.6E+09	
Na2HPO4	2%	240	kg		
CaCL2	2%	240	kg		
total volume	m3	12.48			
waiting time	hr	0			
FILTRATION					
surface area	m2	120			
duration	hr	4			
flow rate	l/sqm*hr	26			
air blowing	°C	ambient			
duration	hr	5			
ULTRAFILTRATION	COV	30000			
surface	m2	120			
input	m3	10			
dry matter	%	2			
output	m3	5			
dry matter	%	4			
filtrate flow rate	l/sqm*h	17			
duration	hr	5			
COAGULATION					
alcohol conc	60%	8.6	m3		
starch	2%	100	kg		

FILTRATION		
surface area	m2	120
duration	hr	4
flow rate	l/sqm*h	28
cake dry matter	%	60
cake weight	kg	3200
PRE-GRINDING		
increase in D.M.	%	3
DRYING		
air inlet	°C	110
air outlet	°C	60
water to evaporate	kg	1184
residence time	hr	0.7
duration	hr	5
DRY ENZYME	kg	2016
Total time	**hr**	**18**

Activity	U/g	1000
Total	activity	2.02E+09
Yield		56%

D - BOTTLE-NECKS: SANITATION AND PRODUCTIVITY

In the actual workshop production schemes as described there are two aspects which are linked together: sanitation and productivity. The yields are far from satisfactory even for gluco-amylase for which it is said that there is no contamination problem.

/ sanitation

It is considered here as a mean to decrease losses in enzyme activity during processing.
Ideally one should observe the following rules:

- in all workshops all doors and windows should be kept closed,
- all parts in contact with enzymes should be in stainless steel and/or in food grade plastic;
- all manual handling should be avoided;
- the equipment should be designed so to have the smallest dead volumes;
- all equipment, including powder storage tanks (bagging) should be washed with bacteriologic agents;
- when possible, the process should be made continuous;
- the processing time should be as short as possible.

/ productivity

Independently of sanitation, keeping enzyme activity at high levels during downstream processing can be obtained by the improvement in equipment performance.

/ actual performances and productions

On the following table are given the yields in activity resulting from the actual downstream processing, in correspondence with the time involved for a whole batch processing:

Enzyme	Yield in activity	Downstream duration (h)
alpha-amylase (solid)	56%	18
glucoamylase (liquid)	80%	8
glucoamylase (solid)	75%	69

It appears as already noted that α-amylase is the most activity-sensitive.

On the next table are reported the maximum annual output for the three enzymes (based on a 3 shifts - 6 days week), the corresponding frame filters filtration and ultra filtration rates. In the last row are given the figures which are obtained nowadays with modern supports.

Enzyme	Production in T	Filtration rate l/h*m^2	UF rate l/h*m^2
alpha-amylase (solid)	900	26	17
glucoamylase (liquid)	2400	9	5
glucoamylase (solid)	600	44	-
	Nowadays rates	40 to 100	40 to 80

Further immediate progress can be achieved in frame filters filtration rates.

A - POSSIBLE IMMEDIATE IMPROVEMENTS

This paragraph concerns all improvements which can be started with minor investments.

Improvements in solid glucoamylase workshop

- filtration rate which is actually around 40 l/h*m^2 has to be increased because, after the 60 m^3 fermentation, the last 15 m^3 batch must be stored 50 hours before being processed. Therefore assessment of other filter clothes is a necessity. These could be first tested in laboratory and then on a smaller size industrial filter.

- when de-caking the filter, the cake chunks should be received in small plastic washable storage tanks and should not be left on the concrete floor. The hot air pneumatic conveying system which flows the cake from ground floor to the drying floor is never washed and might be a cause of contamination because it is not insulated and, particularly in winter, condensation may occur near the outlet. This transport system should be cleaned and ideally withdrawn from the production line.

- in this workshop there is an over capacity in drying which could be used if filtration is improved. At this stage it would be wise in the future to change the 4 dryers for one continuous as is used in the alpha-amylase workshop. This would restrain the numerous manual handling.

With better yields in fermentation gained with the use of better air filtration and further fed-batch technique the productivity of solid gluco-amylase could be approximately doubled.

Improvements in liquid gluco-amylase workshop

- filtration is here again a bottle-neck and test with other filter clothes have to be conducted
Besides, the liquid coming out from the filter has to be drained in a washable light plastic or stainless steel forms easily removable from thereunder.

- flow rate of ultra filtration is also very low and has to be improved. When membranes are cleaned, the water flow rate is about 4000 l/h for 120 m^2, i.e. 33 l/h*m^2. There is a possibility of using more recent and performing type of organic membranes. There exists in the laboratory an ultra-filtration pilot plant of the model used in the workshop: other spiral membranes could therefore be tested provided some fittings and sealing are solved on site.

- it has been noticed that concentrating this enzyme solution by ultra filtration or low temperature evaporation does not lead to the same stability of the solution. This could be due to the high shear stress (pressure and recirculation in pumps) to which enzyme are subjected during ultra filtration. Some enzymes are very sensitive to shear forces (other enzymes are not) and if both operations are ending in giving solutions with the same activity, the room temperature slow relaxation process leading to enzyme denaturation may be the explanation for the less stable ultra-filtered product.

Improvements in solid alpha-amylase workshop

- filtration and ultra-filtration deserve the same observations as for the preceding workshop.

- due to the low yield recovery in enzyme activity, it has been suggested to systematically study the relation between microorganisms counts currently assessed and enzyme activity between each unitary operation of the workshop. This will give the answer if there are other reasons for the loss in activity yield.

- it has also been suggested to study, at the laboratory level, the influence of sodium hydrogen sulfide on the stability of the broth activity at room temperature. This product added at a concentration level of 100 ppm can help in canceling the evolution of a contaminated broth up to ultra filtration in which a part of it will be released in the filtrate.

alcohol distillation should be checked as regards the pH variation which has been observed on several batches.

Working-out Paraben solutions

Paraben is easily brought into water solution with the following procedure:
solubilization of Paraben into propylene glycol heated at 60°C. Cooling down and addition to the enzyme solution.
Example: 0.15 % Paraben
5.00 % Propylene glycol
94.85 % Enzyme solution.
Different proportions may be used according to the enzyme concentration and the desired protection.

Stabilization of enzymes in liquid form

In addition to the usual bacteriologic agents (benzoate's), European producers are usually stabilizing enzyme solutions by adding glycerol to get a final total dry matter content of 50%.

B - WORKING PLAN

It is well-known that the major part of enzymes for food and technical applications, except for a big part of detergent washing products, is sold in liquid form. Therefore an increase in production of enzymes in liquid form does not require extension of drying facilities.

The following work plan is based on the assumption that, provided the fermentation capacity is already available, an increase in liquid form production of alpha-amylase and gluco-amylase could be achieved for the export market with reasonable investments; the cash flow generated by the export sales would then allow more considerable investments to revamp the actual workshops with the goals of increasing export and internal market shares.

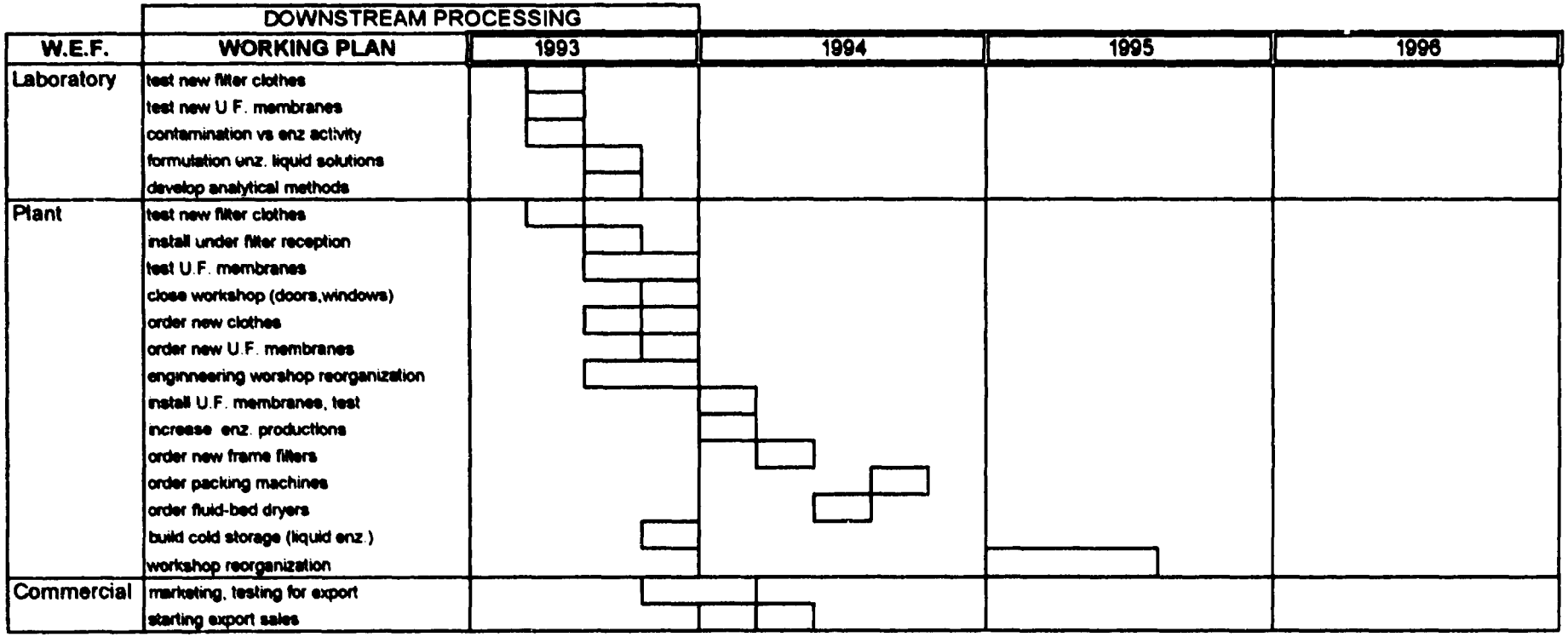
The expert was not informed about the actual and potential production of cellulases at WEF. It must be recalled that a very big market exists in South-East Asia for textile enzymes especially for working jeans clothes. In this sector, two enzymes preparations are sold together: alpha-amylase, for de-sizing, and cellulase, for "stone-washing". The market penetration will be much easier by selling the enzyme couple.

Therefore, it is recommended, in the first years, to reinforce the production tools for liquid gluco-amylase, alpha-amylase and cellulase.

1993 - test of new filter frame clothes,
- test of new ultra filtration supports,
- check for microbiological counts/enzyme activity between each unitary operation,
- formulation and stabilization tests of liquid enzymes solutions,
- install hygienic reception under filters and close workshops,
- develop analytical tools according Food Chemical Codex
- order new clothes,
- install new clothes (and pumps if necessary),
- order new ultra filtration membranes,
- marketing and testing for export,
- engineering workshop reorganization

1994 - install new ultra filtration supports,
- increase production in alpha-amylase workshop for liquid form,
- increase production in gluco-amylase workshop for liquid form,
- increase production in cellulase workshop,
- export sales,
- order new frame filters (all parts in contact with enzymes are in stainless steel or food grade plastic),
- order automatic packing machine for powdered enzymes,
- order fluid-bed dryers.

1995 - workshop reorganization.


DOWNSTREAM PROCESSING
W.E.F.
WORKING PLAN
1993
1994
1995
1996
Laboratory
test new filter clothes
test new U.F. membranes
contamination vs enz activity
formulation enz. liquid solutions
develop analytical methods
Plant
test new filter clothes
install under filter reception
test U.F. membranes
close workshop (doors,windows)
order new clothes
order new U.F. membranes
engineering worshop reorganization
install U.F. membranes, test
increase enz. productions
order new frame filters
order packing machines
order fluid-bed dryers
build cold storage (liquid enz.)
workshop reorganization
Commercial
marketing, testing for export
starting export sales

C - BUDGET ESTIMATE

Budget estimates cannot be worked out precisely without testing the new frame filter clothes and ultra filtration membranes. Furthermore, the quotations have not been received for clothes, frame filters and other equipment
Therefore the figures thereunder will be completed later on:

- monofilament filter frame clothes are thought to work at 5-7 bars feeding pressure and to deliver at least twice the actual flow rate obtained with multifilament clothes (with a good clarification). This would allow either to decrease the processing time or to increase production.
Renewal period would be 9 month instead of 2. Prices are around U$ 110 per square meter.
Equipment of the three workshop filters would represent about U$110,000.

- 86 m^2 frame filters with automatic plate displacement and external drain are U$ 45,000 ex-work. Quotations have been asked for filters with the necessary parts in stainless steel.

- ultra filtration spiral membranes are around U$ 180 per square meter.

ACTUAL APPLICATIONS OF ENZYMES AND MARKET TRENDS

I - SOURCE

Microbial origin:

For cost reasons the majority of enzymes are now obtained from microorganisms fermentation. The main strains now in use are:

Bacillus stearo-thermophilus, Bacillus subtilis, Bacillus licheniformis, Escherichia coli, Pseudomonas sp., Saccharomyces cervisiae, Aspergillus oryzae, and Rhizopus oryzae

A - FOOD APPLICATIONS

I - RAW MATERIAL UPGRADING

Enzyme usage is found in a variety of food industry sectors:

- Corn and others cereals wet processing
- Meat products
- Bread and biscuits manufacture
- Brewery
- Distillery
- Cheese making
- Milk products
- Wine and soft drinks.

These enzymes are mostly of microbial origin. In some sectors they are used for their catalytic properties while in others they are seen as processing aids.

Trends in actual research show an increasing use of enzymes in food industry.

Laboratories are working new processes with beta-glucanases, proteases, lipases and polysaccharidases.

Corn wet milling

This sector is the biggest consumer as regards enzyme tonnage.

Sugar beet

Some plants are using: invertase (inverted sucrose), α-amylase, dextranase (for better sucrose diffusion) and melibiase (sucrose from molasses).

Trends in development :
- more thermostable enzymes such as pullulanase, gluco-isomerase, amylo-glucosidase,
- enzymes which could work in more concentrated solutions,
- enzymes which could hydrolyze starch granules at low temperature.

Drinks

Hydrolytic enzymes are frequently used in the following different processing steps: fermentation, extraction, clarification, and filtration.

Brewery

In beer making enzymes are used in several steps of the process:

- hydrolysis of starch contained in crude grains,
- decrease of wort viscosity and increase in beer filterability,
- elimination of the cold colloidal precipitate due to tannin protein interaction.

Wine making

Pectinases are reducing juice turbidity and are a help for better filtration.

Distillery

Starch is a preferred raw material for alcohol production but it is a prerequisite to transform starch in maltose and glucose.

Soft drinks

Pectinases may be associated to other enzymes like cellulases, hemicellulases and amylases for making easier pressing, clarification and filtration..

Ready to drink products

Tannases are used to suppress colloidal suspension which appear in soluble tea making. Hydrolysis of flavanol gallic esters hinders the formation of precipitating complexes with theine.

There seems to be two major trends in the drinks sector:

- increase in process filtration with cellulases, pectinases, ß-glucanases,
- new types of soft drinks by enzymatic hydrolysis of plant raw materials (see thereunder).

Cheese making

This sector is not important for China and is a difficult one in which to be active because it is considered as strategic by the leading enzyme producers which have put considerable efforts and money on development and research for GRAS status.

Production, from proteins, of peptides fractions for pharmaceutical and/or nutritional use

According to the degree of hydrolysis, the products obtained may address to animal feed, human food or medicines. The two main applications are:
- meat tenderizing (papaine),
- soluble protein hydrolysates.

Vegetal or fish proteins may be hydrolyzed by papaine, bromelin, pepsin and various proteases. These partial digests are used to increase the protein level of acid soft drinks.

Further proteolysis leads to peptides that help to manufacture drinks and foods for patients with digestive disorders, for those which have had intestine resections and for people for which are intolerant to wheat and milk proteins.

Particularly, hydrolysis of whey proteins with pancreatin in a closed loop ultra filtration reactor allows the preparation of short peptide fractions. The fact that such a hydrolysis product is free of single aminoacids results in low osmotic pressure thereby avoiding any dehydration of the gut.

Protease's action may change some functional properties such as protein solubility, viscosity, emulsifying power, heat stability....

Research is actually focused on very limited proteolysis (0.1% degree of hydrolysis) which doubles protein solubility while increasing their gelification properties.

Proteases are used in large scale for producing fish and crustacean digests to manufacture protein hydrolysates which find uses as feed ingredients and flavor extracts.

Animal feed

Hydrolytic enzymes such as amylases, pectinases, beta-glucanases, cellulases, hemicellulases and proteases are starting to be used in feeds as a mean to increase *in vivo* the feed digestibility and as fermentation activators in silages.

Trends in animal and human nutrition:

- increase of functional properties by minor modifications,
- digestibility increase of raw materials.

II - ENZYMATIC SYNTHESIS

Manufacture of products by enzymatic synthesis concerns three sectors of the food industry:

- flavors,
- sweeteners,
- fats.

Flavors

Biosynthesis of flavors may be carried out with microbial cells or with enzymes.

In some companies people have started with whole cells but have now shifted to the use of the enzymes responsible for the transformation.

Microorganisms, such as Aspergillus niger, Escherichia coli, Pseudomonas maltophilia, Acetobacter xylinum, Acetobacter ascendens, Penicillium digitalum, Kluyveromyces lactis, Pseudomonas putida, are able to transform terpens into flavor compounds.

However the choice between a whole cell or free enzymes will have to be studied in each case especially in function of the possible selling price of the final product. This is for instance the case in biosynthesis of B-ionone from carotene: this reaction is obtained with whole cells because the extraction of the responsible enzymes would make the process non-viable.

Trends: The flavor sector is alike to develop this type of production because of the consumer attitude towards more natural products.

Sweeteners

The synthesis of "aspartame" and stevioside have been approached with the help of enzymes.

Fats

In function of water activity, lipases are catalyzing either hydrolysis or the reverse reaction. This sector is highly related to cheese-making in which lipases are used for flavor generation.

B - NON FOOD APPLICATIONS

I - ENVIRONMENTAL APPLICATIONS

This sector has very few industrial applications.

Biomass

Cellulosic by-products are both an important source of carbon and the most important source of pollution.

The use of cellulases in certain conditions allows a good breakdown of the polysaccharides which can be fermented, for instance to alcohol.

Cellulases are constituted of several enzymes: endocellulases or endo 1,4-beta glucanases, exocellulases or cellobiohydrolases et cellobiase.

Beta-glucosidase transforms the products obtained from the preceding hydrolysis in glucose. However, various difficulties have hampered this process to be applied on an industrial basis: low cellulase productivity of the microorganisms, practically no beta-glucosidase in low cost enzymatic preparations and presence of lignin.

Ligninases, which are produced by some microorganisms (Phanerochaete chrysosporium, Streptomyces viridosporus, Streptomyces badius, Coriolus sp.) are breaking down lignin. But the knowledge about these enzymes is not actually sufficient for industrial applications; only some pilot plants have been erected for assessing depollution in the paper industry:

- wood pre-treatment with ligninases leads to a better quality paper with a decrease in chemical pollution,
- when treated with Phanerochaete or Coriolus water effluents of paper making plants loose their coloration.

Decrease in nitrogen pollution

All compounds containing nitrogen are susceptible to give nitrates by biological oxidation, and to the formation of nitrites which are toxic compounds. Some microorganisms are able to transform nitrates in nitrogen, in the absence of air, and in presence of a carbon source such as ethanol or acetic acid. Other microorganisms just transform nitrates to nitrites. These two types of microorganisms are present in water.

Two methodologies are actually developed:
- selection of microorganisms such as Pseudomonas which are growing easily on ethanol or acetic acid, and which possess both nitrate and nitrite reductases,
- immobilization of enzymatic systems nitrate-reductase and nitrite-reductase.

In France there are two sites on which such processes are working.

Elimination of organic products

Enzymatic systems have been used to eliminate phenols with laccases and tyrosinases from Botrytis cinerea and aromatic amines with peroxidase from water effluents, but it seems that it is too soon to apply it industrially because of the enzymes cost.

Trends:

The environmental concern is great and developments of enzymatic treatment should become important. However, for the time being, applications will be restricted to high localized pollution problems such as:

- enzymatic elimination of coloring matters (textile industry),
- margins elimination(olive oil production).

II - CHEMISTRY

Industry has already applied enzymatic synthesis for the manufacture of steroids, antibiotics and prostaglandins but other biotransformations are actually studied.

Steroids

On the thousands of enzymatic reactions on steroids which have been published, only three are applied industrially.

Introduction of a double bond between carbons 1 and 2 is performed with a Δ^2-deshydrogenases. Δ^4-deshydrogenases are forming double bonds between carbon 4 and 5 and allow for instance to obtain from 5α-Androst-1-en-3,17-dione the compound Androsta-1,4-diene-3,17-dione.
Various microorganisms such as Actinomyces sp., Corynebacterium sp., Pseudomonas testosteroni, Steptomyces gelaticus, Streptomyces rubescens possess these deshydrogenases.

Hydroxylation at carbon 11 is the second application. The importance of this reaction is given by the fact that the enzyme is able to check the substrate chirality. this enzyme transforms only one of the chiral form, or, starting from an achiral substrate it produces one of the two possible chiral forms.
Various microorganisms are possessing the necessary enzymes for hydroxylation in 11 α. Others microorganisms, like Curvularia lunata, are able to hydroxylate steroids in 11 ß.

The third group of reactions which are industrially applied are those involved with the steroid side chain cleavage. These reactions produce alcohols, ketones, lactones and acids. However these reactions are non selective.

Uses of enzymes avoid this problem in giving the molecule without its whole side chain or part of it. Arthrobacter globiformis transforms prednisolone in a 20 ß-hydroxy

derivative of hydrocortisone by successively using two enzymes: a 20β-hydroxysteroïd deshydrogenase and a Δ^1-reductase.

These few examples show that the pharmaceutical industry possesses already many enzymatic tools for steroid semi-synthesis, however this potential, being mainly constituted by systems of immobilized cells within various matrices, will be increased by the possibility of using the free enzymes only.

But all the problems are not solved because there is a limit to the usage of enzymes for steroid modifications which is coming from the very low solubility of this products in aqueous medium, and all enzymatic systems are not able to work nicely in organic medium.

Antibiotics

6-amino-penicillanic acid (6-APA) is used as an intermediate in the synthesis of semi-synthetic penicillins like ampicillin or amoxycillin. It is prepared from penicillins by a chemical or enzymatic hydrolysis. The enzyme used is a penicillin amidase extracted from E. coli. The hydrolysis reaction is carried out discontinuously by recycling the immobilized enzyme.

Cyclodextrins

Cyclodextrins have found a use in molecular encapsulation. The cyclodextrin transglycosylase which is produced by Bacillus macerans is used for making cycles from alpha-D-glucopyranose units. From starch one can obtain alpha-, beta- or gamma-cyclodextrins.

Organic acids

The enzymatic production of organic acids with enzymes has to compete with extraction and fermentation processes.
However gluconic, aldonic, L-malic and L-tartaric acids are usually reported as being enzymatically produced on industrial scale.

The immobilized glucose oxydase and catalase allows to produce gluconic acid from glucose.

Oxydases are used to transform mono-, di- and tri-saccharides in the corresponding aldonic acids.

With a fumarase from Brevibacterium ammoniagenes, it is possible to obtain L.-malic acid from fumaric acid.

L(+)-tartaric acid is obtained from maleic acid through cis-epoxysuccinic acid with the help of a hydrolase.

Amino acids

Enzymatic synthesis is well adapted for those amino acids which are difficult to obtain by fermentation like L-alanine. However the main use of enzyme in this sector is for separating enantiomers. Amino acids which are produced by organic synthesis are obtained in racemized optically inactive L and D forms. The separation of the L form is performed by an aminoacylase. L-methionine, L-valine and L-phenylalanine are industrially obtained with this type of enzyme.

5'-mononucleotids

Yeast RNA is hydrolyzed with a 5'-phosphodiesterase to give a mixture of 5'-AMP, 5'-GMP, and 5'-CMP, 5'-UMP.
With a 5'-AMP desaminase, 5'-AMP is transformed into 5'-IMP. 5'-AMP desaminase and 5'-phosphodiesterase may be used in immobilized forms. 5'-IMP and 5'-GMP are separated by chromatography.

Various

Other applications are actually studied in the laboratories:

- synthesis of amidosugars (lipase),
- stereospecific hydrolysis (lipase),
- hydroperoxides synthesis (lipoxygenase),
- amino acids derivatives (aminoacyltransferase),
- obtention of chiral α-diols (epoxyhydrolase),
- glycopeptides synthesis (glycosidase).

III - OTHERS APPLICATIONS

Some sectors of which the technological importance is weak are experiencing the use of huge quantities of enzymes.

Tanning

During tanning, enzymes are used for dressing hides, dewooling and for bating.

Bacillus subtilis alkaline protease is able to hydrolyze the interfibrillar gel of the skins and allows the penetration of water. Bacillus subtilis neutral protease is used for getting some release in the wool while dewooling. It is also used with pancreatin during bating to obtain a leather of very good quality.

Textiles

Gelatin or starch glue are coated on textile fibers to protect them during weaving. After being woven tissues must be desized.
Bacillus licheniformis amylase is used in this operation when textile fibers have been coated by using starch, while Bacillus subtilis neutral protease or Bacillus licheniformis alkaline protease are used for fibers coated with gelatin.

Detergent washing

Bacillus licheniformis alkaline protease is present in many washing powders. By spray-drying it in presence of polyoxyethylene fatty alcohols it's allergenic power has been suppressed.

These products which have wide applications (floor, wall and metallic surfaces washing) may now contain proteases together with cellulases, lipases and amylases.

Specific cleaning products

In these field there are numerous applications. Particularly important are the cleaning solutions sold for organic membranes of ultra filtration and reverse osmosis. However polyvalent cleaning solutions do not exist and a lot of research has still to be done for improving the efficiency of these products.

Development of enzyme uses in chemistry will concern the following sectors:

- fat chemistry,
- detergents and cleaning solutions,
- semisynthesis and synthesis of medicines
- perfumes.

IV - CAPTORS

The large number of commercially available enzymes should, in theory, give the possibility to assay a large number of compounds. These enzymatic assays are largely used for medical diagnosis (search for lactic acid, uric acid, lipids, sugars, urea, and so on..), and for analytical control in pharmaceutical and food industries.
But their use in industrial processes is limited because of their reaction time (from few minutes to hours) and of their short lifetime in solution.

Enzymatic captors do not have these drawbacks.

Application sectors

Biomedical sector

Existing captors are those for glucose, cholesterol and lactic acid determination. Recent multifonctional captors have been developed: from one drop of blood it is now possible to obtain at the same time values for potassium, urea and glucose levels.

Industrial sector

There are several problems to be solved before on-line enzymatic captors are widely used in fermentation plants (wine, beer, milk products, antibiotics,...).

However some are already operational at the laboratory level:

- fish freshness determination by checking for inosinic acid, inosine and hypoxanthine,
- lecithin determination in food products,
- glutamic acid and nucleotides determination in food products,
- and for fat and oil quality controls.

Environmental sector

Captors for measuring dissolved oxygen, nitrates, ammonia and organophophorous compounds are already commercial.

Trends
Research efforts are on :
- increase in reliability and sensitivity,
- multifonctionnal captors,
- development of single use mini captors.

V - BIOMEDICAL

Some enzymes are already well-known medicines:

-as digestive aids: from pancreatic origin.
-as anti-inflammatory substances: chymotrypsine, hyaluronidase and more recently superoxide dismutase,
-in coagulation pathology: streptokinase, heparinase, urokinase, urease, and recently TPA (tissue platelet aggregation factor),
-in discal hernia: chymopapaine.

Other enzymes are being tested as potential medicines:

-uricase: it is able to change uric acid in allantoin which is 100 times more soluble in water,
-a microsomal enzyme of immobilized hepatic cells for kidney elimination of phenolic toxins,
-L-asparaginase (against lymphoblastic and myeloblastic leukemia),
-adenosine desaminase (treatment of children with abnormal immunitary system).

An other approach which is actually studied is to use enzymes which are produced by the human saprophytic bacteria. The enzyme gene is cloned and expressed by E. coli. The gene is then transferred to a bacteria of the usual human flora.

Trends

The main problem to be solved is to master allergenic reactions which are usually met with this type of compound.

However very recent results show that polyethyleneglycol derivatives of enzymes may solve this problem. Genelabs Technologic and Enzon have already two products in clinical trials: a PEG-L-asparaginase (anti-cancerous), and a PEG-hemoglobin (as a blood substitute).

C - MARKET

European market for enzymes
(in U$ million)

	1991	1995
proteases (milk, detergents)	141	187
carbohydrases (starch, drinks, detergents)	63	83
lipases (detergents, cheese)	31	42
pectinases (drinks, wines)	32	42
specialty enzymes	16	21

It is estimated that the food part value (1991) represents about 58% of the market. On a world basis, the food part is around U$ 580 mil (total world market U$ 800-900 mil).
In Europe, the three largest markets are Germany, worth U$ 83 mil in 1991, France at U$ 58 mil, and the U.K. worth U$ 45 mil. Whilst all national markets are expected to grow 20% over the period 1991-1995, the German market will grow by over 50% to be worth U$ 126 mil.

World enzyme producers, which share 90% of the market are:
NOVO NORDISK for 50% (and between 25 to 30% of the food sector),
GIST BROCADES and its USA subsidiary IBIS for 20%,
and GENENCOR (KODAK (USA) and KULTOR (Finnish) joint-venture) for 20%.

I - Food applications

This market is important but suffers from a low profitability; this situation originates from the sharp competition between suppliers on the corn wet milling market (glucose and fructose syrups). Enzymes producers are mainly dependent on forthcoming products essentially focused on cheese and cereals.

Immobilized enzymes are sharing 20% of the world market, but it is foreseen that in a few years this figure will be 35%. This growth will be mainly due to new applications in removing unwanted material such as bacteria, toxins and chemicals from food processing lines and effluent streams.

The biggest market is for HFCS (high fructose corn syrup) (α-amylase, 17 mio U$; gluco-isomerase, 18 mio U$ and glucoamylase, 25 mio U$); however this market is actually growing only with the population growth. On the other hand the biggest market in value is that of rennin worth of U$ 40 mil.

A lot of industry activity has been centered in the U$ 140 mil worldwide chymosin market, the biological replacement for rennet. After PFIZER's gene-spliced Chymax product won FDA approval, the 3-million gallon world rennin market has quickly become more crowded with genetically engineered materials. GIST BROCADES has just gotten the FDA approval for a similar product, and GENENCOR has won GRAS

(generally recognized as safe) for its Chymogen, to be marketed by CHRIS HANSEN LABS.

The other bright spot in enzyme marketing is the baking sector, with NOVO's anti-staling product, Novamyl. The amylase enzyme is said to improve white bread shelf-life by three or more days, and has had similar effects with whole grain breads and hamburger buns. Other enzymes have been used in this application because they tend to cause gumminess. Novamyl is carrying the extra advantage of replacing some softeners, such as mono- and di-glycerides , which have been traditionally added to bread. The potential market for this product is thought to be around US 15 mil.

Profits from these new applications are badly needed to offset problems costs in grain wet milling markets which is now more or less considered as a commodity business.

Therefore the potential for food enzyme expansion appears to be outside traditional markets.

Up to now, enzymes have been non-functional in the foods the consumer buys. They can go beyond just being a processing aid.

Potential to offer functional benefits could be in:
- texture modification,
- antioxidant and
- anti microbial properties.

II - Non-food applications.

Pulp and paper industry

The pulp and paper industry has been actively exploring technologies that allows reduced environmental impact. for instance, use of chlorine products in wood pulp bleaching has been associated with water quality issues, including dioxins. Faced with the need to improve water quality, several mills are currently in the process of converting to enzyme usage.
Xylanase enzymes allow for significant reduction in chlorine consumption without major capital investment.
Cellulases enhance water removal during the production of specialty papers, allowing increase machine speed and improved paper quality.
Several other applications are under development, including pitch control in wood chips, newsprint deinking, microbial growth control and effluent treatment.

Laundry detergents

Usage of enzymes in laundry detergents is continuing to grow with emphasis on non-traditional enzymes. The focus of technical development has moved beyond proteases and amylases to a broader range of enzymes.

GENENCOR has developed an improved enzymatic peracid bleaching system. Using knowledge that bleaching performance of perborate can be improved by using

activators to generate peracids in situ, this company has found that the lipase of Pseudomonas sp. is effective in generating peracids by hydrolysis of triglycerides in aqueous solution containing hydrogen peroxide. However the use of the original enzyme is not cost-effective due to a competing hydrolysis reaction. Therefore they developed several variants, through protein engineering, which demonstrate improved performances.

Although alkaline proteases are still the front-runner in laundry detergents at 75% share of the total enzyme consumption, and amylases and lipases a distant second, each with about 10% of the total, enzyme producers think lipases are the enzyme to watch, because proteases attack grass and blood stains while lipases break down greasy stains.

USA market penetration for enzyme containing detergents is estimated at about 50 percent. 40% powders and 60% of liquids are now formulated with at least one enzyme.

The market for detergent enzymes is expected to expand at 7.5% annually through 1995 to over U$ 110 mil driven by the need to make up mainly for the loss in cleaning ability of phosphates. The lipases market is projected to reach between U$ 10 to 30 mil for the same period.

The next segment for growth may be for products based on cellulases like, NOVO's Celluzyme, which brightens cotton fabrics by eating away microfibers on the surface that give clothing a dull look.

Dish washing detergents

Automatic dish washing detergents (ADD) have undergone sweeping changes in Europe, opening new opportunities for enzymes. The European ADD market is lowering pH levels by reducing metasilicates, removing chlorine and phosphates and replacing them with enzymes and oxygen-containing bleach, because of environmental pressures.

Moreover, the wash temperatures are coming down. At one time the 65°C was preferred, but now half of European consumers are said to prefer economy programs at 55°C. This is favorable to the development of enzymes which remove efficiently stain at relatively low temperature.

There are no enzyme formulated dish washing detergents available in the USA, because despite the strict phosphate control on laundry detergents, ADD have been exempted from such bans for performance reasons.

Backstopping Officer's Technical Comments
based on the work of Mr. P. Bouchez
DP/CPR/88/001/11-53

The expert's technical report covered the downstream processing of the main products of the Wuxi Enzyme Factory, the solid and liquid gluco-amylase and the solid alpha-amylase. After assessing the current situation in the factory he analyzed the bottlenecks, gave suggestions for possible immediate improvements, prepared a workplan for the period of 1993 - 1996 and gave a budget estimate.

As requested by the national project authorities he also prepared a Chapter on the enzyme application and gave a brief market analysis with actual users and future trends.

The substantive backstopping officer's comments on the report can be summarized as follows:

The international market of industrial enzymes is dominated by a handful of highly efficient research-based multinational companies, that are the driving force of this segment of biotechnological industry. In addition to the big companies there are dozens of relatively small companies developing enzymes and other bioactive substances by modern biotechnological techniques such as recombinant technology.

The industrial production of enzymes is by no means new. In the last few decades, however, our recently acquired knowledge concerning biocatalytic capabilities of enzymes and microorganisms has made possible the creation of a new generation of rationally researched and developed biologically based processes and products.

Whenever possible, it is advantageous to use thermo-stable enzymes, as by carrying out the reaction of higher temperatures the reaction rates become faster, the substrate viscosities decrease, the reactant solubilities increase and the risk of contamination decreases.

In the recent biotechnological revolution considerable analysis has been given to the advances in the genetic aspects, as well as in the fermentation technologies. There has been, however, an increasing need for more efficient purification techniques to purify a range of proteins from microbial culture of sometimes involving thousands of litres of fermentation culture.

The expert's report gives the most practical advices at minimum cost to improve the downstream processing techniques and recommendations for a development programme from 1993 to 1996.

Owing to the fact that in most cases any new development on industrial scale would involve capital investment and based on a captive market in China as well as the export potential of quality products, industrial cooperation has been advocated. By analyzing the techno-economic and political environment it is recommended that joint-ventures with foreign industries might be the best alternative of long-term cooperation with the industry.

It is our understanding, that such a joint-venture has recently been established with Synder, Inc., Vacaville, California. It is also our understanding that this joint-venture has been established only for production and marketing of one or two products.

UNIDO strongly recommends that similar joint-ventures should be established for some other enzyme products covering the need of different industrial sectors. Therefore, the new joint-ventures with Wuxi Enzyme Factory will not compete with each other but would strengthen the positions of the mother company in the People's Republic of China and the cooperating companies abroad.

UNIDO is willing and ready to provide advice and guidance to further develop international cooperation with the industry as one of the best options for development in the fast technologically changing biotechnological industry.