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ENZYME PRODUCTS DEVELOPMENT

DP/CPR/88/001

THE PEOPLE'S REPUBLIC OF CHINA

# Technical report: Improvement of strains and fermentation technology in the Wuxi Enzyme Factory *

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

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* 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:

- Improvement of a neutral protease producer Bacillus subtilis strain by mutagen treatment and selection; improvement of neutral protease technology
- Improvement of a cellulase production technology; elaboration of the proper strain maintenance and a selection method
- Lecture about the further possibilities and advantages of technology development
- Consultation about the proper piping of fermenter and seed tanks, emphasising the up-to-date solutions
- Consultation about the proper handling of the recently introduced softwares

The activity began on 28 May 1993 and lasted until 24 June 1993.

Main conclusions and recommendations:

According to the signs of inflation in The People's Republic of China, and the tendency of the economical opening up of the country, the Wuxi Enzyme Factory will encounter soon the problem of the economizing of its production processes. Therefore the development of strains and fermentation technology should be considered to be of crucial importance for the factory's survival.
- The staff of the strain laboratories should continue the strain improvement concerning alpha amylase, glucoamylase, neutral protease and cellulase strains. The improvement work is to be done on the basis of the previous mission and report ("Third Mission to the Wuxi Enzyme Factory: Technical Assesment, Improvement of Production Strains") and on the basis of the current results. The technical circumstances and personal conditions are satisfactory for this improvement.
- There are huge reserves as far as the possibility of technology improvement is educemed. Recently, however, there has not been any proper technical device offered regarding the technological development. This is why I recommend the purchasing or building of a versatile pilot fermenter system, or the renovation of the existing old pilot fermentation plant on the basis of the recommendations of the previous technical report.
- In the case of realizing the investment of the pilot fermenter quick and effective technology improvement should be started towards the elaboration of feed batch technology, which can provide higher fermentation yields and harvests.

# EXPLANATORY NOTES

GOD = glucose oxydase enzyme

CMC = carboxymethyl-cellulose

C1 = cellulase enzyme activity characterized by filter paper hydrolysation to glucose

Cx = cellulase enzyme activity characterized by carboxymethyl-cellulose hydrolysation to glucose

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

OTR = oxygen transfer rate [kg/m3*h]: this is a usual piece of data for characterizing the aeration potential of fermenters/bioreactors

NP = neutral protease

Y = fermentation yield: this gives information about the product quantity (weight or units) which is produced on the basis of one unit of substrate (weight) or one unit of substrate solution (volume)

GA = glucoamylase

BAA = bacterial alpha amylase

CSL = corn steep liquor

# I. INTRODUCTION

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

The location of the mission was the Wuxi Enzyme Factory (now partially Wuxi Synder Bio-Products Co, Ltd, 27 West Tong Hui Rd. Wuxi, 214035 Jiangsu, The People's Republic of China).

The activity began on 28 May 1993 and lasted until 24 June 1993. The present mission was a continuation of the work carried out between 10 November 1992 and 2 January 1993. The objectives of the mission are listed in the job description in the Annex - 1, (page 19).

The original aim of the current mission was to check the recent stage of the strain improvement work which was started during the previous mission. The main purpose of the mission was also to modify the previously elaborated strain improvement methods on the basis of the results gained during the last six months.

The original objectives were modified and new targets were defined after the discussion with Mr Huo Xing Yun deputy director. The reason for this change was that the previously started GA and BAA strain improvement was interrupted by the start up of the new joint venture which brought a reorganisation of the laboratories, tasks and personnel. The initial results of the GA and BAA strain improvement seem to be good and the continuous strain development work will lead to higher activities. Due to the delay, the GA and BAA strain improvement work has not reached yet that phase which would require any further modification.

Parallel with the GA and BAA improvement, the strain laboratory deals also with the improvement of the characteristics of a neutral protease enzyme producer strain (Bacillus subtilis) and its fermentation media.
The improvement of the cellulase enzyme fermentation technology and strain selection has been commenced in a separate laboratory.

The main task of this mission was to superwise and promote the improvement work of these laboratory sections (Annex - 2, page 22).

Concerning the neutral protease improvement, trials were made to develop the effectiveness of the monoculture selection method, and experiments were made for improving the laboratory scale shaking flask fermentation technology.

To facilitate the above improvements four types of cellulase producing strain were delivered ( sources: Technical University of Budapest and the Hungarian Type Culture Collection at the University of Horticulture and Food Industry, Budapest). One of them (Helminthosporium sativum) was used on an industrial scale and therefore could be considered a good producer (Annex-6, page 34).

The further work concerning cellulase was focussed on this strain by making an attempt to elaborate its good maintenance, selection and an appropriate laboratory scale fermentation technology.

As the production section can deal only with batch type fermentation technology, the laboratory flask-size fermentation results can be transferred directly to the production.

According to the modified program a lecture was given on the topic of fermentation technology improvement (Annex-8, page 37), and a consultation was made with the technical management about the proper piping of bioreactors (Annex - 9, page 51). During the lecture and consultation it was emphasised, that the strain improvement and technological development should be considered as inevitable requirements for the achievement of economic production, and consequently for the survival of the factory in the context of an open and competitive economy.

During this mission some advanced personal computer programs were installed as well. The use of the software was explained and shown to Mr Huang Xiao Dong (Technical Development Department) and to Mr Wan Jing Geng (Technical Development Department). The know how transfer was completed by a specific training programme (Annex-10, page 56).

During the previous mission a glucose oxydase producer strain was delivered. An accepted and accurate GOD assay method for this was introduced during the current mission (Annex-3, page 23).

All the objectives of the modified program were attained.

During the mission Mr Huang Xiao Dong facilitated the work by acting as interpretater.

## II. IMPROVEMENT OF NEUTRAL PROTEASE PRODUCER STRAIN, IMPROVEMENT OF FERMENTATION TECHNOLOGY

The improvement of the neutral protease producer Bacillus subtilis strain was started by NTG mutation using the method as it was elaborated previously ( see in the previous report, Annex - 11, pages 88-92) in the case of the BAA B. subtilis strain.

The Petri dish selection was made by using casein in the solid medium for obtaining clarification rings.

However, the comparatively quick growth of the strain hindered the reliable evaluation of the colonies.

The original (I) selection medium was modified for obtaining smaller colonies:

| | I | II | III |
|---|---|---|---|
| | (%) | (%) | (%) |
| NaCl | 0.2 | 0.2 | 0.2 |
| KH2PO4 | 0.25 | 0.25 | 0.25 |
| K2HPO4 | 0.25 | 0.25 | 0.25 |
| Na-caseinate | 1 | 1 | 1 |
| milk powder | 1 | 1 | - |
| glucose | 1 | - | - |
| (NH4)2SO4 | 0.5 | - | - |

pH=7.0

The incubation time was 40 hours at 30 degrees centigrade.

The first - original - variant gave big colonies; whereas the second and third ones gave small, compact ones. The clarification ring was well visible in every instance; in the cases of the I and II variants the clarification rings had a strong dark border. Seemingly, the origin of the border was connected with the presence of the milk powder. It was suggested that the second variant be used in the future, by reducing the milk powder amount to 0.5 % for obtaining a more thin, sharp border.

The same seed and fermentation media were used in the laboratory for the flask-size experiments and in large scale production as well (Annex - 7, page 36 ).

In the first production test, 40 colonies were tried in production flasks. After 36 hours shaking at 30 degrees centigrade and 250 rpm, the neutral protease activity was determined by the well known Folin method ( Annex - 5, page 30). The results showed a wide variation between 620 U/ml and 4682 U/ml. Such a variation could be expected after a mutagen treatment. However, there were 50-70% deviations between the parallel flasks. This was unacceptable; consequently the method was declared to not be appropriate in this form for testing the mutant monocultures. Unfortunately, no internal standard was available ( see in the previous technical report Annex - 12, page 93); therefore it could not be decided wether the mutant strains' instability or the unbalanced fermentation medium was the reason of the high deviations between the parallels.

Since the flasks were showing considerable foaming, and considering that the foam could completely prevent the aeration in shaking flasks, a short meeting was held on the necessity of foam control in flasks, and on the importance of the pH stabilization during shaking. Unfortunatelly, data were not available on the pH profile of the flask-size fermentations of neutral protease.

For the next experiments it was suggested using different volumes of defoamer in the flasks, and to use some pH stabilizer. It was also suggested having at least one flask inoculated by the parent strain (internal standard) so as to be able to compare the mutants' results. In the meantime, it was shown , that the increase in the ingredients' concentration would lead to an augmented final activity.

As a next step, the effect of the defoamer addition was checked with the original strain. In this instance the medium was the same as in the previous experiment.

Results:

| Defoamer (drop/flask) | pH | NP activity (U/ml) |
|---|---|---|
| 1 | 8.23 | 0 |
| 1 | 7.65 | 390 |
| 2 | 6.82 | 312 |
| 2 | 7.47 | 78 |
| 3 | 6.03 | 234 |
| 3 | 7.75 | 780 |

The results showed that, in this case, the defoamer had no considerable effect on production. Nevertheless, these results showed us that the unbalanced composition of the fermentation medium was responsible for the variation in the results and not the unstability of the new mutant colonies.

It was suggested using two drops of defoamer in the production flasks because, generally in the case of a quicker biosynthesis, the foaming was stronger.

The next experiment investigated the effect of increasing of the ingredients' concentrations:

The soybean meal and corn meal contents of the media were modified in four steps; the concentrations of all the other ingredients were the same as in the previous experiments. In the case of the fifth variant a Na2PO3 and NaHPO3 (0.4% each) buffer system was used for the purpose of the pH stabilization.

| | soybean meal (%) | corn meal (%) | pH | NP activity (U/ml) |
|---|---|---|---|---|
| I | 5 | 8 | 8.08 | 780 |
| | 5 | 8 | 8.41 | 780 |
| II | 5 | 10 | 7.45 | 2497 |
| | 5 | 10 | 6.45 | 2809 |
| III | 6 | 10 | 7.97 | 2497 |
| | 6 | 10 | 6.75 | 2575 |
| IV | 6 | 12 | 6.75 | 2731 |
| | 6 | 12 | 6.40 | 2965 |
| V | 6 | 12 | 6.60 | 3889 |
| | 6 | 12 | 6.74 | 3823 |

The results showed that the variation between the parallels became acceptable due to the modification of the composition of the medium. At the same time, the beneficial effect of the pH stabilizer could be detected, since the increasing concentrations gave a much better final activity than that of the original composition.

As Mr Dong reported, there was only a circular type shaker available for these experiments, and previously better results were obtained by using a reciprocal type shaker. This observation lead to the formulation of a hypothesis: that the aeration was not sufficient in our shaking conditions. According to this hypothesis a control experiment was carried out by shaking 50 ml and in parallel 35 and 20 ml of fermentation media in the 250 ml shaking flasks. It is well known that the only driving force of aeration in shaking flasks is the difference between the outside and inside O2 concentrations, given that the diffusion is the only process which transports O2 into the flask. The available surface (determined by the diameter of the flask's neck) and the O2 concentration values were approximately the same during the experiments, even if the constant transport of O2 was used in a smaller volume, the unit O2 supply would be bigger. (In the case of L-lysine fermentation 15 ml was used in 300 ml flasks.)

At the same time, some new variables were tried according to the fermentation media's composition. Parallel with this the volume of the buffer solution was modified and the effect of the alpha-amylase liquification before sterilisation was tested.

Experimental conditions

| | alpha-amylase | soybean: meal | corn: meal | sweet : potato | bran mash | volume | NaHPO4/ Na2HPO4 0.2 mol |
|---|---|---|---|---|---|---|---|
| | [+/-] | [%] | [%] | [%] | [%] | [ml] | [ml/ml] |
| I | - | 4 | 3 | 3 | 2.5 | 50 | - |
| II | + | 4 | 3 | 3 | 2.5 | 50 | - |
| III | - | 6 | - | 12 | - | 50 | - |
| IV | + | 6 | - | 12 | - | 50 | - |
| V | + | 4 | 4 | 4 | 0.5 | 50 | - |
| VI | + | 5 | 5 | 5 | 0.5 | 50 | - |
| VII | + | 6 | 12 | - | - | 50 | - |
| VIII | + | 6 | 12 | - | - | 35 | - |
| IX | + | 6 | 12 | - | - | 20 | - |
| X | + | 6 | 12 | - | - | 50 | 4/1 |
| XI | + | 6 | 12 | - | - | 50 | 3/2 |
| XII | + | 6 | 12 | - | - | 50 | 2.5/2.5 |

The shaking was performed at 32-33 degrees centigrade (instead of the prescribed 30), with 320-340 rpm, for 40 hours.

Results:

| | pH | NP activity [U/ml] |
|---|---|---|
| I | 8.58/8.13 | 312/3043 |
| II | 7.70/7.80 | 312/390 |
| III | 6.30/6.30 | 3121/2653 |
| IV | 6.40/6.40 | 3589/3433 |
| V | 6.40/7.10 | 3277/3902 |
| VI | 6.40/7.10 | 2887/3277 |
| VII | 6.40/6.40 | 4136/3277 |
| VIII | 6.40/6.40 | 2887/4370 |
| IX | 7.10/6.30 | 4448/4370 |
| X | 6.40/6.40 | 3667/3745 |
| XI | 6.40/6.40 | 4058/4370 |
| XII | 6.40/6.40 | 3980/4058 |

From the results it was obvious, that the original medium caused an unstable process and the variation between parallels was too high.

From the III and IV tests it could be seen that the alpha amylase treatment had a beneficial effect.

The V and VI tests showed that the sweet potato powder was not the best raw material for NP production.

The VII, VIII and IX tests gave the result that the reduction of the shaking volume had a good effect on production, and therefore the application of the 20 ml volume was suggested for the further strain selection work.

The results of the last three tests have shown that the phosphate content, (or the pH stabilisation) has a remarkable effect on production, and that the optimum should be obtained by further experiments.

In this way the strain improvement work has achieved a more reliable basis; and the better balanced selection media will enable the staff to select good yielding new strains.

There is one item among the technical devices which needs improving: the temperature regulation of the shaking room was not sufficient, it needs to be repaired by the maintenance department.

## III. IMPROVEMENT OF CELLULASE FERMENTATION TECHNOLOGY, SELECTION OF STRAINS

As a first task the strains were inoculated onto slant agars. Since malt extract was not available, the suggested medium was (the lack of unfermented beer) a 50 % beer containing solid medium (Unfermented beer is often used for the maintenance of moulds):

beer 500 ml
agar 20 g
filled up to 1000 ml by tap water
pH= 7.0

The growth of the strains (observed after three days incubation at 30 degrees centigrade) was satisfactory on this medium. This medium was suggested for the continuous maintenance of all strains.

After inoculating the strains onto slant agars, all further work was made with the most promising Helminthosporium sativum strain.

As an alternative, a new maintenance medium was elaborated for the H. sativum strain:

| | |
|---|---|
| bran | 5 g |
| rice straw powder | 10 g |
| crystal cellulose | 5 g |
| CMC | 5 g |
| $KH_2PO_4$ | 1 g |
| $(NH_4)_2SO_4$ | 2 g |
| agar | 20 g |

filled up to 1000 ml, pH= 7.0

It was expected that some passage on this medium would promote the activisation of the cellulase enzyme synthesis.
The growth on this solid medium (three days, 30 degrees centigrade) also was satisfactory.

The second step of the work was the testing of the seed flask fermentation (Annex-6 page 34). The original aim of this work was to determine the shaking time requested for reaching the late exponential phase of the growth. The growth was observed by microscope.

At the same time as an alternative possibility, two types of defoamer (PPE and BAPZ both of which are polyether based antifoam agents made in China) were tried to check the possible growth repression. The proper inoculation time could not be obtained from this experiment because of the formation of an approximatelly 4 cm diameter mycelial agglomerate in each flask. It must be mentioned here that the rotation speed of the shaker could not be adjusted accuratelly, and the temperature of the shaker room was 33 degrees centigrade ( the temperature requested would be 28 degrees centigrade). The air conditioner of the shaker room was repaired only after the first production test.

Changing the defoamer quality and the amounts used showed that both type of defoamer could be used without any growth repression.

In the next experiment the composition of the seed flask was changed for the elimination of the agglomeration phenomena. The composition of the modified seed flask medium was:

| | |
|---|---|
| bran | 20 g |
| soybean meal | 50 g |
| corn meal | 20 g |
| KCl | 0.5 g |
| MgSO4*7H2O | 0.5 g |
| KH2PO4 | 1 g |
| FeSO4 | 0.01 g |
| (NH4)2SO4 | 2 g |

Defoamer : 0-1-2 drops/flask
filled up to 1000 ml, pH=7
50 ml/500 ml flask

After the evaluation by microscopic observation ( samples were taken at 16, 20, and 24 hours) it was found that the proper shaking time is 20 hours, and one drop/ flask defoamer should be used in the future. The agglomeration was thus ceased, and the mycelial growth was found to be regular.

After optimizing the conditions of the seed flask, the next task was to elaborate a production fermentation medium. Unfortunately, there was no corn cob as a raw material available, therefore it was replaced by rice straw powder, which was known also to have a high cellulose content.

The composition of the modified production medium was the same as it is described in the Annex - 6 (page 34), with the difference that the corn cob was replaced by rice straw powder.

The fermentation experiment showed a repressed growth in the 64 hours age, with an extremely high (8.4) pH. (The normal pH pattern is a descending profile from approximately 5.2 to 4.0-4.2.) It was thought then that the reason for the failure could be the abnormally high temperature in the shaker room. Practically no cellulase enzyme activity could be detected in the fermentation media.

In the meantime a brief consultation was held about the handling and selection of the H. sativum strain. It was emphasized that the strain had been stored for more than five years in a refrigerator without any reinoculation; that is why it might be not homogenuous or the activity could be decreased significantly. To restore the activity a number of "passage" on the modified maintenance medium would be beneficial. The selection method should be elaborated by using CMC as a turbid ingredient in the selection agar medium. This strain has a quick growth capacity, so practically it can overgrow the CMC clarification ring made by the cellulase enzymes. The proper solution for getting compact colonies is to modify the selection medium. It was made previously by using congo-red stain as an ingredient in the medium. The proper concentration should be obtained experimentally. In the literature there are remarks ( see in the previous technical report Annex - 18, page 112, 2./ Hopwood D. A.: " The Isolation of Mutants", a copy was given to the Technical Development Departement) about the benefit of using sodium-deoxycholate for this purpose. The third principal way can be the partial elimination of the easily available sources from the medium. The colony selection should be completed by shaking fermentation tests. The strain gained according to the above method can be mutated by UV or NTG. It was suggested starting with UV mutation.

The air conditioner was repaired at this stage; that is why the next experiment was made at the prescribed 28 degrees centigrade.

Before the next fermentation trial the strain was transferred for the second time again onto the modified solid maintenace medium. It was observed that in this case the growth of the

microbe was considerably faster; the requested incubation time was in fact only two days.

The fermentation trial was made on four type of fermentation media:

| | I | II | III | IV |
|---|---|---|---|---|
| | (g) | (g) | (g) | (g) |
| soybean meal | 10 | 10 | 10 | 20 |
| straw powder | 15 | 10 | 5 | 15 |
| bran | - | 20 | 30 | 40 |
| CaCl2 | 0.3 | 0.3 | 0.3 | 0.3 |
| (NH4)2SO4 | 1.2 | 1.2 | 1.2 | 1.2 |
| KH2PO4 | 1 | 1 | 1 | 1 |
| MgSO4*7H2O | 0.5 | 0.5 | 0.5 | 0.5 |

Two drops of defoamer were added to each flask. The flasks were inoculated with 5% volume 21 hours maturated seed.

The pH was measured at 48 hours,

The pH and cellulase activity were measured at 72 and 96 hours.

Results:

| | 48h | 72h | | 96h | |
|---|---|---|---|---|---|
| | pH | pH | C1/Cx [mg/ml*h] | pH | C1/Cx [mg/ml*h] |
| I | 8.0 | 8.2 | 94.3/ 88.2 | 8.25 | 4.1/ 10 |
| II | 5.1 | 7.6 | 132.3/ 397 | 8.15 | 5.3/ 10.9 |
| III | 5.1 | 5.3 | 147/ 1161.3 | 8.25 | 5.3/ 14.1 |
| IV | 7.4 | 8.3 | 102.9/ 0 | 8.6 | 5.9/ 10 |

The results showed that the rice straw had no effect on the enzyme activity and that decreasing concentrations gave the better results. The quick rise of the pH could be explained by the overgrowth of the mycelia, this was probably because the nitrogen content of the media was higher than optimal.

An assay was made for the first time here, that is why the results showed signs of inaccuracy. The dilution rate was not selected properly. During the test the enzymatic reaction should have been carried out by using a low speed shaker thermostate (40 degrees centigrade). It is very important mainly in the case of the C1 (filter-paper activity) measurement, because a solid and a liquid phase should be reacted. The assay laboratory has no such shaker-water bath, therefore it was suggested to shake well the reaction mixtures every five minutes during the reaction. Nevertheless, the C1 results cannot be as high even in this way as they would be in the case of proper shaking.

In this phase of the work, corn cob was procured through personal connections from the countryside.

On the basis of the last reults a new series of fermentation tests was designed.

The fermentation trial was made on six type of fermentation media:

| | I (g) | II (g) | III (g) | IV (g) | V (g) | VI (g) |
|---|---|---|---|---|---|---|
| soybean meal | 10 | 10 | 10 | 20 | 20 | 20 |
| corn cob | 15 | 15 | 15 | 30 | 30 | 30 |
| bran | - | 5 | 5 | - | 10 | 10 |
| straw | - | - | 5 | - | - | 5 |
| CaCl2 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| (NH4)2SO4 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |
| MgSO4*7H2O | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| KH2PO4 | 1 | 1 | 1 | 1 | 1 | 1 |

Two drops of defoamer was added to every flask.
The production flasks were inoculated with 5% of 20 hours maturated seed.

The pH and cellulase enzyme activities were measured at 72 and 96 hours age:

| | 48 h | | 72 h | | 96 h | |
|---|---|---|---|---|---|---|
| | pH | C1/Cx | pH | C1/Cx | pH | C1/Cx |
| I | 5.1 | -/1088 | 5.8 | 106/529 | 5.6 | 23.5/340 |
| II | 4.4 | -/882 | 4.9 | 82/576 | 6.0 | -/541 |
| III | 4.5 | -/941 | 5.1 | 94/553 | 7.4 | -/494 |
| IV | 8.2 | -/- | 8.5 | -/82 | 8.2 | -/- |
| V | 8.2 | -/176 | 8.5 | 11.8/47 | 8.5 | -/- |
| VI | 8.3 | -/294 | 8.3 | 23.5/117 | 8.3 | -/- |

The results showed that in the case of the concentrated media (IV, V and VI) the soybean content should be decreased to 10 g. Probably this change of composition would prevent the extreme increase of the pH during the flask fermentation by modifying the C/N ratio. (The carbon source in this case was in solid phase, that is why the increasing of the corn cob amount would not be reasonable for this purpose; the proportion of the C source could not be increased on this way.)

As the results did not reach the required level, it is recommended therefore that the following changes should be made:

Firstly, in line with the results obtained by applying the rice straw as raw material, the modified maintenance medium should be changed: the rice straw should be replaced by the equal amount of corn cob. For future work it was suggested making at least 10 passages on this modified maintenance slant agar. After this the cellulase production capacity should be checked again ( by comparing it with the non passaged strain).
Anyway, independently of the results the strain should be selected after the passages have been made. The selection should be made on CMC-Na-deoxycholate or CMC-congo red agar dishes. (The congo-red or deoxycholate stains had not, it should be noted, been purchased until the end of the mission.)

Secondly, the original seed media (first and second consequently) should be tested again. To prevent agglomerate formation, a reciprocal shaker should be applied. Hopefully more vigorous shaking would prevent the agglomerate generation. The other possibility is to increase the corn cob or bran mash content of the medium.) The reason for these changes is the need for the application of a starch free inoculation step before the main fermentation since, given the previous observations, this helps activating the cellulase enzyme synthesis.

Unfortunatelly these experiments could not be made because of the shortage of time, and of the initial obstacles encountered (the temperature of the shaker room and the lack of the raw material corn cob).

After reaching the result of 1000-2000 C1 activity, an industrial experiment can be performed. The recovery of the fermentation liquid should contain one filtration step- this can easily be made by using frame filters- and a concentration step by using 10000 COV UF unit. The product should be preserved by the addition of Na-benzoate (0.5%).

# IV. RECOMMENDATIONS

In the competitive conditions of an open market-economy both the improvements of strains and technology are the most important considerations for the Wuxi Enzyme Factory (and for the Wuxi Synder Bio-Products Co. Ltd.'s as well!) to ensure its continuous succesful existence into the foreseeable future.

## A. Investment for strain improvement

For the time being the strain laboratories are equipped with good shakers, reliable clean benches and all the facilities required to ensure reasonable and succesful strain improvement. The only lacking device is the microscope of an acceptable quality. Therefore it is recommended that a good, reliable Nikon model (Labophot 2) with a moderate price should be purchased (see detailed specification in the previous technical report, Annex 10, page 87 ).

Point A should be an investment from the side of UNDP within the framework of the current project.

## B. Investment for technology improvement

There is not a proper facility for technology improvement in the Wuxi Enzyme Factory. Thus I suggest investing in some necessary equipment for this purpose. Hence it would be useful to renovate and rebuild the stainless steel fermenter pilot plant (see in the previous technical report Recommendations, page 19, Annex - 8, pages 83-85), or else to invest in a new stainless steel pilot fermentation system.

Point B should also be an investment from the side of UNDP within the framework of the current project.

C. Improvement of fermentation technology

All the fermentation technology in this factory is of the batch type. The most up-to-date possibilities for the process direction are the pH control - manual and occasional - and a surplus medium addition manually from separate tanks.

The sugar-based feed batch technology gives multiple results compared with the batch type (see in the previous technical report, Annex-5, pages 32-50, and in this report, Annex-8, pages 37-50). Therefore the commencing of a state of the art fermentation-technology-focussed development is highly recommended.

Point C can be accomplished in the framework of a new UNIDO development project.

V. ANNEXES

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION

## JOB DESCRIPTION

DP/CPR/88/001/11-57

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

**Duration** 1 m/m

**Date required** 2nd Quarter 1993

**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

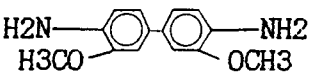
1. Neutral protease - strain improvement, selection
2. Neutral protease - technology improvement
3. Cellulase - improvement of selection and maintenance method
4. Cellulase - improvement of fermentation technology
5. Lecture - improvement methods for feed batch technology
6. Consultation - piping of fermenter and seed tanks
7. Handover of strains
8. Handover of softwares

# DETERMINATION OF GLUCOSE OXYDASE ENZYME ACTIVITY

The determination method is based on the assay method used at the Sigma Chemical Company.

The principle is the measurement of the H2O2 generated during the enzymatic oxydation of the glucose.

D-glucose + O2 + H2O ----GOD---- D-gluconic acid + H2O2

H2O2 +  ----POD---- O-dianizidin (oxydised, polymer, red colour)

/O-dianizidine/ + H2O

GOD= glucose oxydase
POD= horse radish peroxydase

Reagents:

1. 0.05 mol/l sodium acetate buffer solution, pH=5.1

2. Chromogen solution: 1 ml of 0.21 mmol/l O-dianizidine solution is filled up to 100 ml with pH=5.1 0.05 mol/l sodium acetate buffer.

3. D-glucose solution: 1.0 g glucose is dissolved in 10 ml distilled water.

4. Peroxydase solution: 5 mg of horse radish peroxydase enzyme powder should be dissolved in 10 ml distilled water.

Analytical method:

2.4 ml chromogen solution, 0.5 ml glucose solution and 0.1 ml peroxydase solution is measured into a spectrophotometry cuvette. The small vessel is temperated in 35 degree centigrade water bath. 0.1 ml of glucose oxidase enzyme solution is added to the mixture, and is quickly shaken.

The changing of the absorbancy (500 nm) is followed by using the spectrophotometer. The duration is 2-4 minutes.

The GOD enzymatic activity is calculated.

Calculation method:

$$\frac{(A2-A1)*3.1\ [nm/min]}{7.5*\ 0.1\ [cm3\ enzyme]} = \text{GOD activity [ mol unit/cm3]}$$

Where A2, A1 are absorbancies, 3.1 cm3 is the volume of the reaction mixture; 7.5 is the O-dianizidin's molaric absorbancy at 500 nm.

Other information:

1.
The GOD enzyme is an intracellular enzyme of the Aspergillus niger, that is why it can not be harvested from fermentation broth directly. The separated mycelium should be destroyed (using an ultrasonic, cylinder type or ball-mill type homogenizer) and filtrated consequently. The liquid filtrate contains the glucose oxydase enzyme.

2.
GOD can be used for the partial or total elimination of glucose or the biochemical elimination of the oxygen in several food products. In the case of applications GOD functions as a biochemical stabilizer hindering the oxidation processes and the aerob contaminant's growth.

Examples:

- glucose removal during egg-powder production
- dried, packed food products (antioxydant) as milk powder, several nuts, coffee etc.
- fruit juices, drinks (antioxydant)
- vine industry (antioxydant)
- brewery (antioxidant)

It should be noted also that GOD is indispensable in the manufacturing of several medical diagnostic kits (blood glucose assay).

# DETERMINATION OF CELLULASE ENZYME ACTIVITY

Cellulase activity is simultaneously measured by two methods using filter paper and carboxymethyl-cellulose hydrolysis. Both methods are based on the measurement of the glucose generated by the Somogyi method.
The cellulase enzyme preparates are usually characterised according to the above methods by the first (C1) and the second (Cx) types of activities.

Reagents:

- Somogyi reagent

| | |
|---|---|
| Potassium-sodium-tartrate | 30 g |
| Sodium-carbonate (dry) | 30 g |
| NaOH solution (1 N) | 40 ml |
| CuSO4*5H2O solution (10%) | 80 ml |
| Na2SO4 (dry) | 180 g |
| KIO3 solution (1 N) [3.567g/100ml] | 12 ml |
| KI | 8 g |

The potassium-sodium-tartrate and the sodium-carbonate respectively are dissolved in 200 ml hot water; then the NaOH is subsequently added. The CuSO4 is dissolved separately, and the solution is poured slowly into the above mixture whilst stirring. The liquid should be boiled to get rid of the dissolved air.The dry Na2SO4 should be dissolved in 500 ml hot water, and should be boiled to eliminate the traces of oxygen as well. After boiling, the Na2SO4 containing liquid is added to the main part of the reagent's mixture. Finally, the previously dissolved KI and KIO3 should be added to the liquid, then the solution should be filled up to 1000 ml with distilled water.

- 0.1 N Na2S2O3 measuring solution

25 g of Na2S2O3 and 200 mg Na2CO3 are dissolved in 800 ml boiled and subsequently cooled water. In order to prevent microbiological contamination 10 ml of isobutyl-alcohol or 10 ml of amyl-alcohol is added to the solution. The mixture is filled up with cold previously boiled water to 1000 ml. This solution should be stored in a dark cool place.

The Na2S2O3 solution is to be standardized by titration of 10 ml 0.1 N KH(IO3)2 solution using starch solution as the endpoint indicator. The 0.01 N solution is made by 10 times dilution.

- 2 N H2SO4

56 ml concentrated sulphuric acid is added slowly to 800 ml distilled water by gentle, continuous stirring. The solution is to be cooled to 25 degrees centigrade, and filled up to 1000 ml with distilled water.

- 0.05 N acetate buffer ( pH 4.5 )

a./ 0.05 N acetic acid: 2.95 ml of cc. acetic acid should be filled up to 1000 ml with distilled water.
b./ 0.05 N sodium-acetate: 6.8 g sodium-acetate*3H2O is dissolved in water and filled up to 1000 ml with distilled water.

The mixing ratio of the above solutions is two parts of the a./ solution and one part of the b./ solution are mixed properly and the pH is adjusted carefully to 4.5 using acetic acid or NaOH.

- 1 % filter paper suspension

2 g filter paper should be homogenized in 150 ml of distilled water ( the suggested rotation speed of the mixer is 10,000 rpm) for 60 minutes. After homogenization the suspension should be filled up to 200 ml with distilled water.

- 1 % carboxymethyl-cellulose ( CMC ) solution

1 g CMC is transferred into a 100 ml measuring flask. The flask is filled up to 75 % with distilled water and stored at 20-22 degrees centigrade for 24 hours. Then after shaking it is filled up to the 100 ml label with distilled water and the liquid is to be filtered by using a G1 sintered glass filter. The solution can be stored in a refrigerator for 4-5 days.

- 1 % starch solution

1 g soluble starch is mixed in 20 ml cold water and poured into 60 ml boiling water. After cooling and adding two drops of isobutyl- or amyl-alcohol the solution is filled up to 100 ml with distilled water.

Determination of the cellulase activities:

The enzyme activities are determined by measuring the glucose amount which was generated at 40 degrees centigrade over a one hour period.
The activity is expressed as mg glucose/hour units.

For the C1 activity measurement 5 ml 1 % filter paper solution is added into a 150 ml Erlenmeyer flask and 3 ml 0.05 N acetate buffer is added. After incubating the solution in a 40 degree centigrade water bath in order to reach the reaction temperature required, the one ml enzyme solution is introduced. The reaction mixture is to be incubated for 1 hour at 40 degrees centigrade on a shaker ( at least 130 rpm ). Simultaneously a blind sample is prepared and shaken by adding quickly after the enzyme addition a surplus of 1 ml 1 N NaOH solution to the substrate-buffer-enzyme solution. The blind samples are shaken together with the enzyme samples for one hour. The reaction is to be stopped in the flask by adding the 1 ml 1 N NaOH solution at the end of the one hour reaction period. The generated glucose is to be measured by the Somogyi method.

For the Cx activity measurement the reaction circumstances and method are the same as in the case of C1 activity determination, the only difference is the 1 % CMC solution has to be used instead of the filter paper suspension.

Glucose measurement:

5 ml of enzyme reaction mixture sample (ES=enzyme sample) is introduced into a 50 ml wide-necked flask. It is neutralized by using phenolphtalein indicator with 1 N HCl, and 5 ml of Somogyi reagent is given.

Instead of the above mixture 5 ml blind enzyme sample (BE=blind enzyme) is introduced into a separate flask with neutralization and Somogyi reagent as well.

All flasks are kept in boiling water for 15 minutes. After this boiling period the flasks should be cooled using cold water as quickly as possible, and 2 ml 2 N $H_2SO_4$ is added to the mixtures.

All liquids are titrated using 0.01 N $Na_2S_2O_3$ solution; the endpoint should be detected using a starch indicator.

Calculation method

C1 or Cx [glucose mg/ml enzyme sample*hour]=BG(mg/h)-EG(mg/h)

where BG ( blind glucose ) = BE*f*0.294*dilution
and EG ( enzyme glucose) = ES*f*0.294*dilution

f = adjusting factor
BE = ml Na2S2O3 for the blind sample
ES = ml Na2S2O3 for the enzyme sample
dilution = should be calculated on the basis of the originally measured enzyme sample into the reaction mixture ( 1 ml enzyme to the final 10 ml -10*- and as taking 5 ml of the inactivated 10 ml mixture: 10*2=20, so in this case the dilution=20. If a smaller or bigger amount of the enzyme samples are used this number needs to be changed)
0.294 = 1 ml 0.01 N Na2S2O3 measures 0.294 mg glucose

# DETERMINATION OF NEUTRAL PROTEASE ACTIVITY

(Translated and typed by the help of Mr Huang Xiao Dong, Dept. of Technical Development)

## 1. Principle

The neutral protease reacts with casein at a certain pH and temperature. Trichloroacetic acid is added to terminate the reaction and for the precipitation of the residual casein. After filtration and the addition of sodium carbonate Folin reagent is added to the mixture. The developed colour intensity is measured using spectrophotometer. The activity can be calculated on the basis of the extinction data.

## 2. Reagents

A. Folin reagent

100 g Na2WO4.2H2O, 25 g Na2MoO4.2H2O, 700 ml distilled water, 50 ml 85% H3PO4, 100ml concentrated HCL are to be added to a 2000 ml flask equipped with a reflux cooler device. The reaction mixture should be boiled slowly for 10 hours. Then 50 g Li2SO4, 50 ml of distilled water and several drops of thick Br2 solution should be added and shaken. It is boiled again for 15 minutes in a ventilated bench in order to remove sulplus Br2. Then it is filled up to 1000 ml with distilled water and filtered. This golden reagent should be stored in a brown glass vessel. During the operation, 1 part of Folin solution and 2 parts of distilled water are mixed well.

B. 0.4N sodium-carbonate

42.4 g anhydrous sodium carbonate is diluted and filled up to 1000 ml with distilled water.

C. 0.4M trichloroacetic acid

65.4 g trichloroacetic acid is diluted and filled up to 1000 ml with distilled water.

D. buffer solution

0.02M pH 7.5 H3PO4 buffer solution (for neutral protease only) 6.02 g disodium phosphate with 12 crystal water and 0.5 g monosodium phosphate with 2 crystal water is diluted in distilled water and filled up to 1000 ml.

E. casein solution

Accurately weigh 2.000 g casein. Prior to adding proper buffer solution, 0.5N NaOH should be added. The solution is heated in a boiling waterbath and solubilized completely. After cooling it is transferred to a 100 ml volumetric flask and filled up to 100 ml with buffer.

F. 100 ug/ml standard tyrosine solution

0.1 g dehydrated L-tyrosine, should be slowly put into 6 ml 1N HCL and filled up to 100 ml with 0.2N HCL ( 1mg/ml tyrosine solution). Withdraw 10 ml of the solution and then fill it up to 100 ml with 0.2N HCL and gain 100 ug/ml standard tyrosine solution.

3. standard curve

In order to find the relationship between tyrosine solutions and extinction values, first of all, various concentrated tyrosine solutions should be used for reacting with Folin reagent and measure the extinction value. According to these, standard curves can be drawn.

(1). According to the table, various concentrated standard tyrosine solutions should be prepared.

| | C(tyrosine) ug/ml | V( 100ug/ml TS) ml | V(DW) ml |
|---|---|---|---|
| Comparison | 0 | 0 | 10 |
| 1 | 10 | 1 | 9 |
| 2 | 20 | 2 | 8 |
| 3 | 30 | 3 | 7 |
| 4 | 40 | 4 | 6 |
| 5 | 50 | 5 | 5 |
| 6 | 60 | 6 | 4 |

(2). Separately pippet 1 ml of the above tyrosine solution (parallel experiments should be carried out), 5ml 0.4M sodium carbonate and Folin reagent. Keep the reaction mixture for 20 minutes in a 40 degree centigrate waterbath. The extinction is measured at 680 nm wavelength. For blank comparison 0.2N HCL is used. On the basis of these data a standard curve will be drawn.

4.Procedure

1). Preparation

Weigh 2.000 g enzyme accurately, dissolve with buffer solution, and then grind with a glass rod. The supernatant should be poured into a 100 ml volumetric flask; the residue should be dissolved with buffer solution again. This procedure should be repeated until complete dissolving is obtained. The solution should be filled up to 100 ml. The solution is filtered and diluted to a proper rate in accordance with enzyme activity. (The dilution rate should give extinction values in the range of 0.2 to 0.4.)

2). Reference table for dilution rate of neutral protease (powder form)

| Activity | Dilution Rate | First DR | Second DR |
|---|---|---|---|
| 30000 | 2500 | 2g--500ml(250) | 5ml--50ml(10) |
| 40000 | 4000 | 2g--200ml(100) | 5ml--200ml(40) |
| 50000 | 5000 | 2g--500ml(250) | 5ml--100ml(20) |
| 60000 | 5000 | 2g--500ml(250) | 5ml--100ml(20) |
| 100000 | 10000 | 2g--500ml(250) | 5ml--200ml(40) |

3). Assay

a. Put the casein solution into a 40 centigrade waterbath for 3 to 5 minutes.

b. Proceed as follows:

1 ml sample-----preheated for 1-2 min at 40 C waterbath
+
1ml 2% casein solution reaction time: 10 minutes

2ml trichloroacetic acid----shake immediately
filtered after 10 minutes
|
filtrate 1 ml
+
5 ml Na2CO3 solution
+
1 ml Folin reagent-shake and put into a 40 degree centigrade waterbath for 20 minutes
|
measurement at 680 um

For blank comparison, trichloroacetic acid is added first into the sample and then the casein is added.

5. Calculation

An enzyme unit is that amount of enzyme which can liberate 1 ug tyrosine by hydrolysing casein within one minute under the conditions of the assay.

$$A=4/10 * K * E * n$$

where: 4 - Total volume of reaction reagent
10 - Reaction time
K - Colorimeter coefficient which refers to equivalent ug of tyrosine as E is 1.
n - dilution rate

6.Notice

(1) The preparation of casein solution should be strictly carried out, otherwise it will affect the assay results.

(2) For the sake of reliability, casein should be used as a chemical pure reagent produced by Shanghai No.1 Dairy Factory. If using other brand products, a comparable test should be made.

# HANDOVER OF STRAINS, MAINTENANCE AND SUGGESTED MEDIA FOR CELLULASE PRODUCTION

Four strains were handed over:

Trichoderma viridae
Trichoderma reesei
Trichotecium roseum
Helminthosporium sativum

Maintenance suggested: all strains should be maintained on malt agar (slant in test tubes). The incubation period is three days in a 28 degree centigrade termostate. The maturated slants can be stored at 5 degrees centigrade for six months, then they should be transferred to new slant agars.

The Helminthosporium sativum strain was used for industrial scale production in Hungary at Phylaxia Ltd. The production was not really large-scale because of the limited market demands. Recently the cellulase enzyme product of this strain has been used as a component of feed for poultry and swine.

Composition of the seed flask: (g)

| | |
|---|---|
| Bran mash | 20 |
| Sucrose | 10 |
| Corn steep liquor powder | 2.5 |
| KCl | 0.5 |
| MgSO4*7H2O | 0.5 |
| KH2PO4 | 1 |
| FeSO4 | 0.01 |
| (NH4)2SO4 | 2 |
| Defoamer | 0.3 |

pH=7.0, volume 1000 ml, 50ml/500 ml shaking flask
24 hrs, 250 rpm, 28-30 degrees centigrade

| Composition of the industrial seed: | (kg) |
|---|---|
| Bran mash | 120 |
| CaCO3 | 9 |
| (NH4)2SO4 | 6 |
| KH2PO4 | 5 |
| Defoamer | 8 |

pH is not adjusted, volume 3.5 m3
24 hrs, 28-30 degrees centigrade

| Composition of production medium: | (kg) |
|---|---|
| Soybean meal | 200 |
| Corn cob (milled) | 300 |
| CaCl2 | 6 |
| (NH4)2SO4 | 24 |
| Co(NO3)2 | 0.22 |
| KH2PO4 | 20 |
| MgSO4*7H2O | 10 |
| Defoamer | 25 |

pH is not adjusted, volume 20 m3
96 hrs, 28-30 degrees centigrade

# FERMENTATION MEDIA FOR NEUTRAL PROTEASE

| | Seed: | Production: |
|---|---|---|
| | [%] | [%] |
| Soybean meal | 3 | 4 |
| Sweet potato powder | 3 | 5.5 |
| Bran mash | 2 | 3.5 |
| Na2HPO4 | 0.4 | 0.4 |
| K2HPO4 | 0.03 | 0.03 |
| Soybean oil | 0.5 | 1-2 |

The same seed and production media were used for the industrial production and for the laboratory scale experiments (shaking flasks) as well.

# LECTURE ON ADVANCED FERMENTATION TECHNOLOGY (A NEW APPROACH: REPEATED FEED BATCH)

/Summary/

The feed batch fermentation technology is a combination of the continuous and batchwise fermentation technology. Before the complete filling up of the fermentation tank is carried out there is a possibility to harvest nearly maturated fermentation liquid with a considerable product content. In this way, we can feed further our culture, and we have a considerable excess fermentation volume due to the partial harvest (broth-out). By continuing the process with a lower volume, the mass transfer possibilities are usually better, and by properly diluting the remaining part of the fermentation broth there are further advantages, such as: lower product concentration, lower osmotic pressure, lower viscosity and a comparatively younger physiological stage of the culture.

In introducing the main topics, a brief explanation was given regarding the optimization principles of a batch fermentation technology, the proper way to develop a feed batch technology using the basis of the well optimized batch technology.

A short explanation was given concerning the principal and practical advantages of the feed batch technology comparing it with the perfectly optimized batch technology.

It was, shown how to get higher results by using feed batch technology, and how to use the possible methods for the optimization.

Being the main topic of the lecture, the repeated feed batch technology was introduced as the most economical way of developing further a well designed and optimized feed batch technology.

As a matter of crucial importance there are two principal strategies for constructing a repeated feed batch technology.

The first is based on calculation methods, observations and on the evaluation of the physiological stage at the time of the partial broth-out. Calculation methods were explained for designing the proper broth-out time, the broth-out volume, the dilution water/liquid volume and the feed rate after the partial broth-out. The role of the dilution was emphasized during the lecture as a good tool for decreasing the medium viscosity, the osmotic pressure and the product concentration. These changes are leading to a better fermentation yield after the broth-out.

An explanation was given regarding the possibilities of shortening the fermentation time as a compensation of the excess duration. By using this first approach the result comes from a gradual modification of the original feed batch technology.

According to the second strategy or approach, the process is scaled up by a proper multiplication factor and leads to a result in which, at the time of the partial broth-out, we have already reached the available maximal liquid volume of the fermentation tank. After finishing the process completely we reach the maximal free volume again. Consequently, it means principally that this method gives the highest result.

In reality, there are a number of factors which have been hindering the realisation of the designed process. The extention of the capacity is limited mainly by the available oxygen transfer rate (OTR) or the oxygen/mass transfer coefficient Kla.

At Agroferm Ltd, Hungary, the fermentation harvest was increased by approximately 10 % (2000 kg / batch) within the same fermentation time. The fermentation harvest reached an average 24000 kg with peaks of 25-26000 kg but this usually depended on the quality of the main raw material: sugar beet molasses.

At Biogal Ltd, Hungary, penicillin production was increased in the similar - repeated feed batch - way. In this case the broth-out time was relatively sooner (at a younger physiological stage) because of the danger of the irreversible overmaturation of the Penicillium mycélia. The number of repetitions can be higher in the case of antibiotics fermentation because the contamination is limited, the antibiotic producers can "defend" themselves to some extent.

# ADVANCED FERMENTATION TECHNOLOGY

## (A NEW APPROACH: REPEATED FEED BACH)

I. Structure of lecture:

I. Introduction

1./ Optimization of batch technology
2./ Batch ----- Feed batch
3./ Optimization of feed batch technology

II. Repeated feed batch technology

1./ First approach: calculation and design
2./ Second approach: scaling up

III. Advantages, results - limiting factors, obstacles

## I. INTRODUCTION

### 1./ Optimization of batch technology

Tasks are: To improve

Inoculation -
- optimal maturation (exponential phase and highest density))
- optimal volume

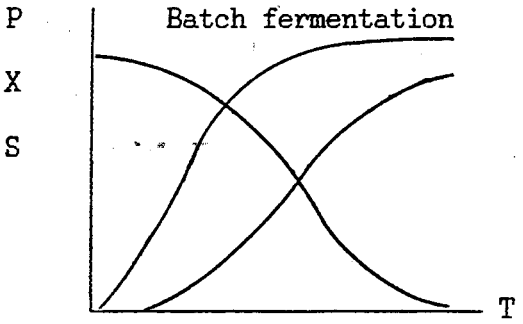
Ratios of substrates
- $S_{1...n-1} / S_{2...n}$

Concentration of substrates
- $\text{Sum} ( S_i )_{i=1-n}$

Aeration

pH

Temperature




2./ Batch -- Feed batch

Method of design: At $(dp/dt)_{max}$ point
$S_{1-..n}$ , $DO_2$
levels are obtained
on this basis we design

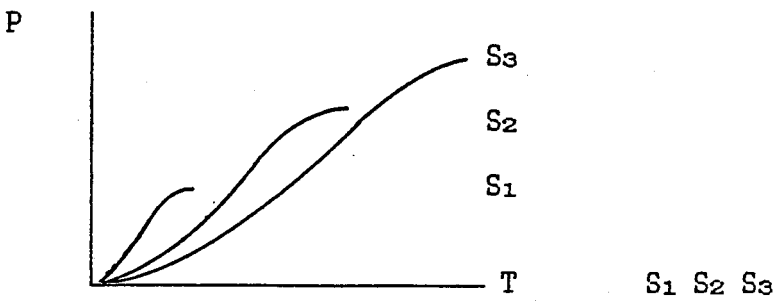
Start/Feed ratio
Volume
Substrate concentrations

Dilution rate
depends on 1/x*dx/dt

Feed rate
depends on $dS_1/dt$

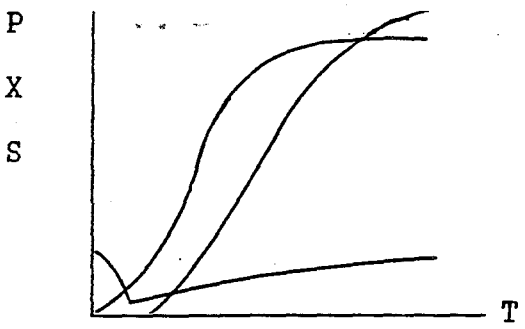
Principle: A./ low sugar/starch concentration
reason: catabolic repression)
B./ high overall sugar/starch input
(by continuous feeding)

Principle A:



Principle B

Feed batch fermentation




The overall S input is very high!

3./ Optimization of feed batch technology

Tasks is: To improve

Ratios of substrates
$S_{1...n-1} / S_{2...n}$
Concentration of substrates
Sum ( $S_i$ )$_{i=1-n}$
Feed rate time-profil
Aeration rate time-profile
PH and T profiles

Aim: the possible highest harvest
within the shortest time

II. REPEATED FEED BATCH TECHNOLOGY

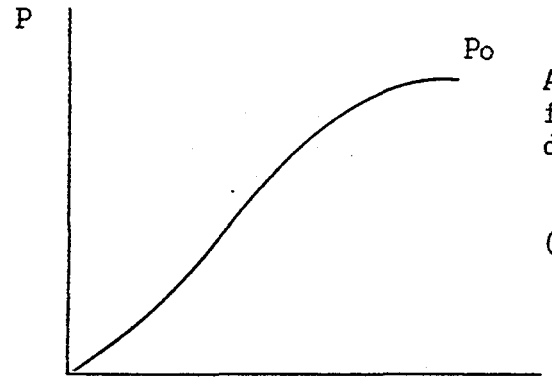
1./ First approach: calculation and design

After optimization - HOW TO GO FURTHER?!
SOLUTION: REPEATED FEED BATCH TECHNOLOGY

POSSIBILITY:

AT THE LAST PHASE OF THE WELL DESIGNED
WELL OPTIMIZED
TECHNOLOGY
THE PRODUCTION RATE DECREASES!!!

OUR TASK IS TO RESTORE THE PRODUCTION RATE




At the last phase of the fermentation, the yield decreases!

( dP/dt is smaller )

If we increase the last phase's yield or production rate:

The harvest from one batch and the factory fermentation yield (FFY) increases !

Principle : to return to a previous physiological stage ( with higher dP/dt) BY DILUTION! BY DECREASING THE FERMENTATION VOLUME

Method : stop before final phase, partial broth-out, dilution, by water feed further


$V_{out}$ = partial broth-out
$V_w$ = dilution water
Vin = feed further

Role of dilution water:

- osmotic pressure
- $K_1a$ (mass, $O_2$ transfer)
- viscosity
- product concentration

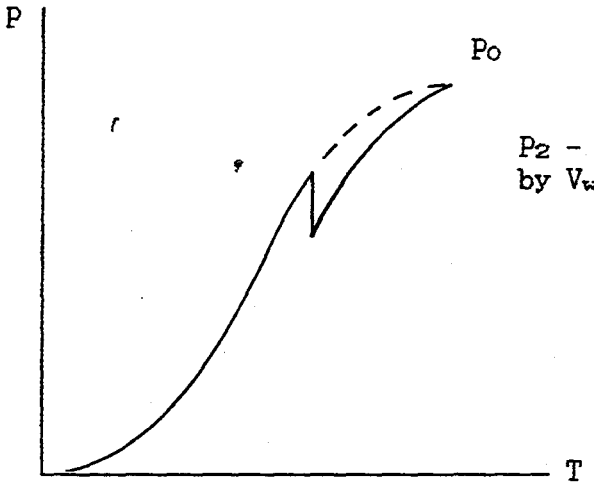
(these factors are hindering production )

It is important to chose the optimal time of broth-out ($T_{bo}$)

Points of view:

economic
- dP/dt should be high enough for extending the high yielding production phase
- P should be high enough for getting big harvest with the partial broth-out

physiological
- activity should be high enough to be able to maintain the high yielding production phase
- X should be high enough to be able to feed further quickly



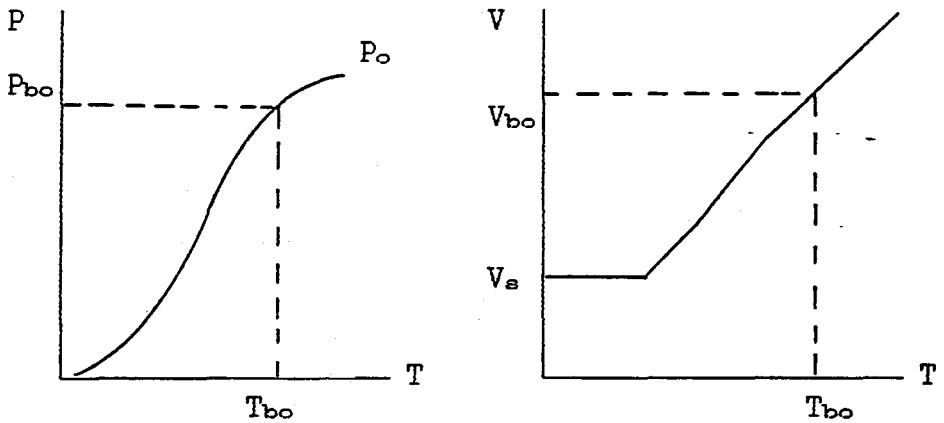

$P_2 - P_1$ = From dilution by $V_w$ dilution water

1./The partial broth-out gives a surplus of harvest
2./After the partial broth-out the
-specific production rate 1/x*(dP/dt) is bigger
-yield (Y) is higher

How to design?

Firstly:

Let's define the exact
point of the broth-out!
(on the basis of the above considerations)




During calculations we are using $V_{bo}$ and $P_{bo}$
(instead of $T_{bo}$).

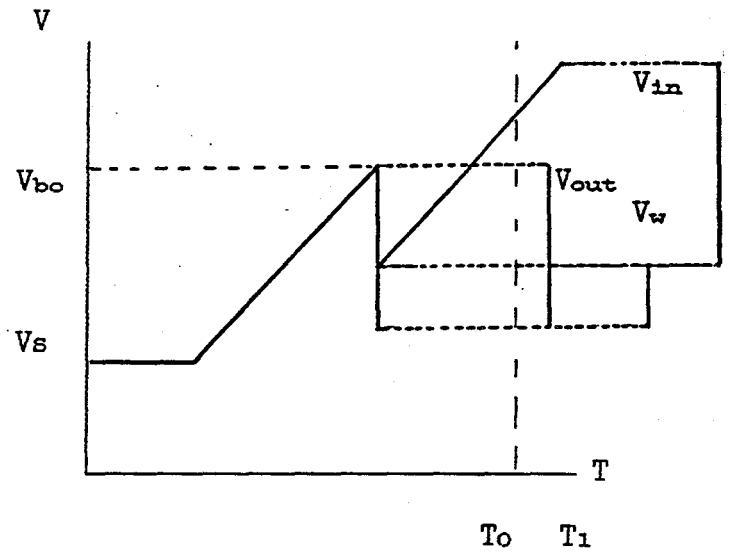
Secondly:

Let's define the volume of
broth-out ( $V_{out}$ )

Points of view: The higher $V_{out}$
the larger harvest

The higher $V_{out}$
the less the remaining biomass
(the further process slows
down)

THE VOLUME OF THE PARTIAL BROTH-OUT DEFINES THE
FURTHER FEED RATE




How to calculate the $V_{in}$, $V_w$ and feed rate after partial broth-out?

*CALCULATION OF $V_{in}$:*

At the end of process the product concentration is $P_o$, the volume is $V_o$. Calculated product is

$$P_o * V_o = G_o.$$

At the time of broth-out the product concentration is $P_{bo}$, the volume is $V_{bo} - V_{out}$. The calculated product is

$$P_{bo}*(V_{bo}-V_{out})$$

We have to produce the difference after the partial broth-out:

| I. | $G_o - P_{bo}*(V_{bo} - V_{out}) = V_{in}*Y$ |
|---|---|

Where Y is the average yield of the process.

From the equation-I the $V_{in}$:

$$V_{in} = G_o/Y - P_{bo}/Y*(V_{bo} - V_{out})$$

*CALCULATION OF* $V_w$ :

From the time of broth-out we reach the final volume on the following way:

| II. | $V_{bo} - V_{out} + V_w + V_{in} = V_o$ |
|---|---|

From the equation-II the $V_w$:

$$\mathbf{V_w = V_o + V_{out} - V_{in} - V_{bo}}$$

From the equation-I and -II by expressing $V_{out}$ and applying $G_o=P_o*V_o$:

$$V_w = V_{in}*(Y/P_{bo} -1) - V_o*(P_o/P_{bo} -1)$$

**This equation shows that the higher yield gives us the possibility of using more dilution water in the process without decreasing the final product concentration.**

*CALCULATION OF FEED RATE:*

At the time of broth-out the volume decreases by the reduction factor:

| III. | $(V_{bo} - V_{out})/V_{bo}$ |
|---|---|

The total biomass is reduced by this factor as well.

The feed rate should be decreased as well by this III. factor:

$$\mathbf{dV/dT = [(V_{bo} - V_{out})/V_{bo}]*(dV/dt)_o}$$

The result of the slower feed rate:
increased fermentation time.

Usually the feed rate can be higher:
the activity increases due to
- the dilution!
- aeration!

RESULTS:

Increased HARVEST
YIELD
and - time...

Further possibilities:

a./ Implementation of water
Multiplication factor:
$( V_{feed} + V_w )/V_{feed}$
Increased feed volume
Increased feed rate
Decreased feed concentration
Same total substrate input

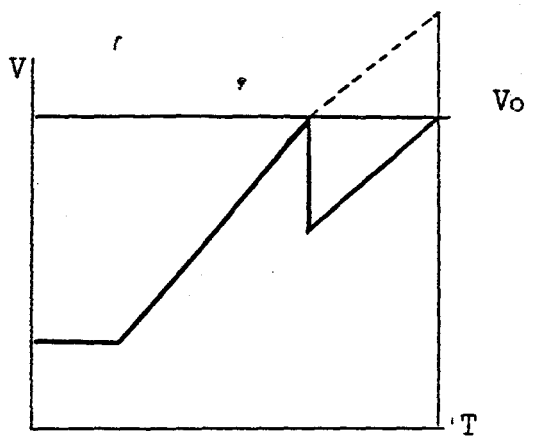
0

b./ The partial broth-out
can be repeated
(if frequency is high
turns out to be a continuous
technology)

2./ Second approach: scaling up

Principle:

The partial broth-out is made just after reaching Vo.
The final broth-out is made also just after reaching the volumetric capacity of the tank -Vo.




The dotted line shows an increased - scaled up - fermentation in the same size fermentation vessel!!

Method: same as in the case of the first approach - partial broth-out, dilution with water, feed further

How to design?

| Scaling up: we use a multiplication factor - X |
|---|

In the case of scaling up, we have to increase the size of the:

- seed volume
- start medium volume
- feed medium volume
- broth-out volume

and we have to increase the
- feed rate
- aeration rate

According to the above - $V_{seed} = X * Vo_{seed}$

$V_{start} = X * Vo_{start}$

$V_{feed} = X * Vo_{feed}$

$V_{out} = X * Vo_{out}$

$V'_o = X * V_o$

| The fermentation time is the same! |
|---|

Let's define the time of partial broth-out

For this purpose we use a ***ratio***:

The feed volume has two parts - before and after the partial broth-out:

$$V_{feed} = [\ V_{feed} - V_{in}\ ] + V_{in}$$

***Ratio:*** $R = [V_{feed} - V_{in}]/V_{feed}$

This ratio shows, how many percent of feed medium is used before the partial broth-out.

According to the graph :

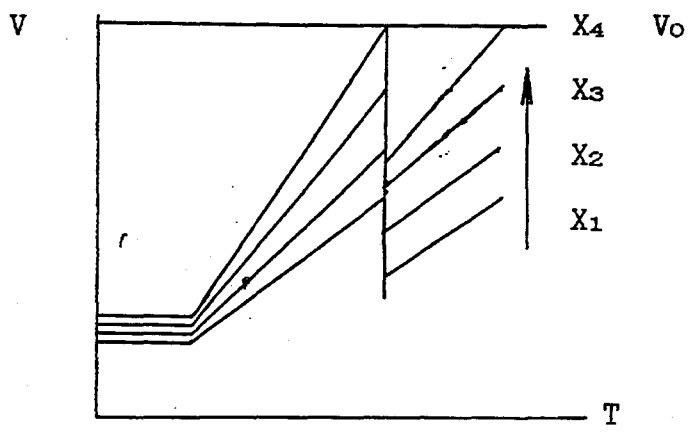
$$V_{start} * X + V_{feed} * R * X = V_o$$

from this equation the $X = V_o / [V_{start} + V_{feed} * R]$

(we can see, that the smaller is R, the bigger is X - the potential scaling up is bigger)

How to obtain these results?!

EXPERIMENTAL OPTIMIZATION !




Where $X_1$ $X_2$ $X_3$ $X_4$

By using this second approach of repeated feed batch technology we can improve the harvest again.

III. Advantages, results - limiting factors, obstacles

MAIN ADVANTAGE: INDUSTRIAL PRODUCTIVITY INCREASES

by the increase of the average dP/dt
by the inrease of the yield
by the increase of the harvest

RESULTS: INDUSTRIAL PRODUCTION

penicillin production Hungary, Debrecen, Biogal Pharmaceutical Co. Ltd.

L-lysine production, Hungary, Kaba, Agroferm Ltd.

OBSTACLES:
- Volume of the seed
- Dilution - can cause osmotic shock

IMPORTANT: LIMITING FACTORS:

- OTR
- $V_{out}$ is big - the remaining biomass is small - the slow feed - results in long fermentation time

# CONSULTATION ON PIPING FOR FERMENTER AND SEED TANKS

During the consultation the main outlines of the piping arrangement were discussed.
As a main principle it was emphasised that the number of the pipelines and the valves should be as low as possible. The more simple arrangement results in less problems during operation and maintenance and less scaling and clogging and therefore it leads to a reduced amount of contamination too.

The sketches shown demonstrate these principles. The arrangement should be designed by applying welded pipeline connections, and all the valves should be good membrane ones. The advantage of the rubber membrane sheet was explained; and the disadvantages of the combined, teflon-rubber ones was mentioned during the consultation.

All equipment should be designed with proper steam-sealing parts preventing the connections from contamination.

A brief explanation was given about the steam seal of the gland packings. In the newly-designed 80 m3 fermenter tanks, the agitator's shaft was sealed with graphite slipping rings. These parts should have proper lubrication, and as a supplement to Mr Fari's suggestions the lubrication must be performed by sterile condensed water. As it was installed in other fermentation plants, the steam should pass through an approximately 5 l stainless steel tank (without any insulation) the outlet of which at the bottom of this small tank should be equipped with steam trap. This arrangement can easily provide the requested amount of condensed water for the lubrication purpose. The advantage of this arrangement is that the heat generation followed by the steam's condensation does not take place in the sensitive packing.

It was demonstrated using drawings that the proper arrangement could contain pipelines with multiple functions as well.

The impact of contamination is usually more dangerous at the initial phase of the large-scale fermentation processes. The possible losses are less if the contamination happens at the final stage of the production fermentation.

That is why the most sensitive equipment (Ist. seed tank) has no bottom valve. Industrial scale automatic bottom valves are dangerous for the fermentation processes because their sterilization is not safe enough. The discharging of the washing water can be performed by using a water injector suction system.

The proper place of the NH3 inlet is the air inlet pipeline. The NH3 inlet can be separated by several valves for safety reasons.

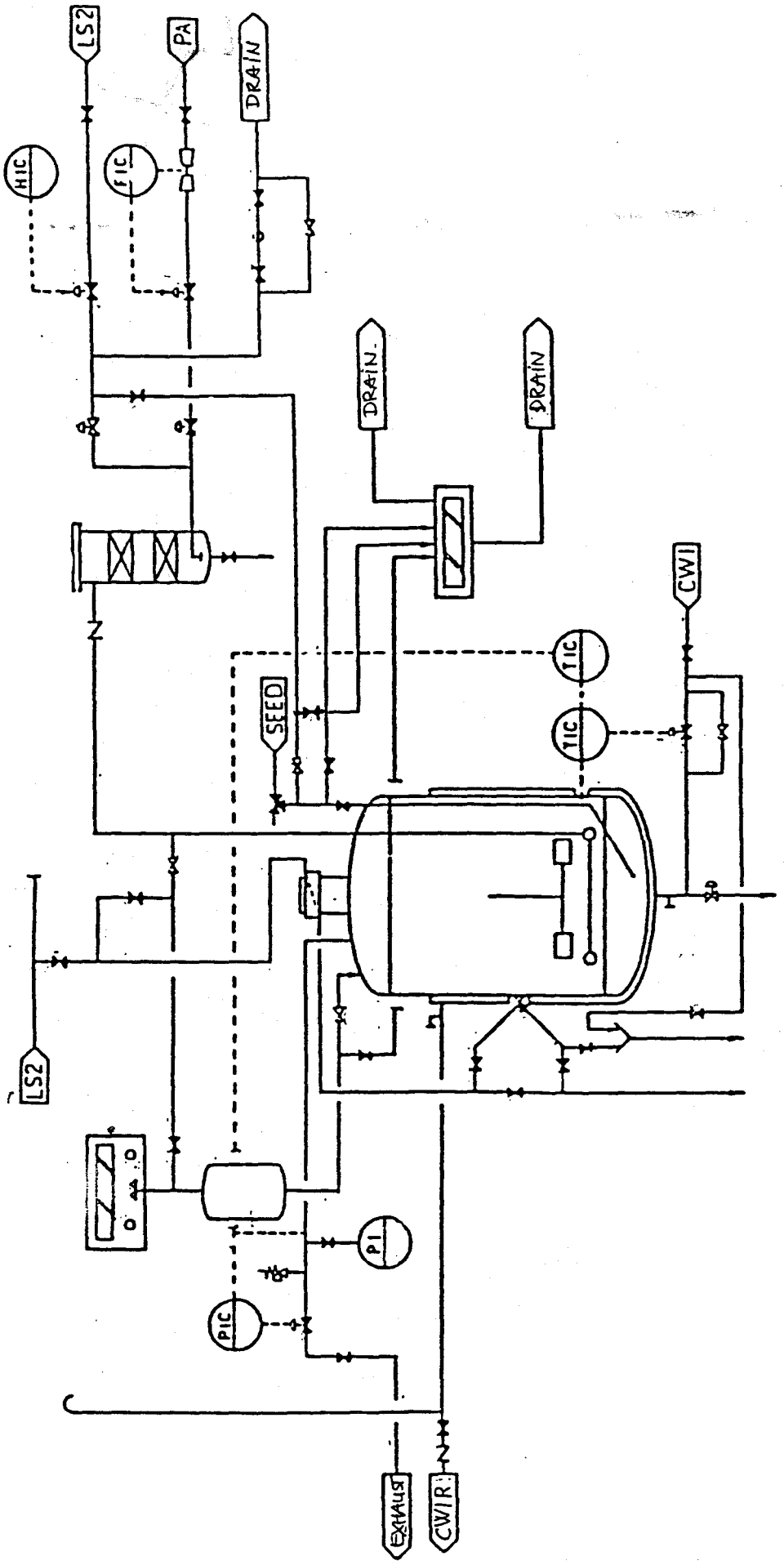
The temperature and pressure control systems are built in by maintaining the possibility of connected operation. During sterilization the temperature and the pressure controls are working in a cascade mode.

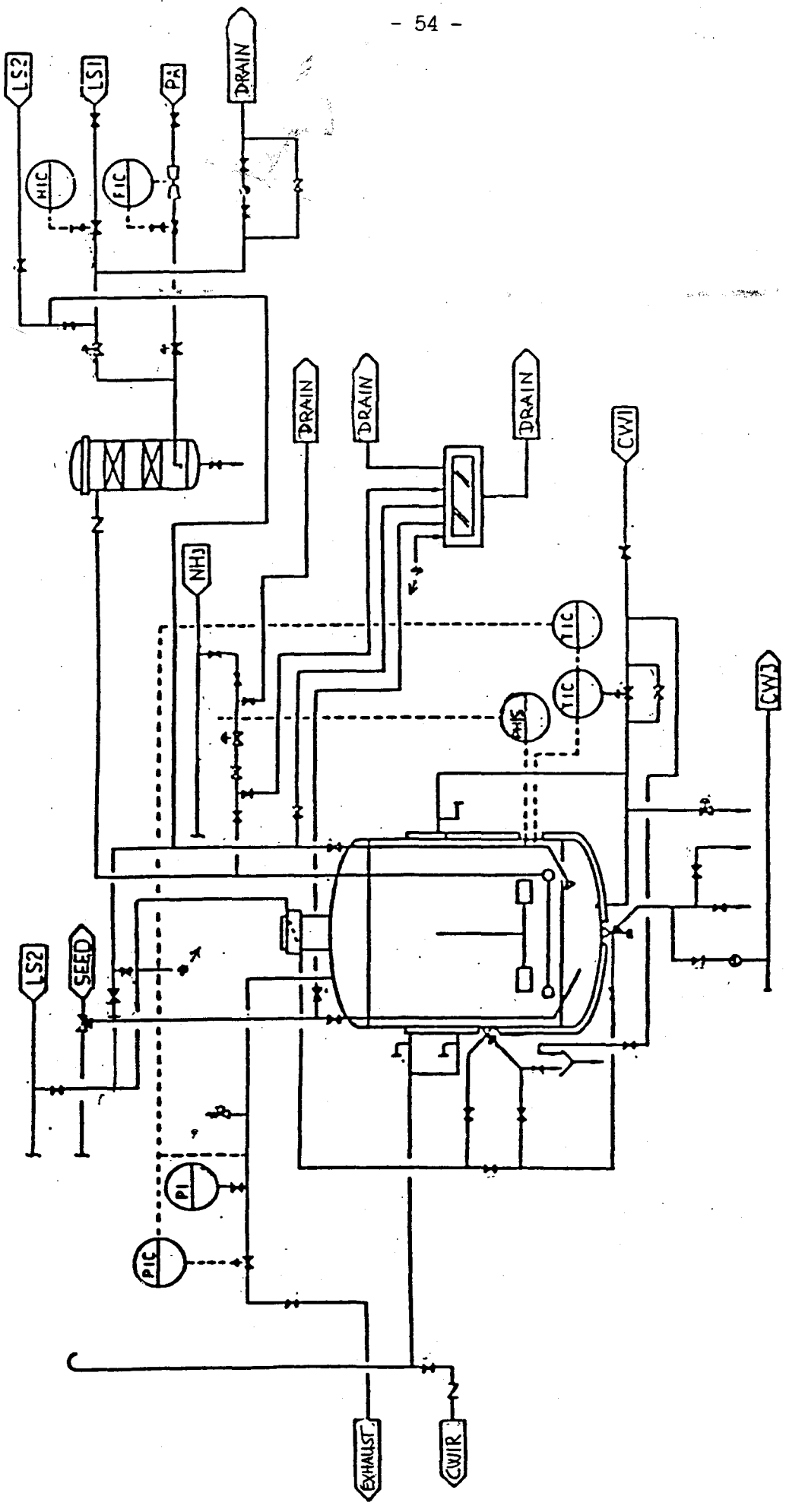
The IInd. seed and main fermenter units have special discharge valves, which are built directly into the tanks body.

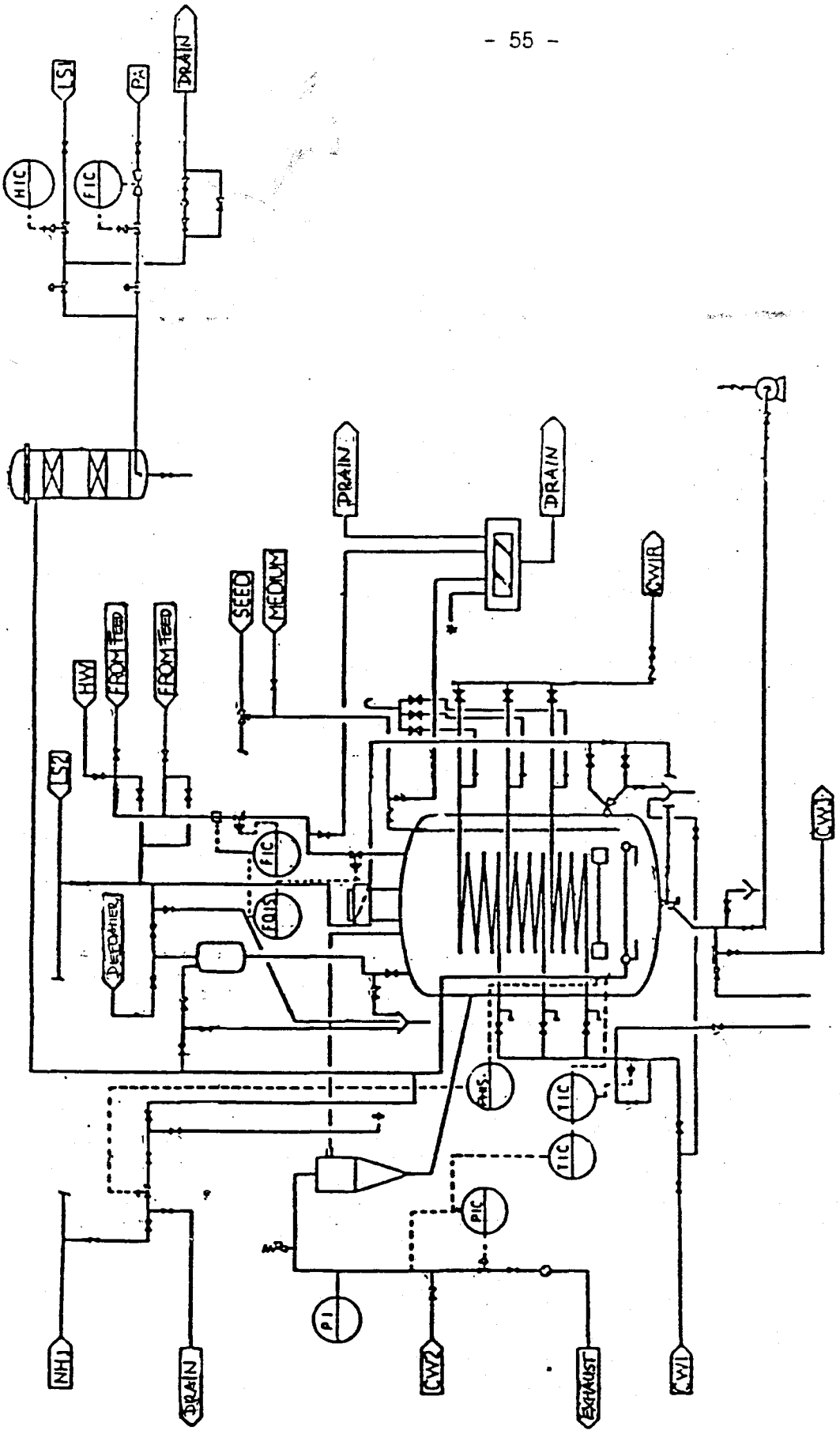
For the proper medium sterilisation the IInd seed equipment is installed with a special steam heating pipeline with a proper silencer on it. As main fermenters are not to be used for medium sterilization, there are no similar parts.

The seed pipelines should be built into the production fermenters as well, because there will be a possibility for inoculation from a production fermentation in the younger age of the fermentation process.

A short explanation was given regarding the handling of the "kasten" - the special manipulator bench for the inoculation of the Ist seed tanks.


LS2
PA
DRAIN
HIC
FIC
DRAIN
DRAIN
CWI
TIC
TIC
SEED
LS2
PI
PIC
EXHAUST
CWR


LS2
LS1
PI
DRAIN
HIC
FIC
DRAIN
DRAIN
DRAIN
CWI
NH3
TIC
TIC
AHS
CWO
LS2
SEED
PI
PIC
EXHAUST
CWIR


LS1
P1
DRAIN
H1C
F1C
DRAIN
DRAIN
CWIR
SEED
MEDIUM
HW
FROM FEED
FROM FEED
LS2
F1C
F01
DEFOAMER
CWI
A1S
T1C
T1C
P1C
P1
NH3
DRAIN
CW2
EXHAUST
CWI

# HANDOVER OF PC SOFTWARE

For easier, user-friendly computer handling a Norton Commander (3.0 version) was installed. All features of the program were demonstrated after installation. After reaching the NORTON directory, the activation can be made by the order "nc".

An up-to-date version of MS WORD (5.5) word processing or edition software was installed as well together with the English spelling, teaching, and "Thesaurus" programs. After reaching the WORD5.5 directory, the activation can be made by the order "word".

Also the last version of the Quattro Pro (4.00) data, spreadsheat and graph processing program was installed. After reaching the QPRO directory, the activation can be made by the order"q".

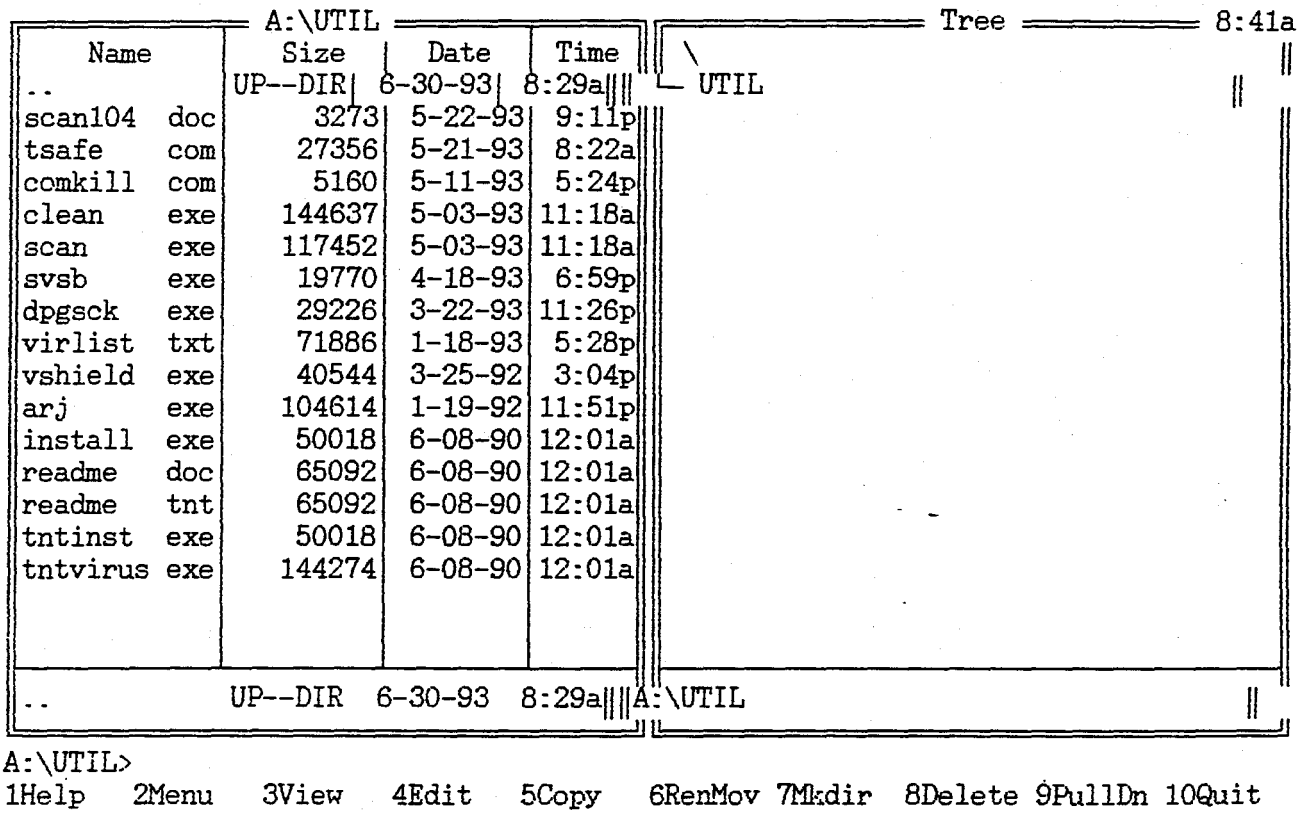
These programs could be very useful and can be handled easily. Nevertheless, all the advantages of these programs could be exploited completely in the case of having a mouse.

In any case some software-virus scanner and killer programs and the easily working "arj" compressing program were installed as well. (The scanning program can recognize and eliminate more than 1500 types of viruses.) The proper handling of the scanning programs are included in their information parts.

The list of the anti-virus programs (together with arj.exe) are shown on the page 57 in the frame of the Norton Commander, gathered into a "UTIL" directory.

One virus FamR [FR] was found in the Chinese 213 system directory in the prta.com file by the scan.exe (9.0V99) scanning program. It could not be eliminated by the clean.exe program, the reason was perhaps that the Chinese program contains some similar sequence like the above virus. The most up-to-date version of the "scan" (104) reported this file as clean.

However, the access of these antivirus programs can be very useful in the Wuxi Enzyme Factory. The parallel existing 213 program (on a floppy disk) was contaminated by the "BLOODY!" boot virus. The tendency of contamination is growing as computer games are more common in The People's Republic of China.


A:\UTIL
Name
Size
Date
Time
..
UP--DIR
6-30-93
8:29a
scan104 doc
3273
5-22-93
9:11p
tsafe com
27356
5-21-93
8:22a
comkill com
5160
5-11-93
5:24p
clean exe
144637
5-03-93
11:18a
scan exe
117452
5-03-93
11:18a
svsb exe
19770
4-18-93
6:59p
dpgsck exe
29226
3-22-93
11:26p
virlist txt
71886
1-18-93
5:28p
vshield exe
40544
3-25-92
3:04p
arj exe
104614
1-19-92
11:51p
install exe
50018
6-08-90
12:01a
readme doc
65092
6-08-90
12:01a
readme tnt
65092
6-08-90
12:01a
tntinst exe
50018
6-08-90
12:01a
tntvirus exe
144274
6-08-90
12:01a
..
UP--DIR
6-30-93
8:29a
Tree
8:41a
\
UTIL
A:\UTIL
A:\UTIL>
1Help
2Menu
3View
4Edit
5Copy
6RenMov
7Mkdir
8Delete
9PullDn
10Quit

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

The expert's report is a very good example to demonstrate the complexity of the technology for industrial enzyme production. It also demonstrates that without a continuous and efficient strain improvement programme, the Wuxi Enzyme Factory cannot compete in the market.

It is therefore our suggestion that a Development Laboratory be set up or the existing Technical Development Department be re-organized to be able to address at least the following issues:

1. Based on the existing domestic market opportunities priority development areas should be redefined.

2. The activity of a Development Laboratory should be focused on:

   - strain improvement and validation
   - process optimization and validation
   - process improvement for fermentation and downstream processing
   - product development (new formulations)
   - quality control of phase and finished products

3. Identification of opportunities in the export market, if any.

4. Identification of potential new partners for cooperation, if that would be the most optimal way for further development.

5. Drastically reduce the percentage of rejected products.

6. Training of employees at all levels in the Factory.

Since fermentation technology is a very complex system and products manufactured by fermentation are the results of this system, the technological know-how is the most important element for successful operation. All factors of the technology, such as production strain, media, maintenance of equipment, essential supplies (sterile air, water, electricity, steam, etc.), the compatibility of the individual items of equipment, etc., should be controlled and optimized. This needs expertise and experience.

The fermentation technology is developing worldwide on fast track. The R&D and its application in fermentation can hardly be followed in the literature. When the People's Republic of China is opening its economy towards the global market, one should clearly appreciate the amount of information to be absorbed. In this context not only the abundance of information in the present report of the expert, but his very positive attitude by offering services and material (strains, software) outside the scope of his job description, should be noted.