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IMPROVED PRODUCTION OF PENICILLIN

DP/CPR/89/021

THE PEOPLE'S REPUBLIC OF CHINA

Technical report: Second visit to the Guangzhou pharmaceutical factory and research institute, July 1992*

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 M. Cole,
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* This document has not been edited.

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ABSTRACT

A new National Project Director (Mr. Liang Zuo Huai) has been appointed. The President of the Guangzhou Pharmaceutical General Corporation and his staff indicated that they would give every assistance. Top priority would be given to getting the new microbiology building operational and to establishing a penicillin V process with at least 70% overall recovery. Deacylation to 6-APA would be dealt with later. As the services to the new building were not complete, attendance by B. Braun to install the new 20 litre fermenters was delayed.

The existing iodometric assay for penicillin V in fermentation samples and solid preparations was critically reviewed. The applications for the new HPLC method being established with the help of Mr. A. E. Bird were discussed.

Results for the fermentation process for penicillin V in the 1000 litre fermenter were studied. The yields were open to debate, in view of uncertainties about the assay methods, the standards used, the p-hydroxy penicillin V content, and the units. The best titre to date of 44,400 units/ml (equivalent to 28,860 µg/ml) may not be a reliable figure. All results in future should be expressed as µg/ml. A recent fermentation after climbing then showed a premature fall. The reasons for this were investigated - possibly contamination or power failure. It was recommended that purity checks be done until the end of the run; methods were discussed. There were difficulties in isolating the penicillin V from this batch; it should have been harvested earlier or discarded. No results were presented on penicillin V acylase, or on the penicillin V yields of different P.chrysogenum cultures.

Methods for P.chrysogenum strain improvement were provided. They will have to be discussed in detail with relevant staff later. Notes were also provided on planning and recording experimental work - quality assurance.

Various pieces of equipment supplied by UNIDO were unpacked and inspected. Aspects of the operation of the Biostat 20 litre fermenters were reviewed. The new building was inspected and suggestions made about its design and operation. Books were presented, covering fermentation and enzyme methods.

In the future, it would be helpful if summaries of work on the penicillin V fermentation and isolation could be presented. The work plan on the penicillin V acylase needs to be discussed. It would be helpful to have a list of staff with their responsibilities.

Attendance at the 7th International Conference on Culture Collections (covering all aspects of microbial metabolites and enzymes) in Beijing 12-16th October, 1992, would make it convenient to plan the next visit around this date.

INTRODUCTION

Dr. F. R. Batchelor, Mr. A. E. Bird (Expert in Instrumental Analysis) and myself met for preliminary discussions in Hong Kong on 4th July, and travelled to Guangzhou on the 5th. We were met and entertained to lunch by Mr. Liang Zuo Huai, the new National Project Director for the penicillin V project, Mr. Liu Guang Tao, Director of Research and Deputy National Project Director, Mr. Li Jian, administration assistant to Mr. Liu, and Mr. Cai Shi Chao (Daniel), from the Foreign Economic Department of the Guangdong Pharmaceutical General Corporation, who acted as translator as on previous visits. There was a general discussion about the administrative aspects of the Project, including the staff training programme on the use of the Braun fermenters.

On Monday, 6th July, we visited the Guangzhou Pharmaceutical Factory, this being my second visit. We were introduced to Mr. He Guo-Wei, who had recently been appointed the new Factory Director and as such had overall responsibility for the Penicillin V Project. Enquiries were made about the progress of various aspects of the Project, including the responsibilities of the various members of staff, and in connection with the latter we requested a staff plan. Consultations in which I was involved continued through to Saturday morning, 11th July.

State of the new building and visit by B. Braun to install fermenters:

We inspected the new building, but it was still in course of construction and quite unsuitable for installation of new equipment for many months. It would have been quite impractical to install and commission the fermenters as the services (electricity, water, steam, air) had not yet been installed in the laboratory where the fermenters were to be located and the floor was still covered in rubble! The building was also unsuitable for setting up the Waters HPLC equipment, but as we were keen to get the HPLC method established for assaying fermentation samples and isolated penicillin V, Mr. Bird suggested it be set up in another laboratory. A suitable one was eventually found.

It was suggested that as soon as possible they should sweep away the lakes of water on floors of the new building, particularly in the area of the microbiological laboratories. I pointed out that it was particularly important that the walls and floor should be dried out quickly so as to discourage the development of sporing fungi (e.g. Cladosporium) which could be a future cause of contamination. Part of the trouble was that the builders kept using the fire hydrants and not turning them off properly.

A FAX was received from UNDP (Jan Mattsson) requesting information on the date for attendance of Braun personnel to install the new 20 l fermenters and on the raw materials required to run them. A reply was drafted (sent by Mr. Cai) explaining that they should not attend until the building was complete, and that all the raw materials were already available in the factory. Initially, the medium used in the 1000 litre fermenter would be used.

Assay methods for penicillins:

Mr. Bird outlined to a group of some 30 people the various methods which could be used for qualitative and quantitative assay of penicillins, drawing attention to the strengths and weaknesses of the various methods. The biochromatographic method (paper chromatogram strips laid on agar seeded with a penicillin sensitive bacterium) had already been demonstrated by Dr. Batchelor and myself but is not suitable for quantitative work. The HPLC method described by Mr. Bird is suitable for both qualitative and quantitative work. It will be valuable for assessing the production of penicillin V and p-hydroxy penicillin V by new strains of P.chrysogenum in flask and tank fermentations, and also for studying the effect of changes to culture medium and cultural conditions. The currently used iodometric method is less specific and gives an estimate of all of the penicillin produced. The HPLC method will be useful for measuring the residual phenoxyacetic acid left in the fermentation and also for following the enzymic deacylation of penicillin V.

Validation of analytical methods and application to fermentation samples:

Mr. Bird, in his presentation on the above topic, emphasised how important it was to ensure that the assay was suitable for the purpose, and explained the principles such as precision, accuracy, linearity, range, specificity, limit of detection and ruggedness. These points were discussed in relation to the methods being used (e.g. iodometric assay for total penicillin) or planned to be used (e.g. HPLC analysis).

The problems of assaying the penicillin V content of fermentation samples was discussed at some length, given the possible presence of p-hydroxypenicillin V and alkylpenicillins, the need to assay samples quickly to avoid decomposition and problems arising from the high content of mycelial solids. It is important to be clear how the results are to be expressed, namely as the amount of penicillin V per ml of whole fermentation or as per ml of culture filtrate after removal of mycelium. Use of the relevant figures in the calculations of extraction efficacy is important. The iodometric assay currently being used measures iodine reacting components after treating with alkali to open the B-lactam ring; it thus measures total penicillin which for a high yielding strain should be mainly penicillin V, but this must be checked by HPLC. The use of the appropriate assay standard, namely penicillin V, of known purity is obviously important.

Sampling the small volumes of fermentation fluids used in shaken flasks can significantly reduce the volume in the flask and thus change the aeration characteristics. Where there is a need to monitor the titre of the fermentation over long periods of time the use of the 20 litre Biostat fermenters will overcome this problem. However, for assessing large numbers of mutants of P.chrysogenum it will still be necessary to use shaken flask cultures, replicating the flasks and the assays to improve reliability of the results.

Progress of 1000 litre scale penicillin V fermentations:

It was difficult to ascertain what progress had been made in the absence of any summary report and the fact that some key staff were unavailable.

The composition of the medium seemed reasonable and used locally available materials: 5% peanut meal, 3% soya bean meal, 2% $CaSO_4$, 0.5% $CaCO_3$, 0.6% $(NH_4)_2SO_4$, 0.9% phenoxy acetate (further 0.9% added after 20 hours). Peanut oil was used as antifoam. Extra $(NH_4)_2SO_4$ was said to be added and also a glucose feed during the fermentation. The initial pH was adjusted to 6.8 with NaOH and the fermentation run at 24° C for 140 hours. The initial air flow was 0.7 v/v/min and was adjusted up to 1.5 v/v/min as the culture grew.

The yield of current 1000 litre fermentations was said to have reached 40,000 µg/ml penicillin V. When I questioned whether this was really µg/ml I was assured that it was. However, discussions which Mr. Bird had with those carrying out the assay suggested that it might be u/ml (international units of penicillin/ml). [See later section]. For such a titre the phenoxyacetate and sulphur contents of the medium were calculated to be adequate.

The priority in the Pilot Plant was said to now being given to the P.chrysogenum fermentation for penicillin V production, and that they had not done any recent runs on the Fusarium acylase enzyme.

Fermentation Pilot Plant Records:

I had asked to see the detailed records of the pilot plant fermentations because, over two days of discussions there had been no presentation of any formal records of results. However, it turned out that the fermentation records were kept in great detail by Mr. Luo, manager of the pilot plant, and Mr. Zu. Some time was spent examining these records to see whether the results were in accord with our expectations. This was indeed the case, except that the titres of penicillin V seemed to climb to very high levels. Some of the results for culture No.2, grown in the 1000 litre fermenter were as follows:-

28 h	76 h	92 h	116 h	140 h
14,000	25,000	29,500	33,000	37,800

(for units see discussion below)

The results for one of the best runs was recorded as 44,400 at 164 hr. On our previous visit we were told that they had recently obtained a higher yielding mutant (Culture No.2). At that time they were not getting yields as high as obtained with their original culture (No.1) and I made suggestions about ways in which they might improve the yield.

As a result of asking what units they were using to express the penicillin V titres, I was repeatedly assured that the assays were given as µg/ml free acid penicillin V. However, the above high figures were thought to be exceptionally good by Dr. Batchelor and this restarted the debate about the way the results were expressed. It was eventually concluded that, in spite of our recommendation that they should be expressed as µg/ml, they were actually International Units of penicillin/ml. To get to µg/ml the above results would have to be multiplied by 0.65. So, the 37,800 value would become 24,570 µg/ml and the 44,400 would become 28,860 µg/ml, assuming the assay standards and procedure were reliable. This aspect was investigated by Mr. Bird (see his Report) and it is by no means certain that the above results are correct because 6-amino penicillanic acid was used as the assay standard and not penicillin V.

Some details for the fermentation run which gave the above results were as follows:-

Inoculum medium:	3% cornsteep liquor 3% glucose 0.5% chalk
Fermentation medium:	3% peanut meal 2% soyabean meal 1% $CaSO_4$ 0.4% $(NH_4)_2SO_4$ 1% peanut oil as antifoam 0.87% (initial) phenoxyacetate 0.5% chalk pH before inoculation - 6.8
Inoculum:	10% of fermentation volume pH of inoculum - 5.3
pH of fermentation:	6 at 4 h; 6.4 at 44 h; 6.2 at 100 h.
total reducing sugar:	before inoculation 1.5 (? units) at 20 h - 0.69
Glucose feed:	after 20 h glucose added: 0.6 litre of 27%; Glucose added if pH >6.3.

Ammonia nitrogen was also assayed.

Progress of current 1000 l penicillin V fermentation:

As a 1000 litre fermentation was being harvested on Thursday, 9th July, the records of the progress of the fermentation were inspected and parts of the extraction observed.

A whole afternoon was spent investigating why the titre of the fermentation was dropping over many hours at a time when it should still have been climbing on its approach to normal harvest time. It was only as a result of Dr. Batchelor investigating the way the iodometric assay for penicillin V was being carried out that the fall in titre came to our notice. The results for the later sampling times had still to be added to the fermenter record sheets I was shown. The titre pattern for this fermentation from 100 hours was as follows:

Hours	100	108	116	124	132	140(harvest)
Thousands of units of penicillin:	24	24	22.7	20.8	18.9	16.0

The normal sugar addition was not made at 108 hours and consequently the pH subsequently rose from 6.3 to 6.7.

We pointed out that the tank should have been harvested early to avoid further losses. As it had deteriorated it was difficult to filter and extract. It should have been sterilised and discharged.

There was confusion as to whether the fermentation had become contaminated. Samples had been taken for a microbiological purity check which was first said to show contamination, and then contradicted. On pressing for details, it turned out that samples were taken only up to 100 hours of the fermentation and that only a test for bacterial contamination was done on agar at 37° C. Apparently a test for fungal contamination was not done because the required lower temperature incubator had broken down. The test for bacterial contamination was said to be negative, but I could not inspect the plates because they had been washed up! I was told that there was a suggestion of fungal contamination by microscope examination. Before I heard the above comments I had spent some time looking at fermentation samples taken just before the extraction was started. I did not see any evidence of gross contamination by unusual looking fungal hyphae. However, the P.chrysogenum hyphae were very variable in diameter, the smaller ones being well stained and containing what looked like oil droplets. These hyphae were joined to the larger diameter hyphae so they did not represent a different fungal culture. There was also great variation in the morphology of the cells; this was not surprising in a high yielding mutant. The mycelium was not clumped into firm pellets, but was rather dispersed. I observed some very small round cells which could have been evidence of early bacterial contamination.

There was a terrible mess around the pilot plant. There were open waste drains and the building works for the new microbiology laboratories were as close as 5 metres. The ambient temperature was 30+ °C and the weather fluctuated between dry and dusty, and soaking wet. These conditions increase the risk of contamination. However, there may have been some other factor which upset this fermentation run, such as the temporary power cut which would have deprived the culture of oxygen, or some other changes in operating procedure, inoculum, etc. which we did not know about. On Friday, yet further discussion revealed that there had, in fact, been a power cut on Tuesday, lasting 5-10 minutes. This would have been of sufficient duration to have seriously depleted the oxygen supply within the fungal cells, thereby adversely affecting the biosynthesis of penicillin which is highly demanding of oxygen.

It was recommended that microbiological purity checks be done right up to harvest and that these should cover both mould and bacterial contamination.

In Calam's book "Process Development in Antibiotic Fermentation (1987) he deals with batch to batch variation and falling production in section 13.2, p.185 onwards. I have already given a copy of this book to the Guangzhou Pharmaceutical Factory, but I had a photocopy made of the relevant pages for Mr. Luo.

As a general comment, it would have been helpful if summary records had been made.

Microscopic examination of current 1000 litre penicillin V fermentation:

A set of stained (malachite green) slides of samples taken at various times (19 to 91 hours) had been prepared for me to examine. I added cover glasses to improve clarity when viewed down the microscope. The sample taken at 19 hours from inoculation was said to be full of pellets but I showed that there were, in fact, few present and suggested that these were probably from the high level of inoculum which was used. There was little evidence of young hyphae but there was a lot of amorphous solid material from the medium (peanut and soya bean flour), as would be expected for such an early sample. The samples taken at 34, 57, 75 and 91 hours showed gradually increasing amounts of mycelium, the amount of growth by 91 hours being very good.

Selection of Antifoam:

Mr. Zu and Mr. Luo were keen to try using polypropylene glycol (P2000 Dow) as an alternative antifoam to peanut oil, but we could not track it down in the catalogue they had. They were recommended to write to Dow Chemicals for information. Peanut oil can be metabolised by P.chrysogenum and act as a precursor in the biosynthesis of unwanted alkyl penicillins, such as penicillin K. P2000 would not be readily metabolised but its antifoam properties and effect on the biosynthesis of penicillin by the Guangzhou Pharmaceuticals strain of P.chrysogenum needed to be investigated. This would be a suitable topic for study in the Biostat 20 litre fermenters prior to larger scale use. [I have now checked the price in the Aldrich catalogue: Polypropylene glycol, average mol. wt. 2000, entry No.20233-9, 500 g. £16.40].

Procedures relevant to strain improvement:

In the absence of the microbiologists (visiting ATCC. copies of my notes on strain improvement were given to Mr. Luo Si Quing (Fermentation Pilot Plant Manager) and Mr. Zu (looking after Braun fermenters). These notes provide details of the objectives of a strain-improvement programme, schemes for mutation, preparation of protoplasts, carrying out hybridisation and selecting improved mutants. Details of culture media were also given. A start was made on going over this information, but it is a major subject and will best be discussed with Mrs. Cheng on my next visit.

Biostat UD 20 litre fermenters:

A long discussion was held with Mr. Zu, who attended the training programme at B. Braun in Germany. We went over various aspects of the layout and operation of the 20 litre fermenters and checked the delivery list to confirm that the various parts had been supplied (e.g. the separate computer).

As Mr. Zu will be responsible for getting the new 20 litre Biostat UD fermenters set up and running, he was asked by Mr. Cai whether he had information about the services needed and details of types of connections. I showed him the product specification provided by B. Braun, detailing the power, steam, air, etc. required. Dr. Batchelor said that the Guangzhou Pharmaceutical Factory had been supplied with information about pipe fittings, etc.

Inspection of extraction process for penicillin V:

Dr. Batchelor asked me to accompany him during an inspection of the progress of the extraction of penicillin V from a 1000 litre fermenter. We made comments on aspects of handling the fermentation broth (removal of mycelium, determination of volume, cleanliness, assay of titre). Recommendations were made for improving the efficiency of the extraction which is said to be only 50%.

On Friday, morning (10th July) various aspects of the extraction process were examined, particularly the filtration stage which was going very slowly, possibly because of contaminating materials and small crystal size for the penicillin V. It was recommended that the size of the crystals be checked microscopically.

Inspection of new microbiology laboratory with the Director:

On Friday, 10th July, following earlier comments about the poor progress with the new building and apparent change in design, a detailed inspection of the microbiology section of the new building was made with the Project Director (Mr. Liang) and the Research Director (Mr. Liu).

The facilities are extensive and should be adequate for the task in hand, if they are properly fitted out and equipped. The walls had been plastered and the wiring was partly done. The ducting was fitted but there were no outlets cut. I was told that the ducts would deliver fresh cooled filtered air to the laboratory areas and corridors; it was said that the air would escape via doors and windows, i.e. the laboratories would be under positive pressure. I questioned whether this sytem would allow an adequate rate of removal of hot air, particularly from the laboratory where the new 20 litre fermenters were to be located, and pointed out that there was another quite separate large duct in the ceiling which could be for exhaust air.

The facilities for incubating the inoculum spore bottles (3 rooms) and for shaking flasks (4 rooms, 4 shakers to be in each; 8 shakers ordered) seemed very adequate, providing all the temperature controls work. There were 3 rooms for the laminar flow cabinets which are to be used when inoculating cultures. Access to these rooms was via sliding double door vestibules to cut down draught and hence risk of contamination. The rooms were separated off by glazed partitioning and could become very warm in use, as had been found in a similar room in the laboratories of B. Braun, Melsungen. I recommended that consideration be given to installing some kind of air cooling system not dependent on blown air which would upset the performance of the laminar air flow cabinets, increasing the risk of microbial contamination.

The water supply had yet to be fitted in the media preparation room. The room was very large with plenty of space for equipment and storage, but the plan to have only one large sink seemed inadequate. Some large island benches had been supplied, their surfaces being covered with stainless steel, which seemed unnecessary and could have been more cheaply and possibly more suitably covered in a plastic material such as Formica.

The room in which it was proposed to install the 20 litre fermenters was large enough for two, and possibly more, but with only one window it might get rather warm. There was debate about using the room next door as this had several windows. However, a lot depends on the design of the ventilation system.

Support from Guangzhou Pharmaceutical General Corporation:

On Thursday, 9th July, we were introduced to Mr. Zhou Ke Ming from the Guangzhou Pharmaceutical General Corporation, who had just been appointed by Mr. Che Ming-Gang to direct the Penicillin Project at the administrative level of his office. He was accompanied by Mr. Ling Shouohu (Vice Director, Pharmacist) and Mrs. Li Xinghua (Deputy Director). Mr. Zhou explained that they wanted to help overcome the difficulties with the Project and said he was committed to getting the new building completed by the end of August. He also said that priority would be given to getting the penicillin V fermentation and extraction operating satisfactorily at the pilot plant scale with extraction recoveries of at least 70%. The deacylation to 6-APA would be dealt with later.

Equipment - delivery check:

Mr. Cai showed me the delivery notes for a LabConco freeze dryer (for microbial cultures) and two autotitrators (for use as pH stats). He said that the equipment was delivered about 6 months ago and had been in store ever since. I confirmed that installation by the suppliers would not be required and that, like the HPLC equipment, they could be used before the new building was complete. I offered to inspect the equipment. This was later unpacked, in the presence of Dr. Batchelor, and seemed to be in good order. An oxygen electrode for a fermenter was also unpacked. It was not clear why some of this equipment had not been put into use. The autotitrator/pH stat would have been useful for controlling the pH of penicillin V/penicillin acylase reaction mixtures.

Quality Assurance:

As there had been a discussion in which the importance of planning work, recording methods and results, and managing the Project had become very apparent, I gave Li Jian a copy of my notes on quality assurance and asked him to copy them for distribution.

Requests made before departure - Meeting with Mr. Che (President):

On Saturday morning (11th July) we were invited to a working breakfast with Mr. Che (President, Guangzhou Pharmaceutical General Corporation), his senior staff and senior staff from the research laboratories. We requested that for our next visit reports and records should be available for our inspection and that the staff should make presentations on each area of work, so that we could assess progress and maximise the usefulness of our advice. Our duty was to assess results, consider problems and give advice.

Books presented:

Mr. Liang and Mr. Liu were presented with a copy of Volume 43 of **Methods in Enzymology** (Ed. J. H. Hash, Academic Press, New York & London, 1975 [£91]). This book is devoted to antibiotics and includes sections on analysis (HPLC, etc), the enzymes involved in the biosynthesis of B-lactam antibiotics and the penicillin acylases.

A copy of **Fermentation, a practical approach**, Eds. McNeil and Harvey, IRL Press, 1990 (£19.50) was also given.

Next Visit:

On my next visit I would hope to find that the Braun fermenters had been installed and to see that they are running satisfactorily. The research programme for which they are to be used can be discussed. I can also discuss strain improvement with the microbiologists and assess progress with the large scale fermentations. All this relates to the penicillin V fermentation, but we should also discuss work on the penicillin V acylase.

I am hoping to attend the 7th International Congress on Culture Collections at the Beijing International Conference Centre, China from 12-16th October, 1992. It would be good if the next visit to the Guangzhou Pharmaceutical Factory could be fitted around these dates.

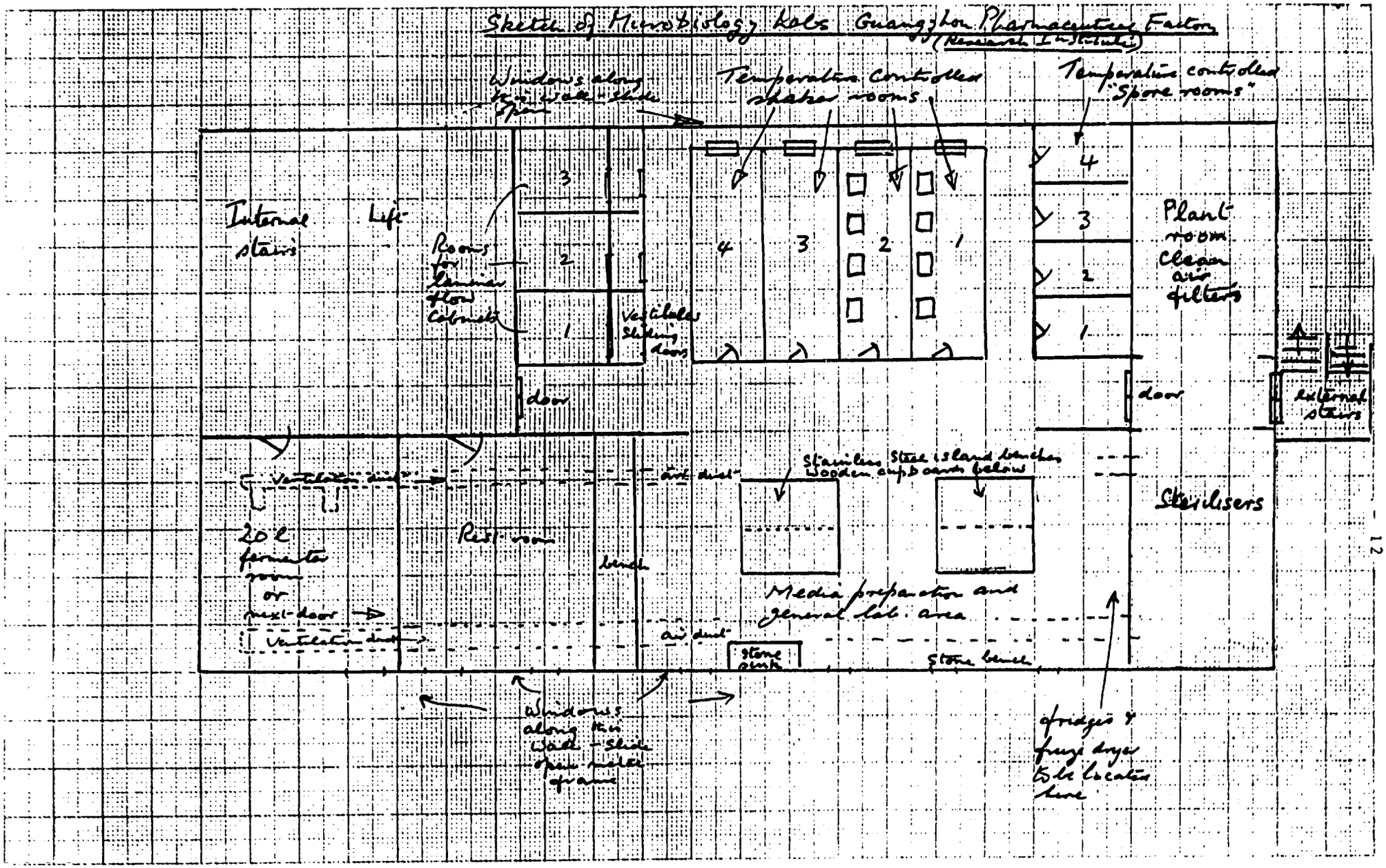
Notes Supplied to staff of Guangzhou Pharmaceutical Factory (copies attached):

1. Objectives and procedures in strain improvement.

2. Quality assurance.

(Dr. Martin Cole)

24th July, 1992


Sketch of Microbiology Labs Guangzhou Pharmaceutical Factory
(Research Institute)
Windows along top of wall-slide open
Temperature controlled shaker rooms
Temperature controlled "Spore rooms"
Internal stairs
Lift
Rooms for laminar flow cabinets
Vertically sliding doors
door
Plant room clean air filters
external stairs
Stainless steel island benches
Wooden cupboards below
Sterilisers
air duct
Ventilation duct
20 l fermenter room
or next door
Rest room
bench
Media preparation and general lab. area
stone sink
stone bench
fridges & fungi dryer & bacteria culture
Windows along top wall-slide open metal frame

Quality Assurance System for

Research Laboratories of the Gangzhou Pharmaceutical Factory

Definition: Quality Assurance System - a formalised management and documentation system for carrying out all activities to an agreed appropriate standard of quality.

Essential actions under a Quality Assurance System:

1. Draw up a plan for what is to be done.
2. Carry out what is in the plan to appropriate standard.
3. Review what has been done so that improvement can be made.

Elements of a Quality Assurance System:

1. A description of the organisation and administration structure and activities.
2. A system for planning and managing a project.
3. A system for operating and maintaining equipment and facilities.
4. Use of standard procedures and analytical methods.
5. A system for recording results and preserving records.
6. A process for auditing the results and reviewing the system.

Required for the Guangzhou Pharmaceutical Laboratories:

1. Staff plan with responsibilities
2. Overall plan for experimental work (equiping laboratories, training in use of equipment, etc. is a separate exercise).
3. Standard operating procedures for microbiological procedures, for calibration and use of equipment including fermenters and for analytical methods.
4. A uniform method for the use of laboratory note books for planning and recording experiments.
5. A system for drawing together the results of a series of experiments so they can be reviewed by management and consultants

Definitions:

Quality System Manual:	Overview, policy statement, management details.
Audit Plan:	Quality policies and procedures specific to a project
Standard Procedures:	Detailed documents mentioned in Quality System Manual
Test method statement:	Working instructions and schedules for project.
Technical Instructions:	Detailed instructions for use of equipment or operation of management systems

References: British Standards:BS 5750 & BS 5882

Objectives of Strain Improvement Programme

Penicillin & Penicillin Acylase

It is essential to define the features which require improvement - yield of penicillin or the enzyme penicillin acylase depend on cultural conditions.

Features to be considered:

Sporulation & type of spores - important in achieving good growth in liquid medium on scale up. Genetic stability required.

Tolerance to elevated temperature, e.g. 30° C v 26° C in in penicillin production.

Rate of growth - more rapid production of product

Tolerance to precursors such as phenoxy acetic acid

Tolerance to metals in water supply

Ability to utilise sulphate in penicillin production

Effectiveness of phenoxyacetate as inducer in Fusarium (constitutive strains)

Tolerance to product accumulation - in penicillin production

Absence of pigment formation - in penicillin and penicillin acylase production

Production of product (penicillin or acylase enzyme) in stirred fermenter compared with plate culture (selection method) or shaken flask

Length of period of sustained penicillin production

Extent of release of acylase enzyme from cells

Yield of penicillin/rate of penicillin production under defined conditions.

Techniques in Strain Improvement

1. Selection of pre-existing component of the population

2. Use of mutagen - far UV light (about 254nm)
   safest/best method

   near UV light at 365 nm after
   sensitisation with 8-methoxypsoralen

   mutagenic chemicals, e.g.
   EMS (ethane methylsulphonate)
   NTG (Nmethyl-N'nitro-N-nitrosoguanidine)

3. Use of resistance to antibiotic or other substances

4. Use of remutation of strains made negative in production

5. Use of an agent to prevent mutation repair (excision repair) e.g. 8-methoxypsoralen

6. Hybridization - formation of heterokaryons and then diploids by growing two auxotrophic haploids (aux 1 and aux 2) together

7. Use of fusion of protoplasts to generate hybrids.

Factors affecting Mutant Yield

Mutagen dose

Mutagen treatment conditions

Physiological state of cells or spores

Sensitivity to mutation

Presence of DNA repair mechanisms (most important)

Length of time for damaged DNA to be converted to heritable mutation and altered phenotype (e.g. 6 hr).

Desirable to carry out mutation on haploid material - for P.chrysogenum use spores.

Method of Assessing Yield in Large Numbers of Strains

Penicillin yield:

1. Plate surviving spores/cells from mutation/selection method onto agar plats. Grow up colonies (widely spaced), make replicate plates and then overpour one plate with agar seeded with penicillin sensitive bacterium (e.g. Bacillus subtilis). Good for detecting negatives and big increases at beginning of yield improvement programme.

2. Subculture survivors into liquid fermentation medium and assay for penicillin production (bio assay, B.subtilis or h.p.l.c.)

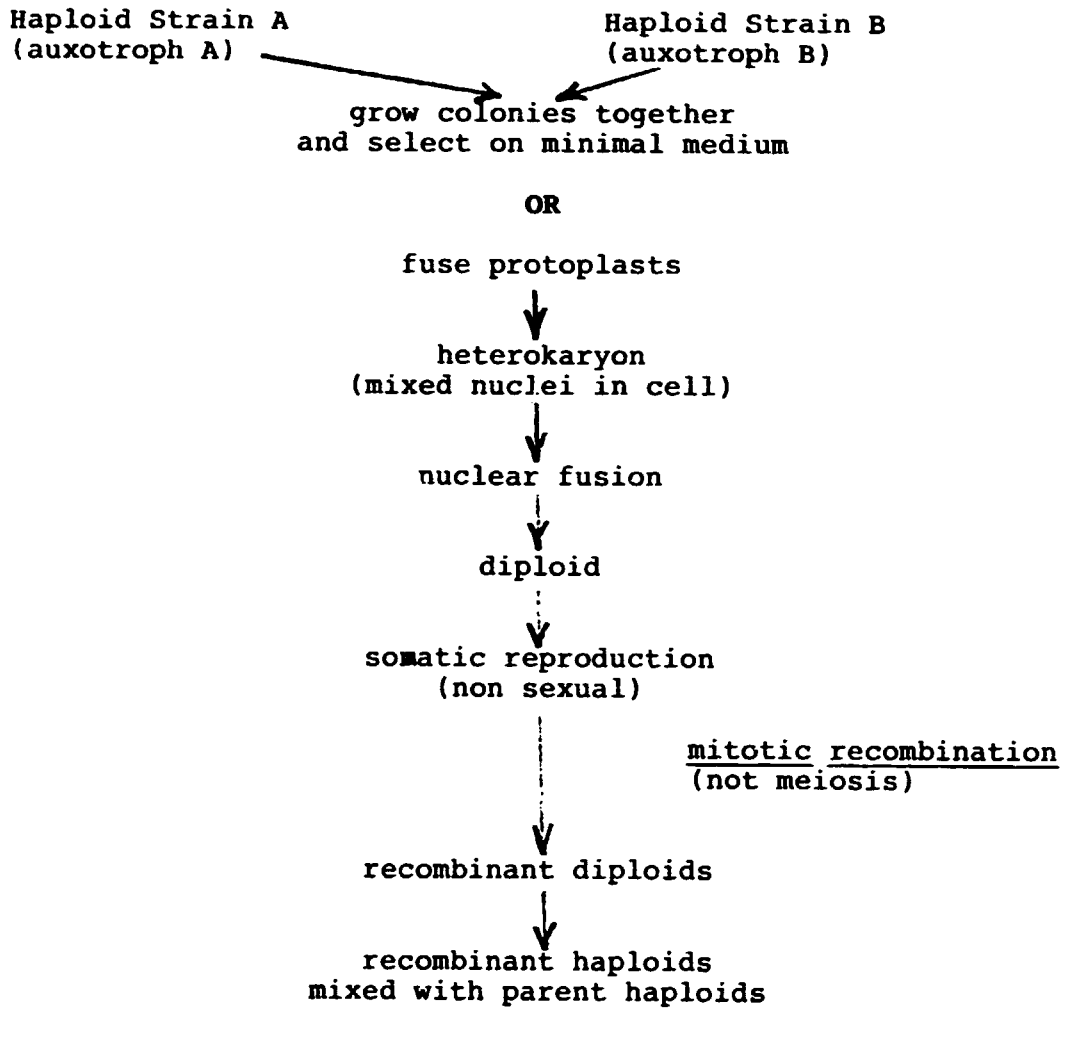
Penicillin acylase:

1. Plate surviving spores onto agar plates. Grow up colonies and make replicate plates. Overpour with penicillin V in agar plus penicillin sensitive bacterium (e.g. B.subtilis)

2. Subculture survivors into liquid medium and assay for penicillin acylase production by measuring yield of 6-APA from pen V over fixed time.

Aim: To test 10,000 mutagenized survivors/week!

FORMATION OF RECOMBINANTS IN P. CHRYSOGENUM VIA PARASEXUAL CYCLE*



5-FU can increase frequency of mitotic crossing over p-fluorophenylalanine and benlate enhance aneuploids.

In P.chrysogenum frequency of diploids 1 in 10^6

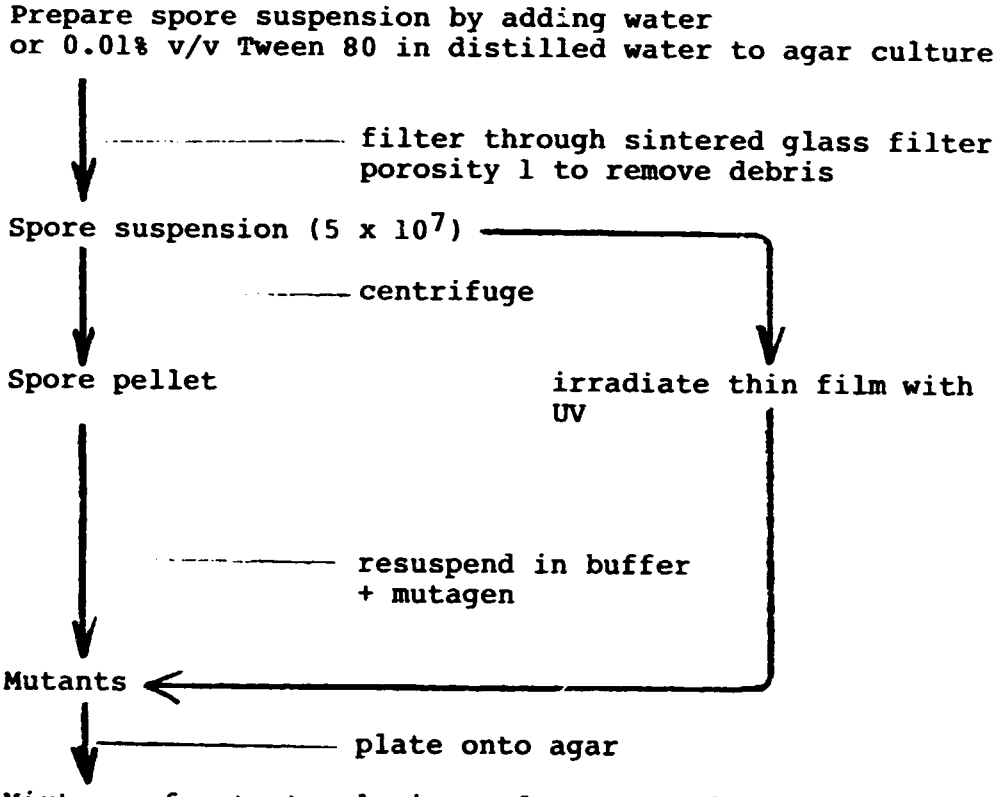
frequency of subsequent segregation 1 in 10^2

*parasexual cycle also occurs in Fusarium oxysporum

Reference: Genetics of Penicillia
by Saunders, G. & Holt, G.
Chapter 3, p.73, in Penicillium & Acremonium
Ed. by J. F. Peberdy, University of Nottingham, England. Plenum Press, New York & London, 1987

PREPARATION OF MATERIAL FOR MUTATION USING HAPLOID CONIDIA (spores) OF P. CHRYSOGENUM

(from Saunders & Holt, 1987)



(Mutation frequencies for surviving spores can be obtained by measuring frequency of resistance to 5 FU or polyene antibiotic such as candidine - after allowing up to 6 hours expression time prior to adding the antibiotic.)

Reference:

Saunders, G. & Holt, G.
Genetics of Penicillia. In, Penicillium and Acremonium, Ed. J. P. Peberdy, Plenum Press, New York & London, 1987

Reference to details of mutagenic treatment conditions for Penicillium chrysogenum with a range of physical and chemical agents:

See: Normansell, P. M. J. et al.
Journal of General Microbiology, 112, 113-126 (1979)

PREPARATION OF PROTOPLASTS OF P.CHRYSOGENUM FOR HYBRIDISATION

(from Saunders & Holt, 1987)

Grow strains for 36 hours in Aspergillus complete liquid medium* at 26° C on reciprocating shaker 200 revs/min

↓ centrifuge

0.2 - 0.5 g. wet weight mycelial pellet

↓ incubate with lytic enzyme for 2-4 hours, gentle agitation (Helix pomatia juice 1% v/v + 5 mg/ml Nova cellulase in 0.6 M KCl)

Cell walls disgested and protoplasts released into osmotically stablised medium

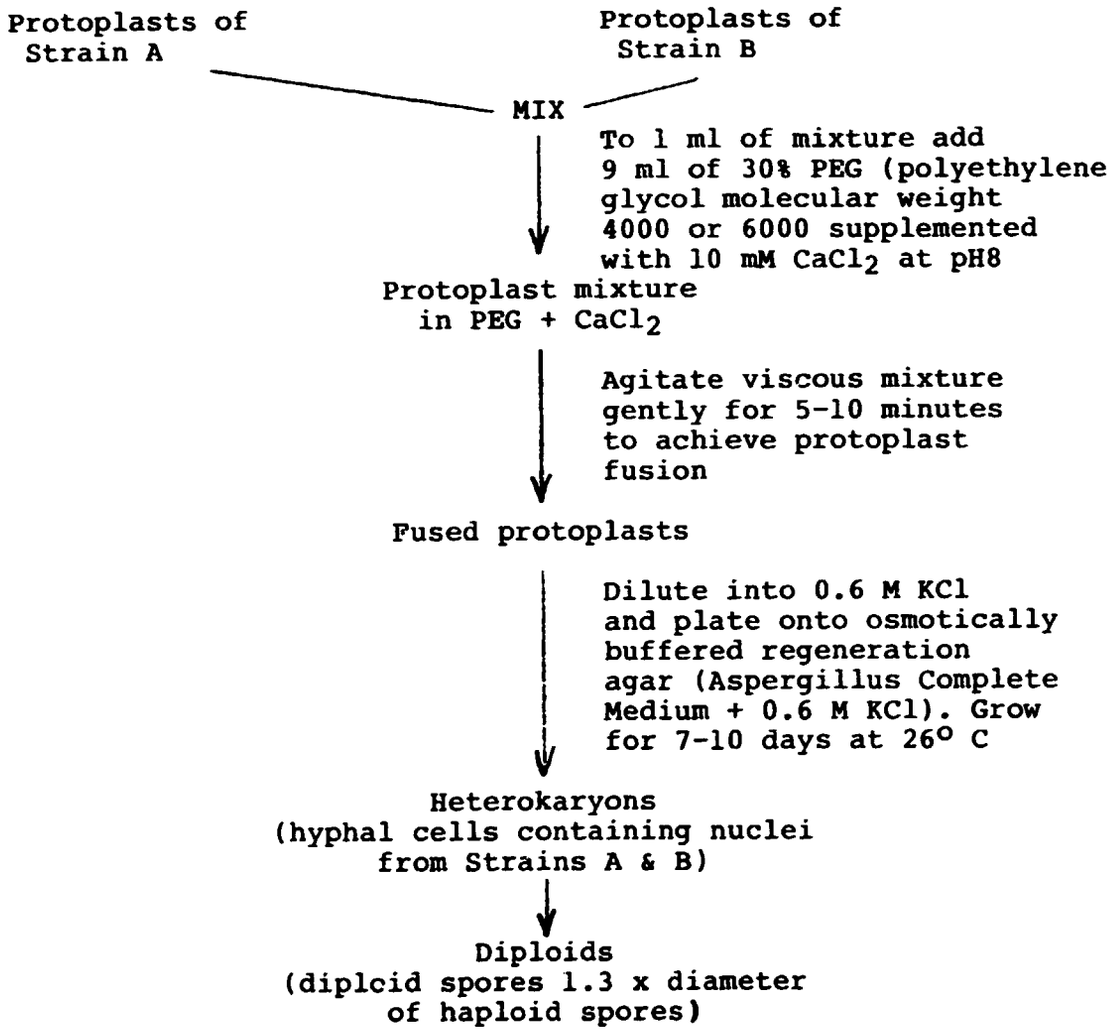
↓ remove mycelial debris by filtering through porosity 1 sintered glass filter

↓ centrifuge and wash three times with 0.6 M KCl

Pellet of protoplasts (e.g. 1 ml of 5×10^7 to 1×10^8 viable protoplast)

* Ditchburn et al, J. Applied Bacteriology, 37, 515 (1974)

HYBRIDISATION USING PROTOPLASTS OF STRAINS A & B
(from Saunders & Holt 1987)



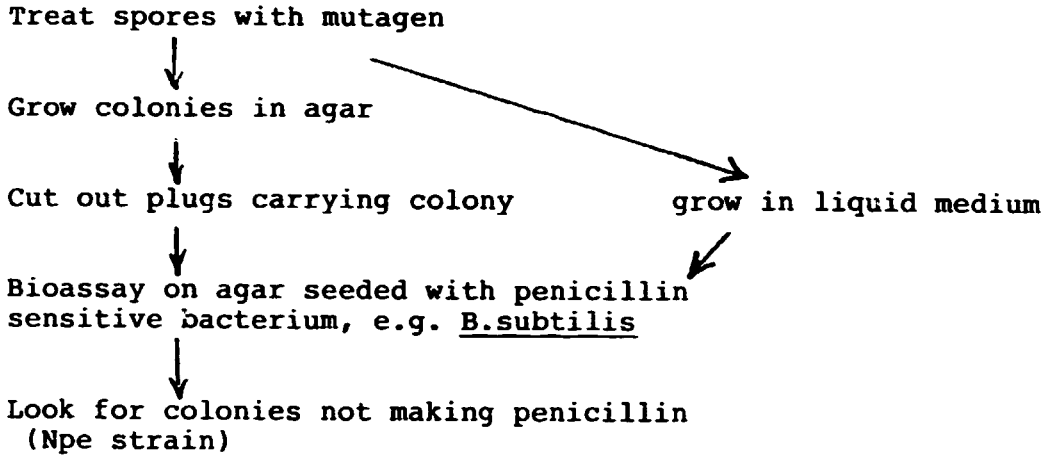
For composition of Aspergillus Complete Medium see Ditchburn et al, Journal of Applied Bacteriology, 37, 515 (1974).

References to fusion of protoplasts see:

Anné & Peberdy, Journal of General Microbiology, 92, 413 (1976)

also Elander, Advances in Biotechnology, 1, pages 3-8, Pergamon Press, Oxford, 1981.

STUDIES ON Npe STRAINS (NEGATIVE PENICILLIN)



Npe mutants are assigned to 5 groups by complementation analysis (crossing negatives to give diploids, etc.).

5 npe genes controlling penicillium synthesis designated:

V) these mutants make tripeptide, therefore they are
W) blocked in ring closure or side chain incorporation.

X) these mutants do not make the tripeptide, therefore
Y) they are blocked in the synthesis of intermediates
Z) prior to the tripeptide

V is blocked at isopenicillin N to penicillin G

W is blocked at tripeptide to isopenicillin N

Y and Z are blocked at aminoadipylcysteine + valine to tripeptide

CULTURE MEDIA USED FOR SELECTION OF AUXOTROPHES AND USE IN HYBRIDISATION

(Ref: Calam, et al. Penicillin: tactics in strain improvement, p.273 in 2nd International Symposium on the Genetics of Industrial Micro-organisms Ed. K. D. MacDonald, Academic Press, 1976)

Minimal Medium	g/l
Glucose	60
$NaNO_3$	3
KH_2PO_4	0.3
K_2HPO_4	0.7
KCl	0.5
$MgSO_4 7H_2O$	0.5
$FeSO_4 7H_2O$	0.01
Agar	20

Complete Medium

	g/l
Lactose	30
Corn Steep Solids	2.5
Solution C	20 ml

pH adjusted to 6.1 - 6.3 with NaOH solution

For slope cultures and general use corn steep solids were increased to 20 g/l

Solution C contained - g/l:

$MgSO_4 7H_2$	2.5
$FeSO_4 7H_2O$	0.6
$MnSo_4 4H_2O$	0.2
$CuSO_4 5H_2O$	0.2

CULTURE MEDIA USED FOR SPORULATION TESTS INOCULUM PREPARATION AND PENICILLIN V PRODUCTION IN FLASKS

(Ref: Calam, C.T. et al, p.273 in 2nd International Symposium on Genetics of Industrial Micro-organisms Ed. MacDonald, K.D. Academic Press, London & New York, 1976)

For sporulation tests (Foster's medium)

	g/l
$NaNO_3$	3
KH_2PO_4	1
$MgSO_4 7H_2O$	0.5
KCl	0.5
$FeSO_4 7H_2O$	0.01
Sucrose	30
$CaCl_2$	40
Yeastrel	1

pH adjusted to 6.8 with NaOH.

50 ml in a 500 ml conical flask inoculated with spores and incubated on a shaker for 7 days to give deep green suspension of spores.

Cultures used: mutants obtained from WIS 51/20, e.g. JH272. (penicillin yield 6000 u/ml)

Inoculum Medium	g/l	Production Medium	g/l
Corn Steep Solids	40	Corn Steep solids	37.2
Glucose monohydrate	25	Lactose	36.6
KH_2PO_4	0.5	$CaCO_3$	6.7
$NaNO_3$	1.5	KH_2PO_4	0.67
$ZnSO_4 5H_2O$	0.04	$MgSO_4 7H_2O$	0.33
$CaCO_3$	10	$NaNO_3$	3.3
$MgSO_4 7H_2O$	0.25	$Na_2S_2O_5 5H_2O$	1.67
		Phenoxyacetic acid	2.2

pH adjusted to 5.3 - 5.5 with NaOH

50 ml. in 500 ml. conical flask

Inoculum - 2 ml. of spore suspension (about 2×10^7 spores).

Grow for 44 hours on rotary shaker, 25° C, to give inoculum culture.

45 ml of medium in 500 ml conical flask + 1 ml of arachis oil + 0.5 ml. white oil.

Inoculum: 5 ml from inoculum culture added to each production flask.

Grow for 5-6 days on rotary shaker at 25° C, 250 cycles/min, 5 cm diameter of rotation.

For 5 litre fermenter the lactose/corn steep liquor medium was used supplemented by a nutrient feed from 72 hours.

B.S.O. Technical Comments on the Reports of Dr. Batchelor (11-01), Dr. Cole (11-02) and Dr. Bird (11-04)

In their reports, the experts present extensive information and sound recommendations for the continuation of the experimental work in GPF.

The initial breakthrough for installation and commissioning of the HPLC and the clarification of the characteristics and problems of every analytical method used or available to be used (potentially useful), proved to be essential for the whole advance of the research and final results of the project.

Dr. Bird's clear recommendations for analytical working procedures, GLP, record-keeping and additional laboratory equipment/items required, are most valuable and should be closely followed.

The supply of reference standards and literature is highly appreciated.

The methods recommended by Dr. Cole for strain improvement and for the conduction of fermentation have to be tried from now on. As strongly recommended, it is essential to maintain a good level of communication between the project team to keep detailed records of the experiments' conditions and results, and prepare summaries of the work done, in order to get the best advantage of the experts! advice and to facilitate all the necessary analysis for the work progress. The measures suggested for the laboratory building finishing/design, especially those related to the avoidance of microbial contamination, should be faithfully followed.

The installation of pending equipment/instruments should be also finished before the next experts' visit.

Work on the Pen V recovery aspects has to be stressed after the installation is finished and fermentation conditions settled, following Dr. Batchelor's recommendations.

The possibility of purchasing the Westphalia centrifuge as pointed out by Dr. Batchelor, will require financial arrangements in the project budget which could be discussed/approved during the next experts' mission in October 1992.

The individual and team work of the experts is regarded as highly qualified and efficient; their personal interest and contribution to the project's success is also recognized.