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ENZYME PRODUCTS DEVELOPMENT

DP/CPR/88/001

THE PEOPLE'S REPUBLIC OF CHINA

Technical report: Third mission to the Wuxi Enzyme Factory Technical assessment, improvement of production strains *

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 I. Balogh, microbiologist

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Vienna

* This document has not been edited.

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ABSTRACT

Title of the project: Enzyme Products Development

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

Objective and duration of activity:
- Technical survey and evaluation of production equipments and procedures in order to determine the actual bottleneck and obstacles of production
- Elaboration and standardisation of strain improvement methods in the case of Bacillus subtilis (BAA) and Aspergillus niger (GA)
- Lectures and consultations for increasing the staff`s awareness concerning the further technological and strain development

Main conclusions and recommendations:
The production of the factory is well organised, the skill and capability of working staff (operators, foremen, section leaders, top management) is excellent. The stainless (!) steel fermenters are of good design, representing high commercial and practical value.

The recent bottleneck of production is the high frequency of contaminations, and insufficiencies in recovery equipment. The contaminations can be eliminated by installing reliable air filters, proper air distributors inside the fermenters, and by the modification of some pipelines.

The obstacles in recovery can be cleared away by changing some equipment and modifying the technology.

The strain improvement methods were elaborated properly, the main task of the strain laboratory`s staff was to continue the work systematically.

For the time being the Wuxi Enzyme Factory does not have any appropriate laboratory or pilot scale fermenters which could be used for fermentation technology optimisation or improvement. The original existing pilot fermentation plant would be suitable for this task after modest renovation and instrumentation.

Therefore, after strengthening the actual production by eliminating contamination and after starting up the strain improvement, I recommend the proper and reasonable instrumentation of the pilot fermenters for being able to improve the fermentation technology towards the high performance feed batch style. Parallely I recommend to invest new pilot scale downstream equipment for improving the recovery technology as well.

EXPLANATORY NOTES

BAA = Bacterial alpha amylase

GA = Glucoamylase

GAU = Glucoamylase activity unit (Annex - 3., page 28)

BAAU= Bacterial alpha amylase activity unit (Annex - 4., page 30) same as the locally used unit

LGAU= Locally used GAU, 1 LGAU = 0.0022352 GAU

NTG = N-methyl-N``-nitro-N``-nitroso-guanidin (one of the most effective and most dangerous mutagen compounds)

UV = Ultraviolet light

COV = Cut off value: the smallest molecular weight, which cannot get through an ultrafiltration membrane

UF = Ultrafiltration
(usually one thousand < COV < one million)

MF = Microfiltration
(usually 0.01 micron < pore size < 0.2 micron)

DO = Dissolved oxygen

CSL = Corn steep liquor

OD = Optical density

Aspergillus niger (A. niger) = GA production strain

Bacillus subtilis (B. subtilis) = BAA production strain

I. INTRODUCTION

The report is based on the work of Dr. I. Balogh / Head of the L-lysine fermentation plant, Agroferm Ltd, Hungary /.

The activity began on 10 November 1992, and lasted until 2 January 1993.

The location of the mission was the Wuxi Enzyme Factory (West Tong Hui Road 27, Wuxi, 214035 Jiangsu, China)

The original aims of the mission were the strain improvement and elaboration of reliable methods for strain maintenance and seed lot preparation (Annex - 1., page 22).

The original objectives were modified after the consultation agreement drawn up in UNIDO headquarters and after the agreement with the Commander-in-Chief of the project (Mr Huo Xing Yun) in the Wuxi Enzyme Factory (Annex - 2., page 25).
The reason for revising the original program was the impossibility of obtaining new production strains within two months. This period is hardly enough for elaboration and optimisation of the proper methods for genetic improvement. So the main task was determined to elaborate proper strain improvement methods and start up the systematic strain improvement work in the laboratory.

The genetic improvement by optimised mutation techniques and high performance selection techniques is the best tool for obtaining new industrial strains for enzyme production. The genetic engineering methods -such as introducing foreign (eg. human) genes into host cells - have limited possibilities in industrial enzyme production (examples, exceptions, explanation in Annex - 6., pages 51, 63-67). That is why during the mission several methods of mutagenic treatment and selection were elaborated, and optimised.
The strain laboratory's staff can improve strains by using these tools systematically.

The microbiological work is naturally intermittent due to the incubation periods which must follow every operation or treatment. This was the reason, why the decision was made to use these intervals for analysis of the actual production technology and equipment.

The modified program contains an attempt to elaborate the method for producing fungal protoplasts of A. niger. The work with protoplasts can be an additional tool of strain improvement.

The modified program also contains the handover of five enzyme production strains (glucoamylase, glucoseoxydase, pectinases - Annexes - 14. and 15., pages 98., 100), which have considerable productivity but they are without industrial importance in Hungary. (From 1960 to 1976 these pectolytic strains were used for pectinase production in Phylaxia, Hungary). The five strains are listed in the Hungarian Type Culture Collection , Budapest.

In addition, it was decided to transfer and install personal computer programs. One of them is a common word processing program (Wordstar), the second is an advanced spreadsheet, graph and data processing program (Quattropro 3.0).
These programs are of high performance in (off line) data collecting, handling and evaluation, and in planning experiments or storing experimental circumstances.

All the objects of the modified working program were attained.

During the mission, Mr. Huang Xiao Dong (technical development department) was helping the work with his continuous attendance and interpretation.

II. ANALYSIS OF PRODUCTION

A. Fermentation plants

Head: Mr. Li De Quiang

There are two fermentation plants (forming the "Production-I" workshop), equipped with well designed stainless steel fermenters. According to the practical experience (considering the height, width of equipment and the agitator's diameter and rotation speed) the aeration is sufficient for the recent fermentations. One of the two plants is equipped with 3 sets of 60 m3 fermenters, the other is equipped with 12 sets of 20 m3 fermenters. Actually, the 60 m3 fermenters are used mainly for GA production, the 20 m3 ones for BAA production.

The usual results of GA production are among 10000 -12000 LGAU/ml as final fermentation activities. These results are not bad at all (cca. 24 GAU/ml), considering the international level. (According to informal information, the recent level of main producers is around 50 -60 GAU/ml.) The production of GA is smooth.

In the case of BAA, the successful fermentation's final activity is about 300 BAAU/ml.

All technological steps, operations are correct, the skill of operators is also of a high level.

For all that, there are certain insufficiencies, causing a high number of contaminations which hinder the successful, smooth production.

The obstacles can be eliminated within a short period.

Concerning equipment and arrangements, there are only instrumentational differences between the two plants. Therefore the next findings are general.

General findings

The biggest obstacle of smooth production is the high frequency of contaminations - mainly in the case of BAA, because of it's nearly neutral pH. The GA fermentation "defends" itself by the low pH (4.0 - 4.5).

The main origin of the contamination is the insufficient air filter system, the second main origin is the horizontally installed air distributor inside the fermenter.

The air filter system's first common filter unit (with cotton wool cartridge) installed just after the process air compressors, can retain only the oil spray from the air. (The process air is supplied with oil-free Atlas-Copco compressors and out-of-date cylinder type ones parallely.) The individual filters are Chinese-made alloy bag filters. The performance of these filters does not meet the sterility requirements at all. These are not deep filters, the thickness of the active layer of filter material is only some millimeters. The pore size is much bigger than 0.2 micrometer (which is required for sterile filtration). They should be replaced by reliable ones as soon as possible (Annex - 5., page 32).

The horizontal air sparger is the worst solution of the possible arrangements. Even in those cases when the fermentation broth does not contain starch, these air distributors are clogged partially after some fermentation cycles. The sticky clogged material cannot be cleaned away anyhow. This material, due to it's lower water content and sporulating contaminants, is a usual source of systematic contamination. The proper solution to this item is to modify the arrangement (Annex - 5., pages 36 and 46).

Concerning the sterile work, there are many smaller technical items about piping, arrangement, valves and the operation style, which are absolutely necessary for preventing contamination - good engineering and manufacturing practice in this field (detailed information in Annex - 5. page 37). To mention the most important items, the high amount of defoamer destroys the oxygen transfer, therefore small sterilisable defoamer tanks should be installed for proper (intermittent) addition during the fermentation process. This manual foam prevention gives the opportunity to use higher fermentation volumes in the fermenters. Therefore this investment results in a higher yield and volume in the same time.

It would be beneficial to modify the sterilisation time from 45 minutes to 60 minutes. In the case of batchwise sterilisation, this period is used in Europe in cases of starch-based mediums.

It would be necessary to replace the seed transfer line, and fix it above the fermentation tanks. It would be important to maintain the steam seal continuously in the seed transfer line, and in other connection points which are out of operation during the fermentation process (all in annex - 5., page 37).

According to local experience, there are difficulties with cooling the fermentation tanks in summer time. The 60 m3 fermenters are installed with an external cooling surface as well as in a well designed arrangement. There is not enough room to install new internal cooling coils. That is why the best solution seems to be - to increase the overall cooling capacity of the cooling system by installing additional water coolers in the recycled water cooling line. (This results in decreasing the temperature of the cooling water.)

The fermenters are not insulated. The lack of insulation can cause contamination due to the locally lower temperature during the sterilisation period. Otherwise, the lack of insulation can cause trouble in maintaining the fermentation temperature in cases of hot outside temperatures. (This requires additional cooling capacity.)

As an addition to the general findings, the fermentation media for both GA and BAA fermentation seems to be very simple. There are no trace elements, the potassium and phosphate supplies seem to be insufficient (Annex - 16. page 108). Proper optimisation of industrial media could result in significant increase of activity.

Instrumentation

In the case of the 3 X 60 m3 fermentation plant, much data is displayed in the instrument room: temperature, pH, pressure, rotation speed, air flow, heat generation and DO. The temperature is controlled through controlled valves. The DO measurement system is out of work, due to the lack of DO probes. (In this plant there is a good possibility to control the pH - manually - by using gaseous ammonia, introduced into the inlet air pipeline. In the case of automatic control, this would be an up-to-date solution.)

The instrument panel is connected through an interface to a personal computer which is installed with a variant of CC-BIOS data collecting software. The software was made by the experts of the local Institute of Light Industry. The computer's task would be to collect, store and express in graphs the data of fermentation batches. The software could not work recently because of some insufficiency of the program. It would be necessary to have the software repaired - the author is available. In this case the graphs of the collected data should

be modified according to the graph represented in Annex - 17. (page 109) in two separated groups of data. (Recently the program has been providing seven individual graphs in one display, the Y axes are too short for visual evaluation.) If the manual input could be enabled by modification of the program, the laboratory analysis results could be collected as well.

After these modifications, the software would be able to collect and store data on a really high level. The computer, interface and instrumentation of the fermenters are of high commercial value, therefore proper repair of the software is recommended.

The delivered word and data processing software was installed on this personal computer. These programs could be good tools for off-line data handling and analysis. Demonstrations and explanations were given for getting acquainted with this software.

In the case of the 12 X 20 m3 fermentation plant, there is no automatic temperature control, the temperature is regulated manually by using mercury thermometers. The automatic temperature control is an absolute requirement, the measured temperature data must be registered by proper chart recorders. The air flow is estimated only by checking the pressure gauges of the exhaust air pipeline. The cheapest but much more reliable solution would be to buy small portable mechanical air flow meters which are used in meteorological institutes. This type of instrument is very cheap, the air flow can be adjusted by inserting it into a slot of the exhaust pipeline fairly well, until the factory will be able to finance instrumentation at a higher level.

The basic and most urgent modification would be to install new and reliable air filters for every fermenter and seed tank. Only on this basis, can the high amount of contaminations be eliminated.

B. Recovery plants

GA (workshop - 3), Head: Mr. Zhong Jian Guo

BAA (workshop - 2), Head: Mr. Qiu Jian Xing

Recovery of GA

In the case of GA fermentation the contamination frequency is very low. Therefore the fermentation liquid due to it's low pH can be treated with a stable recovery yield.

For producing a liquid product, the first step is the filtration by frame type filters after addition of diatomite. The filtration process is smooth, but the batchwise manual handling of the filters needs a rather long time.

The filtrate is concentrated in a spiral type UF (COV=500000) unit. The liquid product is stabilized by sodium benzoate. The average activity of this product is 50000 LGAU/ml. The recovery yield of the process is 65-70 %.

The frame filters could be replaced by a continuous up-to-date vacuum drum filter equipped with a washing section. In this way, the most of the losses could be recovered.

For producing the solid product, the fermentation liquid is treated by (NH4)2SO4 addition. In this way, after filtration the filter cake contains the mycelia and the precipitated enzyme as well. The filter cake is dried in a hot air chamber dryer. The average activity of the solid product is 50000 LGAU/ml. (Same as in the case of the liquid product, not higher, because of the presence of the mycelia.) The recovery yield of the process is 75 %.

The frame filters could be replaced by a vacuum drum filter in this case as well. For drying the filter cake, a good fluid bed dryer would be more effective.

Recovery of BAA

In the case of BAA fermentation broth, the contamination frequency is very high.

The first technological step after the broth discharge from the fermentation tank is precipitation. This is made in separate tanks by adding 2.2 % of CaCl2 and 2.2 % of Na2HPO4. After the precipitation the pH is readjusted to 7.0.

Due to the frequent contamination, usually the pH is decreasing dramatically after the broth discharge. This leads to an uncertain pH value during precipitation, which can cause losses at this technological step. (Even under normal circumstances, the precipitation has it`s significant loss as well.)

The precipitate is eliminated by frame filters. These types of filters are comparatively good for filtration of mycelial fermented broths, but for this task they are not appropriate at all. The biomass, the precipitate and sticky remains of broth are clogging the filters very quickly, therefore causing a big loss of liquid during the frequent disassembling and cleaning.

These first technological steps could be solved by using a high speed industrial separator equipped with an automatic sediment discharge system.

The next step is ultrafiltration, (COV=30000) in this case as well.

After this concentration procedure, the BAA containing liquid is enriched by starch, and precipitated by alcohol addition. In this case, the solid precipitate is the BAA containing phase. This technological step is considered as being the second frequented point of loss.

After filtration (on frame filters again) the filter cake is dried in a hot air chamber dryer. This last procedure could be solved by using a fluid bed dryer. The usual average activity of the product is 2000 BAU/ml. The overall yield of the recovery process is under 50 %.

At present, with no possibility of significant technological changes, it would be beneficial to add some bacteriostatic compound to the fermentation liquid just before the broth discharge. 0.5 % of sodium-benzoate would prevent any further contamination, and this compound is widely used in the food industry as well.

The second modification is the washing of frame filter clothes, not only by water, but by using some disinfectant chemical compound, in proper concentration (eg. NaOCl solution).

With reference to Mr Joyeaux's report (his first mission in this project) there are suspicions about proteinase which can destroy the BAA protein molecule during downstream processes. To check this out, it would be necessary to realize the whole downstream process of BAA in the laboratory by sampling and measuring BAA and proteinase activities after every step. If the effect of protease is significant, even the UF concentration can represent a big loss, provided that the protease is concentrated by the UF membrane as well.

After this experiment, depending on the result, a proteinase deficient microbe could be improved easily as Mr Joyeaux suggested, but according to our experiments in the strain laboratory, this microbe cannot grow with the lack of organic nitrogen supply.

It means, that without protease, some protein hydrolysate (as pepton) or CSL should be added to the fermentation media, and according to the prices of raw materials, this question seems to be more than technical. Therefore it would be very important to know much more about the role of proteinase in the recovery processes. Anyway, there are some methods for reducing proteinase activity by modifying the fermentation medium's composition. If necessary, based on the results of the suggested laboratory scale experiment, the modification of the fermentation medium can be performed.

The final bacterial cell concentration can be reduced in the case of GA and BAA as well. The proper method is the microfiltration, where the pore size is smaller than the smallest spore, usually 0.2 micrometer. The equipment is the same as in the case of ultrafiltration, but due to the bigger pore size, the mechanical resistance of the membranes is much smaller than in the case of UF. The process results in a practically sterile liquid.

C. Pilot fermentation plant

Head: Mr. Zhou Xi Liang

The pilot fermentation plant contains two sets of 3 m3 stainless steel, two sets of 1.2 m3 and four sets of 0.5 m3 carbon steel fermenter tanks.

The instrumentation is insufficient, there is only an automatic temperature control by controlled cooling water valves. According to the items of piping , air filtration and valves, there are the same deficiencies as in case of the production workshop.

The instrumentation, to a reasonable and useful degree, is absolutely necessary to enable the technology improvement by these pilot fermenters.

The suggested investments of instrumentation:

- To install a pH measuring facility in the case of 1.2 and 3.0 m3 tanks. In the case of 3.0 m3 fermenters, pH controllers and controlled valves are required (gaseous ammonia connected to the air inlet pipeline).

- DO measuring facility in the case of 3.0 m3 tanks.

- Two sets of programable logic controller and peristaltic pumps for performing feed addition through silicon rubber hose to the 3.0 m3 fermenters.

- Two sets of chart recorders for collecting pH, temperature and feed rate data (in the case of DO, the continuous registration is not required).

The instrumentation to the above extent (Annex - 8. pages 81-85) gives the possibility to perform batch and feed batch fermentations as well in 3 m3 fermenters, at a useful level, which can be reproduced in industrial scale fermenters.

The financial requirement for this investment is fairly modest compared to the commercial prices of sophisticated, fully computerized devices.

The piping, air filtration system and valves should be replaced/ reconstructed in the same way as mentioned in the case of industrial fermenters (Annex - 5., pages 45-47, Annex - 8., pages 83-85).

According to the above planned system, two sets of 0.5 m3 tanks would be used as feed tanks from where the feed medium would be transferred by peristaltic pump to the 3.0 m3 "main fermentation tanks". The other 0.5 m3 and the 1.2 m3 tanks would be used as first and second seed tanks. This arrangement enables a parallel operation as well as one with the two lines. The chart recorders must be of 6 channels. One recorder can collect all the data from one fermentation line: pH from 1.2 and 3.0 m3 tanks, temperature from 0.5, 1.2 and 3.0 m3 tanks, and feed rate from a 3.0 m3 tank.

The aeration rate could be adjusted by a portable rotameter. The feed tank's operation does not need temperature registration.

D. Quality control laboratory

Head: Mr. Wu Bing Yan

The quality control laboratory covers the tasks of systematic raw material and final product quality control. The third task of this department is the so-called "after sell service". They keep contact with purchasers, in cases of new customers, they explain the proper use of the product and even help to perform and optimise industrial experiments as well.

The quality control laboratory keeps samples for subsequent checking in case claims about quality arise.

The equipment of the laboratory is modest but meets the requirements. The activity measurement methods are based on iodometry. The BAA's measurement method is the same as in Europe (Annex - 4. page 30). In the case of GA, the local method is different. (The quality control laboratory uses 2 % starch solution as substrate, at pH= 4.6, at 40 degrees centigrade.) The laboratory compared the local (LGAU) and the Miles (GAU, Annex - 3., page 28) determination methods. The conversion factor between the local and Miles activity units was obtained :
1 LGAU = 0.0022352 GAU.

In the case of BAA determination, the quality control laboratory does not use color standards for comparison. In spite of this, this inaccuracy is well balanced by the high skill of the laboratory assistants.

In cases of strain improvement work, a high number of new mutants should be tested by shaking flask fermentations. The quality control laboratory would be able to collaborate with the strain laboratory by sharing the task of activity determinations.

III. MICROBIOLOGICAL LABORATORIES

A. Production laboratory /seed/

Head: Mrs. Fan Xiao Ying

The production laboratory provides seed for the fermentation plants.

B. subtilis

In the case of B. subtilis, they are starting with opening the freeze dried tube. The first step of the procedure is to screen colonies by shaking flasks. From the best colony, slant agar subcultures are inoculated, and the maturated bacteria`s sterile water suspension is transferred to flat type slant agar (Roux) flasks. From one lot, the production laboratory obtains 30 -40 pieces of Roux flasks. 10 % of these flasks are tested by shaking flask experiments, the remaining flasks are used for inoculation of seed tanks. Before seed tank inoculation, a cell suspension is made in the Roux flask by using sterile water, and the suspension is introduced into the seed tank.

This seed production procedure is well designed and reliable .

According to the result of the first screening steps, the standard deviation is about 5 %. It means, that the strain is stable. This first step is useful for preventing any contamination which can be introduced from the freeze dried tube.

The sterility and productivity checking of the seed by using 10 % of the seed is an accepted standard method in fermentation industry.

The inoculation by using spore suspension is satisfactory in the case of B. subtilis. Due to it`s well known very short generation time ( 20 minutes ), a vegetative or higher volume inoculation would give only one or two hours advantage. ( The seed tank`s cultivation time is 20 hours in the case of B. subtilis.)

The only modification to be suggested is to increase the number of one lot. The method is very simple: suspension should be made by using 10 % sterile glycerol solution from a Roux flask culture.

The suspension should be dispensed by 3 mls to test tubes. A high number of these test tubes can be stored in a deep freezer (- 70 degrees centigrade), and if requested, one tube should be used for the inoculation of one new Roux flask, which can be transferred to a seed tank after one day of incubation. The distribution of the glycerol containing suspension can be made by using a sterilisable dispenser syringe, lacking this, pipettes can be used equipped with a sterilisable silicon rubber cap.

A. niger

In the case of A. niger, the production laboratory uses freezed ascospores' suspension for making subcultures on sterile bran in test tubes. The maturated test tubes are inoculated in Roux flasks onto sterile bran as well. In these flasks, after incubation, a huge amount of spores are generated. 10 % of these flasks is used for production and sterility tests. The remaining flasks are used for inoculation of seed tanks, after sterile water addition for obtaining spore suspension.

The freezed suspension was made after a previous selection step.

This method is reliable and safe as well.

Howewer, in the case of fungi there are examples of significant modifications. Modifications can be obtained and maintained by using vegetative cells. Therefore independent of the strain improvement by mutaganesis, it would be very important to try whether it would be possible to gain positive modifications and maintain them or not. In some cases, there are very successful GA fermentations with a result of 15000 LGAU/ml (the usual is about 10000-12000 LGAU/ml). The task would be to plate sterile samples from the final stage of these good fermentations onto Petri dishes - and after incubation the colonies should be tested.

The crucial point of this work is to prevent sporulation. By using Czapek-Dox agar media, the sporulation is delayed. So the subcultures should be made before sporulation, and the flask's inoculation should be made from test tubes before sporulation as well. This way, principally the positive modifications can be maintained.

A positive result of this experiment would highlight a more successful (but less convenient) seed production technology. In this case, the inoculation should be performed by vegetative cells by using a shaken flask (vegetative) culture.
Mrs Fan Xiao Ying has promised to make this experiment.

B. Strain laboratory /strain improvement/

General findings

The strain laboratory's task is to improve the production strains. The laboratory is equipped with two shaker rooms, two sterile rooms with clean benches. In the temperated shaker rooms there is only one good and reliable shaker (made by New Brunswick) with a capacity of 40 x 300 ml flasks. The other two shakers are out of date and low speed ones. It would be highly recommended to install at least one more shaker with high capacity, for supporting the strain improvement program. The sterile rooms are of modest installation, but due to the good UV lamps and the systematic cleaning made by the staff with benzalconium-bromide, the level of sterility is meeting not only the research, but the production requirements as well. (The sterility was checked by exposure of Petri dishes.)

The microscope of the laboratory is insufficient for strain improvement work. The visible picture is of low quality, unclear and the sharpness is unsatisfactory. An investment of a cheap but high quality binocular microscope (equipped with planachromat objectives) would be appreciated.

The spectrophotometer of the laboratory is under repair, the installation of the new HPLC has not been finished yet, because of the lack of some spare parts.

The style of work and preparation of sterile media, glassware and tools are careful, accurate and the skill of the staff is very good as well.

Freeze drying

After installation of the equipment, the staff of the strain laboratory started up freeze drying of strains. The handling of the freeze dryer is properly carried out, the freeze drying conditions are well selected.

However, some modifications were made after consultation. Originally the freeze drying was made by using cells harvested from the exponential phase. The cells are very sensitive in this

growth phase, therefore during freeze drying the lethality rate is comparatively high.
After consultation, maturated spores (in the case of B.subtilis) and ascospores (in case of A. niger) were used for this purpose resulting in higher survival rates.

As a freeze protecting agent, 25 % diluted sterile milk is used. The sterilisation of milk was made previously by autoclaving at 120 degrees centigrade for 10 minutes. This is insufficient for killing spores, but the time cannot be extended because of the burning of the milk.

The proper method (according to Tyndall) was introduced: as a first step, the milk was maintained for 30 minutes in a 100 degree centigrade water bath for killing the vegetative cells. The treated milk was stored for one day at room temperature. This period enables the germination of all survivant spores. After this, a 10 minute autoclaving leads to a real sterility, because all living spores have already turned to vegetative form. Liophilisation (freeze drying) was made for 24 hours at - 4 degrees centigrade.

Strain improvement

For strain improvement by mutagen treatment, a theoretical basis was given in the second lecture (Annex - 6. page 52) . A general scheme as a practical approach was elaborated (Annex - 12. page 93), and copies of several literature were handed over.

For introduction of a high performance method for strain improvement all steps of the procedure were optimised: proper fermentation media, shaking and inoculation circumstances were elaborated for obtaining cells which are in an exponential phase (B. subtilis) or in a germinating phase (A. niger). Proper mutation conditions (distance from UV source, exposure time, NTG concentration, reaction time) were optimised. Proper selection solid media and shaking flask fermentation conditions were elaborated (procedures in Annex - 11. page 88).

This work required a long time, because the proper results could be obtained only by performing many pre-experiments for each step.

According to the literature and industrial experiments, after some (2-10) successful mutation steps, made by one type of mutagen agent, it is beneficial to change to another type of mutagen. In the case of the recent improvement, the first agent suggested is UV. The change to NTG is reasonable if (further) success cannot be reached.

The strain laboratory should continue the mutation - selection work systematically. Usually a high number of colonies should be tested until good results are achieved.

Formation of fungal protoplasts

The procedure was demonstrated as a tool borrowed from the methods of cell level genetic engineering. In the recent phase of the strain improvement, protoplasts can be used for mutagen treatment as an additional possibility (Elaborated method in Annex - 13. page 96).

Generally protoplast fusion is used for introducing secondary characteristics as a low foaming property or advantages at filtration. These characteristics have to be restored usually after many successful steps of strain improvement for higher production capacity (theoretical approach was given in the second lecture, Annex - 6. page 67).

Handover of strains

Five strains were handed over as :

/Strain/	/Product/
Aspergillus niger	glucoamylase
Aspergillus niger	glucose-oxidase
Aspergillus niger	pectinase
Aspergillus awamori	pectinase
Aspergillus foetidus	pectinase

These strains are no longer of industrial interest in Hungary. Nevertheless, some of them (pectinases) were used as industrial strains between 1960 and 1976. All are listed in the Hungarian Type Culture Collection at the University of Horticulture, Budapest. These strains can be good starting points for further strain and technology improvement (Annex - 14., page 98).

At the request of the chinese colleagues, the determination methods for several pectinase activities were translated into English, and a good and simple laboratory method was given for producing pure pectin from apple. This pectin can be used as a

reliable substrate for pectinase enzyme activity determination (Annex - 15., page 104).

The local method for pectinase assay is based on reducing sugar measurement (Somogyi method). The delivered pectinase determination methods were used successfully in Hungary for a long time, and several activities can be measured by them. These activities are responsible for the fruit juice clarification effect, which can be used in the canning industry. Therefore it would be necessary to find out the exact correlation between the local and the delivered methods.

Practical advice was given about demonstrating and checking the clarification effect of pectinases: apple juice can be made in the laboratory by using an electric mixer from apple and water. The clarification performance can be observed by measuring the time required for a certain degree of sedimentaion in a calibrated glass cylinder (and should be compared with the control apple juice sample). This approach is very useful, because the main purpose of application of pectolytic enzymes is the clarification of several fruit juices, or the increasing of the liquid yield from fruit smashes.

IV. RECOMMENDATIONS

On the basis of findings at production workshops and laboratories, the recent bottleneck of production is the high frequency of contaminations, and high losses at recovery. Therefore the first part of the recommendation is connected with the strengthening of the present production. The second part is connected with investments for technology and strain improvement. The third part is connected with further attendance of experts and training suggested.

A. Strengthening the recent production

1./ In the case of industrial fermenters and seed tanks new individual air filters should be installed. In the case of industrial fermenters an automatic temperature control system should be installed (Annex- 7., pages 79-80).

2./ All horizontal types of air distributors should be replaced by safe ones (Annex - 5., pages 36, 46) as soon as possible.

3./ Piping should be modified (at least seed transfer lines according to Annex - 5., page 45).

4./ In the case of BAA, 0.5 % of sodium-benzoate addition to the fermented broth would help in preventing further contamination during recovery processes. The addition can be repeated at recovery steps.

5./ The frame filter textiles should be washed with diluted NaOCl solution between each batch, to prevent contamination.

6./ The 3 X 60 m3 fermentation plant's data collecting system should be repaired by modification of the personal computer's software.

The first point should be an investment from the side of UNDP. Points 2. - 6. should be the tasks of the Wuxi Enzyme Factory.

B. Investments for technology and strain improvement

1./ The strain improvement laboratory should be equipped with an additional high capacity shaker to enable the selection of a high number of new mutants, and a reliable microscope (Annex - 10., page 87).

2./ The fermentation pilot plant should be equipped with air filters and instrumentation at a reasonable but high performance level to enable improvement of fermentation technology (Annex -8., pages 83-85).

3./ Piping must be modified in the fermentation pilot plant (Annex - 8., pages 83-85).

4./ For elaborating new downstream technology with a higher recovery yield, new pilot-scale downstream equipment should be installed (Annex - 9., page 86).

Points 1, 2, and 4 should be investments from the side of UNDP.

Point 3 would be the task of the Wuxi Enzyme Factory.

C. Attendance of experts, training

1./ The continuation of a training program abroad would be beneficial to the factory. There are some capable young engineers like Mr Huang Xiao Dong, who could help the further improvements with an increased performance after training.

2./ Knowledge of the English language is an inevitable necessity in this field but there are only 3-4 persons in the factory who speak English. The management of the Wuxi Enzyme factory should promote young capable graduates to participate in English language courses.

3./ The international technical - scientific literature should be followed and surveyed continuously. The computerized weekly literature alerting service is highly recommended. If there is no other possibility, even the ASCA Institute for Scientific Information can be chosen (MTA Könyvtár Informatikai Igazgatóság, Budapest V., Akadémia u. 2. Pf 7. 1361 Hungary).

4./ After six months, the latest stage of strain improvement should be evaluated by a UNIDO expert, and further tasks should be elaborated.

5./ The role of protease (pages 8-9) in the case of BAA recovery should be discovered in laboratory-scale experiments, directed by a UNIDO expert.

6./ The fermentation media, independent from any further investments, (Annex - 16., page 108) should be optimised in shaking flask experiments concerning trace element, potassium and phosphate supply. These experiments require the direction of an expert from UNIDO.

7./ In the event that the investments suggested are realized, the fermentation and recovery technology should be improved on by the assistance of a UNIDO expert. The main goal in the case of fermentation technology is to elaborate high performance feed batch processes.

Points 2 and 3 are tasks for the Wuxi Enzyme Factory.

Point 1 should be organized and financed by UNDP/UNIDO.

Points 4, 5 and 6 (+7) require at least 4 weeks attendance of a UNIDO expert in 1993.

V. ANNEXES

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION

November 1992

JOB DESCRIPTION

DP/CPR/88/001/11-57

Post title Microbiologist, consultant in genetic engineering for improvement of strains

Duration 2 m/m

Date required 3rd Quarter 1992

Duty station Wuxi, Jiangsu Province, People's Republic of China

Purpose of project Improvement of technology for the production and development of enzymes at the Wuxi Enzyme Factory.

Duties

1) Screening of the existing production strains at the Wuxi Enzyme Factory;
2) Introduction of a proper seed lot system for maintenance of the strains' stability;
3) Introduction of freeze-drying for production strains;
4) Improvement of at least 2 selected production strains by genetic methods and optimisation of the recipe of raw material media composition and ways of preparation;
5) Demonstration of different techniques for improvement of the production characteristics of bacterial and fungal strains;
6) Participation in organizing and conducting a workshop/seminar on industrial enzyme production;
7) Preparation of a technical report with recommendations, as well as material specifically prepared for the above workshop/seminar.

..../..

Qualifications Microbiologist, microbial physiologist, microbial geneticist, genetic engineer with Ph.D. experienced in improvement of production strain characteristics, either by conventional or genetic engineering techniques.

Language(s) English

Background information

Based on the overall importance of the utilization of enzymes in a wide range of industries, industrial enzymes (in addition to antibiotics and aminoacids) are the most important products of the new biotechnological sector.

In Wuxi, Jiangsu Province, the wine factory and brewery were established in 1956. Enzyme production was introduced in 1965, and in the same year the factory was renamed; since then it is called the Wuxi Enzyme Factory.

On a site of 50,000 m^2, the buildings cover 31,000 m^2. The total fermentation capacity is about 420 m^3. There are 2 fermenters of 60 m^3, 12 fermenters of 20 m^3 and several smaller ones of 5 and 0.7 m^3.

The factory is staffed by 785 employees, among them 95 professional staff including engineers, technologists, economists, etc. The graduated technical staff with university degrees (75 persons) consist of chemical and mechanical engineers (3 senior engineers, 36 engineers, 3 assistant engineers), biochemists, biotechnologists, chemical analysts, microbiologists, etc.

In 1987, the annual output was 10,500 tons of enzymes, which represented about 25% of the total domestic production. The most important enzyme products are as follows:

alpha-amylase, glucoamylase, lipase, immobilized glucose isomerase, penicillin acylase, proteases, beta-amylase, pectinase, etc.

Approximately 80% of the total production output of the factory consists of only three products:

alpha-amylase, glucoamylase and proteases.

The Wuxi Enzyme Factory was the first in 1965 which developed domestic production of alpha-amylase. There are about 200 enzyme factories in the country, among them around 150 are small manufacturers. More than 30 factories of pilot or industrial scale were established with the assistance of the Wuxi Enzyme Factory. The Director of the Wuxi Enzyme Factory is the Chairman of the Association of the China Enzyme Producers. The Association convenes at least once a year to discuss relevant issues of the domestic enzyme industry. These meetings are always held in different cities. In 1989 it was held in Shanghai. The work of the Association is supervised by the Department of Food Industry in the Ministry of Light Industry.

.../..

Background information (continued)

A total of 15 industrial enzymes are produced in the country out of which 11 are produced in the Wuxi Enzyme Factory. Other important manufacturers are: Shanghai New Type Fermentation Factory and Tianjin Li Hua Food Factory. The products of the Wuxi Enzyme Factory have a good reputation. Two of them are evaluated as "good quality product" by the Ministry of Light Industries.

The Wuxi Enzyme Factory is the top manufacturer in the People's Republic of China. However, the average yields of production are far behind the yields obtained at the international level. Most of the technologies used in the factory are outdated, the modern in-process monitoring equipment is lacking. Only alpha-amylase complies to the standards of food grade quality.

The Research and Development Division of the factory is poorly equipped. In particular, modern instrumentation for organochemical analysis is missing. The factory has several joint research and development agreements with leading institutions such as the Microbiological Research Institute of the China Academy of Sciences, Beijing, the Qing-Hua University, Beijing, the Microbiological Research Institute of Shanghai and the Nanjing University, etc.

MODIFIED PROGRAM

/Copy/

Attn. Mr Huo Xing Yun
Director
Commander-in-Chief of the project

Working Schedule

Items:

1. General visit to departments
2. Elaboration of methods for strain improvement
3. Optimisation of freeze drying procedure
4. Improvement of seed lot systems for BAA and GA production
5. Elaboration of method for obtaining Aspergillus niger protoplasts
6. Lecture on strain improvement and genetic engineering
7. Lecture on up-to-date fermentation technology and industrial technology improvement
8. Consultation, and visits at production departments
9. Handover of strains, and information about them
10. Handover of personal computer programs, demonstration of handling

Time schedule:

1. 10. 11. 1992. - 14. 11. 1992.
2. 18. 11. - 31. 12.
3. 18. 11. - 25. 11.
4. 01. 12. - 31. 12.
5. 23. 11. - 30. 11.
6. 20. 11.
7. 25. 11.
8. Intermittently
9. 20. 11.
10. 30. 11.

Details of work:

1. Interview with every department leader, visiting every production workshop and laboratory

2. Optimisation of liquid cultivation circumstances for obtaining high density suspension of exponential (BAA) / germinating (GA) cells
Optimisation of UV treatment method (distance, exposure time) for getting mutants in large numbers
Introduction of safe and high performance method for obtaining NTG mutants
Development of first selection method on agar plates
Development of second selection method in shaking flasks

3. Changing of cell preparation method, modification of freeze protective agent`s sterilisation for longer lifetime and absolute sterility

4. In the case of BAA , elaboration of method for obtaining large number of equal seed tubes, modification of seed system
In the case of GA, improve the seed system towards liquid inoculations into production seed tanks, and to recover modified strains from the best fermentations

5. Producing protoplasts from Aspergillus niger by using snail (Helix pomatia) and/or Novozyme (Trichoderma viridae) enzyme, explanation on possibilities

6. Theoretical approach, development possibilities, practical use of methods

7. Theoretical approach, technical requirements, development possibilities

8. In all fermentation, recovery/downstream workshops, and laboratories (fact finding, consultations)

9. The strains concerned:

Aspergillus niger	GA
Aspergillus niger	Pectinase
Aspergillus niger	Glucose-oxidase
Aspergillus awamori	Pectinase
Aspergillus foetidus	Pectinase

10. Word processing program "WORDSTAR", data processing program "QUATTROPRO", the programs will be installed into the - IBM-compatible personal computer, the proper handling will be demonstrated

DETERMINATION OF GLUCOAMYLASE ENZYME ACTIVITY (MILES)

Reagents:

- 2 mol acetic acid solution (dilute 116 ml of cc. acetic acid to 1000 ml by distilled water)

- 2 mol NaOH solution (dissolve 80 g of NaOH with distilled water to 1000 ml)

- pH=4.2 acetic acid buffer solution (add 50 ml of distilled water to 200 ml of 2 mol acetic acid, and as much 2 mol NaOH solution, as required to reach pH=4.2 -indicated by pH meter- and dilute the solution to 1000 ml by distilled water)

- 1% phenolphtalein solution (dissolve 1 g phenolphtalein in 60 ml ethylalcohol, and adjust the volume to 100 ml by distilled water)

- 4% starch solution - as substrate (add 20 g of soluble starch to 300 ml of boiling water, boil by vigorous shaking for 3 minutes, after cooling to room temperature, add 30 ml of acetic acid buffer to the solution, and fill it up to 500 ml by distilled water)

- Schoorl reagents
  A: 69.3 g of CuSO4*5H2O dissolved in 1000 ml of distilled water

  B: 346 g of Sodium-potassium-tartarate*4H2O dissolved in 350 ml of distilled water, after complete dissolving fill it up to 1000 ml by distilled water

  30% KJ solution (300 g KJ dissolved in 900 ml of distilled water, add 1.5 ml of 2 mol NaOH and fill it up to 1000 ml by distilled water)

  26% H2SO4 solution (add 145 ml of cc. H2SO4 slowly and by stirring and cooling to 700 ml of distilled water, and fill it up by cooling to 1000 ml)

- glucose standard (10 g of glucose dissolved in 100 ml of distilled water, filled up to 1000 ml)

- 0.1 N Na2S2O3 (1 ampoulle of titrisol diluted to 1000 ml by distilled water)

- 1% starch indicator (5 g of soluble starch and 150 g NaCl dissolved in 500 ml of distilled water)

Proper dilution of enzyme samples: to 0.15-0.6 GAU/ml

Analytical procedure:

A./ Enzyme reaction
1. 50-50 ml of starch solution must be heated up to 60 C centigrade in 100 ml volumetric flasks, by maintaining them in 60 C centigrade water bath for 15 minutes.
2. Add 1 ml of enzyme solution to the starch substrate, shake well and in the meantime start the stopwatch. As blind test, use 1 ml distilled water instead of enzyme solution. Put back both volumetric flask into the water bath.
3. After exactly 60 minutes incubation, take out the flasks from water bath, and by using two drops of phenolphtalein indicator add 2 mol NaOH solution to the mixture until pink colour appears.
4. Cool the flasks to room temperature, and fill them up to 100 ml by distilled water.
5. Reducing sugar content must be measured by Schoorl method.

B./ Schoorl method
1. 10-10 ml of sample and blind must be poured into 300 ml Erlenmeyer flasks, and add the reagents in the following order:
   15 ml of distilled water
   10 ml of solution A
   10 ml of solution B
2. After mixing, the reaction mixtures must be boiled for two minutes. After 2 minutes of boiling, cool the flasks to room temperature.
3. Add 10 ml of KJ solution, and 10 ml of H_2SO_4 solution.
4. Titration with 0.1 n thiosulphate solution until yellow colour appears. Then add 2 ml of starch indicator and continue titration until the dark blue colour turns a light bluish-white.
5. For controlling the procedure, 5 ml of glucose standard solution must be used instead of enzyme reaction mixture, according to B/1.-4. points. 15.47 ml of 0.1 n thiosulphate solution corresponds to the 5 ml glucose standard solution.

One glucoamylase unit (GAU) is able to generate 1 g glucose from starch within one hour in the above conditions.

DETERMINATION OF BACTERIAL ALPHA-AMYLASE ENZYME ACTIVITY

Principle:

Standard starch solution is to be hydrolised by alpha-amylase enzyme sample. The reaction time is measured until a certain degree of degradation. The endpoint is checked by using iodine reaction, the color is compared to a standard.

Reagents:

- acetate buffer solution : 164 g Na-acetate (dehydrated) is dissolved in 800 ml of distilled water. The pH is adjusted to 6.0 by cc. acetic acid, then the mixture is filled up to 1000 ml.

- substrate solution: 8 g soluble starch is suspended in 25 ml distilled water, and the mixture is transferred to 270 ml boiling water. After two minutes of boiling, the mixture is cooled to room temperature, and 25 ml of acetate buffer is added. After adding two-three drops of toluene, -for preventing contaminations - fill it up to 400 ml with distilled water. By storing it at 4-5 degrees centigrade, the lifetime of the solution is one week.

- basic iodine solution: 11 g crystal iodine and 22 g KJ are dissolved in 500 ml volumetric flask. After adjusting the volume we prepare two solutions:

A: 8 g KJ is dissolved in 15 ml of basic iodine solution. Filled up to 200 ml with distilled water.

B: 20 g KJ is dissolved in some distilled water, and 2 ml of basic iodine solution is added. Filled up with distilled water to 500 ml.

- dextrin solution: 0.6 g dextrin is dissolved in 1000 ml distilled water. After adding 2-3 drops of toluene, lifetime at 4 degrees centigrades is one month.

- enzyme solution: the liquid or solid product is diluted by distilled water to adjust activity to less than 5 U/ml.

- standard color solution: 3 ml of solution A and 0.6 ml of dextrin solution is mixed. The red-brown standard color is stable for 3 hours.

Analytical procedure:

The substrate and enzyme solutions must be thermostated at 60 degrees centigrade. 20 ml of substrate solution and 10 ml of enzyme solution are mixed in a 50 ml Erlenmeyer flask. The mixture is thermostated at 60 degrees centigrade water bath. After 3 minutes we take out 0.6 ml of sample and transfer it to a test tube which contains 3 ml of solution B. The sampling and testing with solution B is repeated every 0.5 minutes, until the color obtained is equal to the standard color solution. The time required must be measured by stopwatch. By getting closer to the endpoint of the reaction the sampling frequency should be higher.

Calculation:

Activity [U/ml or U/g] = 60 * k / E * t

t= reaction time in minutes
k= the starch amount in 20 ml solution (0.4 g)
E= the volume or weight of the solid enzyme introduced into reaction in the 10 ml enzyme solution

One unit of alpha-amylase can hydrolyse 1 gram starch at 60 degrees centigrade during one hour.

LECTURE ON ADVANCED FERMENTATION TECHNOLOGY, EQUIPMENT AND INDUSTRIAL OPTIMISATION

Structure of the lecture:
1./ Basic software
2./ Basic hardware
3./ Secondary software
4./ Secondary hardware
5./ Technology optimisation- industrial approach

1./ Basic software

If we compare the main principles of batch and continuous fermentation technology (page 39), we can see, that the batch fermentation provides continuously changing conditions for the microbe, when the mainly research-used continuous fermentation technology can provide longterm steady conditions for the microbes.

During batch fermentation, the substrate level is decreasing by a certain profile, which seems to be the inverse of the biomass (cell concentration) curve.

According to the product= f(time) curve, we can find in every case an inflection point, where the production rate (dP/dt) has a maximum value. Concerning the microbes' physiological stage, the conditions of production are optimal only at this point.
[That is why we can optimise a batch fermentation only according to the final result. In the case of even a very well optimised batch fermentation, the conditions - substrate levels - are optimal only for a short time.]

There is an other widely known phenomena, which can be modelled by using different carbon source levels in batch fermentations. We can see on the graph (page 39), that the production rate is the highest in the case of the lowest carbon source level, but in this case, the final product concentration is the smallest one. In the case of a higher carbon source level, the production rate is slower, but the final product concentration is much higher.

This phenomena originated from the cAMP level in the cell, because in case of a high level sugar supply, the cAMP level decreases hindering the overall enzyme synthesis of the cell, regulating the RNA-polymerase operation (page 60-61). This phenomena is called catabolite- repression.

In the case of continuous fermentation technology, we can maintain optimal conditions for the high rate production. It means, that we are feeding continuously the fermentation process

by a sterile, substrate containing liquid, and we are discharging fermentation broth with the same flow rate. This procedure results in steady substrate concentration, cell density and product concentration level. This process is very good for discovering the microbe's requests for optimal, high rate production, but not so easy to maintain it for a long time. The main problems are the increased danger of contamination, and the media composition should be punctually adjusted to the requirements of the biomass, so it is solved by using fine chemical compounds.

To solve the contradiction between dP/dt and P as requirements, the continuous fermentation technology would be a good solution, but besides some exceptions (yeast, single cell protein) the sterility and other requirements (media composition cannot be so accurate being based on raw materials) are hindering the industrial development and use of this type of technology.

By turning back to the batch fermentation, it seems to be impossible to solve the question by batch fermentation, so as to meet the two requirements in the same time : high production rate (dP/dt) , and high final product concentration (P).

The proper industrial solution of the task is the feed-batch technology, which can unify the advantages of the batch and continuous technology in one process. The main principle of the procedure is to use the same fermentation volume which is used in cases of batch fermentations. There is continuous feeding during the process, in order to maintain an optimal low carbon source level in the fermentation broth, but there is no continuous discharge, therefore after reaching the final operation volume of the fermenter tank, the feed addition should be finished, and after the substrate levels are decreased, the fermentation process should be stopped (page 40).

This method is widely used at leading fermentation companies. The feed batch technology gives the possibility not only for maintaining the highest production rate for a long time, but enables the use of much more carbon source in the overall process. Therefore, the final product concentration is much higher than in the case of batch fermentation.

The phenomena of catabolite repression is general among microbes, so the feed batch fermentation technology is useful in the case of primary and secondary (!) metabolite production as well.(Detailed list and explanation was given on several technologies, strains and products as alcohol, lysine, glucoamylase, bacitracin, penicillin, oxytetracyclin, glutamic acid) The catabolite repression's importance is increased in the case of catabolic enzymes as glucoamylase and alpha-amylase.

The usual method of feed batch fermentation is made by inoculation of a so-called start medium, which has a low volume and sugar (carbon source) content. The task of this first part of medium is to enable quick biomass growth. After the carbon source content has decreased to the level which is optimal according to the production rate, the feed medium addition starts. The feed addition's flow rate should be adjusted to the actual requirement of the microbes in order to maintain the optimal carbon source level. The feed addition proceeds until the final working volume of the fermenter is reached.

2./ Basic hardware

During the fermentation process we have to maintain optimal conditions for the microbe. For maintaining optimal conditions, the minimal criteria is to maintain the optimal pH. This can be solved easily by automatic pH control. (In some cases the pH can be controlled manually as well, depending on the properties of strain and medium.)

The foaming should be controlled by intermittent addition of a defoamer (page 42). If there is no possibility for foam control, all the defoamer required for the process must be added before the sterilisation, and this destroys the oxygen transfer, the results are poor. In the case of further foaming, operators can reduce the air supply only - with the same result - or the broth is discharged (spoiled) through the exhaust pipeline. The measured and controlled air supply (air flow rate) is required as well.

For realisation of feed-batch technology, we need an electromagnetic type flow meter, a programmable logic controller and controlled valves. This arrangement is good for performing certain feed rate profiles which should be designed (and improved) by the technologist. The feed rate is following the programmed profile, independently from the fermentation process.

The other more sophisticated variant is the regulated feed addition. Usually the exhaust air's oxygen or carbon dioxyde content can be the regulation parameter for the feed rate control. The exhaust air's composition gives a quick and reliable answer on the carbon source level in the fermentation broth.

Generally, the first variant is more simple and less expensive, and can be reliable in the case of standard fermentation circumstances.

3./ Secondary software

Secondary software means operations and working discipline. Only the main items are enumerated.

One of the tasks of main importance is to prevent contamination. The proper washing of fermentation and seed tanks is an absolute requirement. The tanks should be filled with water up to the manhole, and the vessel must be agitated and aerated in order to dissolve all solid particles from the inside wall and exhaust pipeline of the equipment. The washing procedure should be performed for two hours. The washing should be made after every batch.

The tank sterilisation is not less important. The empty vessel should be sterilised for three hours at 120 degrees centigrade. In the case of good insulation, we have to use a relatively low steam flow, so the energy consumption is not so high.

Explanation was given on the proper technology of medium sterilisation (page 40). The normal period of heat (120-122 degrees centigrade) maintenance is 60 minutes in the case of starch -based media. The heat degradation is not significant in this case. Only in the case of sugar-based media should the period be shorter. In the case of amylolytic enzymes this question is very important. The longer period is necessary because of the higher viscosity and relatively wrong heat transfer properties of the fermentation media. The "heating -up" and "cooling" periods are important as well. The agitator should work during all the sterilisation processes including these periods. The so-called "cold points" can be found by using surface- thermometers.

During fermentation process, all steam seals should be under steam pressure and slight steam outflow is required.

The seed transfer line must be kept under steam pressure all the time, except the periods of seed transfer.

4./ Secondary hardware

Secondary hardware means all equipment, piping and arrangements which are necessary for sterility.

Filtration is a basic item in this aspect. Two types of safe and reliable filter systems can be suggested, membrane filters or glass fiber filters.

The first type is a so-called absolute filter, because the pore size of it is less than the size of the smallest spore. Usually the pore size is 0.2 micrometer. These types of filters are very sensitive, concerning the solid particles and humidity brought by the process air. Therefore the installment of filters requires expensive investments of air dehumidifier, and there must be prefilters before each individual filter. (Main dealers: PALL Italy, MILLIPORE USA etc.) The deterioration of these types of

filters occurs dramatically, due to the thin layer of membrane material. In this case (after some waste batches) the filter cartridge can be replaced very easily.

The second type is a so-called deep filter. In this case, the pore size of it is much bigger than a microbe. Therefore the filtration is performed by adsorbing particles in the depth of the filter, and not on the surface, as in the case of membrane filters. The type suggested is stuffed with prefabricated glass wool sheets, and the cover of the filter vessel is pressing down the layers. In cases of good design (the size of filter must correspond to the air flow rate required) these types of filters are as reliable as membrane types. The glass fiber filters do not need any air pretreatment, and prefiltration. In case of deterioration (after some years) the contamination appears gradually, and concerning the operation principle of these types of filters, the contamination appears at the end of fermentations for the first time. (Dealer: Yamato Riken Japan) In Agroferm's lysine factory this type of filters are used with very good results. During one year (380 batches) only one waste fermentation was caused by contamination. Considering the size of fermentation tanks (240 m3) and the neutral pH of fermentation and sugar containing (sensitive) media, these statistics are really very good.

The second item of main importance is the air disributor inside the fermentation tank. This part of the equipment is considered usually as being responsible for disperging the air. This is a false idea. On this basis designers are drawing several types of horizontal pipelines with holes (perforation) on them. In the case of industrial size fermenters this arrangement is a source of systematic contamination. The horizontal line is stuffed - clogged after some fermentation cycles by the sticky remainings of fermentation broth. In the case of starch-based fermentation media this danger is higher. The proper arrangement is one or more branch of pipeline which leads under the agitator. These pipelines must not be horizontal, the decline suggested is 30 degrees. The dispersion of air is the task of the agitator impeller, so there are not any difficulties about the performance of such an arrangement. The declined position of the distributor pipeline(s) enables a good self cleaning during sterilisation and washing procedures.

About piping: All steam pipelines must lead downwards for the proper drainage, seed lines must be above the tanks for being able to sterilize and handling them successfully. All horizontal lines must be built in a slight declined (2 degrees) position for being able to get rid of condensate, and for easier cleaning. Steam seal's condensates should be collected in illuminated, transparent (window) collectors for preventing high humidity inside the building (page 45).

For inoculation, the most reliable and acceptable device is the "kasten" (sketch, explanation provided on page 46). This arrangement enables safe inoculation with a higher amount of liquid as well.

In case of sterile fermentations the agitator`s shaft gland packing should be steam-heated, this part being a frequented point of contamination too.

At least, the first valves (the tank`s neighbour valves) should be membrane type ones. All valves of the seed transfer line should be membrane type ones too. The seed line can be equipped with teflon type three-way-valves as well, which is a safe and convenient solution.

In the case of high fermentation tank volumes (according to an estimation of above 100 m3, this is a question of economy because of the high expenses of the equipment) an automatic continuous steriliser system is appreciated.

5./ Optimisation of technology

The usual way is to optimise in a pilot scale fermenter, and afterwards: scaling up - or realisation in industrial fermenter. Generally, all the parameters cannot be reproduced exactly in industrial fermenter tanks. In industrial fermenters due to their significant height, the influence of hydrostatic pressure (up to 1 Bar) is considerable on the process. The solubility of gases is depending on the partial pressure (Henry`s law, page 48). The other phenomena of great importance is the homogenity. In huge vessels such as 100-200 m3 , such a level of homogenity like in 1-2 m3 cannot be reached.

According to the usual approach, optimisation is dangerous in an industrial scale because of jeopardizing the smooth production. Nevertheless, in cases of careful changes (max. 15 %) of parameters or component concentrations, the experiments can be carried out safely.

Several mathematical models and methods are available. Generally, these methods are useful in the initial part of research and not in the case of technology improvement on an industrial scale.
The main principle for industrial optimisation is to look for limiting factors. Liebig`s minimum rule is valid in the case of microbes as well. However, these factors are not independent in most cases. By discovering limiting factor/s, we can modify the ratio between factors, and we can reach a "relative optimum". In this case, the ratios between factors are optimal.
The main contradiction is, that we cannot optimise further. Any step made, is a deviation from the optimum obtained.

The solution of the contradiction is to modify all factors or some of them together. (E.g. enrichment of carbon source, nitrogen source and O2 supply together)

The most simple but highly successful method is to "withdraw water" from the fermentation media. This means concentrating all components. This way we can get a real optimum.

How to step towards feed batch? We have to look for the dP/dt maximum, and we have to identify the substrate levels in this point. The carbon source level obtained should be maintained during feed-batch process. For the first approach, we have to use the original overall substrate amounts which were optimal in the case of batch fermentation. We have to keep the optimal ratios between the components. The distribution to start and feed volumes must be made carefully, technical tasks as heating, cooling, intermediate bearing's lubrication must be considered. The ratio between start and feed volumes depends on the strain properties as well.
The feed period's length (with maximal dP/dt) and the feed medium's volume are in relation to the amount of harvest (page 50). That is why the start medium's volume and carbon source content should be as low as possible, in this way we can get a longer feed time, and a bigger free volume for feed medium.

Usually feed batch technology gives new possibilities for optimisation. The task is to look for limiting factors again, and the main task is to increase the production rate. Generally , by using feed batch technology, the overall carbon and nitrogen source supply can be higher than in the case of batch culture, due to the long term high metabolic activity and production rate.

In the case of Agroferm Ltd., the further optimisation of the Japanese feed batch lysine fermentation technology resulted in a 40 % increase of harvest in 1992, after two years of industrial optimisation.

ADVANCED FERMENTATION TECHNOLOGY / EQUIPMENT AND INDUSTRIAL OPTIMISATION

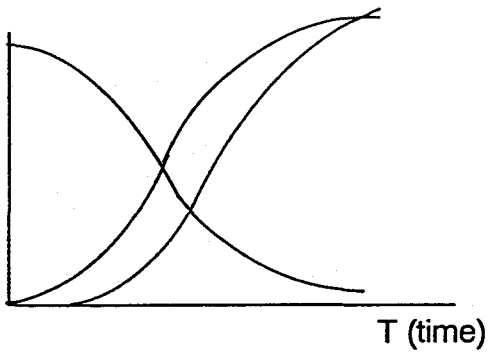
(Sketch of lecture)

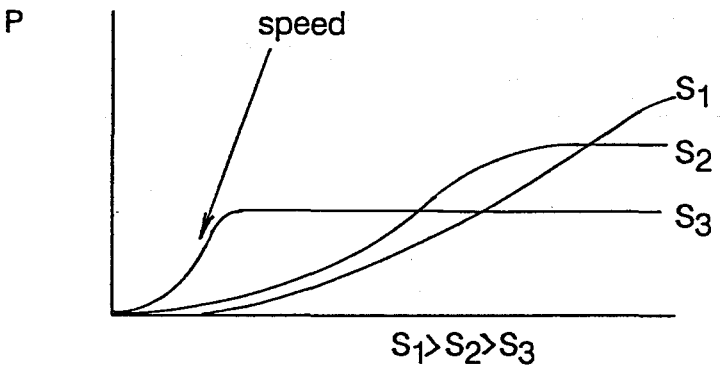
1./ BASIC SOFTWARE
2./ BASIC HARDWARE
3./ SECONDARY SOFTWARE
4./ SECONDARY HARDWARE
5./ TECHNOLOGY OPTIMIZATION

1./ Basic software

Properties of BATCH fermentation

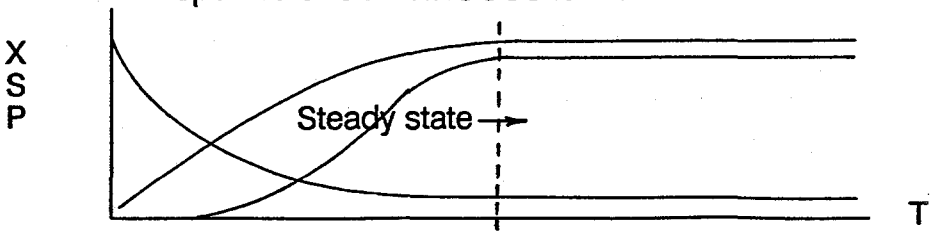
X (cell density)
S (substrate)
P (product)





You can have an optimum batch wise -- but you have physiological optimum for 10 minutes only!!!!!!!!!

(Phys. opt.: all conditions are optimal)
Properties of CONTINUOUS fermentation



Steady state can be optimal

STEADY STATE:
1. CONDITIONS are steady
2. PHYSIOLOGICAL STATE is steady

(1.: composition- fine chemicals- exactly follows the requirements of the microbe)

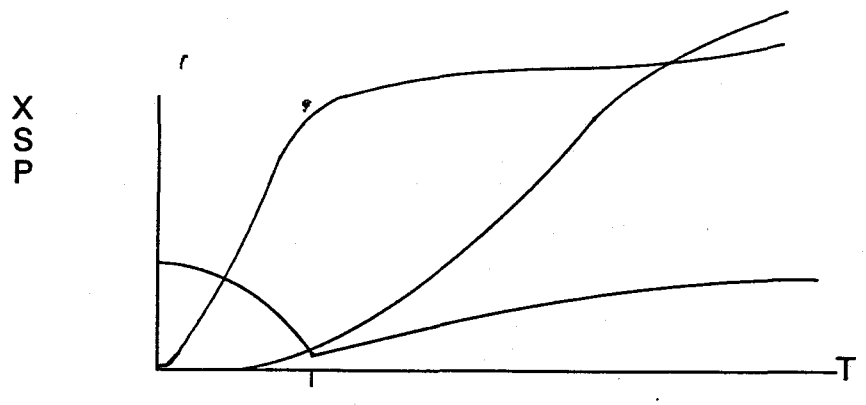
We are sorry: cont. ferm. --- only limited area e.g. yeast, SCP

WHY? ----------CONTAMINATION
RAW MATERIALS (because composition cannot follow exactly the requirements increasing, decreasing concentrations of components)

Main task: TO MAINTAIN OPTIMAL CONDITIONS as long as it is possible - how can we do it?!

INDUSTRIAL SOLUTION OF THE ABOVE : FEED BATCH FERMENTATION TECHNOLOGY

PROPERTIES OF FEED BATCH FERMENTATION



Feed in -time

ADVANTAGES:

1./ ALL CONDITIONS can be kept OPTIMAL
for LONGER PERIOD

2./ LOW CARBON SOURCE LEVEL - high speed enzyme synthesis
Reason: GENETIC REGULATION of enzyme synthesis , origin:
Szentgyorgyi-Krebs or CITRIC ACID CYCLE : AMP --- ATP reaction-- detailed explanation

3./ OVERALL CARBON SOURCE AMOUNT can be much HIGHER -
HIGH cell density
HIGH product concentration

Can you imagine 30% starch containing (batch) medium?!

MY EXPERIENCE:

- alcohol production by yeast, and Zymomonas mobilis- industrial
- GA production by A. niger laboratory
- lysine production by Corynebacterium glutamicum industrial
- bacitracin production by Bac. licheniformis laboratory

- penicillin production by Penicillium chrysogenum industrial
- oxytetracycline production by Streptomyces rimosus industrial
- dihydroxyacetone production by Acetobacter suboxydans laboratory, pilot

As summarizing: secondary (!) and primary metabolites, enzymes, transformations, Streptomycetes, Fungi, Bacteria

REASON: ALL PROCESSES ARE BASED ON ENZYMATIC ACTIVITIES, this regulation is G E N E R A L !

2./ Basic hardware

To keep steady conditions!!!

1. DEFOAMER addition: should be controlled - manually, automatically

If not: case a./ Broth is foaming out

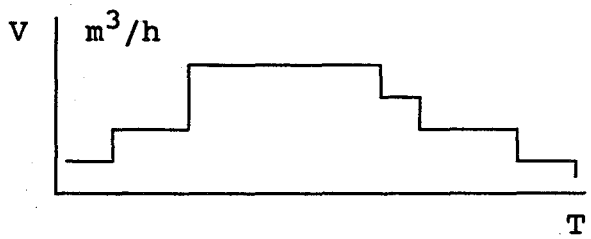
case b./ Oxygen transfer rate (OTR) is insufficient!

2. PH CONTROL - automatic, (sensor controler, controlled valve)

   NaOH, KOH, H_2SO_4, Acetic acid, best: NH_3 gas

3. FEED: case a./ directed, programable logic controller, electromagnetic flow-meter, controlled valve

Advantage: time-profile can be programmed:



case b./controlled: e.g.
exhaust gas O_2/CO_2 meter, controller, controlled valve,

Advantage : self regulation

4. Of course: T°C must be controlled, and AIR FLOW must be controlled too

FEED ADDITION - THERE ARE SPECIAL PHYSIOLOGICAL SIGNS OF CARBON SOURCE ACCUMULATION - TO AVOID

3./ Secondary software

responsible for 10 % of contaminations

OPERATIONS, DISCIPLINE

a./ empty sterilisations - after every batch 120 ^{o}C , 3 hours

b./ washing - by filling up with water, agitation aeration 2 hours

c./ medium sterilisation (except sugar) for 60 minutes 120-122 ^{o}C
charging (loading) temperature!
cold points - surface- touching thermometers
agitation during, heat up, maintaining and cooling phases

d./ during operations, fermentation:
STEAM SEALS

e./ SEED LINE : under steam pressure all the time (except seed transfer)

4./ Secondary hardware

responsible for 90% of contaminations

a./ AIR FILTRATION of great importance!

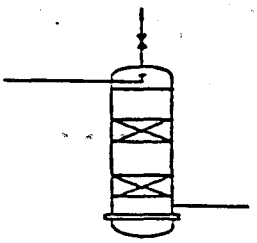
PRINCIPLES OF FILTRATION: Absolute

Deep

Absolute filters: membrane filters (microfiltration)
dealer: PALL, Millipore etc.

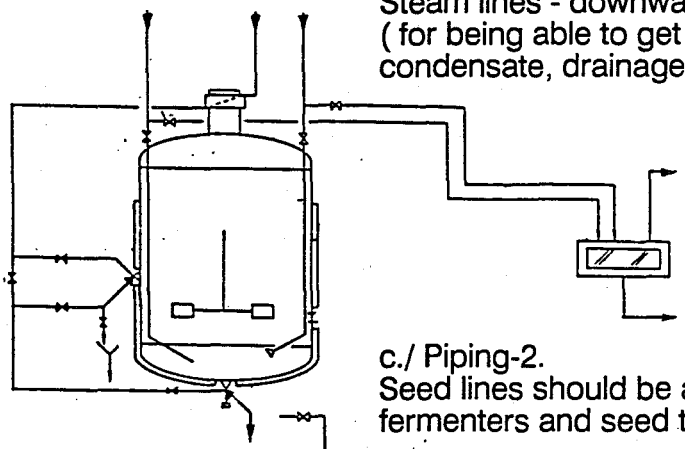
Deep filters : glass fiber (wool or fabric) filters
dealer: Yamato Riken (Japan)

My opinion: DEEP type is more reliable
LIFETIME is longer!
LONGTERM EXPIRY:
glass fiber

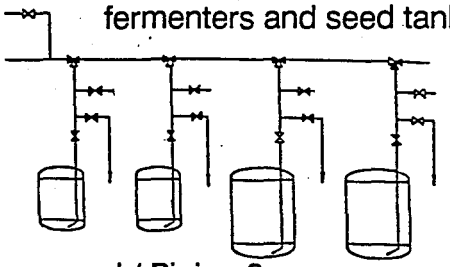


MEMBRANE type LIFETIME is shorter
EXPIRY:SUDDENLY!

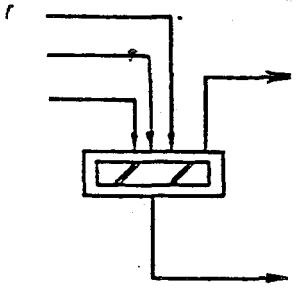
b./ Piping-1.
Steam lines - downwards
(for being able to get rid of
condensate, drainage)



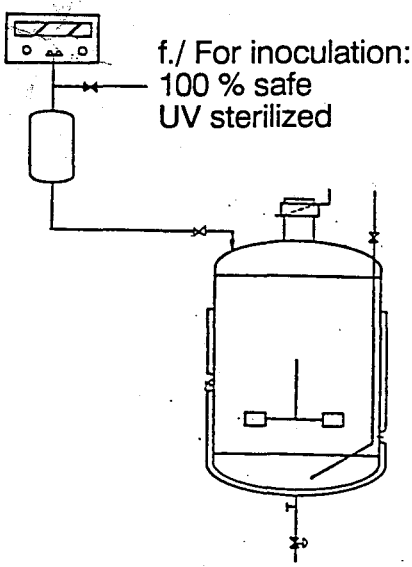
c./ Piping-2.
Seed lines should be above the
fermenters and seed tanks!



d./ Piping-3.
„Horizontal" lines : 1-2
degree slope!!

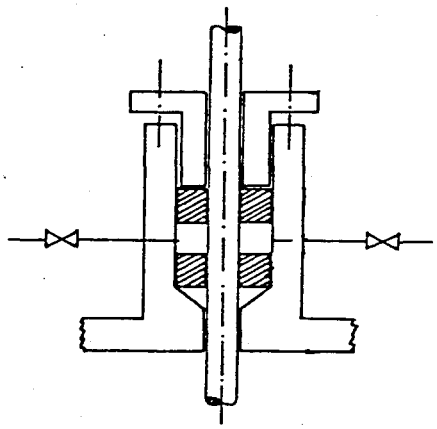


e./ Piping-4.
Steam seals: Condensate to
transparent drainage collectors
(illuminated) prevents high
moisture content in the building



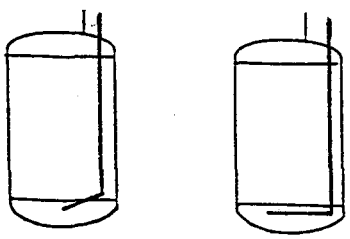
f./ For inoculation: KASTEN
100 % safe
UV sterilized

g./ GLAND PACKING - steam heated



h./ AIR SPARGER in case of starch

containing medium: no horizontal
part-danger of clogging



AIR DISPERGATION IS THE TASK OF THE AGITATOR!

i./ VALVES

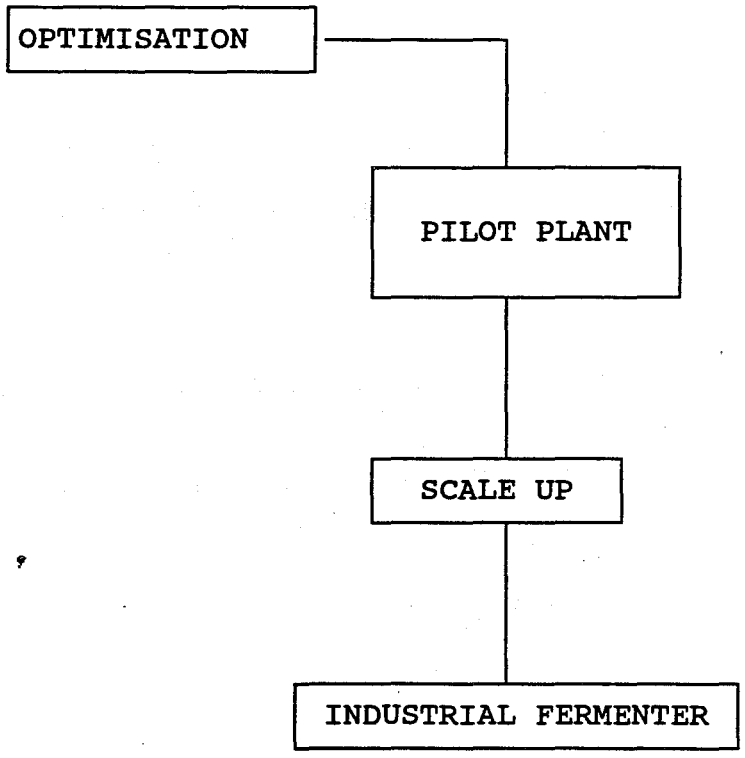
1st valves (at least) -
MEMBRANE TYPE

seed line: all should be
membrane type, three ways valves!

PERMANENTLY CHECK LEAKY VALVES

g./ in case of high volumes
(overall capacity more than
200 m^3) CONTINUOUS STERILISER
WITH HEAT RECOVERY SYSTEM

5./ Technology optimisation



AFTER ALL PARAMETERS ARE REPRODUCED, THE OPTIMUM MUST BE REACHED AGAIN!!

What is the reason?

PILOT H = 1.0 m
D = 0.5 m

INDUSTRIAL H = 10 m
D = 3.5 m

Hydrostatic pressure
reaches 1.0 BAR

1./ DISSOLVED GASES

Conclusion: solubility of gases -
Henry's law

$$C_i = K * P_i$$

C_{CO2} and C_{O2} are different

2./ HOMOGENITY is not the same

in our 240 m^3 fermenters
temperature difference = 4 ^oC

OPTIMISATION IN PRODUCTION FERMENTER = DANGEROUS?

JEOPARDIZING THE SMOOTH PRODUCTION?

NO!!!!!

+/- 15 % CHANGES !

OPTIMISATION METHODS: mathematical optimisation
Useful at university, research institute, Ph.D. thesis , scientific papers etc.

MAIN PRINCIPLES:

1./ Liebig's Minimum Rule

LIMITING FACTOR(S)

Task: to discover them!

Factors are not independent:
$P = f(T, P, X_i, O_2, CO_2, n, \text{etc.})$

2./ RELATIVE OPTIMUM

ratio/proportion of factors are optimal!

HOW TO GO FURTHER?!!!

IF ALL FACTORS ARE OPTIMAL YOU CANNOT GO ANY STEP FURTHER

ANY STEP YOU MAKE WILL BE A DEVIANCY FROM OPTIMUM!

3./ FACTORS to increase/ decrease

TOGETHER (ALL FACTORS, OR SOME)
keeping the optimal ratios

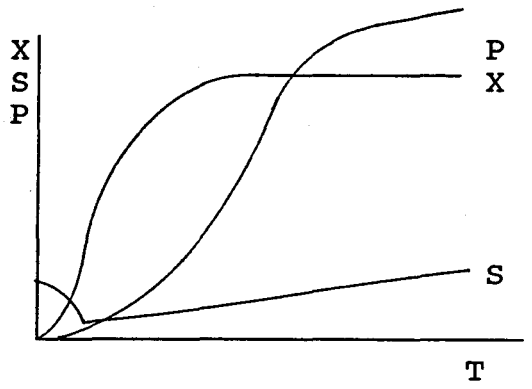
THIS WAY YOU CAN GET REAL OPTIMUM!

4./ After reaching BATCH OPTIMUM step towards FEED BATCH

BATCH: look for $(dP/dt)_{max} = d(dP/dt)/dt = 0$

- to add THE SAME TOTAL SUBSTRATE first!
- KEEPING RATIOS - you can INCREASE/DECREASE

Construction:



Ratio: START/FEED mediums
volume,
C- source
time

Time:
Start: shorter
Feed: longer (more substrate, longer time at max. production rate)

TECHNOLOGY MAINTENANCE, DEVELOPMENT PROCEEDS FURTHER-

AFTER CONSTRUCTION OF FEED BATCH- LOOKING FOR LIMITING

FACTORS AGAIN

LECTURE ON STRAIN IMPROVEMENT, GENETIC ENGINEERING

/Abstract/

A detailed review was given on genetics in order to enable a better understanding of the main topics. (Primary, secondary and the third level of DNA`s organisation, a brief explanation was given on transcription and protein synthesis, pages 55-59).

The first item of main importance concerning industrial enzyme production is the regulation of enzyme synthesis on gene level. It had been mentioned in the first lecture that certain catabolites are repressing the synthesis of catabolic enzymes by causing lack of cAMP in the cell. This phenomena is the main theoretical explanation of the feed batch technology`s higher performance (pages 60-61).

A detailed review was given on the several kinds and methods of genetic engineering techniques, and theoretical basis of them (Enumeration of principal recombination possibilities, explanation on the importance of reverse transcriptases, page 59).

An explanation was given on our limited possibilities in this field, hence genetic engineering`s main target to introduce foreign (usually human) DNA into the host cell, recombine and express it. Our task is to increase the copy number and activity of our enzymes, this target can be reached by mutagen techniques and selection, on a much easier way as all fermentation firms are dealing with strain improvement in their laboratories.

Aspergillus niger`s genom as being eukaryote is very big and sophisticated, the genetic mapping is unsolved till now. So there is not even a theoretical possibility to deal with it by genetic engineering methods.

In the case of Bacillus subtilis, the mapping is partially solved, there are good vectors as well.

Explanation on thermostable alcaline protease (95 degrees centigrade) as a result of "protein engineering" (page 64).

In the case of BAA, such a goal can be reached principally, the main question is the huge financial background for the improvement. The recent level of thermostability (60-70 degrees

centigrade) seems to be good even for the most up-to-date hydrolysis processes as well (Explanation on HUNGRANA's maise based izosugar producing technology at Szabadegyhaza, Hungary, page 64).

A short review was given on cell level genetic engineering possibilities, such as protoplast fusion and using the parasexual cycles of Ascomycetes (page 67).

An explanation and review was given on the types and effects of mutation, mutagens and discovered types of repairement mechanisms (pages 69-72).

As a second item of main importance, a detailed explanation was given on the difference between spontaneous and induced mutagenesis, proper selection techniques, importance of optimisation of induced mutagenesis.

Generally, the frequency (probability) of natural mutations among microbes is 10-6 - 10-7. (There are certain deviations depending on the genus) The estimated (!) probability of a better yielding mutant among mutants is 10-5. The multiplied probabilities are forming a very low frequency: 10-11 - 10-12. It means, that by "natural selection" (without induced mutation techniques) the practical probability of success is =0.

In the case of a well optimised mutation technique, the survivant cells are nearly all mutants. The probability obtained is much higher = 10-5. The crucial question is the proper sreening or selection method. The selection steps should be standardized tools for increasing the probability.

By using a good, purposeful selection method, the probability can be increased to 10-3 - 10-1, or even 1 (!). (Probability can be 1.0 in cases of selecting antibiotics or other antimetabolite resistant mutant strains, on antimetabolites containing solid mediums. In this case, the growing and appearing colonies are all resistants, because all the other cells were killed by the antimetabolite.)

The first selection level should be the solid medium on Petri dishes. The second level is the shaken flask fermentation, the third level is the jar (laboratory scale) or pilot fermentation.

Examples were given on Petri dish selection techniques (antibiotics, aminoacids, enzymes, auxotrophy, resistancies, page 74).

So as to delete the high number of auxotrophs (which are not

useful in our case), the organic N source should be eliminated from the selection medium in the case of Aspergillus niger, and the medium should not contain glucose, lactose and glutamic acid, because these compounds are causing catabolite repression. The natural starch in selection medium is highly appreciated, because the visible liquifaction zone diameters give a good possibility for selection. (As being in correlation with the amount of amylolytic enzymes produced)

In the case of Bacillus subtilis, for reducing the number of auxotrophs among the colonies, we should make an organic-N-poor medium, based on a low concentration of pepton only. (This microbe cannot grow well without an organic nitrogen source supply.)

The number of tested colonies must be high: 4-500 from one mutation cycle, and 30-40 colonies must be picked up by selecting them by the turbidity clearance zones on solid media. The colonies selected must be inoculated at least to two test slant agar test tubes.

The flask shaking is an important step as well. In cases of poor conditions, the improved new strain cannot show us it's high capacity. Maximal attention should be paid to aeration (rotation speed, shaken volume, shape of flasks), buffer, and medium composition. These conditions should be optimised and standardised.

The best way is to use our parent strain at every shaking selection as an "internal standard". It means, that the parent strain should be shaken at the same time and in the same medium and under the same conditions, to obtain a good basis for comparison of the new mutants.

All the processes are based on high numbers and probabilities, so the higher the number of colonies tested, the sooner a good result can be reached.

An explanation was given on special selection techniques in the case of genetic engineered strains (page 77-78).

A principal (uncontrolled) possibility was mentioned as an advanced, high performance selection tool in the case of densely covered Petri dishes. The refined (HPLC) glucoamylase isoenzymes could be used in order to get polyclonal antibodies (eg. from a rabbit).

With a well constructed RIA, the presence and location of a better producer strain could be proven by using autoradiography. (This project could be performed only by

cooperation with an institute which is familiar with RIA construction and can deal with radioactive compounds.)

An explanation was given on vegetative modifications. Maintenance and stabilisation of positive modifications can result in a 20-30 % increase of production in the case of fungi.

The proper procedure is to harvest fermentation broth from the most successful fermentations, and after dilution and plating, colonies can be obtained. The colonies should be selected by shaking: the parallely stored slant agar cultures should be used for shaken seed culture. The main point for this type of selection is to prevent ascospore production, because this leads to reversion of the modification.

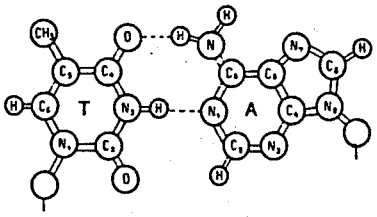
Therefore, the Czapek-Dox medium should be used for this type of work because it delays the sporulation. If successful, the seed production technology should be revised on the basis of these results.

STRAIN IMPROVEMENT, GENETIC ENGINEERING

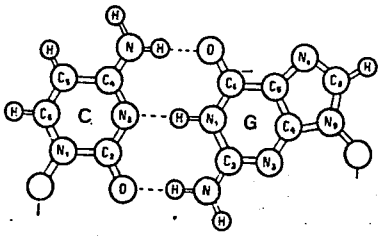
(Sketch of lecture)

1./ Structure of genetic INFO

Nucleotide bases



Timine — Adenine

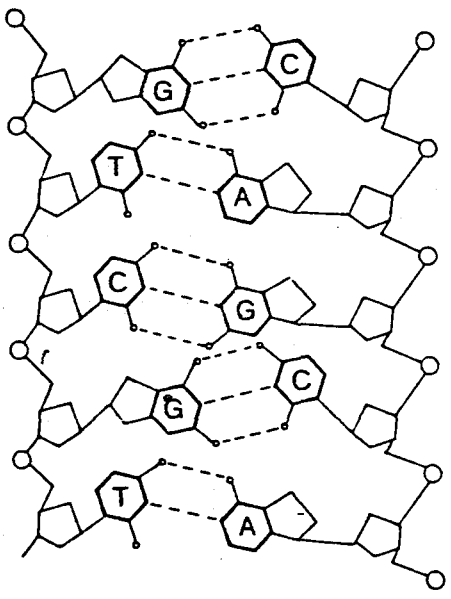


Citosine — Guanine

are able to form
light hydrogen bonds

FIRST LEVEL OF STRUCTURE

H - bonds — Covalent bonds

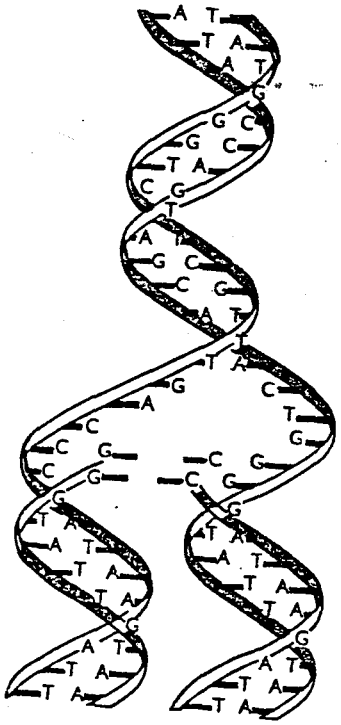


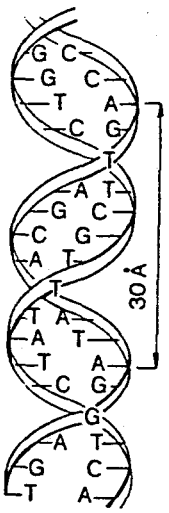
Every amino acid has
his own triplet

(maths: 4*4*4 = 64
variants - but
practically we have
only 21 amino acids)

Smallest information unit: TRIPLET = AMINO ACID

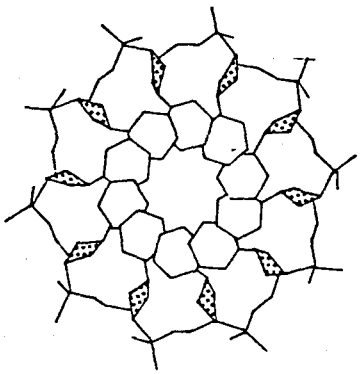
SECOND LEVEL OF STRUCTURE





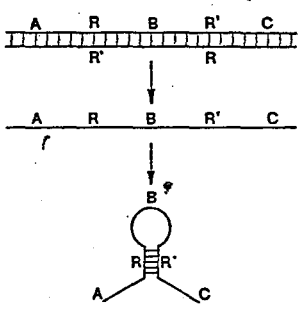
Helix
(double)

Wattson-Crick
Nobel - price



DNA

THIRD LEVEL OF STRUCTURE

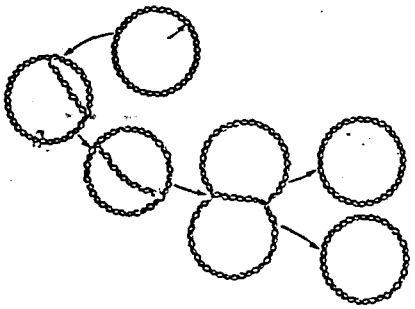


Palindromatic
sequences
(hairpin curves)

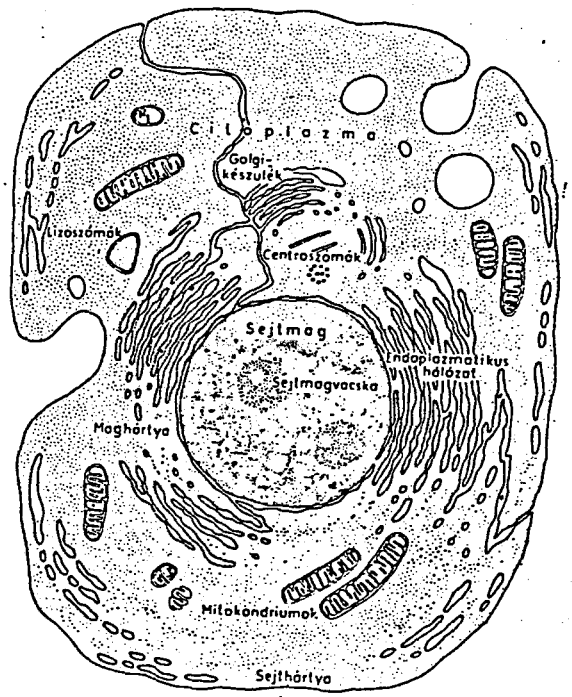
1. ABCDEF
2. FEDCBA
(Inverse sequences)

Role: information points for
ENZYMES (DNA- polymerase, - ligase etc.)
CONTROLLERS (repressors, inducers etc.)

FOURTH LEVEL OF STRUCTURE

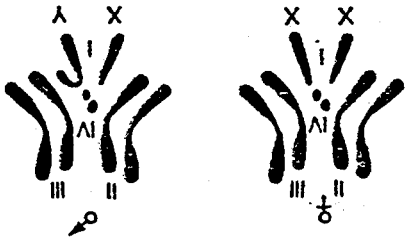


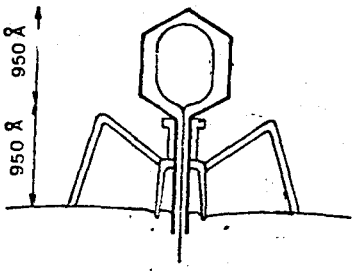
Plasmids



Cell organs

Chromosomes





Phages or viruses

FUNCTION OF DNA:

Information source:

-built up proteins
-cell organs
-biochemical procedures
-control of procedures at several levels
-multiplication
-inheritage
-repair
-etc.

RECENTLY: Protein synthesis

DNA ⟶ Protein/Enzyme

DNA ------- Messenger RNA (mRNA)
(Sense strand,
mute strand)

Ribosome ----- Transfer RNA (tRNA)

PROTEIN

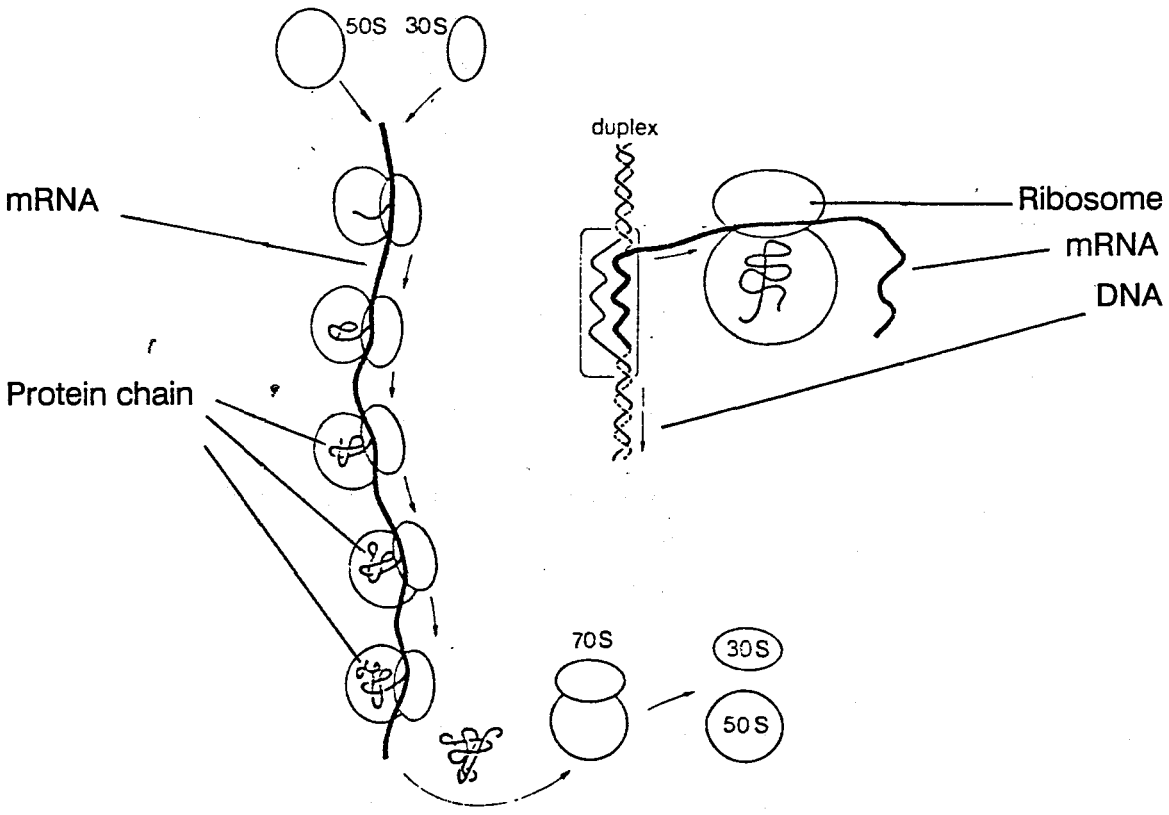
---------- DNA

= TRANSCRIPTION
RNA - polymerase

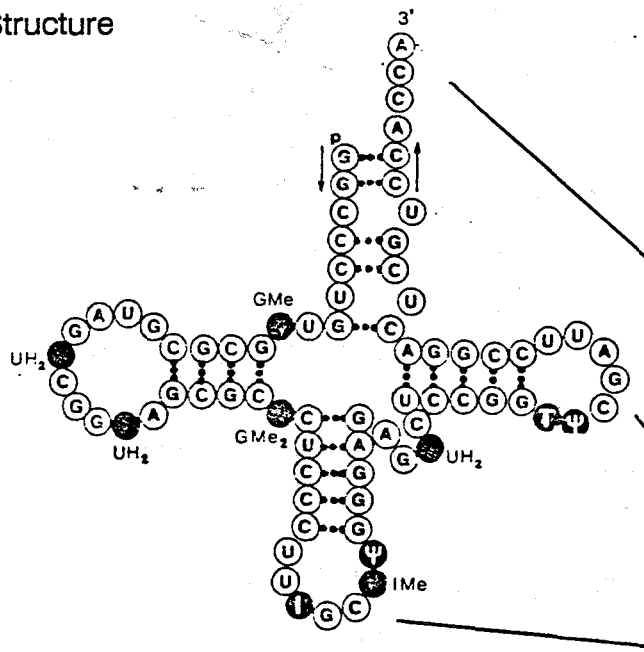
i e i e e i e e
--|---|-|--|--|-|-|-- Hn DNA (heterogen DNA)

= MATURATION
e: exon
i:intron (meaningless)

e e e e e
---|----|---|--|-- mRNA



tRNA Structure



Bonds: amino acid

Ribosome bonding structure

Triplet Anticodon

3' — C C G — 5' kodon

A------T
G------C
T------A

EVERY AMINO ACID HAS IT'S OWN tRNA

(Central dogma of genetics: -Wattson-Crick:
DNA ⟶ RNA ⟶ PROTEIN)

1970's: TEMIN: Dogma failed-

RNA ⟶ DNA

DNA Reverse transcriptases

- Good tool in genetic engineering
- Evil enemy : causes cancer

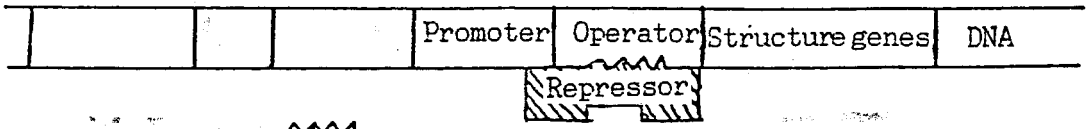
"GENE" means: info of property
"GENOM" means: all info of organism

If TRIPLET is WORD
The OPERON is SENTENCE

WHAT IS OPERON, WHY IS IT IMPORTANT FOR US?

OPERON: GENE LEVEL REGULATION

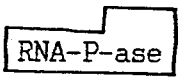
Case"A":



repressor protein

catabolite activation protein

cyclic adenosine monophosphate (cAMP)

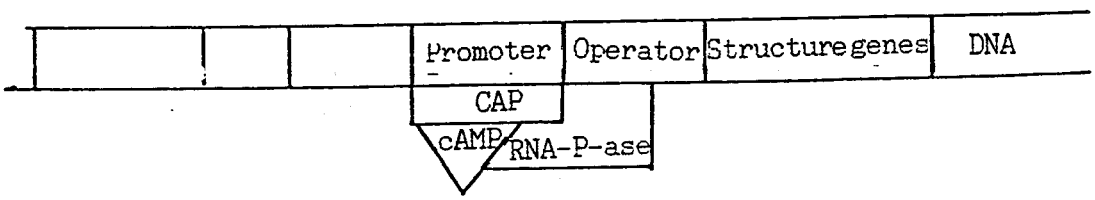


RNA polymerase

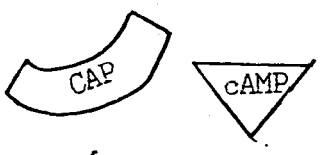
(promoter: info point for RNA P-ase,
operator: linking point for repressor)

NO INDUCTOR (EFFECTOR) : REPRESSED ENZYME SYNTHESIS

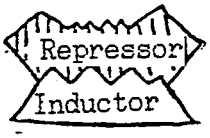
Case "B":

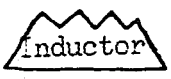


Transcription



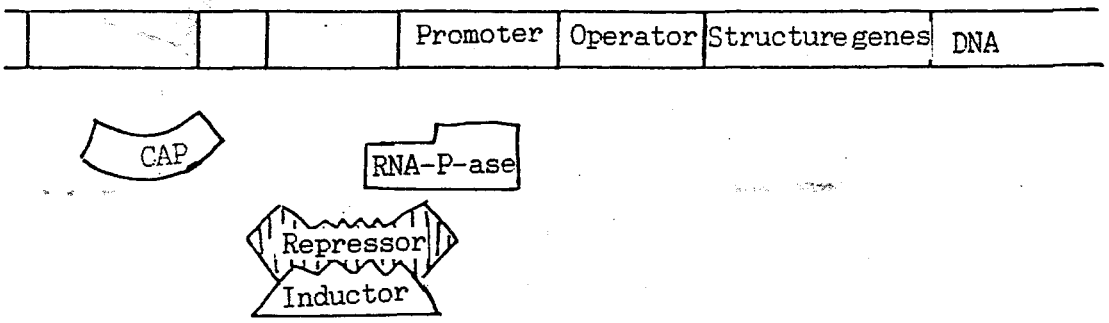
Inductor (Effector)





IN PRESENCE OF INDUCTOR: INDUCED ENZYME SYNTHESIS

Case "C":



IN THE LACK OF cAMP , THE CATABOLITE ACTIVATOR PROTEIN CANNOT BOND: REPRESSED ENZYME SYNTHESIS

Catabolic repression !!!!!!

Low sugar conc.---High cAMP conc. High sugar conc.——Low cAMP conc.

2./ Change of genetic INFO

Change:	Genetic engineering (recombination)	molecular level
		cell level
	Mutations	natural
		induced

Gen. Eng. at molecular level = artificial recombination

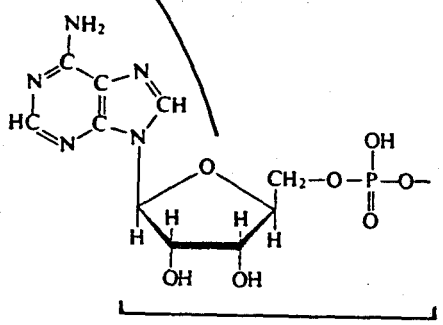
FOREIGN DNA ---------- CONNECTS TO PARENTAL (HOST) DNA

MUTATION
GENETIC ENGINEERING → SELECTION

STRUCTURE:
1. Basic information
2. Change of gen. info.
3. Selection
4. What-to-do- conclusions

1./ DNA Genetic Information

Desoxyribose "D"



Desoxyribose phosphate "D---P"

Chain D---P---D---P---D---P---D---P---D---
A T G A C

TASKS:

1./ Introduce foreign DNA
2./ Recombine (connect) + stabile, heritable
3./ Express!!! Cell must produce the product

INTRODUCING METHODS, TECHNIQUES + VECTORS

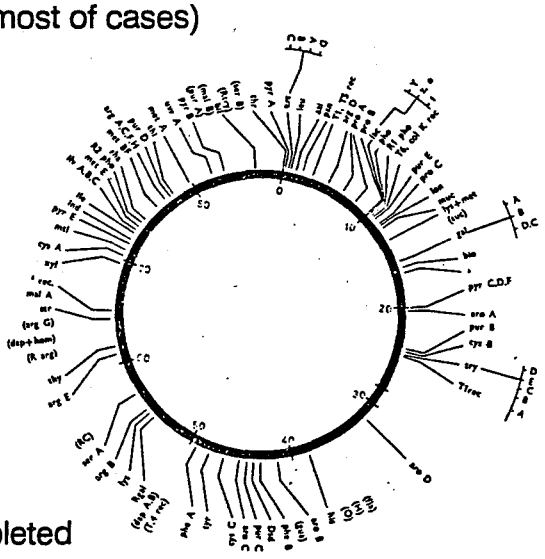
Competition- pure DNA + competent cells
Transduction- phags (bact. viruses)
Transformation- plasmids
Transfection- sexual, parasexual processes (eg. conjugation)

Recently: Electroporation method

These methods are for absolutely foreign products:
like:
gonadotrop hormone,
insulin

Usual " HOST ": E. coli

Basis: Complete knowledge of host genom
of vector genom
foreign gene (in most of cases)



Complet discovery of E. coli genom took 10 years!

Bacillus subtilis: genetic mapping has not been completed

THERMOSTABLE ALCALIC PROTEASE

Bac. thermophylus

1./ thermophylic bac. bears originally 60° centigrades
2./ engineered enzyme now: works at 95° centigrades

The strain is selected for heat tolerancy!

Importance : DETERGENT INDUSTRY

Protein engineering: glycine

is replaced by

cysteine

cysteine: strong -S- bonds!!

RESULT: Increased stability

IN THE CASE OF BAA: PRINCIPALLY O.K.

1./ STEP: Thermopyl bacillus selection
(fermentation around 45-50° centigrades)
2./ STEP: Engineered enzyme

Reason: industrial task
Task: liquefaction of starch

Hardest starch liquefaction :
in jet heaters 120-150° centigrades
maintenance time: 10-20 seconds

Enzyme deteriorates, but: liquefaction is complete
(izosugar production at Hungrana Ltd. Hungary)

WE COULD DO, BUT:

Financial requirements of such a project are enormous

Instruments required:

- DNA sequence analyzer
- Ultrasonic cell breaker
- Electrophoresis equipment
- High speed centrifuges
- Two dimension gel electrophoresis equipment
- Isotope laboratory + instruments safety design, radioactive compounds
- Electroporation equipment

MOREOVER:

+ (knowledge of literature)

+ (vectors)

SORRY TO SAY, BUT THE CONDITIONS HERE ARE NOT SATISFACTORY FOR THIS TYPE OF PROJECT.

IN ADDITION: A. niger: EUKARYOTE

IT'S GENOM is approx. 100 times bigger!

CONSEQUENCE: (MOLECUAR LEVEL)

OUR OPPORTUNITIES ARE

VERY LIMITED

IN MOL. LEVEL GEN. ENG.

WHAT SHALL WE DO ANYWAY ?

BIG FIRMS: Enzyme., antibiotics, etc.

- Genetic engineering - cell level
- Mutation techniques
- Advanced, large scale selection

GENETIC ENGINEERING - CELL LEVEL

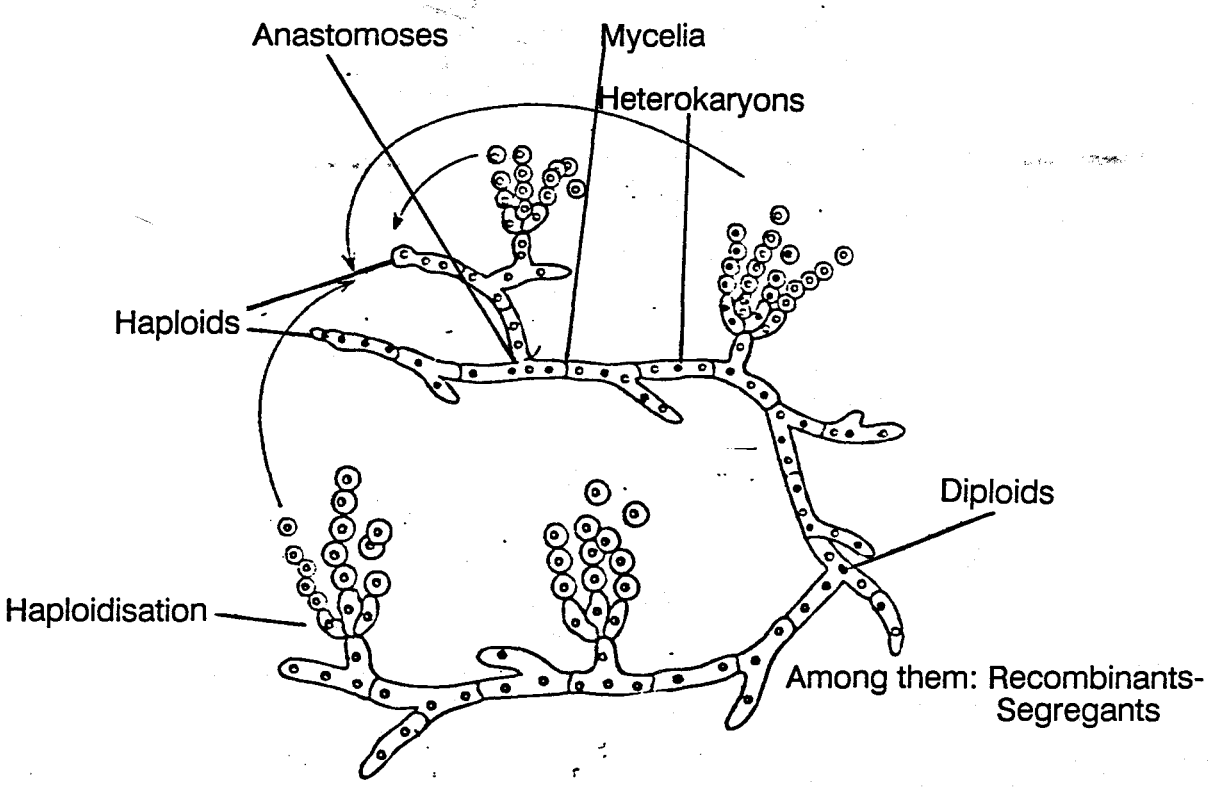
Protoplast formationlysozyme, snail enzyme
trichoderma enzyme

→ Protoplast fusion PEG

→ Protoplast regeneration "CM"

RECOMBINATION OCCURS! → SEGREGATION (segregants)
(recombinants) among them: new combinations

USUALLY FOR SECONDARY CHARACTERISTICS : less foaming, easier filtration etc.



SOMETIMES: Mating types "Thallism"

Anastomoses - frequency is very low

Probability can be increased by protoplast fusion

THESE TYPES OF RECOMBINANTS (FUSANTS) ARE USUALLY GOOD FOR

SECONDARY CHARACTERISTICS

AS BETTER FILTRATION, LESS FOAMING

NOT FOR INCREASING PRODUCTION CAPACITY

ABOUT PROBABILITIES:

Let's talk about mutations

> FOR THE TIME BEING: MOST EFFECTIVE TOOL
> IN INDUSTRIAL STRAIN IMPROVEMENT
> (FOR ENZYMES)

FREQUENCY/PROBABILITY of natural mutations

$P1 = 10^{-6} - 10^{-7}$

very low

Let's tell: among mutants

$P2 = 10^{-5}$ is useful

$P1xP2 = 10^{-12} - 10^{-11}$

This is a case when to select without mutation techniques

> " To pick up one certain piece of rice ---
> ---- from 100 tons of rice"

This is the success probability of "natural selection"

IN THE CASE OF INDUCED MUTATIONS: NEARLY ALL SURVIVANTS ARE MUTANTS

$P1 = 1.0$ $P2 = 10^{-5}$ --- $P1xP2 = P2$

If we try every mutant: by fermentation test

The probability is $P = 10^{-5}$

> Selection methods are of great importance

With good selection techniques: P will be:

VERY HIGH !!!!!! $10^{-4} - 10^{-1}$

DEPENDING ON THE METHOD, PRODUCT , STRAIN

> " TO PICK UP ONE CERTAIN PIECE OF RICE--
> ---FROM A SMALL CUP OF RICE "

INDUCED (ARTIFICIAL) MUTATION:

——► HIGH NUMBER OF MUTANTS

several types of mutants:

Genom mutants
+,-,x,/
number of chromosomes

Chromosomal mutants
sequence of genes as:
deficiency, deletion
inversion
duplication
translocation

Gene mutants
point mutants - the
linking property of
nucleotide base changes
transition
transversion

Transition pirimidine ——► pirimidine

purine ——► purine

(T ——► C)

(A ——► G)

Transversion pirimidine ——► purine

purine ——► pirimidine

(T ——► A)

(C ——► G)

raster or frameshift mutation - only one or some nucleotides

deletion
insertion

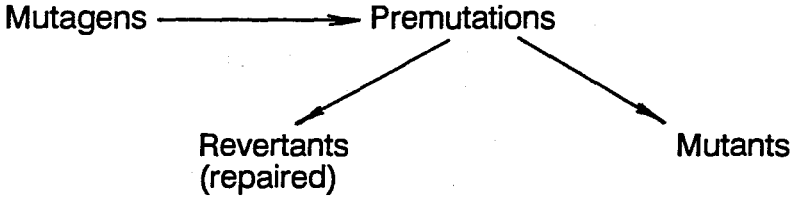
Explanation:

C A T/G/A T C A G G C A
/--------FRAME

C A T A T C A G G C A
/——➔FRAME

Frame of triplets moved to the right:

encoding new amino acids
result: new protein



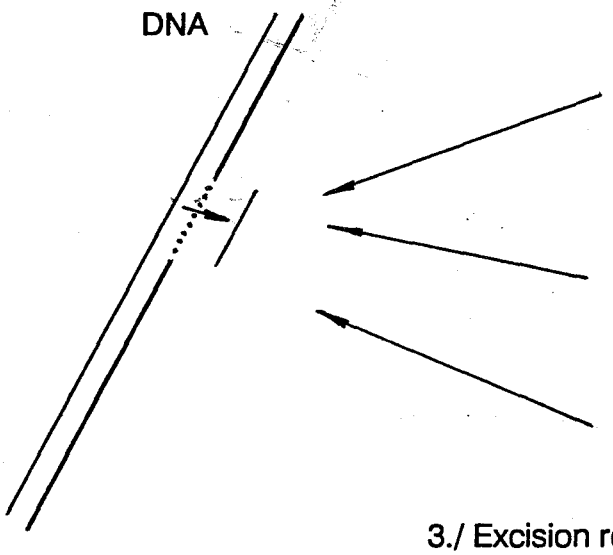
Example:

1./ Photoreactivation after UV

visible light -- enzyme activation

2./ Excision repair after UV

uvrA, uvrB, uvrC genes'
endonucleases



Endonuclease

cuts off the destroyed DNA part

DNA polymerase-I

rebuilds the lacking part

DNA nucleotide ligase

links the new part

3./ Excision repair after alkilation, dezamination

4./ Post replicative repair after multiplication!!

5./ Adaptive repair (repair capacity can increase up to a threshold)

TYPES OF ARTIFICIAL MUTATIONS:

- pirimidine dimers (cannot couple)
- alkilation, dezamination (cannot couple or causes mistakes)
- insertion of mutagens (frameshift)
- cuts off one chain of DNA
- cuts off both chains of DNA

TYPES OF MUTAGENS:

PHYSICAL

CHEMICAL

AGENTS

PHYSICAL:

UV — "NEAR" UV 300-400 nm

HIGH FREQUENCY 200-300 nm

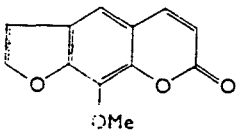
X - ray

gamma - ray

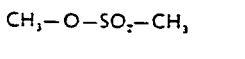
beta - ray

CHEMICAL:

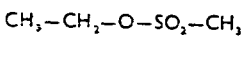
8 - methoxypsoralen



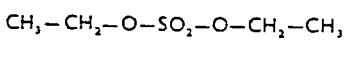
methyl-methane-sulphonate



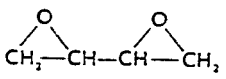
ethyl-methane-sulphonate



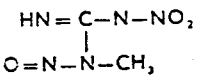
diethyl-sulphate (DES)



diepoxybutane (DEB)

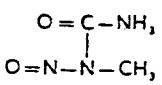


N-methyl-N'-nitro-N- nitrosoguanidine (NTG)

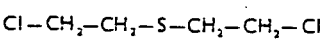


EXCEPTION IN PERFORMANCE AND DANGER

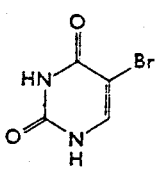
diasomethane



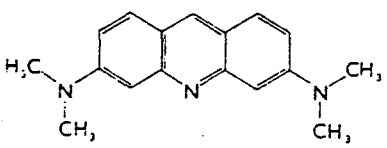
N-methyl-N-nitrosourea (NMU)



di-(2-Cl-ethyl) sulphide (mustard-gas)



5-Br-Uracil

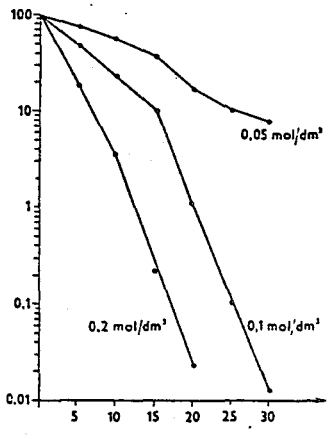


Acridinorange

DIFFERENT EFFECTS---- DANGEROUS COMPOUNDS ---- CANCEROGENS

TASK: OPTIMISE!!!

Survivant cell concentration



Time of treatment

High lethality----------High % of mutants among survivants

USUALLY: 99.9 % OF LETHALITY

(e.g. : $5x10^8$ cell/ml -------- $5x10^5$ cell/ml)

AFTER TREATMENT: S E L E C T I O N

" SCREENING"

1./ Petri dish
2./ Shaking flask
3./ Lab. fermenter

1st level: Petri dish

1.1. Easiest: resistance
(e.g. antibiotics)

1.2. Harder : auxotrophy
(e.g. aminoacids)

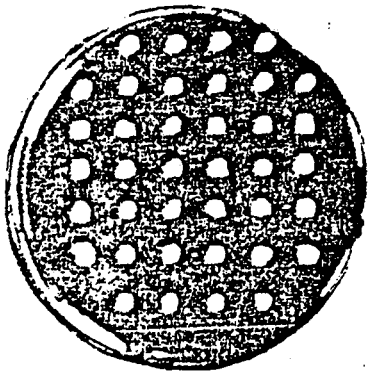
1.3. Hardest: " + variants"
(e.g. enzymes)

1.1. Resistance

Medium contains
penicillin

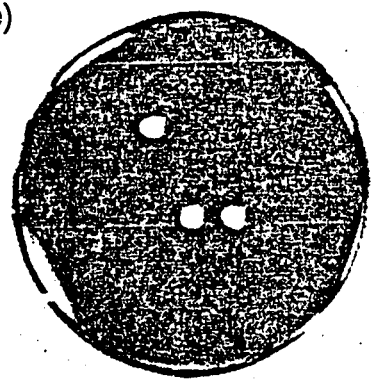
```
colony which grows: resistant to
     penicillin
```

1.2. Auxotrophy



"replica" method

(e.g. methionine)

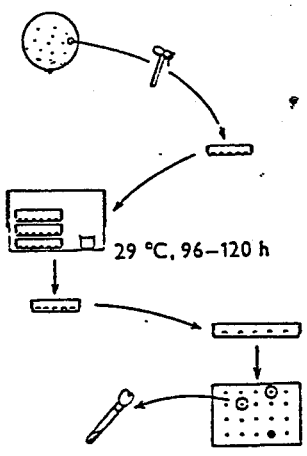


medium with methionine
CM - complete medium

medium without methionine
MM - minimal medium

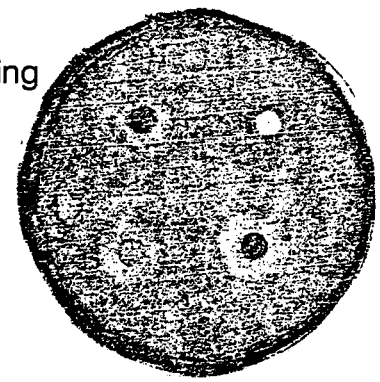
```
colony which cannot grow without
methionine : auxotroph ( met⁻)
```

1.3. " + variants"



Medium contains sensitive microbes
(antibiotics) - lethal ring
Visible substrates
(enzymes) - transparency ring
Several stains
(depending on product)

"Agar disc method"



Transparency

In the first two cases: solid medium selects only

Third (our) case: we have to select too!

Systematic longterm work

Selection medium must be optimal:

GA: without glucose!
(catabolic repression)

glucose, glutamic acid, lactose
represses the GA operon!!!

We have to exclude the auxotrophs in our case
(unusual mutants)

Solution: CzD
medium

2nd level of screening: shaking flask

Optimisation:
- volume
- C/N balance
- buffer
- defoamer
- minerals
- concentration

TO GIVE THE MICROBE THE HIGHEST CHANCE TO SHOW IT`S CAPACITY!

Big task remained: how to measure high number of samples?

We can increase probability by screening a high number of mutants

Quick analysis methods - standard is our parent strain's shaking flask.

3rd level of screening: laboratory or pilot fermenter

here: must be improved

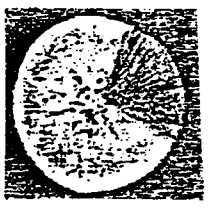
CELL LEVEL GENETIC ENGINEERING:

Screening (selection) in case of fusant fungi

There are only natural markers (real markers can be dangerous for the production capacity)

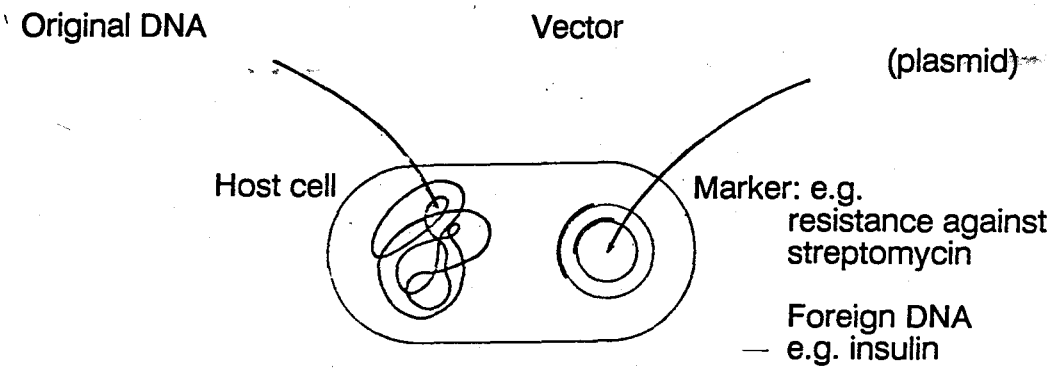
Mainly in research...

Diploids: larger spore size
Segregants: (inducable) sectors of colony



Inducer e.g. parafluoro - phenylalanine

Screening in case of molecular level genetic engineering



? Transformation -- detect the resistance

? Implementation -- detect the foreign DNA
(radioactive RNA test)

? Expression -- detect the product
(radioactive immuno assay
RIA on the product)

(Phantasy??!!:

GA -------- HPLC fine preparation

Monovalent (or multivalent - izoenzymes-)
POLYCLONAL ANTIBODIES (rabbit)

Radioactive compound

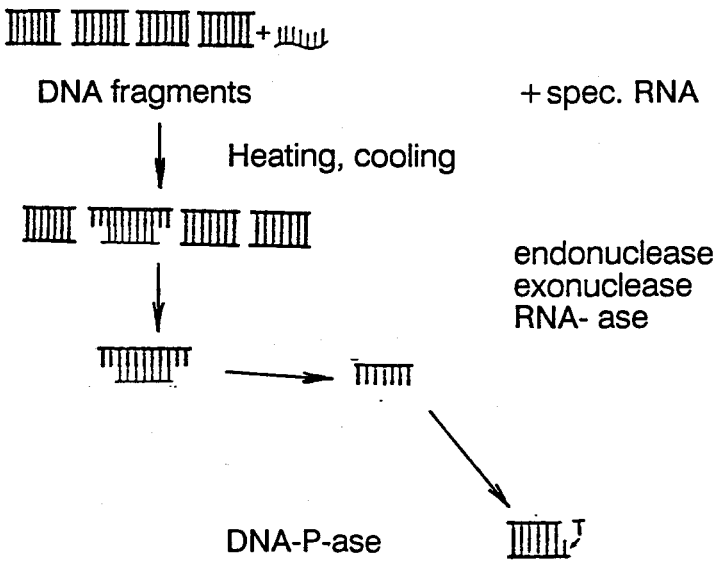
Autoradiography RIA test on GA !!!!!

Possibility: 10^6 cells/ dish
Increased probability!!!!

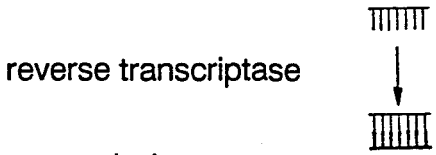
"Genetic surgery"

The synthesis of the DNA (to be introduced into the host)

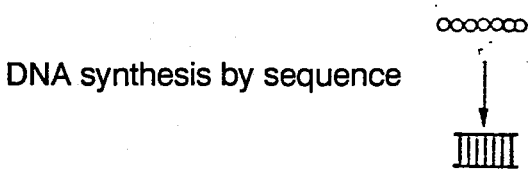
A./ Isolation



B./ Spec RNA



C./ Protein Sequence analysis



EQUIPMENT LIST FOR SMOOTH PRODUCTION BEFORE TECHNOLOGY IMPROVEMENT

Air filters:

(one individual filter has two layers of cartridges)

Cartridge sizes and the required number of cartridges in the case of a 3 x 60 m3 plant:

	diameter/mm/:	height/mm/:	Estimated price:
3 x 60 m3	1300	440 + 220	150000 Yen
2 x 8 m3	600	440 + 220	30000 Yen
2 x 0.76 m3	340	440 + 220	9000 Yen

12 x 20 m3 plant:

12 x 20 m3	850	440 + 220	61000 Yen
7 x 5 m3	600	440 + 220	30000 Yen
7 x 0.76 m3	340	440 + 220	9000 Yen

The housing of the filter cartridges should have a smaller diameter than the diameter of the prefabricated glass fiber sheets. The inside diameter of the housing should be designed according to the description of the manufacturer.
The cap of the housing should press the cartridges down by supports, the extent of compressure required is 10 %.

Manufacturer: Yamato Riken Kogyo Co. Ltd.
Address: 59, 2 chome Wakabayashi-cho, Yao-city, Osaka, Japan
Telephone: Osaka 0729 (49) 4081 , Tokyo 03 (608) 5566

Temperature control:

(In the case of the 12 x 20 m3 fermenters only, the other fermenters are equipped with automatic temperature control.)

12 sets of indicating temperature controllers
Type: SLCD-151,-171,-251,-271
Estimated price: 1800 USD
Manufacturer: Yokogawa Electric Corporation
Address: 9-32,Nakacho 2-chome, Musashino-shi, Tokyo 180 Japan
Telefax: 0422 550461
Phone : 0422 541111

12 sets of electric temperature sensors
Type: PT 100
Estimated price: 500 USD

Manufacturer: Yokogawa (as above)

12 sets of controlled valves, diameter 80 mm
Estimated price: 850000 Yen

7 sets of controlled valves, diameter 50 mm
Estimated price: 600000 Yen

7 sets of controlled valves, diameter 35 mm
Estimated price: 400000 Yen

Manufacturer: Nihon Koso Co. Ltd.
Address: 1-5-11 Nishishinbashi Minato-ku Tokyo, Japan
Telefax: 03 3580 8667
Phone : 03 3580 8611

EQUIPMENT LIST FOR IMPROVEMENT OF FERMENTATION TECHNOLOGY

Air filters:

(one individual filter has two layers of cartridges)

Cartridge sizes and the required number of cartridges in the case of a pilot fermentation plant:

	diameter/mm/:	height/mm/:	estimated price:
2 x 3.0 m3 (4 sets)	400	440 + 220	13000 Yen
2 x 1.2 m3 (4 sets)	340	440 + 220	9000 Yen
4 x 0.5 m3 (8 sets)	300	440 + 220	6000 Yen

Manufacturer: Yamato Riken Kogyo Co. Ltd.
Address: 59, 2 chome Wakabayashi-cho, Yao-city, Osaka, Japan
Phone: Osaka 0729 (49) 4081 , Tokyo 03 (608) 5566

pH measurement/ control:

4 sets of pH indicators/controllers
Estimated price: 1800 USD

5 sets (one spare) of pH probes
Estimated price: 1500 USD

4 sets of pH transmitters Type: PH8FT or TF21
Estimated price: 1500 USD

Manufacturer: Yokogawa Electric Corporation
Address: 9-32, Nakacho 2-Chome, Musashino-shi, Tokyo 180 Japan
Telefax: 0422 550461
Phone : 0422 541111

2 sets of (NH3) controlled valves, diameter 0.5``

Estimated price: 400000 Yen

Manufacturer: Nihon Koso Co. Ltd.
Address: 1-5-11 Nishishinbashi Minato-ku, Tokyo Japan
Telefax: 03 3580 8667
Phone : 03 3580 8611

DO measurement system:

2 sets of DO electrodes (+spare membranes, electrolite)

Estimated price: 1500 USD

1 set of DO indication instrument

Estimated price: 3000 USD

Manufacturer: Ingold Messtechnik AG
Address: CH-8902 Urdorf, Switzerland
Telefax: 01 736 2636
Phone : 01 736 2211

Feed addition:

2 sets of controllable peristaltic pump, capacity: 10-50 l/h

Estimated price: 2000 USD

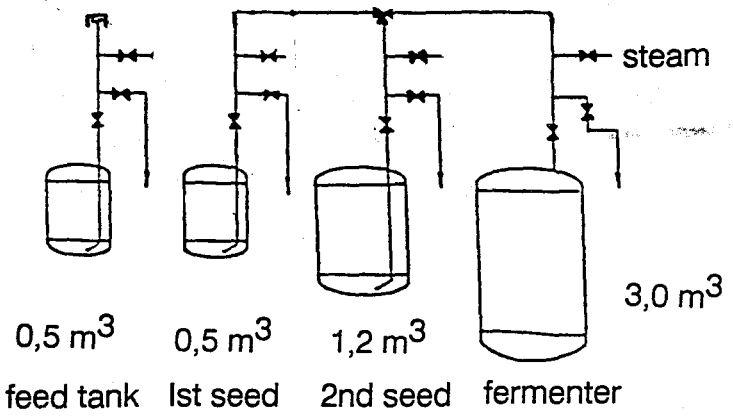
Manufacturer: Smith & Nephew Wattson-Marlow
Address: Falmouth, Cornwall TRII 4RU, England

2 sets of programable logic controllers

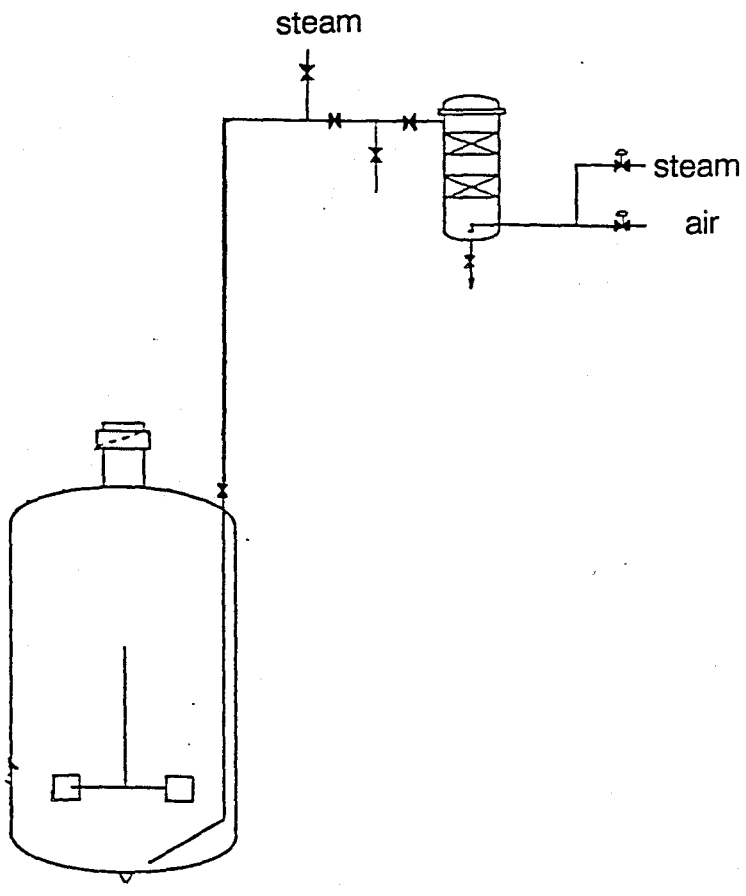
Estimated price: 1500 USD

Manufacturer: Yokogawa (as above)

1./ Seed transfer line



2./ Air filters (every tank)



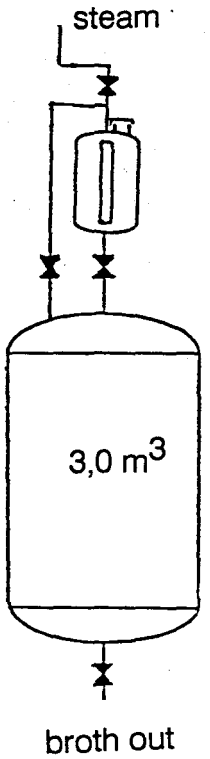
Samples: 0.5 m^3, 1.2 m^3 ------- from seed line
3.0 m^3 ------- from broth out line

Exhaust air: measured by portable rotameter

3./ Automatic temperature control --- installed

4./ Defoamer system

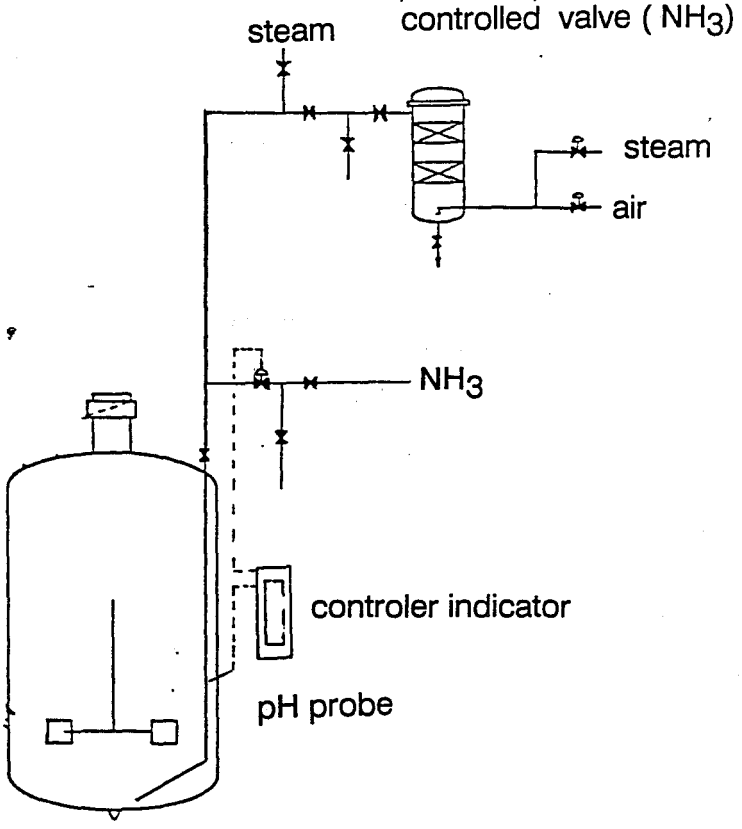
(Empty sterilisation,
filling by sterile defoamer)



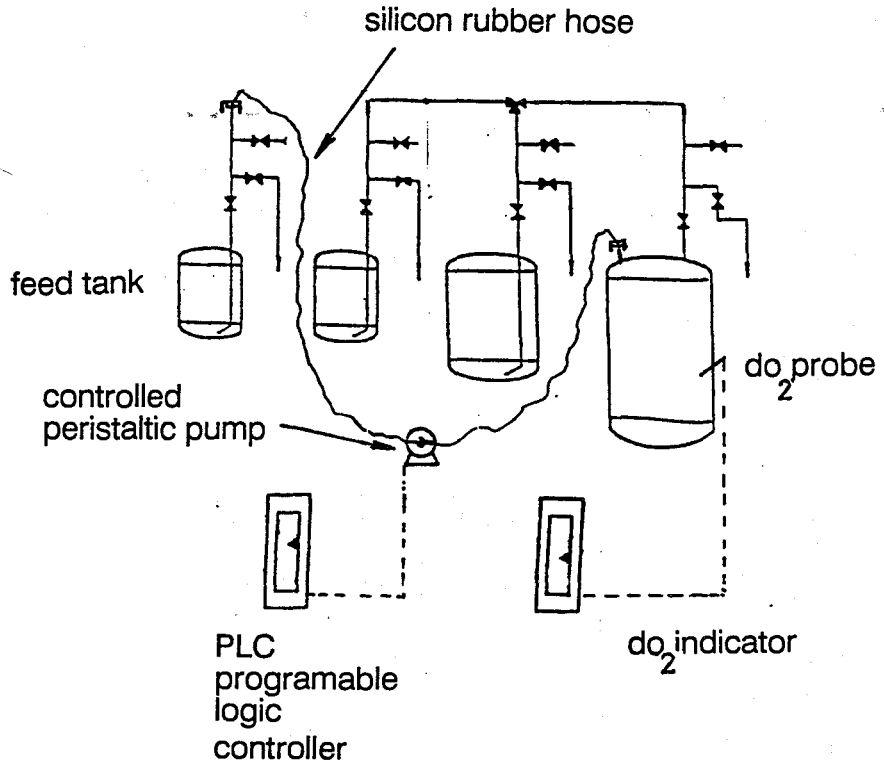
Only for 3 m_3 tanks

5./ Instrumentation (pH)

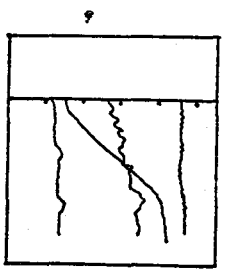
1.2 m^3 : pH probe, indicator
3.0 m^3 : pH probe, controller/
/indicator,
controlled valve (NH_3)



6./ Instrumentation (feed, DO)



7./ Data collection: 2 pieces of chart recorders (each with 6 channels)



recorder

pH	1.2 m^3
pH	3.0 m^3
T	0.5 m^3
T	1.2 m^3
T	3.0 m^3
FEED	3.0 m^3

EQUIPMENT LIST FOR ELABORATION OF ADVANCED DOWNSTREAM PROCESSES

1./High speed separator, pilot scale (capacity required 500 l/hour of B. subtilis fermentation broth), with automatic sediment discharge system

Estimated price: 30000 USD

Manufacturer: Alfa-Laval Unimex AB
Address: S- 14780 Tumba, Sweden
Phone : + 46 753 65000
Telefax: + 46 753 60141

2./Pilot scale vacuum-drum filter (capacity required 500 l/hour of A. niger fermentation broth), with washing section

Estimated price: 12000 USD

Manufacturer: Filtrox
Address: Mothgasse 25, A- 1190 Wien, Austria
Telefax: 0222/374215
0222/371552
Phone: 0222/37163439

3./ Pilot scale UF equipment (capacity required 500 l/hour of filtrated/ separated fermentation broth), with spare membranes 30000, 500000 and microfiltration, membrane area: 9 m2,

Type: Pilot Plant M37/M38
Estimated price: 35000 USD

Manufacturer: Dow Danmark A/S DDS Separation Systems
Address: Stavangervej 10. P.O.Box 149, DK-4900 Nakskov, Denmark
Telefax: + 45 53923111
Phone : + 45 53922799

4./Pilot scale vacuum fluid bed dryer (capacity required 10 l/hour water evaporation)

Estimated price: 40000 USD

Manufacturer: Glatt AG, Maschinen & Apparatebau
Address: Kraftwerkstrasse 6, Ch-4133 Pratteln, Switzerland
Telefax: + 61 821 8411
Phone: + 61 821 4481

EQUIPMENT LIST FOR LABORATORY / STRAIN IMPROVEMENT/

1./ Binocular microscope, phase contrast condensor, planachromat objectives, ocular 10x, 15x,
objectives 10x
20x

40x
100x (immersion)

Type: LABOPHOT 2

Estimated price: 50000 AS

Manufacturer: Nicon
Address: Optoteam, A-1183 Wien 18, Gersthoferstrasse 93.
Telefax: 470 20 50 /20
Telex : 114650 opto

2./ Laboratory shaker (capacity 200 x 300 ml Erlenmeyer flasks)

Estimated price: 3000 USD

Manufacturer: New Brunswick
Address: New Brunswick Eurotech, 12 Black Acre Close
Amersham,Bucks HP7 9EW
England
Telefax: 04 94-724308

ELABORATED STRAIN IMPROVEMENT METHODS

Exponential stage cells from B. subtilis

Fermentation medium for obtaining cells from exponential phase:

/Component/	/ % /
yeast extract	0.2
pepton	1.0
NaCl	0.2
glucose	3.0
beef extract	0.3
KH2PO4	0.05
K2HPO4	0.05

pH= 7.0

Shaking volume: 30 ml/ 300 ml baffled Erlenmeyer flask

Shaking temperature: 37 degrees centigrade

Shaking speed: 240 rpm

Harvest : at 7th hour of culture
(as being the last phase of exponential stage - checked by graphic evaluation of OD /670 nm/ data)

Cell concentration : 1.4 x 10+9 cells/ ml
(measured by decimal dilutions and plating on Petri dishes)

Germinating cells from A. niger

Fermentation medium for obtaining germinating cells:

/Component/	/ % /
sucrose	3.0
NaNO3	0.3
KH2PO4	0.1
KCl	0.05
MgSO4	0.05
FeSO4	0.001

pH= 6.6

Shaking volume: 30 ml/ 300 ml baffled Erlenmeyer flask

Shaking temperature: 32 degrees centigrade

Shaking speed: 240 rpm

Harvest: at 13th hour of culture
(germination time was checked by microscopic observation)

Cell concentration: 2.7 x 10+7 cells/ml
(measured by decimal dilutions and plating on Petri dishes)

Mutagen treatment of B. subtilis cells by UV

Optimal exposure time: 35 minutes

Distance from UV source: 15 cm

Lethality obtained: 99.9 %
(survivant cells' concentration was measured by decimal dilutions and plating on Petri dishes)

Mutagen treatment of A. niger cells by UV

Optimal exposure time: 40 minutes

Distance from UV source: 15 cm

Lethality obtained: 99.9 %
(survivant cells' concentration was measured by decimal dilutions and plating on Petri dishes)

Mutagen treatment by NTG

Less than one mg NTG is measured into a dry test tube. (The test tube's weight was measured previously.) The crystals should be dissolved by pH=6.0, and 0.2 mol of phosphate buffer in order to get a 600 gamma/ml NTG solution in the case of A. niger and 300 gamma/ml in the case of B. subtilis.

In the meantime, 3 ml of broth containing exponential B. subtilis or germinating A. niger cells are centrifuged for 30 minutes at 3000 rpm.

After discharging the supernatant, the cells are suspended in 3 ml of pH=6.0 phosphate buffer, and 3 ml of NTG solution are added to the cell suspension. After careful (!) mixing the treatment is performed for 30 minutes at 30 degrees centigrade.

At the end of incubation, the cells are washed by using a centrifuge for 30 minutes at 3000 rpm and resuspendation by 3 ml of pH= 6.0 phosphate buffer.

Lethality obtained: 80 % in the case of B. subtilis
87 % in the case of A. niger

(survivant cells' concentration was measured by decimal dilutions and plating on Petri dishes)

Selection on Petri dishes - B. subtilis

After proper dilution the mutant cells (corresponding to the lethality rate), are plated on a solid media.

Selection media for B. subtilis:

/Component/	/ % /
pepton	1.0
MgSO4	0.01
FeSO4	0.01
NaCl	0.2
starch	1.5
(NH4)2SO4	0.5
KH2PO4	0.05
agar	2.0

pH=7.0

The low concentration of organic nitrogen sources is useful for getting rid of the majority of auxotrophs.

After incubation reaching a 1-2 mm diameter of colony size, the colonies are replicated onto the same solid media by using sterile wooden sticks in a systematic geometrical arrangement. A marked paper disk is used under the dish for fixing the positions. After the subsequent incubation, the colonies are each in nearly equal distance from one another. The colonies' BAA production rate can be detected by visual observation and by evaluating the clarification zone's diameter surrounding the colonies. (The BAA enzyme molecules diffusing from the colony cause the clarification of the turbid starch.)

The selected colonies should be inoculated onto two slant agars parallely.

Selection on Petri dishes - A. niger

After proper dilution the mutant cells are plated on solid media (Petri dishes)

Selection media for A. niger:

/Component/	/ % /
$NaNO_3$	0.3
KH_2PO_4	0.1
KCl	0.05
$MgSO_4$	0.05
$FeSO_4$	0.001
starch	1.5
pH= 6.6	

This medium does not contain any organic nitrogen sources, therefore excludes all the auxotrophs.

The selection procedure is the same as described in the case of B. subtilis. For the replica formation, the "selection media for A. niger" should be used.

Selection by shaking flask fermentation

In both cases the fermentation media is the same as the production fermentation media (Annex - 16., page 108). In the case of A. niger, the medium preparation is performed without BAA addition.

In the case of A. niger, the conditions of cultivation are:

Shaking volume: 40 ml/ 300 ml buffled Erlenmeyer flask

Shaking speed: 240 rpm (rotary shaker)

Temperature : 32 degrees centigrade

Duration : 120 hours

Usual results: 8-10000 LGAU/ml

In the case of B. subtilis, the conditions of cultivation are:

Shaking volume: 50 ml/ 500 ml Erlenmeyer flask

Shaking speed: 250 rpm (reciprocating shaker)

Temperature : 37 degrees centigrade

Duration : 48 hours

Usual results: 330-350 BAAU/ml

By testing the mutant colonies' productivity, the shaking experiments should be made parallely with the parent strain's flask shaking, providing a reliable basis for comparison and evaluation.

GENERAL METHOD FOR STRAIN IMPROVEMENT

Tasks:	Methods:
1./ To obtain cells in high density (100 million-one billion cells/ ml) in exponential phase. In the case of Ascomycetes, to obtain cells in the germinating phase	In the case of Bacteria, a shaking flask medium must be improved with transparent (non turbid) ingredients. The proper cultivation time is obtained by measurement of optical density, and by graphic evaluation. The latest exponential stage must be selected. In the case of Ascomycetes the proper germination stage (before branch formation on the mycelia) can be selected by microscopic observation. The cell concentration must be measured by plating decimal dilutions, and subsequent counting of the colonies.
2./ To perform mutagenesis to a 99.9% of lethality as in the case of UV, X-ray, gamma-ray and chemical mutagens. In the case of NTG, 10-80 % of lethality is satisfactory	The irradiation dose can be modified by the exposition time, and by the distance of the radiation source. (The dose is in quadratic function with the distance) The rate of chemical mutagenesis can be modified by the concentration of the mutagen agent, and the reaction time. In the case of UV irradiation, special care must be paid

to the photoreactivation. Incubation should be made by excluding the visible light. The lethality ratio must be evaluated by counting the survivant cells' concentration by plating decimally diluted suspensions on petri dishes, and subsequent counting of the appearing colonies.

3./ Screening of Petri dishes

There are a wide variety of screening methods, depending on the strains, products and the aim of selection.
In the case of amylolytic enzymes, the best selection tool is a turbid, starch containing solid media, where the starch liquefaction zones are transparent. The diameter of these zones is in correlation with the enzyme production level of the colony. Therefore, colonies of higher capacity can be selected by visual observation o f the clarification ring's diameter. The selectivity of the medium can be largely increased by shortening (BAA) or eliminating (GA) organic nitrogen sources from the medium. This way, nearly all the auxotroph mutants which are usually decreased producers, can be excluded.

4./ Screening by shaking flasks

The conditions supplied for the microbe must be as good as possible. We have to give the chance to the possible high producer strains for being able to show us their capacity.

	By adjusting the shaking volume and the shaker's speed we have to reach (if possible) the industrial fermenter's Kla (specific oxygen transfer) value. The most simple method is the checking of the production level in the shaken flasks. The medium composition should be optimised.
5./ Screening by laboratory scale or pilot fermentations	The technology and medium should be optimal for the parent strain.
6./ Adjusting the technology to the new strain	The better yielding strains have usually different requirements, that is why in most cases further results can be gained by media optimisation.

FORMATION OF PROTOPLASTS FROM ASPERGILLUS NIGER, REGENERATION

Reagents:

- physiological buffer solution:

NaCl	0.9 mol	
CaCl2	0.01 mol	
citrate-phosphate buffer	0.05 mol	autoclaved

- snail digestion enzyme (from Helix pomatia) produced by the biological Center, Szeged, Hungary

- cellulolytic enzyme complex (from Trichoderma viridae) originated from NOVO, refined and delivered by Sigma

- 30 % sucrose solution, autoclaved

- regeneration medium:

NaCl	0.9 mol	
agar	0.2 %	autoclaved

- medium for fusion :

NaCl	0.9 mol	
CaCl2	0.01 mol	
polyethylene-glycol (PEG-6000)	30 %	autoclaved

Procedure:

Approximately 0.2 g mycelium is harvested from the fermentation broth, at the 40th hour of shaking. (According to our experiments, corresponding to the data in literature, the age of mycelium has a high influence on the success of protoplast formation.)

The mycelia is introduced into 2 ml of physiological buffer solution , then 30 mg of snail enzyme and 30 mg of Trichoderma enzyme are added to the suspension. The digestion reaction is performed at 34 degrees centigrade, by gentle shaking (50 rpm). A loopful sample is taken after 30 minutes. If the formation is not completed, the reaction should be performed

until the protoplasts can be detected by microscopic observation. In our case, the protoplast formation was complete after 30 minutes.

Protoplasts are separated by carefully introducing the 'protoplast ' mixture onto a layer of 30 % sucrose in a centrifuge tube. After centrifuging at 3000 rpm for 15 minutes, the protoplasts can be harvested carefully by pipette from the intermediate layer between the sucrose and the supernatant liquid.

For regeneration , protoplasts are diluted by a regeneration medium, and poured onto the usual solid media which must also contain 0.9 mol NaCl for preventing autolysis. After incubation, regenerated cells form normal colonies.

With separated protoplasts, even from different strains, protoplast fusion can be made. Thus in the case of Aspergillus, we cannot apply the usual markers (auxotrophy) the spore colour and size can give information about the heterokaryons. The fusion can be carried out by the well-known method of introducing protoplasts to a 30 % polyethylene-glycol (PEG-6000) and 0.9 mol NaCl, 0.01 mol CaCl2 containing liquid. The fusant protoplasts should be handled very carefully because of their large size and mechanical sensitivity.

HANDOVER OF STRAINS, HANDLING, INFORMATION

Suggested solid media for the glucoamylase, glucoseoxydase and pectinase producing strains:

Unfermented beer (malt extract from beer factory) adjusted to 5% as dry material content: 1000 ml

glucose	10 g
peptone	10 g
agar	30 g
	pH=7.0
sterilisation:	120 C, 30 minutes

Inoculation and fermentation media:

Glucoamylase:

inoculation medium:

corn meal:	45 g
corn steep liquor:	94 g
CaCO3:	3 g
alpha-amylase:	1.8 ml
	pH= 6.0, volume: 300 ml

fermentation medium:

starch hydrolysate:	2160 ml(60% dry material, by BAA)
corn steep liquor:	180 g (50% dry material)
CaCO3:	60 g
(NH4)2SO4:	120 g
defoamer:	1 ml
KH2PO4:	0.1 g
	pH= 6.0, volume: 6000 ml

Glucose oxydase

inoculation medium:

sucrose:	150 g
corn meal:	60 g
Ca(NO3)2:	22.5 g
MgSO4:	0.38 g
CaCl2:	0.75 g
FeSO4:	0.03 g
citric acid:	2.25 g
KH2PO4:	7.5 g
	pH=4.5, volume 300 ml

fermentation medium:

sucrose:	300 g
corn steep liquor:	120 g
Ca(NO3)2:	45 g
MgSO4:	0.8 g
KCl:	1.5 g
FeSO4:	0.03 g
citric acid:	4.5 g
KH2PO4:	15 g
defoamer:	5 ml

pH=4.5, volume 6000 ml

Pectinases

inoculation medium:

sucrose:	50 g
corn steep liquor:	20 g
Ca(NO3)2:	7.5 g
MgSO4:	0.125 g
KCl:	0.25 g
FeSO4:	0.005 g
citric acid:	0.75 g
KH2PO4:	2.5 g

pH=5.6, volume 1000 ml

fermentation medium:

extracted sugar beet slices:	200 g (from sugar factory)
sugar beet slices extract:	1000 ml (dry material content 4%)
(NH4)SO4:	50 g
MgSO4:	5 g
KH2PO4:	5 g
FeSO4:	0.02 g
Co(NO3)2:	0.02 g
MnSO4:	0.01 g
ZnSO4:	0.01 g

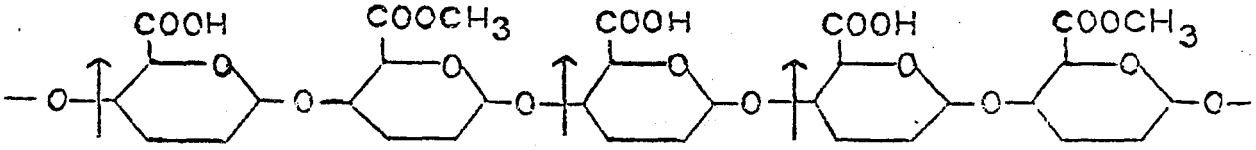
pH=5.6, volume 10,000 ml

(For pectinases, the medium composition must be reconstructed by using for example orange waste powder which is available in the People's Republic of China, and has a high pectin content as well)

ENZYMATIC ACTIVITIES, DETERMINATIONS

Determination of endopolygalacturonase activity

Principle:
The enzyme causes random cleavage of the polygalacturonic acid chain, therefore the viscosity of the pectin substrate solution decreases.
The end-products of the reaction are di-, tri-, tetra-, and penta-galacturonic acid fragments, and oligomers.



Endopolygalacturonase unit:

One EndoPG unit (Uepg) is that enzyme quantity, which causes the 75 % decrease of viscosity in 1000 ml of 0.5 % Na-polypectate solution at 50 degrees centigrade.

Chemicals:

- Na-polypectate (Serva)
- Na-acetate
- Acetic acid
- 96 % ethylalcohol

Tools:

analytical balance, pipettes, volumetric flasks, Ostwald's viscosimeter

Reagents:

-pH=4.5 acetate buffer: 430 ml of 0.2 mol Na-acetate (27.2 g Na-acetate*3H2O in 1000 ml of distilled water) and 570 ml 0.2 mol acetic acid (12.65 g acetic acid in 1000 ml of distilled water) should be mixed properly.

- substrate : for 1000 ml of substrate solution, 5 g of Na-polypectate is added into a dry Erlenmeyer flask. 96 % of ethylalcohol is poured onto the Na-polypectate until properly soaked. Then 500 ml of distilled water is added quickly and shaken vigorously, preventing sticking of the substrate. The final volume should be adjusted to 1000 ml by adding distilled water.

- enzyme solution: 100 mg solid enzyme should be dissolved in a volumetric flask, and filled up to 10 ml.

- preparation of the 10*, 100*, 1000* diluted enzyme solutions: 1ml of enzyme solution or 1 ml of fermented broth should be dissolved with a pH=4.5 acetate buffer.

Analytical procedure:

2.5 ml of (diluted) enzyme solution and 25 ml of 0.5 % Na-polypectate solution are transferred to a 50 ml Erlenmeyer flask. The flask must be covered by an Al or "parafilm" membrane. The flask is kept in a 50 degree centigrade waterbath. The control should contain 2.5 ml of distilled water instead of an enzyme solution. After exactly 60 minutes, the reaction should be stopped by quick cooling to 25 degrees centigrade. For viscosimetry, the Ostwald's viscosimeter should be maintained in a 25 degree centigrade waterbath for 30 minutes before measuring. After washing the viscosimeter with the actual sample, 7.0 ml of the sample is added into the viscosimeter. After sucking up the downflow time is measured by using a stopwatch. One sample should be measured three times, the deviation should be within 0.2 seconds.

Relative viscosity:

$$V_{rel} = \frac{T_s}{T_w}$$

Ts= time for sample
Tw= time for water

Specific viscosity:

$$V_{sp} = V_{rel} - 1$$

Decreasing in relative viscosity:

$$B` = [\ Vspc - Vsps\ ]*100/\ Vsps$$

Vspc= specific viscosity of control
Vsps= specific viscosity of sample

The measurement and evaluation is made by graphic interpolation.

X series: log A
Y series: B`

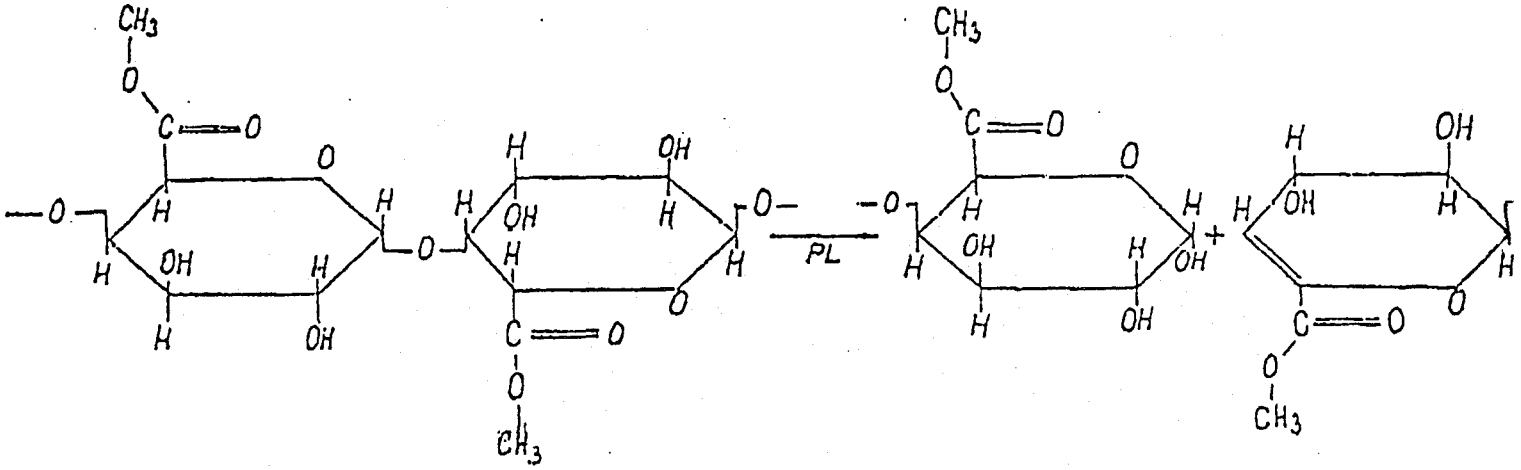
"A" means the originally used enzyme volume (e.g. in the case of 1000* dilution, 0.0025 ml of enzyme solution, or fermented broth).

After getting the first result, we have to adjust the dilution of the enzyme solution in order to get points on the graph which are close to the B`= 75 value. It means that we have to make several new dilutions by using the basic 10 ml solution, or fermented broth. The nearest points around the B`=75, should be connected with a line. The point of intersection with the B`= 75 horizontal line gives us the logA data, from which the activity can be calculated by the dilution (which would correspond to the logA value). Otherwise: A= the activity of the enzyme sample.

Specific viscosity can be measured by any other type of viscosimeter as well.

Determination of pectinlyase activity

Principle:
The pectinlyase can disassemble the pectin polymer chain only between methylated galacturonic-acid units. The cleavage is made by transelimination, so in the product unsaturated bonds can be obtained. The double bonds, due to their absorbancy peak at 235 nm, can be detected by spectrophotometry.



Pectinlyase activity unit: one unit generates one micromol of unsaturated-galacturonic-acid-methylester in one minute at pH=6.0 and 40 degrees centigrade.

Chemicals:

- 70 % estered pectin (Pomosin GMBH)
- citric acid
- 96 % ethanol

Reagents:

- buffer: pH=6.0 McIlvaine Buffer (368 ml 0.1 mol citric acid solution - 21.01 g/ 1000 ml- and 631.5 ml 0.2 mol Na2HPO4 solution -35.61 g Na2HPO4*2H2O/ 1000 ml - are mixed properly

- substrate: 10 g Pomosin pectin is washed by 96 % ethanol into a dry Erlemmeyer flask. Pour onto the mixture 700 ml of buffer quickly and mix it vigorously. The last 300 ml will be added by mixing and maintaining the mixture in a 50 degree centigrade water bath for one hour. The substrate solution can be stored in

a refrigerator for one week.

- enzyme solution: from a solid product, we usually make a 1 mg/ml solution by using analytical balance. The solution is made by a McIlvaine buffer.

- preparation of the control samples: for making blind samples, 5-5 ml of every sample is poured into test tubes. For proper inactivation of the enzyme, the test tubes are maintained in a 100 degree centigrade water bath for two hours.

Analytical procedure:

0.2 ml of an enzyme solution (blind samples are used in the same way parallely) is pipetted into 15 ml test tubes. After adding 5 ml of substrate solution and mixing, the mixture must be kept for 15 minutes in a 40 degree centigrade water bath. After incubation the samples are cooled by ice water, and the volume is adjusted to 10 ml by adding 4.8 ml of 0.15 % HCl solution. After mixing, the extinction must be mesured at 235 nm. (The extinction values must be inside the 0.3-0.8 interval.) For obtaining correct data, the acidified samples together with the blind samples, must be diluted accurately at the same dilution ratio.

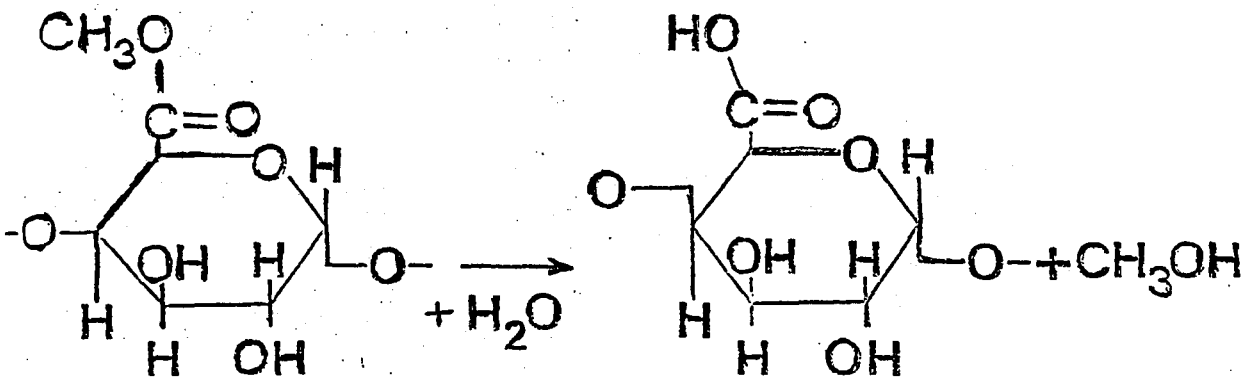
Calculation of activity:

$$Upl = (Es-Ec) * 10000 * D / 5500 * 15 \ [micromol/ min]$$

Es = extinction of sample
Ec = extinction of control
5500 = molaric extinction coefficient of unsaturated galacturonic-acid-methylester
15 = time of reaction (minutes)
D = dilution

Determination of pectinesterase activity

Principle:
The pectinesterase can hydrolyse the methylester groups of the polygalacturonase polymer. The liberated carboxy groups can be titrated by NaOH solution.



Activity unit: one unit hydrolyses one microequivalent of ester bonds within one minute at 30 degrees centigrade.

Chemicals:
- Na-acetate
- acetic acid
- 70 % methylated pectin (Pomosin GMBH)
- 96 % ethanol
- phenolphtalein indicator
- 0.1 n NaOH solution

Reagents:
- acetate buffer solution: 500 ml of 0.05 n Na-acetate (6.8 g of CH3COONa*3H2O/ 1000 ml distilled water) and 500 ml of 0.05 n acetic acid (3.15 ml 98 % acetic acid / 1000 ml distilled water) are mixed properly.
- phenolphtalein indicator : 1 g phenolphtalein is washed into a

100 ml volumetric flask by 96 % ethanol and filled up by distilled water to 100 ml.
- substrate : 10 g Pomosin pectin is washed into a dry Erlenmeyer flask by using 96 % ethanol. Add 700 ml of acetate buffer by vigorous shaking, and fill it up with buffer to 1000 ml. Maintain the mixture in a 50 degree centigrade water bath until completely dissolved , usually intermittent shaking is necessary.
- enzyme solution: 500 mg of solid enzyme is measured by analytical balance, and filled up to 500 ml by a 4.5 pH acetate buffer in a volumetric flask.
- control sample solution: 25 ml of enzyme solution is maintained in a 100 degree centigrade water bath for 2 hours.

Analytical procedure:
5 ml of enzyme solution, 5 ml of buffer solution and 20 ml of substrate solution are pipetted to a 50 ml Erlenmeyer flask. (The preparation of the control samples is performed in the same way) The mixtures are kept in a 30 degree centigrade water bath for 15 minutes. The reaction is stopped by cooling the mixture in ice water. After cooling, the titration is performed by 0.1 NaOH in the presence of a phenolphtalein indicator.

Calculation of the activity:

$$Upe = A*F*100/\ t*e\ [mval/min]$$

A = titration difference between the sample and control (ml)
F = adjustment factor of 0.1 n NaOH
100 = number of microequivalent ester bonds corresponding to 1ml of 0.1 NaOH
t = reaction time
e = amount of enzyme used for the reaction (g, or in the case of fermented broth ml)

Preparation of pectin from an apple (laboratory method)

This method is useful for preparing a substrate for enzyme determination methods.

1./
The apple must be cleaned and cut into 0.5 cm cubes. With the addition of 100 ml acetone, it must be mixed for obtaining a fine, thin smash. (It is best to use an electric mixer.)

2./
By using a rectification cooler, the smash must be kept in a 100 degree centigrade water bath fo 20 minutes.

3./
Without cooling we have to filter the smash by using a sintered glass filter (G-4) and vacuum flask.

4./
After filtration the filtrate should be washed first with acetone, and three times with alcohol, and once again with acetone.

5./
The washed material should be dried carefully (40-50 degrees centigrade).

The preparate contains some proteins and polysaccharides which are not soluble in acetone and alcohol, but the level of these compounds is very low. That is why this preparation method is used for analytical procedures as well.

FERMENTATION MEDIA

	GA [%]	BAA [%]
Corn meal	14-16	8.5
Soybean meal	4	5.5
Bran mash	2	-
Na2HPO4	-	0.8
CSL	2	-
(NH4)2SO4	-	0.4
CaCl2	-	0.2
alpha-amylase	6-10 BAAU/g starch	-

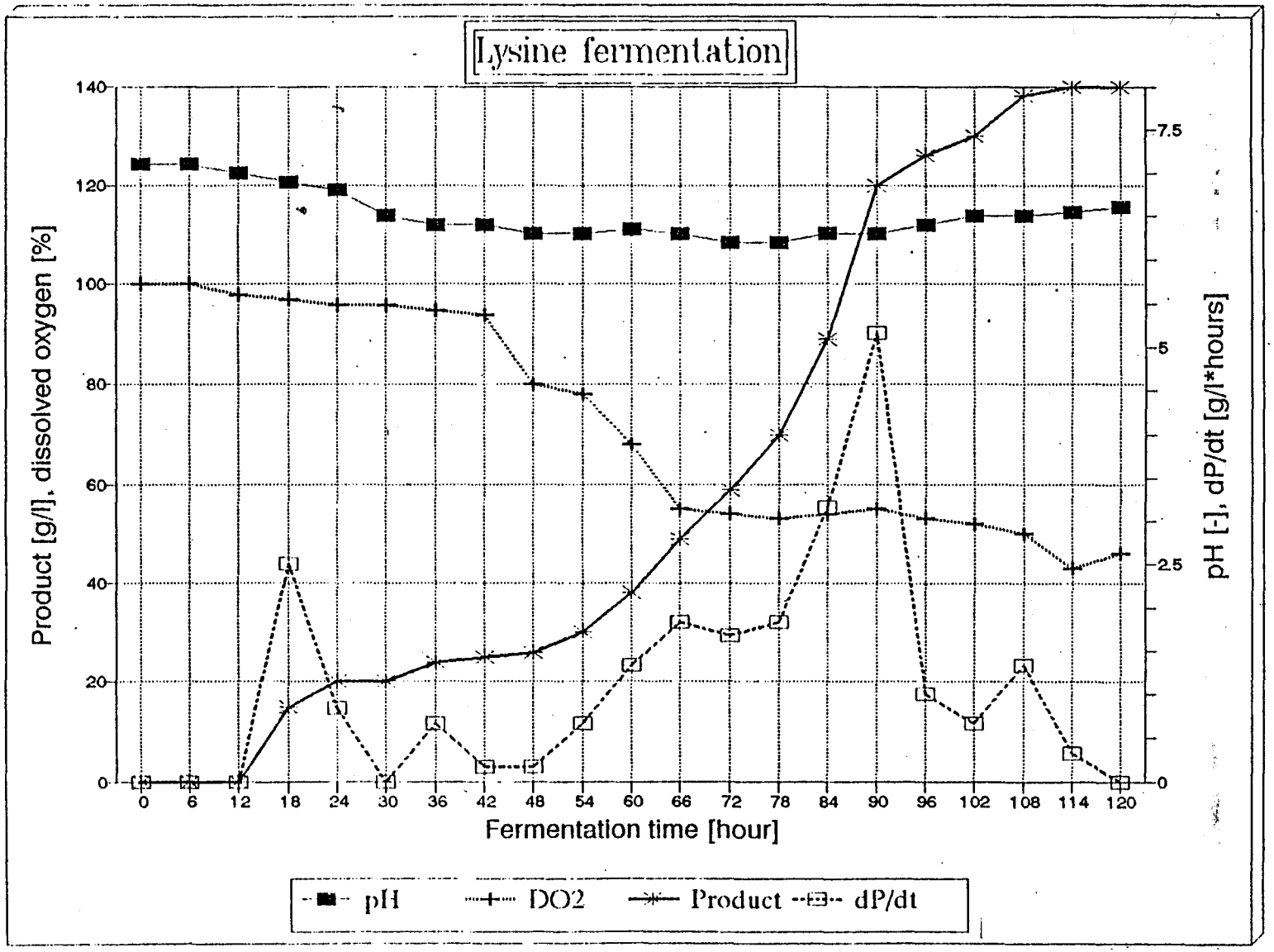
pH is not adjusted before inoculation

HANDOVER OF PC SOFTWARE

Demonstrating the delivered data processing software, some samples are shown:

In the case of the "QUATTROPRO 3.0" data processing program which is an advanced variant of "QUATTRO" , one graph (with no real data!) and one spreadsheet are demonstrated on pages 110-111.

Both programs (word and data processing) were introduced to Mr. Hang Xiao Dong.


Lysine fermentation
Product [g/l], dissolved oxygen [%]
pH [-], dP/dt [g/l*hours]
Fermentation time [hour]
0
6
12
18
24
30
36
42
48
54
60
66
72
78
84
90
96
102
108
114
120
0
20
40
60
80
100
120
140
0
2.5
5
7.5
pH
DO2
Product
dP/dt

Fermentation data

Time [hours]	pH [-]	DO2 [%]	Product [g/l]	DO2*1000 [mg/l]	dP/dt [g/l*h]
0	7.1	100	0	550	0
6	7.1	100	0	550	0
12	7	98	0	539	0
18	6.9	97	15	533.5	2.5
24	6.8	96	20	528	0.833333
30	6.5	96	20	528	0
36	6.4	95	24	522.5	0.666667
42	6.4	94	25	517	0.166667
48	6.3	80	26	440	0.166667
54	6.3	78	30	429	0.666667
60	6.35	68	38	374	1.333333
66	6.3	55	49	302.5	1.833333
72	6.2	54	59	297	1.666667
78	6.2	53	70	291.5	1.833333
84	6.3	54	89	297	3.166667
90	6.3	55	120	302.5	5.166667
96	6.4	53	126	291.5	1
102	6.5	52	130	286	0.666667
108	6.5	50	138	275	1.333333
114	6.55	43	140	236.5	0.333333
120	6.6	46	140	253	0

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Backstopping Officer's Technical Comments
based on the work of Mr. I. Balogh
DP/CPR/88/001/11-57

The expert's report gives a very good example for an extensive in-plant training course. By assessing and analyzing the production processes and equipment, as well as the activities in the microbiological laboratories, one can clearly understand the potentials and opportunities for immediate, medium and long term development in the Wuxi Enzyme Factory.

The prerequisite for this development is to establish a system for quality assurance. By building-in quality into the product, by introducing the good manufacturing practices the production consistency would improve.

Good manufacturing practices (GMP) not only mean computerization of the in-process control and production and final quality control administration, but first of all GMP changes the manufacturing strategy. The vision for this strategy is the quality.

All production and quality control procedures, techniques should be validated, but in biological production systems it can only be achieved if one builds up the product in compliance with the new regulations. At least the following systems should be validated:

1. Production facilities (buildings, equipment, utilities)
2. Personnel
3. Production strains
4. Production systems (up-stream and down-stream processing)
5. Formulation and fermentation (packaging)
6. Quality assurance and control systems (purity, efficacy, stability, etc.)

The report gives technical details on the enzyme activity screening systems, the strain improvement methods and the up-stream processing (fermentation) of the enzymes.

Parts of the report can even be used as training manual and, therefore, gives a very good example how, through UNIDO, generic information and know-how can be provided by a private firm to a company currently belonging to the Government sector. This know-how might be instrumented in the negotiations for establishing joint-ventures by the Wuxi Enzyme Factory in the future.