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THE PEOPLE'S REPUBLIC OF CHINA

Technical report: Third and fourth visits to the Guangzhou pharmaceutical factory and research institute, October 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, expert in industrial microbiology

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* This document has not been edited.

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ABSTRACT

The Expert in Industrial Microbiology made two visits to the laboratories (2-9th October and 23-31st October 1992). An international microbiology conference was attended in Beijing and a visit made to see Mr. Nygard at the UNIDO offices there.

Staff had moved into the new microbiology laboratories, although the ventilation and shaker rooms were not complete and much finishing and cleaning work had still to be done. There is plenty of space for key operations but there is a need for new basic equipment such as incubators, autoclave, pH meter, balance, etc.

The Braun 20 l fermenters had been installed in a separate room. Various testing and calibration procedures were carried out but there were difficulties with the PC, with contamination of air lines with foam and with the inoculum transfer equipment. Most problems were overcome but foaming during sterilisation needs attention. A faulty control valve has been returned. A full length fermentation run has still to be carried out. A service engineer from B. Braun Biotech spent one week helping with problems and training staff.

New culture freeze-drying equipment was set up and training given. Preservation, using liquid nitrogen, was explained but could not be set up. For both methods ancilliary equipment is required. Staff from the Guangdong Institute of Microbiology, Guangzhou, presented posters on these methods at the conference in Beijing; a visit to their laboratories could be useful.

Talks were given on methods relating to Penicillium chrysogenum strain improvement, e.g. mutation, replica plating, selecting auxotrophs and making genetic recombinants. Methods for selecting and evaluating strains were discussed in detail and a start made on setting up UV mutation.

To improve the specificity of the iodometric assay for penicillin in fermentation samples penicillinase could be used. An old preparation was tested and found inactive. If fresh enzyme cannot be obtained it was suggested it could be made, using Bacillus cereus 569/H.

The latest 1000 l penicillin V fermentation run, E68, gave a high titre at 140 hr by iodometric assay. After cooling and adjusting to pH 4.7 it was readily filtered and penicillin V obtained as crystalline material by slow adjustment to pH 2.5.

PART I

Covering Period 2nd-9th October, 1992

1. Introduction.

Travel and Communication Difficulties: Mr. A. E. Bird (UNIDO Consultant on analytical methods) and I travelled together and arrived in Guangzhou a day later than planned because of travel difficulties.

Prior to setting out we had problems communicating by FAX or telephone with the Po Lian Development Company Offices in Hong Kong or with the Guangzhou Pharmaceutical Factory (GPF) and, as a result, no train tickets had been purchased for us for the journey to Guangzhou. The China Travel Agency in Hong Kong had no tickets available for Sunday, Monday or Tuesday but did have seats on the Monday morning ferry (catamaran) service from Hong Kong to Guangzhou. We elected to take this route, but it meant we had to stay an extra night in Hong Kong (expensive) and arrive in Guangzhou at lunchtime on Monday, 5th October.

Staff in Guangzhou only knew on Monday morning, 5th October that we were coming as a result of a FAX they received from Mr. Nygard, UNDP, Beijing, who forwarded a copy of my itinerary to Mr. Li Jian at the GPF.

Meeting at Guangzhou Pharmaceutical General Corporation Offices on 5th October: On our arrival, a meeting was immediately arranged at the Guangzhou Pharmaceutical General Corporation's offices, 45 Sha Mien North Street, which are close to the White Swan Hotel where we were staying. Present at the meeting were:-

- Mr. Che Ming Gang (President, GPGC)
- Mr. Yuan Zheng (Director, GPGC)
- Mr. Cai Shi Chao (Daniel - Senior Engineer, GPGC, our technical translator)
- Mr. Liu Guang Tao (Director of Research Institute of the GPF)
- Mr. Liang (GPF Penicillin Project Director)

Our travel and communication problems were discussed. Mr. Cai gave us a 24-hr FAX number for his office and recommended we use this in future. He also gave us his office and home phone numbers:-

24-hr FAX GPGC offices Sha Mien St.		8861913
Mr. Cai's office	Tel:	8854609 ex. 20, 26
Mr. Cai's home	Tel:	8880943

There was a general discussion about the progress of the Penicillin V Project, and of the consultancy work to be done. It was agreed that we would be collected from our hotel at 8 a.m.

We were presented with copies of a book describing the many enterprises (factories, laboratories, shops) which made up the Guangzhou Pharmaceutical General Corporation and their amazing range of products, particularly herbal remedies.

UNIDO Travel Fellowships: The microbiologists who had UNIDO travel fellowships to the USA were unable to go to the laboratories of the American Type Culture Collection for training in culture preservation methods as had been hoped, because it was said they could not accommodate them. Instead they spent several weeks studying various microbiological topics at the University of Maryland. [In a later discussion I had with a Dr. Roblin of ATCC, I was told that ATCC could provide training in culture maintenance but was not able to help with the wide range of topics in the original request.]

I had suggested that it could be advantageous for a microbiologist from the GPF laboratories to attend the VIIth International Congress of Culture Collections meeting on micro-organisms which was being held in Beijing (12-16th October) and which I was attending. It was stated that they applied too late to get a place. This was a great shame as it was an excellent conference and would have been an opportunity to meet Chinese and foreign microbiologists.

Just as we were arriving, several members of staff of the GPF were setting off on UNIDO Travel Fellowships to Europe, some to a Swedish institute of technology; this group included Mr. Luo who was responsible for the microbiological pilot plant, the person working on the pencillin acylase and one of the analysts.

In future, it will be important to try to plan UNIDO consultancy visits so that key staff are not missing on UNIDO organised tours abroad. Although this did not cause too much of a problem this time, my last visit, unknown to me until I arrived, clashed with the research microbiologists being in the USA. Also, it is vital that the content of the study tour is relevant to the work or responsibilities of the staff being sent.

I was asked during this consultancy visit to the GPF laboratories to set up the freeze-drying and other equipment and demonstrate its use for preserving micro-organisms, whereas the original plan was that such training be given by the ATCC who have an international reputation in this field.

Preliminary meeting at Guangzhou Pharmaceutical Factory on 6th October: We met Mr. He Guo Wei, the Director responsible for the factory, who said he thought the research environment had improved and that he would like us to comment on all aspects of the work during our visit, including staff and equipment. We were taken to inspect the new building with Mr. Liu. My comments on this are in a separate section of this report. We understood from Mr. Cai that the main product of the factory had ceased to be profitable and that this was making it difficult to pay for improvements, equipment and facilities.

2. Progress with the construction and fitting out of the new microbiology laboratories:

Considerable progress had been made with the construction work, allowing staff to start using some of the new laboratories, although many of the facilities, for example, constant temperature rooms for shaken flask cultures and air ventilation systems for laboratories, had still to be completed. Although the floors and walls were of good materials, the general finish was poor due to careless workmanship, no attempt having been made to clean spilt materials off floors or stone work benches. The system of sinks was of ancient design and sometimes consisted of large cast concrete and tiled troughs with a hole smashed in the bottom to let the water run away, via open channels. It would have been far more satisfactory and probably cheaper to have fitted stainless steel kitchen-style sinks with drainers, and connected the outlets via pipework to the main waste water pipes. The underbench cupboards were poorly made and all doors will have to be refitted.

The floors, laboratories and main corridors and stairs were extremely dusty when first inspected, but attempts were made to clean them with water and mops. There are inadequate power points around the general microbiology laboratory area, making it difficult to connect current, let alone future equipment. There is a great need for new equipment, such as incubators, small centrifuges, autoclaves, balances, etc.

The room set aside for the Braun 20 l Biostat UD fermenters had been well fitted out, with supplies of steam, chilled water and compressed air. The pressure of the latter was inadequate for operating the valves on the fermenters, so this was achieved using a compressed air cylinder. The two Braun fermenters had been installed shortly before I arrived, but the location of the computer was unsatisfactory, being too close to the fermenters. When Mr. Khoo of Braun Biotech International, Malaysia, arrived to service the fermenters and provide further instruction, the computer was moved to the other side of the room away from steam and water.

One of the three sterile transfer rooms was in operation with a safety cabinet and seemed to be operating satisfactorily, although the fresh air supply via a small pipe from the compressed air system was not adequate for the purpose of supplying fresh cool air.

One of the three or four spore culture rooms was in operation with a temperature control unit. It contained a large number of P. chrysogenum spore bottles. While I was visiting the laboratories, the shaker rooms were cleaned and a shaker installed in one of them.

Work was continuing on the ground floor (plastering and flooring) where the plan is to relocate the pilot plant, currently housed in a very old building alongside. Outside, at the front of the building, a large area of concrete paving has been laid, but there are still open waste drains round the building, which in the long term must be routed below ground.

I and my visiting colleagues were allocated a room as our office, next to the Director's general office. These facilities were much appreciated.

3. Progress with operating the Braun 20 l fermenters (starting 6th October, 1992).

Initial problems with fermenters: Mr. Zhu Ding Xing was in the process of calibrating the pH electrode on one of the fermenters when I arrived. I asked him how he was getting on with the task of getting the fermenters operational. He reported that the link between the digital control units (DCUs) on the fermenters and the computer (AST Premium Cupid -32™) was not working and a disc drive was found to be faulty on installation. The fermenters could be operated automatically, via the DCUs but not via the computer and no data could be stored or processed. One of the DCU display panels was not illuminated, making it difficult to see - this seemed to be due to a poor electrical connection and was rectified. One of the

solenoid air supply control valves was said not to be working properly. Guangzhou Pharmaceutical staff contacted the Braun offices in Hong Kong, requesting someone to come to help solve the problems. Miss Edith Cheung, a Biotechnology Sales Executive of B. Braun Medical (HK) Ltd. came and took note of the problems. She corrected some and said she would make arrangements for a fermenter expert from B. Braun to visit.

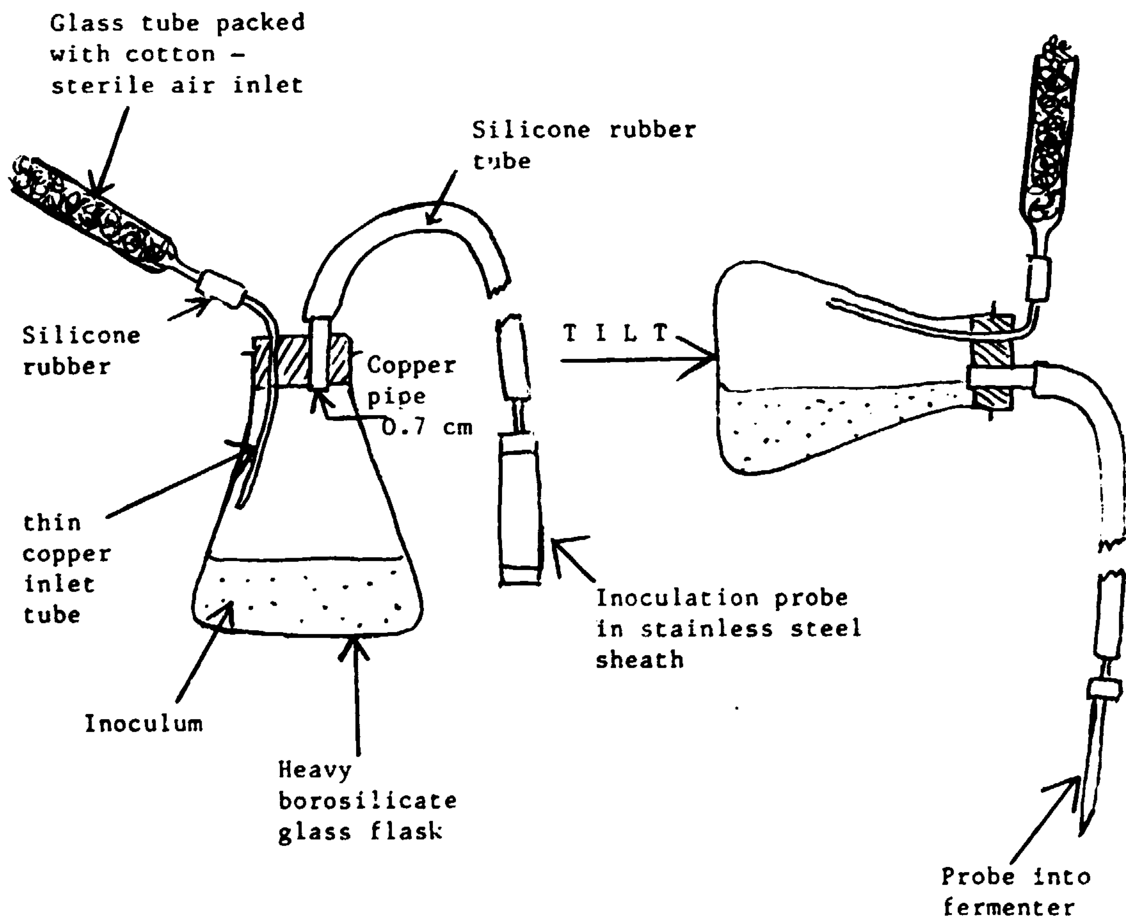
First attempt at sterilisation: After calibrating the electrodes, using the pH 4.3 and 7 buffers supplied by Braun, and marking the 20 l volume line on the front glass viewing panel, one of the fermenters was charged with the soyabean/peanut meal medium, as used in the 1,000 l fermenter, and put through the automatic sterilisation cycle (steam heated via jacket and temperature maintained at 121°C for 30 mins before entering cooling cycle). However, there was a discrepancy between the reading in the thermometer in the tank and the reading on the DCU, the latter reading low, e.g. 26° v 33° and 114° v 121°. I suspected a calibration error as the sterilisation cycle operated when the thermometer read 121°.

As the medium contains solids, I felt that a check should be made to see if the medium had been satisfactorily sterilised. However, the outlet port was blocked, presumably with medium solids, but was cleared by blowing steam back through the valve. A sample was then taken, using the accepted procedure, directly into an agar culture tube. No growth was noted after several days' incubation.

First attempts at inoculation of fermenter: Miss Chen had transferred the contents of several shaken flask cultures of P.chrysogenum to the inoculum transfer bottle (pre-sterilised and fitted with transfer tube and stainless steel probe). Using standard aseptic techniques the inoculum tube was satisfactorily connected via a septum port in the top of the fermenter. However, on raising the bottle above the fermenter, the inoculum would not run in under gravity. The arrangement and size of the tubing meant that a siphon could not be established; also air might not have been able to flow into the bottle at a sufficient rate via the sterile filter. Applying air pressure (cycle pump newly purchased for the purpose!) via the air filter did enable the transfer of some inoculum, but then the tube blocked and the method had to be abandoned.

Redesign of inoculum transfer bottle: Inspection of the bottle revealed that the pipework was blocked by mycelium and solids and that it would be necessary to redesign it. The training programme at B. Braun's laboratories had involved an E. coli culture in a medium which did not contain solids. At that time, it was pointed out - that it was important to use a filamentous organism such as P. chrysogenum, as several aspects could be different including stirring, aeration and sampling.

In the new design the outlet port would need to be of large diameter (at least 0.5 cm) to avoid blockage, and not dip into the contents of the bottle. Also the air inlet port would have to allow easy entry of filtered air. The following was constructed, tested with inoculum in a subsequent fermentation run and found to be satisfactory.



<u>Glucose feed for pH control - first run:</u> A glucose feed bottle had been connected via a pump on the fermenter to be triggered if the pH rose during the fermentation. Owing to the over addition of NaOH solution (pH indicator papers used) the pH was 8.0 after sterilisation, with the net result that all of the glucose feed was added prematurely. It was recommended that the medium be put into the fermenter and the pH adjusted to 6.8 (from 6.2) with dilute NaOH solution prior to sterilisation. The glucose feed pump should then only cut in when the growth was well established and metabolism induced a rise in pH.

4. Composition of glucose feed solution (reducing sugar solution):

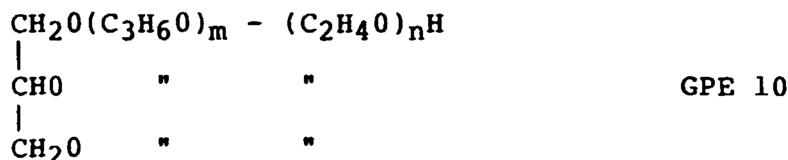
The glucose solution was a dark brown mobile liquid which Mr. Zhu said that he had prepared from starch, by the following process: A 50% starch suspension in water was heated to 90° C for 1.5 hr, cooled to 60° C, the pH adjusted to 4.0 and a hydrolytic enzyme from the Wuxi enzyme factory added. After 48 hours, a brown liquid preparation was obtained, having a 48% reducing sugar content.

It was suggested that this material be called a reducing sugar solution, as it was clearly not all glucose. It was kept chilled but after a few hours standing on the bench at 26° C it started showing signs of fermenting. The advantage of using such a material is presumably that it would be cheap for a large scale; however, it should be produced freshly and to a defined standard.

5. Antifoam for use in Braun 20 l fermenters:

Further details of where to buy the antifoam polypropylene glycol P2000 were given to Mr. Zhu (Aldrich catalogue entry 20233-9, 500 g £16.40). My earlier suggestion that Dow Chemicals be contacted had been followed up and their Hong Kong office had supplied 500 g. gratis. Unfortunately, the whole batch was added to a 1,000 l fermenter and was said to have not worked very well; however, the final concentration would have been less than 0.1%. It was suggested that a further small sample be obtained for experiments on its effects on foam and penicillin yield in the 20 l fermenter, in comparison with other agents. It may be too expensive for large scale use.

In a trial sterilisation run carried out during my visit three weeks later, when Mr. Khoo, an expert from B. Braun was present, the peanut/soya bean medium (usual medium) was observed to foam when the temperature reached about 90° C. The sterilisation heating cycle had to be stopped to prevent the foam rising into the air outlet filter. A possible explanation is that the peanut oil antifoam which is included via the medium, ceases to be in a form which has antifoam properties (e.g. dissolves in the medium). It was, therefore, necessary to find another agent which could be used. A request was made for a silicone antifoam as it was understood that this was used in the factory for the lincomycin fermentation. However, the liquid material supplied did not look like a silicone oil. It was called GPE 10 and was obtained in China. Mr. Liu looked up its composition which was of a glycerol polyalkylether type as follows:-



This agent, when added to the medium (100 ml in a 20 l fermenter) acted as a very effective antifoam during sterilisation and permitted the cycle of 121° for 30 mins with stirring to be completed without foaming. However, this was a very high concentration to use, so trials would have to be done to find the lowest amount which would control foam and have no adverse effect on the penicillin fermentation. Mr. Liu indicated that it was not suitable, but I am not sure why.

6. Second attempt at running the Biostat UD 20 l Fermenter:

The fermenter was charged with the standard peanut/soyabean medium as before. Mr. Zhu set up the sterilisation cycle but was concerned that the pH was not correct, although he had adjusted the pH in the fermenter as I had suggested (pH 6.8). It was decided that the electrode should be recalibrated. As part of the calibration of the oxygen electrode it was set to zero at the end of the sterilisation cycle. The 100% air saturation point was to be set when a steady state had been reached by blowing sterile air through the uninoculated medium.

The Digital Control Unit was set to introduce air at the rate cf 0 l/min at the end of the sterilisation cycle, but the actual flow rate was only 5 l/min and fell to zero as shown by the rotameter. I asked if foam might have escaped from the tank during sterilisation and got out along the pipework to the exit air filter to block it. It was acknowledged that this was a possibility. Inspection of this filter revealed that it was blocked, thus preventing escape of air. It was replaced and the airflow of 20 l/min achieved. Clearly, it is imperative to prevent foaming during sterilisation.

Having run into the above problems it was decided to practise the use of the new inoculation bottle and then abandon the run. After sterilising the bottle, it was charged with about one l of seed stage culture by combining the contents of several shaken flask cultures grown using a corn steep liquor medium. The transfer bottle worked satisfactorily.

7. Discussions with Mr. Cai for translation and communication to Mr. Liu:

Seminars on strain improvement for penicillin V: It was agreed that I should give a series of talks on how the performance of their P. chrysogenum strains might be improved by mutation and genetic recombination methods. Methods of assessing performance would also be discussed. These talks would be given during the second part of my visit at the end of October. Mr. Cai borrowed my notes and diagrams so he could study them. These had been left with the Guangzhou laboratories following my previous visit and were attached to my report. However, they had not been discussed in any detail in the absence of the microbiologists on a visit to the USA.

Publication about sporulation of P. chrysogenum: On a previous visit there had been discussion about the formation of spores or chlamydospores in suspension culture. A paper relating to this had appeared in the Chinese J. of Antibiotics, 17 (1) page 1, 1992. A copy was given to Mr. Cai.

Presentations by GP research staff on progress with penicillin V project: It was suggested that it would be very helpful to Dr. Batchelor when he arrived if the senior staff could give progress reports, describe problems and outline their proposals for future work. It was agreed that a meeting be arranged for Saturday, 10th October.

List of suppliers of equipment: A list of addresses, telephone and FAX numbers of equipment suppliers in UK, as given in the September issue of Process Biochemistry, was given to Mr. Cai.

International Mycological Institute Book on Culture preservation: As Mr. Cai indicated that the GP laboratories did not have literature on culture preservation, I gave Mr. Liu my copy of the above book, details of which are given in the section "Publications supplied". Page 14 onwards in this book describes methods for preserving cultures by freeze drying and p.20 onwards describes liquid nitrogen preservation.

8. Culture preservation equipment:

I was asked to help unpack and set up the LabConco freeze dryer and Edwards high vacuum pump. The location of the equipment was agreed but it could not be tested until suitable electrical plugs were connected.

I went over the method of setting up the equipment but running it would have to be left until my next visit. Also silicone grease or oil was needed for the high vacuum connections. The need to keep the equipment free from dust was emphasised.

Two liquid nitrogen flasks had already been unpacked but one of the ampoule carriers was found to be broken.

9. Progress with the current 1,000 litre penicillin V fermentation and extraction:

On Friday, 9th October arrangements were made for the collection of a preharvest sample (164 h) for iodometric assay of total penicillin and for HPLC assay of penicillin V (by Mr. A. E. Bird) [32,000 u/ml, by HPLC, 37,000 u/ml by iodometric]. I made a quick inspection of the progress of the extraction when Dr. Batchelor arrived in the afternoon. The mycelium was filtered off fairly quickly at pH 4. The filtrate was adjusted to pH 2, but the resulting precipitate of penicillin V was very fine and difficult to filter. Microscopic examination revealed very small crystals and some other solids. It was thought that the temperature might be too low and the acid added too quickly. Also, there could be better removal of unwanted solids at pH 4. Chaplin & Bucke in "Enzyme Technology", p.53, refer to the use of Whatman CDR-cell debris remover - as being useful to bind to unwanted negatively charged material. CDR consists of fibrous cellulose with diethyl aminoethyl functional groups, and could be tested as a floculating and clarifying agent (Whatman catalogue has been supplied - £23 for 500 g. may be too expensive).

10. Review Meeting between GP research staff and UNIDO Consultants - Mr. Batchelor, Mr. Bird and Mr. Cole on 10th October, 1992:

As mentioned earlier, I had requested that a meeting be held when Dr. Batchelor arrived so he could be updated by GP staff with progress and problems. Unfortunately, although requested in advance, no summary reports or data such as graphs, tables, etc. of results were provided by GP staff. However, Mr. Liu provided the detailed record sheets of the recent 1,000 l fermentation runs. Only in the last two or three weeks had penicillin V titres in the region of 40,000 u/ml been obtained. Although this was encouraging, it was felt that there was room for improvement. Currently, they are carrying out about six runs a month. It appeared that a second vegetative seed stage had been introduced between spore suspension and the final tank, as I had suggested. Titres were still being expressed as u/ml (rather than µg/ml) and there is uncertainty about the reference standard used.

In response to a question about what we wanted to know, Dr.Batchelor said he would draw up a list of items such as: run number, strain used, date, final titre (units or µg/ml and reference standard used), final pH, fermentation time at harvest, results of tests for evidence of contamination, any unusual feature in run (power cut, change in medium composition, unusual pattern of precursor or sugar addition, etc.). As no summary data were available about the yields at various stages (mass balance) in the extraction, again a table would be drawn up by Dr. Batchelor setting down what was required.

GP staff expressed concern that the P.chrysogenum mycelial pellets seen in the 1000 l fermentation were not of large enough size, the presumption being that large pellets were desirable. I am not sure where they got this idea from, but I pointed out that small pellets could be desirable from the point of view of improved oxygen transfer.

Inspection of the fermenter record sheet for the last completed run, inoculated on 30th September 1992, revealed the following results:-

Tank No.2 Culture No.2 (902) Run No. E-64

Sample time (hours)	28	44	68	106	124	148	164
Titre (thousands u/ml iodometric)	10.0	15.4	21.4	31.5	36.0	40.2	42.4

These results are almost certainly an over-estimate of penicillin V titre, because Mr. Bird had found that a sample assaying at 37,000 u/ml by iodometric assay for total penicillin gave a value for penicillin V of 32,000 u/ml by HPLC assay; however, it was still to be investigated whether the samples had been treated in identical ways prior to assay (see report by Mr. A. E. Bird).

Mr. Cai made it clear that work on the penicillin acylase had been stopped and no 6-APA is to be made. The programme would concentrate on making penicillin V for use as an end product and not an intermediate.

The objectives for the second part of my visit (2 weeks time) were agreed, namely: to set up culture freeze drying equipment and demonstrate its use, discuss other methods of preservation (e.g. liquid nitrogen), help with establishing methods for using the Braun Biostat UD 20 l fermenters, describe and, where possible, demonstrate methods for carrying out strain mutation and other methods of strain improvement. Mr. Liu agreed to get a 254 nm UV light source set up for my next visit for use in determining kill curves and making mutants. A request was made for a multipoint inoculator for replica plating work.

PART II

Covering period 23rd-31st October, 1992

11. Second Part of Consultancy Visit - Introduction.

After attending the 7th International Congress for Culture Collections in Beijing and visiting various cities in China, I returned to Guangzhou for consultancy work from 23rd - 31st October. On Friday, 23rd I met Mr. Cai at his office, discussed items of interest from the conference and planned work for the coming week, including equipment required.

I had met Dr. Richard Roblin of the ATCC in Beijing and he indicated that they could provide a specific one week training course on preservation of P. chrysogenum.

Mr. Cai informed me that a technical expert from B. Braun Biotech in Malaysia would be visiting the laboratories starting 26th October, to deal with problems with the equipment and to give a training course on its use. Mr. Cai showed me a FAX from Dr. Batchelor in which he mentioned that the iodometric assay was giving results which were higher than the HPLC. I suggested the use of B-lactamase, (penicillinase) to generate the penicilloic acid from penicillin V, rather than addition of caustic soda, might provide a more specific method (see next section).

12. Problems with assay of penicillin V in fermentation samples, using the iodometric assay - Possible use of penicillinase (B-lactamase):

On 24th October I was given a letter which Mr. Bird had left for me at the end of his visit to the GPF Research laboratories. In this letter he indicated that using the iodometric assay a sample of uninoculated sterilised culture medium assayed as though it contained a substantial amount of penicillin V. It seemed that some component was reacting with the caustic soda and generating an iodine absorbing material. Mr. Bird suggested that use of penicillinase instead of caustic soda might overcome the problem, and asked me to suggest a source of the enzyme. I discussed this with Mr. Liu, but he did not know of a source in China. I indicated that it should be possible to obtain it from Sigma or one of the other suppliers of biochemicals, but he had no catalogues. I therefore recommended that a culture of Bacillus cereus 569/H which is a widely used source of the enzyme be purchased. This organism, which produces a mixture of B-lactamases constitutively (i.e. without induction) can be grown in a nutrient broth medium in shaken flask culture to give a potent preparation of B-lactamase.

I pointed out an article on this enzyme in Vol.43 of Methods of Enzymology (copy previously presented to Mr. Liu). This article (p.640) gives the NCTC as a source of the organism, NCTC 9945 (National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT, United Kingdom). The organism is also available under the number NCIMB 8933 (National Collection of Industrial and Marine Bacteria, 23 St. Machar Drive, Aberdeen, AB2 1RY, United Kingdom). It is possible that the organism is available in China, for example from the Beijing or Guangdong Institutes of Microbiology.

The B-lactamase of B.cereus 569/H is available as a mixture of types I and II (Catalogue No.191489) from ICN Biomedicals, United Kingdom (FAX 0494 473162). It is supplied as a lyophilised powder, costing £44.75 sterling for 5,000 units [1 unit = amount of enzyme which will catalyse the hydrolysis of 1 µmole of penicillin per minute at 25°C]. As this activity probably relates to penicillin G as substrate, the activity against penicillin V could be slightly different, and would have to be determined by experiment.

Assuming the activity against penicillin V to be the same as against penicillin G, 386.5 µg of penicillin V would be hydrolysed to penicilloic acid by 1 unit of enzyme in 1 minute. Thus, 10 units should convert 3.86 mg in 1 minute. Given that this could be near the maximum amount of penicillin V in a 1 ml sample of fermentation broth after the 1/10 dilution recommended, 10 units of enzyme should be enough to destroy all of the penicillin V in the diluted sample within a few minutes, and should, therefore, be suitable in place of caustic soda (NaOH) in the iodometric assay. Mr. Bird sent a FAX to the GPF saying that he had found that Sigma supplied penicillinase, £35 for 5,000 units.

During a discussion with one of the microbiologists (Miss Chen) about the method for cultivating B. cereus to make penicillinase, it came to light that she had some old vials of this enzyme. These had been obtained from the Medicine Detection Unit, Jinan City, Shandong Province in 1987. Although the material looked brown and sticky, arrangements were made to carry out a test of its activity against penicillin V, using the iodometric assay. The contents of a vial were dissolved in 5 ml water and 1 ml of this solution added to 5 ml of a 1 mg/ml solution of penicillin V at pH 7. Iodine uptake values were determined on reaction mixtures after 0, 15, 30, 45 and 60 minutes. As there was little evidence of an increased iodine uptake with time, it was concluded that the enzyme was virtually inactive. Miss Chen had carried out a test on the enzyme in 1990, using a penicillin fermentation sample as substrate. Activity was measured by bioassay. Although there was some evidence for destruction of the bioactivity of the penicillin at the higher penicillinase concentrations, it was far from complete, suggesting the enzyme preparation was of low activity.

I recommended that attempts be made to obtain a fresh sample of the enzyme, or purchase the culture of B. cereus so that a crude preparation of the enzyme (culture supernatant after centrifuging off cells) could be made when required.

During discussions about the iodometric assay, it was said that the volume of filtered fermentation broth sample used in the assay was only 0.1 ml. I pointed out, as Mr. Bird had already done, that if the concentration of penicillin V in the sample was high there could be very large errors when taking such a small volume. A larger sample of whole broth should be taken, diluted into water and a proportionately larger volume of the diluted sample used in the iodometric method.

13. Further Progress with Establishing the Methods for Running the Braun 20 l Fermenters (starting 23rd October):

Mr. Zhu had succeeded in establishing the link between the computer and the Digital Control Units on the fermenters by giving each fermenter a number, so it could be recognised by the computer. This should have been done by the installation engineers. However, there were other problems, it being impossible to get an adequate airflow through one of the fermenters. I came to the conclusion that this was as a result of a blockage between the sparger and the sterile filter for the incoming air; the filter was heavily contaminated with dirt on the inner or fermenter side where the air should be clean and sterile. The outlet air filter was also contaminated. The pipework was disconnected and the air sparger found to be blocked with culture medium. Mr. Zhou reported that the fermenter had foamed during the sterilization cycle but this does not readily explain how medium was sucked back down the incoming air line. It is important not to overfill the fermenter and to use an adequate antifoaming agent. It may be necessary to use a culture medium which does not contain solids which can block the small holes in the air sparger. It might be possible to use a medium based on cornsteep liquor as a source of organic nitrogen. These problems were to be discussed with the Braun engineer (see below).

14. Visit by Engineers from B. Braun - problem solving and training:

On 26th October, Mr. H. S. Khoo, a service engineer from B. Braun Biotech, Asia Pacific Headquarters, Penang, Malaysia, arrived with representatives from the Hong Kong, Guangzhou and Beijing offices. He checked the computer link with the DCU and adjusted the parameters so as to match the DCU/actual values with what was shown on the PC screen. He went through the control system and reported all OK before setting about trying to solve the problems with the air flow blockages. By this time, we found a similar problem with the second fermenter, i.e. blockage in the air inlet side. Mr. Khoo said it was important to maintain an air flow of at least 15 l/min during the sterilisation cycle, to prevent medium getting into the sparger line. This airflow should be automatically shut off during the heating phase, but restored during the cooling cycle so as to prevent a vacuum developing in the fermenter. Mr. Khoo recommended fitting in the condensation return line a disc with a small hole in it to help alleviate the problems we were having. He had suggested this to B. Braun designers in Germany but so far they had not incorporated it. He also recommended that care be taken to replace all rubber 0 rings when pipework was disconnected and that such rings, including the one on the top of the fermenter, be smeared with silicone grease to make an airtight seal.

In a discussion about the unsuitability of the Braun inoculum bottle, Mr. Khoo said that Braun do make a bottle with large ports which should be suitable for our purpose. I asked for the bottles which had been supplied to be replaced. Miss Cheung, the representative from Hong Kong, said she would arrange this. Mr. Khoo clearly had experience of using the Braun fermenters for fungal fermentations being carried out by other customers in Asia.

As Mr. Khoo was not satisfied with the functioning of the main air inlet control valve on one of the fermenters but was unable to repair it with the tools he had, Miss Cheung arranged for it to be returned to Germany. A bypass pipe was put in place of the valve so the fermenter could be used manually.

During the rest of the week, Mr. Khoo conducted an extensive training programme on the use of the equipment, particularly the computer control system. The calibration of all electrodes was checked.

15. Talks and Demonstrations on Culture Preservation and Methods:

Throughout the week 24-31st October, a series of talks and demonstrations was given to a group of microbiologists. These covered the practical aspects of culture preservation, relevant to *P. chrysogenum*, assessment of penicillin V productivity of different strains and preparation of mutants and recombinants. Miss Ching (English teacher) and Miss Xiong Li Hong (one of the microbiologists) acted as translators when necessary.

Culture Preservation:

Aspects of culture preservation and recommended methods for freeze drying (lyophilisation) and storage in liquid nitrogen are given in **Appendix 1**.

At several stages in the methods, improvisations were needed because of the unavailability of materials or equipment. The use of skimmed milk was suggested as a suspending agent as other components were not available. The cryopreservation system cannot be set up until containers for the vials, safety visors and gloves are obtained and arrangements made for a supply of liquid nitrogen.

Existing Preservation methods:

It was stated that they used the mineral oil method. I assumed that this was the standard technique involving covering agar slope cultures with liquid medicinal paraffin; however they said they suspended spores in this agent. Later it was stated that *P. chrysogenum* spores were suspended in 20% sucrose for longer term storage (months). I suggested they continue to do the latter but in parallel with the new methods I had described. Storing spores in sterile soil had been tried but found to give poor viability. This is an established method but requires the selection of a suitable soil.

Operation of Freezer Dryer:

The Edwards high vacuum pump and LabConco freeze dryer were made operational. A very satisfactory vacuum of less than 10 µHg at a temperature of just below -50°C was obtained, with all taps closed. A test freeze drying of a sample of glucose solution was carried out. The technique of plugging, labelling, sealing and opening glass ampoules was demonstrated. However, sealing under

vacuum could not be carried out because a suitable gas flame was not available. It will be necessary to buy a portable propane or butane gas torch for this purpose. Another problem is the lack of availability of a cardice and acetone bath to freeze the contents of the ampoules. A bath of alcohol cooled to -18°C in the deep freeze was used instead.

A dense suspension of P. chrysogenum spores was prepared in milk sterilised by autoclaving (skimmed milk with cream removed). Aliquots were transferred to sterile glass ampoules and frozen. Freeze drying was successfully carried out overnight to give a dry plug of material in each tube. Details of the procedure are given in **Appendix 1**. The contents of two of the ampoules were reconstituted in water and plated out on agar for a viability check.

16. Use of far UV light (about 254 nm) to generate mutants of P.chrysogenum (See Appendix 2):

A 30W UV lamp was set up in a cabinet. A P. chyrosgenum spore suspension in water was prepared and put in a Petri dish on a magnetic stirrer just below the lamp. First of all, an experiment was carried out to determine the length of exposure required to give a kill rate of around 99.9% (i.e. 0.1% survivors). Although the culture plates used to assess numbers of survivors had not had adequate time to give clear colonies, it looked as though too high a kill rate was obtained at the shortest time. If this was the case the procedure would have to be repeated with a greater distance between the lamp and spore suspension.

An experimental method was described for searching for survivors which had undergone mutation and no longer made penicillin. Such cultures would be blocked at one of the many steps in penicillin biosynthesis.

In my previous report I mentioned that five types of penicillin negative mutants (npe) had been described:-

V and W (make tripeptide precursor but blocked in ring closure or side chain incorporation)

X, Y and Z (do not make tripeptide, therefore blocked in synthesis of intermediates.

Penicillin negative mutants can be back mutated to productivity, sometimes with beneficial results. However, the preparation of negative mutants would provide good practice in mutation and selection methodology, details of which are described in **Appendix 2**. The methods can be used in the selection of mutants which are, for example, tolerant to a high concentration of phenoxyacetic or

phenylacetic acids, or can produce penicillin at higher temperature. In such cases the colonies surviving the mutagenic effects of UV (or other mutagen) are grown on agar containing the selective agent or on culture plates incubated at higher temperature (e.g. 28°C compared with 24°C) prior to assessing penicillin yield.

17. Preparation of auxotrophs and use in genetic recombination studies via heterokaryon formation or protoplast fusion:

A talk was given outlining the principles and methods for making auxotrophic mutants and their use in preparing recombinant strains via heterokaryon formation or protoplast fusion methods. Written details of the procedures were given to the GPF staff during my previous visit and were incorporated in the report I prepared.

Auxotrophs are mutants which require the addition of a particular amino acid, vitamin or nucleoside, etc. to a minimal medium to permit growth. Such strains can be used to generate genetic recombinants. A strain requiring phenylalamine can be made to undergo genetic recombination with one which requires, for example, lysine for growth. The combination of the genes from the two strains cancels the requirement for addition of amino acids and so the recombinant will grow on the minimal medium, whereas the parents will not. During this process various genes, other than, those involved in the nutrition of the organism, can get exchanged and it is hoped that this might give a useful hybrid of the characters in the parents used for the cross.

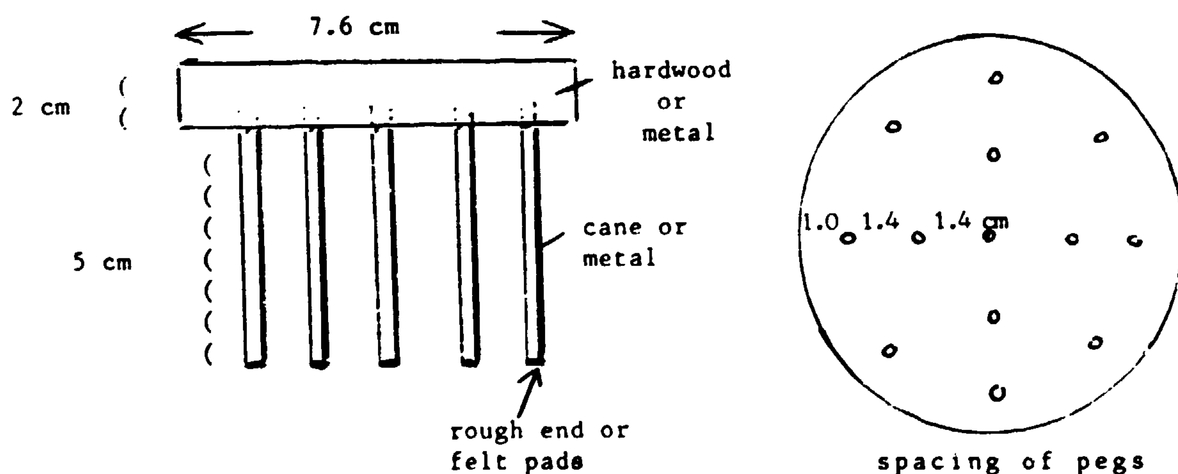
It will be some time before techniques in the microbiological laboratories have advanced to the point where such methods could be carried out. Consideration would have to be given to the characters which needed improvement and the strains required for genetic recombinantion could then be selected accordingly. I would recommend that for the initial strain improvement work should focus on selecting mutants resistant to various agents such as penicillin V, its precursors or heavy metals, or tolerant to higher temperatures.

18. Multi-point inoculator for use in replica plating:

In the mutation and selection experiments described above, it can be very useful to use a multi-point inoculator to transfer spores of colonies from one agar Petri plate to other plates (replicates). If the position of the inoculator is kept constant one of the duplicates can be used for assay of penicillin yield by overpouring with agar seeded with B. subtilis, while the other is used to recover the strain which gives the largest antibiotic zone.

An alternative use is in the selection of a mutant strain (auxotroph) which will not grow on a particular minimal medium. If the orientation of the multi-point inoculator is maintained, the culture which will not grow can be recovered from the master plate in which complete medium was used. Replica plating of the auxotroph onto minimal media containing different amino acid could reveal which amino acid was required.

The design of a multi-point inoculator was discussed with Mr. Liu and other staff. It was suggested that prototypes be made to test the design and available materials. The size must be such that it will fit a standard Petri dish or plate. It must stand up to autoclaving. A suggested design was as follows:-



19. Procedure for Comparing the penicillin V Productivity of Different Strains of P.chrysogenum (See also Appendix 3):

The currently used strain of P.chrysogenum (No.2 in my reports but designated 902 by GPF staff) was claimed to produce lower amounts of p-hydroxy penicillin V but no data has been presented to support this. Also, an earlier strain, PC5, was said to have been selected as being more tolerant to phenoxyacetic acid (about 1%) in agar, but at this concentration it is more likely to be a random selection of the original isolate. I recommended that attempts be made to isolate a strain tolerant to at least 5% phenoxyacetate in agar. Such a strain would permit the addition of all of the phenoxyacetate at the beginning of the fermentation and may give a higher titre of penicillin V.

Comparison of strains requires a high number of replicate fermentations and assays, and is best done in shaken flask culture. It has been stated by Calam (p.176 in his book "Process Development in Antibiotic Fermentations) that at 10% SE, 4 replicates (flasks) would be needed to reveal a 15% increase in yield and 12 replicates for a 7.5% increase.

When carrying out laboratory scale (shaken flask) comparisons of the strains described above or when comparing any new strain (mutant, recombinant) with its parent, at least four flasks should be assayed at each fermentation sampling time. For a more stringent test ten flasks should be used for each strain. The numbers of fermentation samples requiring assay can be reduced by bulking pairs of samples. Both HPLC (for penicillin V and p-hydroxy penicillin V) and iodometric assays of total penicillin should be used. A possible design for such an experiment is outlined in **Appendix 3.**

During discussion of such an experiment it was stated that GPF staff only used a volume of 20 ml. of culture medium in a 250 ml conical flask. I think this is too small a volume. Although this will provide a high aeration rate, evaporation over long fermentation times will give a false indication of concentration of penicillin V formed. I recommended that 40-50 ml be used in each flask, that the cotton stoppers should not be so tight as to restrict aeration, and that the fermentation should not go beyond 7 days. A shaken flask fermentation should be used to assess rate of production of penicillin by a strain and not duration of production. A stirred, aerated and fed tank fermentation is better for maintaining prolonged production times.

20. Progress of 1000 l penicillin V fermentation, Run E68, compared with previous runs:

At 76 and 82 hr the iodometric assays were recorded as 35,860 and 35,550 u/ml respectively (21,600 and 21,415 µg/ml using the conversion factor of 1.66 u/µg). These results are much higher than the previous run at the same sampling times (23,000-25,000 u/ml) and makes one wonder about the reliability of the assay. The standard penicillin V K salt used was said to assay at 1.38-1.56 u/µg (83-94%); presumably the relevant correction factor was applied to the results. However, by 106 and 116 hr the assays were 40,000 and 40,960 u/ml. These are also much higher than Runs E66 and E67, but more like E63 and E64. The assay on the 116 hr sample was done by the improved procedure of diluting the whole broth by 1/10 and using a larger volume in the iodometric assay. The results for the recent runs were plotted in the same way as done by Dr. Batchelor for earlier runs. This allows the changes in pattern to be seen easily. It was recommended to the GPF staff that they should plot the results as they are obtained.

The tank run E68 was harvested at 140 hr by which time the iodometric assay was quite high at 44,850 u/ml (27,000 µg/ml total penicillin). HPLC assays were not carried out because of shortage of filters. Microscopic examination revealed good growth with small pellets.

I was informed that it was thought that the two previous runs, E66 and E67, which had lower titres, had fungal contamination. This conclusion was based on microscopic examination which revealed long mycelial filaments, not usually seen. However, these filaments could have been <u>P. chrysogenum</u> hyphae which were growing independently of pellets. I have observed similar hyphae which are attached to the more stunted, fatter cells often seen as the culture matures. I recommended that a test for fungal contamination be done by plating out a later stage sample (e.g. 120 hr) onto cornsteep agar or peanut meal agar, etc and incubating at 25°C. It is interesting to observe the form of growth by microscopic examination in a fermentation but it is not a reliable test for presence of contaminants.

A test for bacterial contamination is best done by taking a sample and inoculating it into nutrient broth or onto nutrient agar and incubating at 30°C. The nutrient broth test was done on E66 and E67 but was reported negative. When such purity tests are done an occasional bacterial or fungal contaminant may be seen as a result of a microorganism falling in accidentally during the test. Therefore, only gross contamination in the test should be taken as evidence of the tank being contaminated.

Run E68 had 0.9% phenoxyacetate at the start (solid in with medium), followed by 0.2% (33 l of 6% solution, 2 kg.) at 36, 68 and 96 hr; this seems satisfactory. The inoculum was 15% vegetative growth, which would have got the run off to a flying start. The reducing sugar feed must have been satisfactory judging by the titre.

21. Isolation of penicillin V from 1,000 l tank (Run E68):

At 140 hr the contents of the 1,000 l fermenter were transferred to a chilled tank and adjusted to pH 4.3 using 30% sulphuric acid; a check on the final pH revealed it was 4.7. In future, 10% sulphuric acid is to be added via a spray bar, to reduce possible acid destruction of penicillin. The mycelium was filtered off easily and rapidly.

For one sample of about 100 ml of culture filtrate, at 10-12°C, the pH was rapidly reduced to 2.5 by adding 30% sulphuric acid. This resulted in a milky appearance but no pronounced precipitate. Microscopic examination revealed some platelike crystals and some oil droplets which could have been penicillin V which had not crystallised. A second sample of culture filtrate, which by now had warmed up, had its pH reduced to 2.5 by slow addition of less concentrated sulphuric acid (10%) with continuous stirring. A precipitate formed readily without a milky appearance in the bulk of the liquid. The clear supernatant was decanted off and the solid washed twice with cold water. Microscopic examination revealed that the solid was crystalline. The material was allowed to dry and a sample given to Mr. Bird for analysis. This was consistent with the material being penicillin V (free acid) with some minor impurities.

The bulk of the chilled culture filtrate at 13°C was adjusted to pH 2.5 by addition of 10% sulphuric acid (not 30% as previously) with stirring over 0.5 hr. The temperature was then allowed to warm gradually to 18-20°C to encourage crystal growth. The solid was readily filtered off in the filter press.

At this stage I had to leave the GPF to return home.

Meeting with Mr. Jostein Nygard, JPO
United Nations Development Programme, Beijing

16th October 1992

While in Beijing I took the opportunity to visit the UNDP offices at 2, Dongqijie Sanlitun, Beijing. I met Mr. Ian Davies (UNIDO Country Director) and had a long talk with Mr. Nygard as he is the Junior Professional Officer covering the GPF penicillin V project.

Mr. Nygard was not aware of any changes to the project on penicillins. I said that my understanding was that priority was not being given to making penicillin V, i.e. development of methods on a lab and pilot plant scale, including strain preservation, strain improvement, analytical methods for penicillin V as isolated solid and in the fermentation; work on 6-APA was stopped.

Other items discussed were:-

a) Some of the visits abroad by GPF staff on UNIDO travel fellowships had not achieved their desired objectives (e.g. training in culture preservation); it was important that this did not recur.

b) It could have been beneficial if some staff had attended the 7th ICCC Conference in Beijing. I had been unable to meet Dr. Jong of ATCC at this meeting as he did not present the paper scheduled in the Programme (Dr. Jong has visited the GPF labs).

c) My next visit to the GPF labs, after returning at the end of October, should be in the New Year. Adequate funds were available.

d) The penicillin V Project could be a long one, rather as expected, bearing in mind the complexities of the situation (technical aspects, getting equipment running, assay methods, culture improvement, etc.).

e) At the 7th ICCC, I met Ms. Virginia Campbell from UNIDO, Vienna, (Biotechnology and Genetic Engineering Unit, Department for Industrial Promotion, Consultations and Technology).

f) I suggested that the penicillin V Project at the GPF labs could benefit from extra money for items such as pH meters, incubators, laboratory autoclave, domestic refrigerator, electronic balances, etc.

Attendance at 7th International Congress of Culture Collections meeting in Beijing

12-16th October, 1992

This meeting was jointly organised by the World Federation of Culture Collections (President: Mrs. Barbara Kirsop) and the China Committee for Culture Collections of Microorganisms (Chairperson: Dr. Da-Kang Song), and was devoted to all aspects of micro-organisms, including their isolation, identification, preservation, classification and economic properties such as commercially important enzymes and bio-active metabolites.

I had arranged to attend this conference independent of my consultancy work for UNIDO and the GPF. However, there were topics of relevance to our work at the GPF, particularly culture preservation techniques. It was a pity that it was not possible for one of the microbiologists from GPF to attend as they would have made useful contacts with other microbiologists and learnt things relevant to their own work.

A poster (P1-S1-09, p80 in Book of Abstracts) on the preservation of filamentous fungi, yeasts and bacteria in liquid nitrogen was presented by S. E. Deng, L. H. Yiang, D. Li, Y.E. Hong and X.C.Xu from the Guangdong Institute of Microbiology, Guangzhou (100 Central Xianlie Road, Guangzhou 510070). They used 10% glycerol as the suspending medium, finding it better than 5% DMSO. The physiological characters of these organisms were better preserved by this method, than by successive transfer on agar slants stored at 4-10° C.

Two other posters presented by staff from the same Institute described lyophilisation as a preservation method. The Poster by Q.P.Wu and X.Y.Zhou (Abstract P1-S1-06, p.79) described the use of 10% defatted milk as the suspending agent for L-malic acid producing fungi such as Rhizopus and Aspergillus. The poster by L.H. Yang, Q.P.Wu and S.Y.Tang (Abstract P1-S1-25, p.84) compared lyophilisation with other methods for preserving glucoamylase producing fungi.

My suggestion to the GPF staff for the preservation of P. chrysogenum are in accord with the observations reported in the above posters. I strongly recommend that a microbiologist from the GPF research laboratories should visit the Guangdong Institute of Microbiology, as it is in the same city. They will be able to see the equipment used and discuss practical aspects of the methods which are similar to the ones they are trying to establish in their own laboratories.

Publications supplied to Guangzhou Pharmaceuticals Institute of Microbiology

1. The Preservation and Maintenance of Living Fungi by D. Smith and A. H. S. Onions, 1983, International Mycological Institute, Surrey, TW20 9TY, UK. £10.

2. Catalogue of Whatman LabSales Ltd., St. Leonard's Road, 20/20 Maidstone, Kent ME16 0LS. Tel: 0622 674821

   Gives details of chromatographic materials, electrodes, general laboratory equipment.

3. Photocopy of article "Genetics of the Penicillia" by G. Saunders and G. Holt, Chapter 3, p.73 in Penicillium & Acremonium. Ed. J. F. Peberdy, Plenum Press, London, 1987.

4. Photocopy of article "Differentiation of P.chrysogenum and Penicillin Biosynthesis" by Hang Yao-qun et al. Institute of Antibiotics, Shanghai No.3 Pharmaceutical Factory, Chinese J. of Antibiotics, 17, No.1, p.1 (1992).

Publication received from Mr. Cai Shi Chao, Guangzhou Pharmaceutical General Corporation

Book detailing the various factories and products for which the Bureau of Pharmaceutical Administration of the above corporation is responsible.

Address: 45 Sha Mien North Street, Guangzhou, China.
Tel: 885712
Fax: 861913 and 861751

APPENDIX 1

Preservation Procedures for Penicillium chrysogen cultures

PREPARATION OF MASTER STOCK CULTURES

1. The main stages in culture preservation:

   a) Prepare a healthy freshly grown culture which is producing spores

   Use media and cultural conditions which have been shown to give a satisfactory spore inoculum when tested for pencillin production in shaken flask culture.

   b) Carry out culture preservation procedure:

   Use several different procedures.

   c) Recover organism from preserved form:

   Use appropriate method.

   d) Check viability and penicillin production of recovered culture:

2. Types of preservation methods:

   a) Freeze drying (lyophilization) of spores.

   b) Liquid nitrogen (vapour phase) storage of spores

   c) Medicinal liquid Paraffin (mineral oil) protection of slope culture

   d) Freezing in 20% glycerol at -80°C for spores or agar culture discs.

   e) Dry sterile soil for spores.

3. Procedure for freeze-drying P.chrysogenum spores:

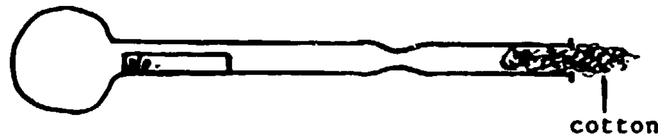
a) Grow culture on medium which has been found to give a good yield of viable spores.

b) Prepare a dense spore suspension in suspending fluid. Use freshly grown culture which has produced mature spores.

Examples of suspending fluids (sterilise at 120°Cfor 20 mins.

INOSITOL/MILK (Parton & Willis of ICI)	Inositol	5 g
	Skimmed milk	10 g
	Distilled water, make up to 100 ml.	
SKIMMED MILK	Skimmed milk	12%
	Distilled water	
DEXTROSE/TREHALOSE	Dextrose	5%
	Trehalose	7%
	Distilled water	

c) Label sterile ampoules or vials with culture number and date



d) Dispense 0.2-0.5 ml of spore suspension into the ampoules using a Pasteur pipette. Keep residual suspension for spore count, viability count and purity check. Push loose cotton plug below constriction on tube.

e) Freeze contents of ampoule in deep freeze or by rotating in a bath of very cold alcohol (-20° C or lower). Use cardice and acetone if available.

f) Put ampoules onto freeze dryer manifold. Open one tap at a time; open tap as soon as ampoule attached to ensure vacuum achieved quickly and tube does not thaw.

Use silicone grease on connections between adaptors, ampoules and manifold to ensure no leaks.

g) Run freeze dryer overnight - check that vacuum is as high as possible. Check vacuum gauge regularly, and also temperature gauge on refrigerator.

h) Using a gas flame, heat constriction on ampoule and when glass soft,pull so as to seal the end of the ampoule. Leave other sealed end on manifold. Repeat for all ampoules.

i) Store ampoules in a cool, dark place.

j) Recover culture from dry ampoule by scoring glass near cotton plug, snap tube, remove cotton plug and add 0.2-0.5 ml sterile distilled water. Wait 20 min. for spores to absorb water (rehydration).

k) Determine viable count on recovered spore suspension. Compare with initial viable count. Use spore suspension to inoculate tube cultures for preparation of spore inoculum for shaken flask culture. Carry out penicillin V fermentation. Compare yield at 4, 5 and 6 days with culture before freeze drying.

l) Repeat k above at 3 or 6 or 12 months to determine effect of storage.

NOTES:

a) Prepare a sufficient number of freeze dried ampoules for future work programme. Suggest use whole manifold, i.e. 24 for a batch. Use more if procedure found satisfactory.

b) Freeze dry each culture type, i.e. Culture No.1 and Culture No.2 and any other improved culture.

c) Keep records in a hard back book of how cultures were prepared and when they were freeze dried.

d) Write clearly on the labels and make sure they do not come off the ampoules.

e) Try to avoid burning the cotton plugs when sealing tubes.

f) Throw away any ampoule which is not properly sealed.

g) If purity check on original spore suspension shows contamination, reject whole batch of ampoules.

SAFETY:

Wear safety spectacles when handling glass under vacuum.

4. Procedure for storing P.chrysogenum spores in liquid nitrogen:

   a) Grow culture on medium which has been shown to give a good yield of viable spores.

   b) Add suspending fluid and make suspension of spores.

      Suspending fluid: 10% glycerol in water - sterilize at 120°C for 20 min.

   c) Dispense 1 ml. aliquots of spore suspension into sterilized 2 ml. polypropylene tubes with internal screw caps and label OR make tubes from polypropylene drinking straws (2.5 cm long, seal one end in flame, sterilize in Petri dish at 120° for 20 min) and add spore suspension with Pasteur pipette. Seal end with flame, place tubes in small plastic bottle and label. Keep some of original spore suspension for viability and purity checks.

   d) Pre-cool bottles or tubes to 5°C. Place bottles or tubes on carrier rods (canes) - attach securely and lower rods into liquid nitrogen container so that they are suspended in the vapour phase above the liquid nitrogen.

   e) Remove two tubes. Allow to thaw in covered box. Open tubes and carry out viable count and penicillin V production test as in k) above (freeze drying method). Compare with original culture.

   f) Repeat above after 3, 6 or 12 months to determine effect of storage.

NOTES:

a) Prepare sufficient tubes for future programme.

b) Keep records of stored cultures in a hard back book.

c) Ensure tubes cannot drop off carrier rods.

d) Use self adhesive labels which will not come off tubes or place label inside bottles.

e) Do NOT use glass bottles or tubes - they could explode on warming.

SAFETY:

a) Wear full face protection when handling frozen tubes and liquid nitrogen.

b) Wear protective gauntlets (gloves over wrists) when handling liquid nitrogen.

c) Put liquid nitrogen containers in well ventilated place.

5. Procedure for storing P.chrysogenum cultures under medicinal liquid paraffin:

a) Grow culture on agar used for sporulation, such as peanut or soya bean meal agar.

Grow until agar slope well covered with sporing hyphae. Do not use old cultures.

Grow culture in glass tubes with cotton plugs or in glass bottles with screw caps. Label containers with self adhesive labels.

b) Sterilize liquid paraffin by heating at 140° C for 3 hours (or by autoclaving at 121° C for 15 mins three times).

c) Cover agar slope with liquid paraffin at room temperature. Ensure the agar is covered to a level at least 0.5 cm above the agar.

d) Store paraffin agar slopes in cool place or at 5°C. Keep the tubes upright so that agar stays covered with paraffin.

e) Recover sample from paraffin slopes by using a sterile metal cutter or loop to cut out a small piece of agar from below the liquid paraffin. Place this on the side of a Petri dish containing the agar used for sporulation. Draw the agar piece to the middle of the dish leaving oil behind.

Wait several days for hyphae to grow away from under the drop of liquid paraffin. When culture well established use to inoculate working spore slopes.

f) Check recovered culture for pencillin V production.

NOTE:

This is a widely used basic method for fungi (used at the Beijing Institute of Microbiology, along with freeze drying and liquid nitrogen).

APPENDIX 2

Use of replica plating method to detect penicillin negative producers following mutation with UV

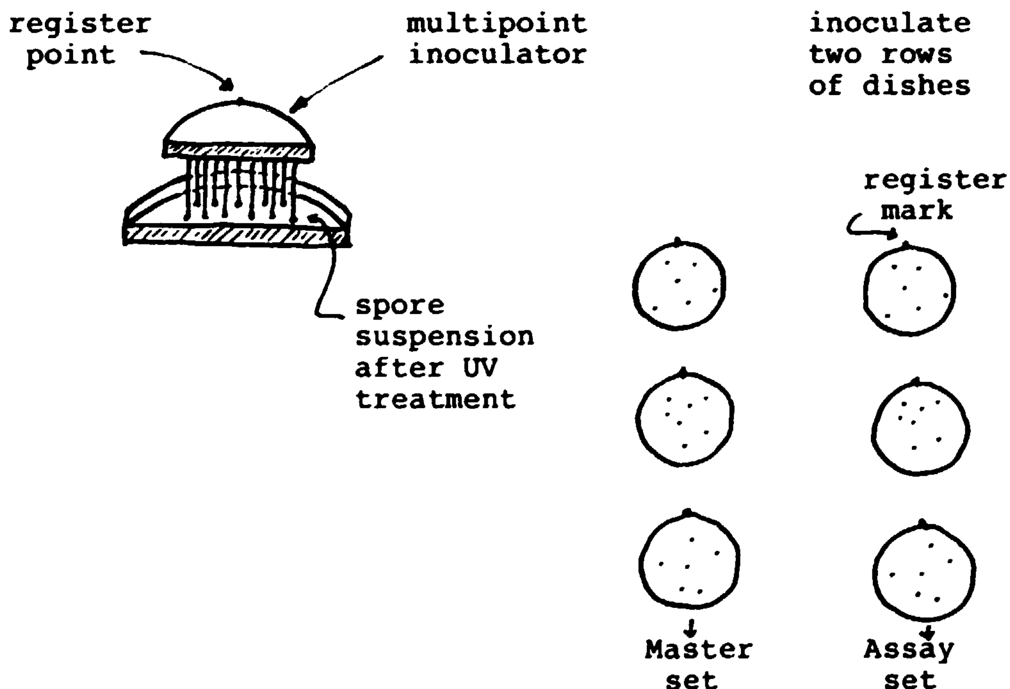
a) Determination of UV dose required for high kill rate using 30 W UV lamp available in GP laboratory:

Place petri dish containing 5 ml of 10^6 spores/ml 5 cm from lamp. Remove 0.1 ml sample at 1, 2, 4, 8 and 16 min. and spread on separate agar plates containing CSL/agar (do exposure by turning light on and off). Choose an exposure time giving at least 99.9% kill (0.1% survivors) for use in next experiment.

If kill rate too high increase distance of petri dish from lamp and if too low decrease distance or increase exposure time.

b) UV mutation and selection of penicillin V culture:

Using conditions giving 0.1% survivors, as determined above, treat another spore suspension and use the multi point inoculator to transfer it neat and at 1/10 dilution on to peanut/soya bean/agar + phenoxyacetate, or CSL/agar + phenoxy acetate.



Use multi-point inoculator to inoculate two rows of plates keeping the inoculator in the same orientation (position) - use register point and mark edge of dish. Inoculate a large number of dishes (20 - 100). Choose conditions which give clearly separated colonies after a few days' incubation. Before colonies are sporing heavily cover the assay set of dishes with agar seeded with *Bacillus subtilis* (penicillin sensitive), incubate overnight and note any colonies which do not produce a bioactive zone. Recover these colonies from the duplicate master plate and retest on agar and in liquid medium to confirm negative penicillin production.

The above experiment is an exercise in methods but negative strains can sometimes be back mutated to make higher yields of penicillin.

APPENDIX 3

Experiment in shaken flasks to compare P.chrysogenum Cultures 1 & 2 using the iodometric assay to determine total penicillin, and HPLC analysis to determine amounts of penicillin V and p-hydroxypenicillin V

Cultures:	P.chrysogenum strains 1 and 2 (PA5 and 902)
Seed stage:	Peanut/soya bean meal medium* inoculated with spores and grown until good growth obtained (3 - 5 days, 25°C) in shaken flasks (40 ml. in 250 ml flask).
Fermentation stage:	Peanut/soya bean meal medium* + phenoxyacetate * usual composition Inoculate 35 ml in a 250 ml flask with 5 ml of seed stage (5%). Shake at 25°C on a rotary shaker. Do not have the cotton stoppers too tight - allow air to get in.
Sample times for assays:	Sample at 5 and 7 days. Take 5 ml samples from each of two flasks of fermentation stage. Pool these two samples and dilute to 50 ml. Repeat with two other flasks.

Sample	5 ml 5 ml	5 ml 5 ml
Combine	10 ml +	10 ml +
Dilute	40 ml water ↓ filter ↓ assay two samples	40 ml water ↓ filter ↓ assay two samples

Assay methods a) Iodometric assay of total penicillin

b) HPLC assay of pen V and p-OH pen V

Objectives of experiment:

a) to compare total penicillin productivity of the two cultures in shaken flasks.

b) to see whether strain 2 makes less p-OH pen V than strain 1.

c) to see which culture makes pen V the faster.

NOTE:

Shaken flask cultures are not suitable for obtaining the highest titres which a strain can make. This is best done in a stirred, aerated fermenter, where nutrients and precursor can be fed in gradually and pH controlled.

Backstopping Officer's Technical Comments on the Report of Mr. M. Cole

To carry out developmental work in the pharmaceutical industry, one has to adhere to certain principles. First of all any product development or process improvement work should be properly designed, scheduled, managed, monitored and evaluated.

The expert has not specifically listed his recommendations but it is very clear from his report that the product (penicillin V) has to be developed in such a way that it complies to the GLP required for achieving consistency of the results. In a biotechnological development project, the following basic design should be applied:

1. Establish a seed lot system
2. Validate master seed
3. Validate working seed
4. Optimize culture media
5. Optimize fermentation conditions/parameters
6. Validate quantitative testing of product (HPLC)
7. Standardize techniques to generate mutants of high productivity
8. Optimize downstream processing (recovery, printing)
9. Prepare reference preparations, reagents, culture media, fine chemicals, etc.

Since the origin and characteristics of the penicillin V producing Penicillium chrysogenum strain of GPF have not been made available to the experts, neither the experience published in scientific literature nor comparative studies could be used for improving the productivity of the strain. One of the main responsibilities of the GPF Research Institute should be the continuous strain improvement.

As soon as a new strain of higher productivity is generated and its stability is confirmed, a new cycle of the development project shown in the above should be commenced by establishing a master seed. According to international experience, approximately 10% annual increase of productivity can be achieved by traditional selection techniques such as UV light and chemical mutagenic agents.