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STRENGTHENING OF PESTICIDE DEVELOPMENT CENTRE

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INDIA

Technical report: Findings and recommendations*

Prepared for the Government of India
by the United Nations Industrial Development organization,
acting as executing agency for the United Nations Development Programme

Based on the work of K.A. Cook, consultant
in microbiological formulation

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1. INTRODUCTION

The Pesticide Development Centre at Udyog Vihar, Gurgaon, on the outskirts of Delhi was set up with the goal to strengthen and support the expansion and improvement of the pesticide industry within India, and to facilitate economic production and formulation in the various sectors. The Centre was created with funds provided by UNDP/UNIDO and the government of India, and comprises basic research and development laboratories and training facilities in various aspects of formulation development, manufacture and quality control of pesticides.

With the increasing environmental pressures placed on some currently used chemical control agents, and with the continuing problems of the development of resistence to chemicals, there has been an increased interest in the use of biological control agents and there is an All India coordinated research project in this area.

The purpose of my consultancy period at PDC was a) to advise on the setting up of microbiological facilities which would enable the centre to become involved in the formulation and development of biological control agents and b) to assist in the formulation of medium and long term research programmes in this area.

2. THE POTENTIAL FOR BIOLOGICAL CONTROL AGENTS

Before PDC embarks on any programme related to biological control agents it is important to answer a number of key questions:

1) Are there opportunities for the use of microbial agents in agriculture in India?

Specifically one would like to identify pests that cannot be controlled by normal chemical means, pests which are currently controlled by chemicals that are likely to be withdrawn from the market as environmental concerns lead to the development of more prohibitive legislation, pests which are beginning to develop resistance to chemicals currently used, and pests that are currently only controllable by the use of excessively expensive chemical agents.

2) Are suitable biological agents available or in development?

A source of active biological material is required before any development work can occur. PDC is not intended to be a laboratory involved in the initial discovery of biological agents, and does not have the facilities to become involved in this area

3) Is there a demand for formulation of biological agents in India?

PDC must ascertain whether there is a market for the type of service it intends to offer, and indeed, the type of service that it intends to offer. It is obviously counter productive to set up a facility for formulation of biological agents if other institutes are already doing such work themselves. It will almost certainly be necessary for PDC to find out what potential collaborators would require in terms of a development service, and then to advertise its capabilities and facilities as widely as possible.

4) Will it be possible to register such biological agents in India ?

A number of contacts that I made expressed the view that registration was unlikely to be easy because of concerns over toxicity of biologicals to non-target organisms. A relevent example here was the concern over the possible toxicity of Bt products to silk worms.

3.THE ROLE OF THE PESTICIDE DEVELOPMENT CENTRE

A prime requirement for PDC will be a source of biological material to formulate, and such material will have to be obtained from laboratories and institutes involved in the discovery and evaluation of new materials.

I suggest that academic institutions and other government funded research institutes are the most likely sources of material for the following reasons:

a) Industry within India may collaborate but I suspect that those with an interest in biological control will have in-house expertise and wish to maintain confidentiality and develop their own materials

b) Industries outside India are unlikely to be interested in collaboration because of problems of confidentiality and intellectual property rights which are difficult to guarantee, especially in view of India's position with regard to international patent agreements

c) There are a number of institutional laboratories in India that are interested in biological products, both in the area of biological control, and in the use of microorganisms for nitrogen fixation in agriculture, composting, phosphate mobilisation etc. In all cases the efficacy of products is likely to be enhanced by the application of appropriate formulation technology which does not seem to be available, or which is very basic and hence amenable to improvement

d) There are a large number of academic laboratories in Europe and the USA which are investigating biological control and are happy to have interested laboratories look at their products. In many cases field testing stations are not readily available and the opportunity to test promising organisms in a realistic situation could be welcomed. Many of the organisms reported to be effective as biological control agents have failed to perform in field studies in spite of good results in the laboratory. As few of these organisms are either novel or patentable then I suspect that the appropriate laboratories would be willing to provide them in return for access to the results from any field or formulation# trials conducted.

I therefore recommend that the overall subject of biological control in India is thoroughly reviewed before any course of action is decided upon, and that strong links are formally set up with appropriate scientific institutions. I would suggest the following approach by PDC scientific management:

a) Liaison with appropriate government departments to obtain a directory of all of the government sponsored research projects in the microbial biotechnology area. I have initiated such conversations and a summary of my discussions is attached (Appendix 2).

b) Initiation of discussions with key personnel from the appropriate institutions. An effective approach here would be to follow up the initial personal contact with a series of seminars, a short workshop, or a small conference to discuss the overall approach within India to the development of biological control capabilities. Such meetings are already held as part of the all India Programme and it would be useful if PDC could become involved in this programme and attend the coordination meetings.

4.DETAILED DEVELOPMENT PROGRAMME

If the requirement for a formulation activity for biologicals is confirmed from the survey suggested above, and a source of potential control agents is available, then a detailed programme can be worked out. I recommend that a basic formulation activity is set up, in parallel with a specific programme on mosquito control agents, and make suggestions for a longer term role for PDC in this area.

A. Basic studies

While basic studies are not normally carried out by development laboratories, there are definite reasons why they are necessary in this area. While the types of studies I am recommending all almost certainly being carried out by research teams in industry this information is unlikely to be made readily available as in many cases the actual formulation process used is likely to form the main basis for patenting of a product - it will often be difficult to patent a specific microorganism. Little work is going on in academic laboratories in this area as it is generally considered to be too "near market" to gain governmental research support in most western countries, and also because it requires a multidisciplinary approach and the type of formulation resources that are unlikely to be found in a normal academic laboratory.

It is therefore advisable to generate certain basic information in house at PDC in order to facilitate further studies.

i) Toxicity of formulation ingredients

The effects of the normal range of formulation chemicals on a representative collection of microorganisms should be examined. Here I would suggest using a range of typical microorganisms e.g. common Gm +ve and Gm -ve strains (e.g. Bacillus and Pseudomonas), a yeast, and a typical

sporulating fungus such as Aspergillus or Penecillium. Representative Fusarium, Trichoderma and Streptomycete species would also be valuable in view of their reported activities as biological control agents.

This range of organisms should not present major problems as inhibition tests can be run in a number of systems which will allow a high throughput for comparatively little effort, and require no major capital expenditure or expensive consumable items.

Such tests could also be used for assessing the toxicity of other chemicals to microorganisms, and hence a spin off could be in the environmental testing area where new chemicals should be assessed for their toxicity to non-target microorganisms. Although this type of testing is not a requirement in India it would be advisable to monitor any detrimental effects on microbial innoculants as these are used increasingly as fertilisers (e.g. nitrogen fixing and phosphate mobilising microorganisms). Similarly once a microorganism is used to control another microorganism (as in the use of antagonists for biological control) then it will become more relevant to ensure that such an agent does not have any detrimental effects on non-target microorganisms.
Hence inhibition testing as an activity would fit in well with other quality control tests currently offered by PDC to industry.

ii) Toxicity of formulation procedures

The second area of study would be to look at the effects of general formulation procedures on microbial viabilities. Here one would be concerned with the effects of temperature, pressure, shear stress, dehydration, pH, dehydration etc. Such a study will help identify procedures that could be suitable for formulation of microorganisms, and will certainly quickly identify those that are too harsh.
Experimental procedures suitable for assessing the effects of both formulation materials and formulation processes on microorganisms are described in Appendix 1.

iii) Design of formulations for specific environments

Different formulations will be required depending on the environment in which the material is expected to work. Aqueous applications, for example, will require different properties to applications designed to adhere to leaf surfaces, or to disperse in the solid matrices of the soil environment. Four basic types of environment could be envisaged, namely soil, water, air, and surfaces (e.g. leaves, insect cuticles). While there will be a degree of product specificity in these environments it should be possible to consider some generic types of formulations suitable for the different environmental conditions that will be encountered.

A detailed discussion with the biologists at PDC identified soil and surfaces (foliage) as the prime targets for formulation because of the importance of the pests in these environments. The major soil borne pests were identified as nematodes, locusts, grasshoppers, termites, grubs and a variety of soil fungi. Foliar pests of importance were brown plant hoppers, spodoptera, heliothis, and amsacta, in addition to a number of pests of stored grains. Hence it would be sensible to look at generic formulations for soil penetration, and for adhesion to foliage and grain.

The basic studies suggested above can be carried out even in the absence of specific targets. They are , in my opinion, essential activities for a number of reasons. First they will provide the background knowledge required to be able to devise formulations for specific products as they arise, and avoid a long period of familiarisation each time a new type of product is presented. Secondly, they will provide a basis of continuity to the research and development effort. It is obvious with current activities at PDC that when specific problems are not being studied, then very little use is made of the manpower resource - it is very inefficient to have what is effectively a contract organisation having no basic activity to revert to at times when contract work is not forthcoming. Conversely the operation of an active and ongoing programme will increase the credibility of the group and should actually attract more customers to use the expertise being developed.

B. Specific studies

There is an identified need for alternative agents to control mosquitoes in India, and a number of government funded projects have been set up to look at the possibilities for biological control using species of Bacillus thuringiensis and Bacillus sphaericus. From my discussions with scientists involved in the projects and with government officials coordinating such projects, it is evident that there is little activity or experience on formulation of such agents. Given this basic interest and PDCs past experience with these organisms, then they present the most likely target for an immediate formulation programme. An additional advantage is the considerable amount of work being carried out on Bt in other parts of the world for the control of different insect pests. Some Bt strains are already in commercial production and many are under development. However the use of Bt for mosquitoes control has received relatively little attention in the west. There is, however, an interest in the control of mosquitoes in utility waters in certain areas and this could be a profitable outlet for any product developed.

1. The role of Bacillus spp in mosquito control

Historically the PDC programme was initiated in response to an enquiry from the Anna University, Madras, who were looking at B. sphaericus 1593M and needed assistance with formulation.

PDC experimented with wettable powders and floating dusts initially but both presented problems. The former tended to sink and the latter did not spread well. Work subsequently concentrated on the use of surface spreading oils. These essentially consist of a stable suspension of active ingredient in a non-toxic oil phase containing lyophilic surfactants dispersed in a hydrophilic aliphatic alcohol. On contact with water the droplets of the formulation spread spontaneously, with a pressure of 60 dynes/cm2, into a microreticulum. This spreads over the surface due to the initial repellancy of it's threads, and eventually breaks into evenly distributed microglobules containing the active ingredient.

Bt was provided as a spray dried powder and was mixed with a pluronic surfactant (butylene/propylene oxide block copolymer), and an oil (vegetable, liquid paraffin etc). The bacterial preparation was added as 20% by weight (20 parts /80 parts of formulation ingredients).

This mixture was mixed in a Silverson (wet) mill, treated in a dyno mill and emerged as a liquid slurry.

Among the advantages of such a formulation are the maintenance of the active material in the larval feeding zone, the lack of effects on oxygen supply to non-target species (as contrasted to the use of continuous oil films), and the decrease in photodegradation of the active ingredient because of it's encapsulation in an oil film.

Three different microbial preparations were formulated:

1) B.sphaericus 1593 (Anna University, Madras)
2) B.sphaericus 2562 (Univ. of California, Riverside)
3) B.thuringiensis H-14 (Univ. of California, Riverside)

All formulations were monitored by bioassay. No specific microbiological analyses were carried out. The bioassay consisted of applying a known dose of the formulation to the surface of water contained in a rectangular tray. The test was conducted at 27C with 3rd instar larvae and mortality was measured at 24 and 48h with Bt and Bs preparations respectively.

The results indicated the overall superiority of the B. thuringiensis preparations - they acted faster on all mosquito strains used and gave better or comparable mortality rates to Bs. Furthermore Bt was active against A. culicifacies which was poorly controlled by Bs.

2. Comments on the previous studies

The advantages claimed for Bs over Bt included environmental persistence, and a greater biological specificity (it has little effect on predaceous non-target mosquitoes). However few of the researchers I met with had seen data confirming this and some clarification is required.

Little information was given to PDC staff on the microbial preparations provided, and it was unclear whether the spores used were viable or not. Similarly there was no data on storage stability and the preparations used were of different ages. Basically the different strains were compared on the basis of weight of active ingredient in the formulation (active ingredient being the material supplied by the collaborators). In addition the original materials provided were not tested for activity before formulation.

Given this data it was obviously not possible to give a quantitative comparison of the different preparations used, and all that can be concluded is that all preparations contained some activity after being formulated. Similarly the lack of data from the unformulated control make it impossible to tell if formulation increased or decreased the activity of the organisms.

3. Future programme for the evaluation of Bacillus species

There are certain actions that must be taken before any further work is initiated:

a) The literature on Bs and Bt for mosquito control should be thoroughly reviewed with a view to answering the following questions:

- What are the relative toxicities of Bs and Bt to mosquitoes? One should attempt to make a quantitative comparison based either on microbial viabilities or concentration of active protein toxin.

- What are the relative toxicities of Bs and Bt to non-target insects, especially silk worms ?

- Are there any data on the survival of Bs and Bt preparations in aqueous environments ?

b) Contact should be made with the other Indian laboratories studying these problems with a view to avoiding any overlap of work, and to cooperating in areas of mutual interest. We have identified the locations and the workers - the main contact should be with Lucknow and Madras and the names of appropriate workers and further details are given in the section on discussions outside PDC (Appendix 2).

c) Discussion with relevant government departments should be initiated to determine what are regarded as the regulatory hurdles for such a product - it is important to identify the chief concerns so that they may be addressed as part of the development programme

The overall objective of the above actions is to determine whether there is a role for PDC to play in this area, or whether the appropriate development work is already in progress. There is no point in wasteful duplication of effort within the country.

Assuming there is a role for PDC, then one must examine the rationale for continuing work with Bs as it is apparently an inferior control agent in activity terms, and it is far from clear whether it does present any actual environmental advantages over Bt. This could be a key point in product registration and hence a discussion with the regulatory authorities on their views in this area should be initiated before any work commences.

4.Detailed programme

The overall programme should have three major objectives:

a) To identify the most appropriate biological control agent to be used

b) To produce the appropriate control agent in a physiologically active state by the cheapest and most consistent means possible, and to be able to store active cultures in a viable form

c) To formulate the control agent in such a way that the maximum culture viability is maintained, while optimum performance in the field is achieved

It should be remembered that the microorganisms themselves will constitute the product - thus any loss of viability of microorganisms caused through formulation will represent a loss of product and contribute to an increase in the overall cost.

Identification of the control organism

The merits of using Bs versus Bt must be examined before any formulation is attempted. Formulation work will be time consuming and the appropriate organisms should be selected before the formulation stage of the project is initiated.

While the information in the literature is likely to give an indication of whether Bs or Bt is the best organism to use, nevertheless it is likely that the biological effectiveness of different candidate strains will need to be checked in the PDC laboratories before any formulation work starts. This is especially important as it is likely that strain differences will be encountered, and one should thoroughly understand the biological characteristics of the particular strains provided by the various laboratories before any decisions are made.

Such strain evaluation involves two distinct types of activities - the evaluation of the toxicity of the strain to a number of target mosquito species, and also the assessment of toxicity to key nontarget species. This latter evaluation is crucial as it seems to be the stumbling block to registration in India - the greatest concerns about the use of such products are based on their potential toxicity to silk worms. It is therefore a prerequisite to any formulation programme that a silk worm screen is set up at PDC. At present screens are available for mosquito, spodoptera, amsacta, and some stored grain pests.

This setting up of additional screens is possible but poses a few problems. The biological laboratory area is not large and space is not used as effectively as it could be. The presence of desks for staff within the laboratory area not only reduces the amount of working space available, but also constitutes a health and safety hazard. This will become a more acute problem when formulations of microorganisms are evaluated in the biological section. It should then be treated as a clean working area and a number of the precautions recommended for microbiological laboratories will need to be taken. These will include the wearing of laboratory coats, the ban on eating and drinking etc (see Appendix 3 on microbiological safety). These regulations can not be easily followed while laboratories are also doubling as office accommodation.

Looking at the general use of space in the PDC buildings I would recommend that the empty office adjacent to the ground floor administration office, and indeed a part of the existing administration area, could be used to provide office accommodation for the biologists. This would release valuable laboratory space.

It must be remembered that if PDC intends to provide an overall service to participating industries, then a varying selection of biological screens must be set up to cater for the different types of products to be evaluated. While the creation of completely new biological laboratories cannot be justified until PDC has established itself in the scientific community, and evaluated it's future role, nevertheless in the short and medium term some extra space must be made available.

Given the increased use of their existing facilities the biological section should be able to expand their current screening capacity to cover the

requirements for a programme on mosquito control agents.

It is outside the scope of this report to discuss the set up of biological screening activities in any detail.

Production of microorganisms

The institute is already in contact with a number of laboratories which have provided them with Bt and Bs cultures in the past. However these cultures are now old, have not been stored in a suitable way, and were very poorly documented when first received.

Initially then, I would recommend that a discussion is instigated with the collaborating laboratories and a new set of cultures generated by them. At that time they should be asked to fully document the procedures used to generate the cultures and to specify the growth form of the preparation provided (ie vegetative cells or spores) and the number of viable units present in the material provided. They should also be asked for their recommendations on culture storage techniques, maintenance media and any other relevant information. It is important to have a comprehensive dialogue at this early stage in order to be able to generate cultures reproducibly with activity comparable to that described by the laboratory which carried out the initial biocontrol experiments.

With this information it will be possible for the microbiologist at PDC to maintain basic cultures for use in the provision of bulk material for subsequent formulation studies.

Initial studies should concentrate on storage of microorganisms as different strains can react differently to different storage techniques. It is a fairly straightforward job to store an organism under a variety of conditions and to periodically check that activity is still present. There are a limited number of storage techniques which are suitable for microorganisms and key strains should be stored in a variety of ways on the assumption that activity is unlikely to be lost in all cases.

Common modes of storage are listed below and in my own laboratory all key strains are stored by all of the techniques named until definitive information on the most appropriate made has been obtained:

- Liquid nitrogen: this is used for more difficult strains and is most likely to preserve activity. Cultures are stored as frozen liquids in a cryostat which is kept filled with liquid nitrogen. Unfortunately this method is both material and labour intensive as a constant source of liquid nitrogen is required and cryostats must be filled on a daily basis, especially where ambient temperatures are high

- Freeze drying: this is one of the most frequently used methods and microorganisms can survive in the freeze dried state for long periods of time with little maintenance

- Storage on solid medium: here cultures are kept on slopes of solid medium and subcultured at regular intervals to ensure that a fresh viable culture is always available. While acceptable for routine organisms maintenance it is not suitable for secondary metabolite producers, as the

ability to produce matabolites tends to be lost over extended periods of subculture if the exact physiological requirements of the individual culture are unknown

- Glycerol: many cultures are very well preserved by storage in a 15% glycerol solution at -20C or lower. This is often the method of choice with secondary metabolite producers

With well studied microorganisms such as Bt and Bs there is sufficient literature on storage stability to guide workers in this area, but caution should always be exercised as bad storage conditions are one of the most frequent causes of loss of interesting leads in research laboratories

Optimisation of growth

It is important to be able to generate active material on a routine basis, and this is not always a simple matter. For example if a microorganisms is active by virtue of its ability to produce an active metabolite, then it is likely that metabolite production will vary with the growth conditions used and, indeed it is not uncommon for microorganisms to completely loose the ability to produce a metabolite. This emphasises the importance of understanding the impact of growth conditions on the activity of the organism.

Once a consistently active culture can be produced then it is possible to embark on growth optimisation studies. This is usually achieved by experimentation with the constituents of the growth medium with respect to culture yield and activity. It is important to understand something of the mode of action of the organism under study as optimisation of growth and, for example, secondary metabolite production may be mutually exclusive objectives - some metabolite production is not growth related. Work with Bt and Bs cultures should be facilitated as the crystal formed within the cells is easily observed microscopically. It is evident that some technique for estimating the numbers and activity of toxic crystals would greatly facilitate work on process optimisation. However at present it looks as though bioassay techniques are likely to be the best currently available, although literature regarding alternatives should be carefully followed.

In this specific study on mosquito control I would suggest that it is important to look at locally available sources of nutrients with a view to at least considering in situ production of a control agent by local farmers or distributors. The UNDP/World Bank/WHO 1982 publication previously cited contains a considerable amount of information on Bt production and the availability of suitable carbon sources.

The fermentation equipment recommended elsewhere in this report (Appendix 4) will be adequate for small scale process optimisation, and for the production of larger patches of material for field testing. It is in this area that a period of training for the new PDC microbiologist would be most profitable. Such training, in conjunction with the specific instrumental training course provided by the instrument manufacturers would equip the microbiologist to carry out a programme such as that described.

Formulation of microorganisms

Given a strain with the appropriate biological characteristics, which can be effectively stored and produced consistently in reasonable amounts, then research on formulation can commence. I must emphasise that it is pointless to embark on a programme of formulation if the basic data on strain evaluation and growth optimisation is not available.

There is no real science of microbial formulation but it is evident that a multidisciplinary approach is essential. The microbiologist and the formulation chemist must collaborate on the selection of formulation procedures and the monitoring of microbial survival, and the biologists must advise on control specifications and test the effectiveness of the formulated material.

I have listed both common formulation chemicals and processes in Appendix 5 and a microbiologist will be able to highlight those processes which are unlikely to be suitable for microorganisms. Basically microbes will survive within defined physical and chemical limits and hence certain extremes should be avoided. Limit conditions are briefly listed below:

Temperature : Microbes are generally active at temperatures between 10C and 40C. Below this they will survive but in a quiescent form, and above this they will normally be killed. Short exposure to higher temperature may be acceptable but prolonged exposure, as practised in certain chemical formulation techniques, should be avoided

Pressure and shear: While microorganisms can often survive extremes of pressure, processes of pressurisation and depressurisation can rupture cells - even slow periods of decompression can lead to losses of viability. Hence processes employing extreme pressures should be avoided especially those which involve pressurising a solution and then forcing in through a small aperture or nozzle. In such cases there is not only decompression but also a shearing effect exerted on cell walls which can actually lead to cell rupture.

pH: While there are microorganisms which are tolerant to extremes of pH these should generally be avoided. Optima for growth of bacteria tend to be between 5 and 8.5 although there are many exceptions.

Chemicals: The reaction of microorganisms to the presence of different chemicals can vary widely but one can make certain generalisations. Solvents at high concentration can effect cell membranes and even dissolve them. Other chemicals interfere with specific enzyme reactions and thus will inhibit microbial growth. This is a complex area and each case must be considered separately. The text books recommended (Appendix 6) will have lists of toxic chemicals against descriptions of the different microbial genera and these should be consulted. Moreover in my section on basic research programmes I have recommended a programme to evaluate the toxicity of a variety of commonly used formulation chemicals to a range of microorganisms.

Given consideration of these factors it should be possible to select formulation processes which are at least theoretically likely to be suitable for microbes. Actual effects must then be confirmed experimentally.

Amongst those factors which need to be examined are a number that have given other workers problems. Certainly Bt products have suffered from a lack of UV stability and this area should be examined with any new formulations developed. It is also of considerable interest to determine whether formulated microorganisms are actually dividing in the field or whether they are decaying rapidly ie is their activity due to preformed toxic crystals or are they continually forming new crystals under field conditions.

Finally any evaluation must be made against competitors products, both biological and chemical. Certainly DIPEL and JAVELIN (both BT preparations), and TALCORD (permethrin) should be used as comparisons.

C. Longer term programme

This will be dependent on the results of the surveys suggested in the first part of this report, and on the role which PDC wishes to adopt in relation to other institutions. However certain facilities will be required if the biological formulation activity reaches larger proportions and it is necessary to consider these requirements in any longer term planning strategy.

1) Fermentation scale up

For the reasons given earlier I would not recommend the purchase of larger scale fermentation equipment in the short, or even medium term (5 years perhaps), Even if the demand is identified I would still suggest considering various other options initially.

If we look at the way in which industrial companies already involved in the production of biological agents operate, then it is evident that large scale production of biological material is often contracted out to professional fermentation companies. This removes the necessity for a large investment in capital equipment, while utilising the expertise of organisations with ongoing experience and involvement in fermentation processes. In principle this is analogous to a chemicals company contracting out synthesis of an agrochemical or an intermediate, and this is fairly common practice. In view of the capacity which must exist in the pharmaceutical sector in India I would suggest that appropriate links are made and the possibility of having such organisations grow up larger batches of material is considered. This will remove the necessity to purchase large scale equipment, hire extra staff etc.at PDC. Furthermore it would be inadvisable to have such fermentation equipment running in a chemical scale up plant at PDC - the provision of facilities for sterile operation and the higher standards of cleanliness and safety required could be prohibitively expensive if the whole existing PDC plant room had to conform to them.

b) Formulation scale up

If the existing facilities are to be used for the large scale formulation of microorganisms in the future then the overall design and safety of the area will need to be reconsidered. This will apply especially to any equipment that produces aerosols as a constant exposure of workers to even innocuous microorganisms can result in allergic responses especially where sporulating fungi are concerned.

Once some idea has been gained with respect to the suitability of different types of equipment for microbial formulation, then the health and safety aspects of operating such equipment with microorganisms should be carefully considered. Certainly it may be necessary to isolate appropriate pieces of equipment and to improve local ventilation, air filtration etc., and it will certainly be necessary to provide a much cleaner working environment to avoid contamination of product with unwanted microorganisms. Again, given these constraints it may be wise to look at the formulation capacity of drug manufacturers, although almost certainly they will be concerned with the isolation and formulation of microbial products rather than of the microorganisms themselves. One could also look at the industrial processes employed in the brewing industries.

c) Storage facilities

Irrespective of the size of fermentation equipment used the resulting live microbes will need to harvested and stored in a form in which their viability will be maintained. On a small scale this is accomplished by freezing cells, or by various drying procedures (freeze drying, spray drying). In many cases this storage will be an integral part of the formulation procedure.

I have recommended the purchase of an appropriate continuous flow centrifuge to facilitate harvesting of the cells which will be generated from the fermenter proposed for PDC. In general larger fermenters simply require larger scale equipment to carry out the freezing and drying processes mentioned above.

It is worth emphasising that different organisms will react in different ways to different storage techniques and it should never be assumed that a certain technique will always be effective - it should be monitored for each organism under study.

d) Capability to conduct field trials

At present there is a limited capability to conduct field trials without outside assistance/ involvement, and this is restricted to local terrestrial environments. Trials on pests in aqueous environments (mosquitoes for example) will therefore require cooperation with other organisations, as will larger scale terrestrial trials. Again it would be wise to form links with organisations in different regions that have local field stations etc, and also with local farmers who may be prepared to cooperate given some guarantees that they will not lose the value of their crops. This is the way in which most large organisations conduct trials over a broad area.

I assume that there are well established channels/ organisations for testing normal synthetic chemicals in agricultural situations and PDC should avail themselves of these facilities.

5. DEVELOPMENT OF MICROBIOLOGICAL CAPABILITIES AT PDC

A. Levels of possible operation

The nature and extent of the facilities required will obviously be dependent on the definition of the longer term role of PDC in biological control

activities within India. However a number of different scenarios may be envisaged depending on the level of future interest - these are described below as different levels of activity.

Level 1

It is evident that a basic microbiological capability is necessary to allow even simple evaluation of formulation procedures used for biological agents. There is a basic requirement to enumerate microorganisms and to assess their viability in order to validate any formulation process. The operations essential for support of this basic formulation work can be listed:

- detection of microorganisms

- enumeration

- estimation of viability

- culture of microorganisms in liquid media

Given a capability to carry out such determinations then it will be possible to compare formulation techniques, to look at the effects of formulation chemicals on cell viability, to assess the shelf life of products, to produce material for formulation trials, and to monitor microbial viability and survival in field trials.

Level 2

This would involve the ability to culture microorganisms in both batch and continuous modes and would allow :

- growth of larger batches of material for trials - production of consistent batches of material - optimisation of yield of microorganisms

- assessment of the suitability of local nutrient sources

- studies on the effects of microbial physiology on formulation

Level 3

This would give a capacity for large scale production of microorganisms for larger field trials and for possible commercial use, and would involve the running of large scale fermentation equipment and plant.

B. Equipment and facility requirements

1. Equipment for a basic microbiological laboratory

A basic microbiological laboratory is required for all of the operations discussed and this would also accommodate the basic fermentation equipment required for level 2 operations. Detailed plans for such a laboratory are discussed later in this document (Appendix 8).

The equipment required for this laboratory is largely standard but the more specific microbiological equipment is detailed below. Specific fermentation equipment is discussed later.

Microbiological cabinets

These are effectively enclosures in which a sterile environment is generated by passing filtered air over the working areas. This is generally brought about by passing air through a series of microbiological filters and hence into the cabinet. Cabinets are normally of 3 types:

1) Sterile air is passed through the cabinet to the outside environment thus protecting the cabinet contents from contamination, but not protecting workers from any microorganisms in the effluent air.

2) Non - sterile air is sucked into the cabinet, thus protecting the worker from any microorganisms inside the cabinet but not preventing contamination of the microorganisms in the cabinet working space.

3)Sterile air passes from the top to the bottom of the cabinet to give a curtain which both prevents entry of non-sterile air into the cabinet and the exit of microorganisms to the outside of the cabinet.

The 3rd type of cabinet is the most useful for normal operations as it ensures both worker and product protection.

Autoclaves

It is essential to be able to sterilise chemicals, media and equipment in any microbiological laboratory. The size and type of autoclave required will be dependent on the volume of material that needs to be processed. For small scale sterilisation a standard pressure cooker is adequate as this will hold 4-6 small bottles of media, sample bottles etc. To ensure adequate sterilisation such a pressure cooker should be operated at a pressure of 15psi which will give a working temperature of 121C.

A pressure cooker obviously has limited usefulness, especially when sterile materials for liquid culture are required. Larger scale autoclaves come in various shapes and sizes but those of intermediate size are normally free standing with cylindrical chambers of up to 50cm diameter. Such a vertical autoclave will normally be equipped with a built in steam generator.

Still larger autoclaves tend to have horizontal chambers and these can be of varying sizes but would normally be capable of holding larger glassware and equipment, and generally of taking larger loads. Such autoclaves are normally designed with trolleys to facilitate loading of heavier items. If large 20-40l culture vessels are routinely handled then it is advisable to have a small hoist close to the machine. These autoclaves will normally operate on steam that is either locally or remotely generated.

Incubators

Microbiological incubators are simply temperature controlled cabinets - they should be well insulated and should have the facility for cooling as well as

heating. This is especially important in India where the ambient temperature could be above the optimum growth temperature for the organism under study. Such cabinets are normally constructed of a non-corrosive material which is easily cleaned and can be chemically sterilised if necessary (in case of microbial spillages). Both static incubators (for incubation of plates), and shaking bed. rotatory incubators (for liquid culture growth) will be required. The shaking incubators should be capable of holding flasks of up to 2l capacity and again should be enclosed and provided with facilities for both heating and cooling.

Microscopes

There will be a basic requirement for a high power, high resolution microscope, for examining individual microbial cells for direct enumeration, microbial identification etc. In addition a lower power stereo microscope is required for examining colony morphology of microorganisms growing on solid media. Associated camera equipment is useful for recording microscopic observations. Suitable microscopes are available and are currently located in the formulation and biological laboratories. These are not regularly used and can be relocated to the mew microbiological laboratory.

Light bed

Enumeration of microorganisms normally involves their culture on solid medium in Petri dishes followed by counting of individual colonies once they have grown on these plates. This counting process is facilitated by the use of a light bed - this is essentially a platform on which plate can be stood and illuminated from behind. Plates are normally viewed through an associated magnifying glass positioned above the bed, and some electronic counting device, for recording microbial colonies as they are counted, is useful. A light bed is available in the biology laboratory although the counting pen associated with it needs repair.

Addresses of appropriate suppliers, detailed cost estimates, and recommendations for purchase of specific equipment are detailed in Appendix 7. All suppliers catalogues and formal quotes were lodged with the General Manager at PDC.

Standard equipment

This is largely non-specific and is listed for the sake of completeness:

Bench centrifuge
Rotatory mixer
Bunsen burners
Sterilisation oven (to operate at 300C) Water bath
Electronic 5 figure balance
pH meter
Refrigerator
Freezer
Pipetted canisters
Pipette washer
Water still
Metal sterilisation containers
Basic UV/visible spectrophotometer

Glassware and chemicals

In addition to normal materials used in a chemistry laboratory there will be a requirement for a large selection of conical flasks and Petri dishes for microbial culture, and for small McCartney bottles and medical flats for storing both medium and cultures. Similarly a plentiful supply of pipettes of sizes ranging from 1ml to 20ml will be routinely needed and these will need to be plugged (in the neck) with cotton wool and sterilised. A selection of micro-pipettes will also be required for biochemical work.

Most chemicals needed by the microbiologist will be readilY available in chemical laboratories with the exception of stains for microscopy, and of premixed microbiological culture media.

2. Fermentation equipment

Whatever the original source of any biological material used it will undoubtedly be necessary to culture it for normal formulation studies, and to scale up culture to provide sufficient material for field studies. A complete specification and justification for suitable equipment is given in Appendix 4.

I have advised that the fermentation equipment purchased by UNDP for PDC is of an intermediate size, and that the larger scale (300l) vessel originally budgeted for is inappropriate at this stage of development. The two vessels that I have suggested PDC purchase (20l and 35l) will be more than adequate for the programme at it's current stage of development. It will be suitable for growing up smaller amounts of material for optimisation of formulation studies and investigations into the effects of microbial physiology on formulation stability, but will also be suitable for preparing sufficient material for field trials. Material can in fact be continuously generated by operating the recommended fermenter in continuous culture mode. In addition there is always the option of growing up material in batch culture in 20l vessels. Batch culture possibilities should certainly be examined during development as it is quite possible that a biological agent could be produced in situ on a farm using local carbon and nitrogen sources for culture growth (see later discussion).

3. Pilot plant fermentation equipment

Installation of such equipment would require extensive capital investment and increase in staffing levels which I do not believe is appropriate at this stage of PDC's development. Detailed discussion in support of this view is given in the section dealing with the long term programme possibilities for PDC.

4. Facilities

Construction of a microbiological laboratory

One of the basic problems in converting the selected laboratory into a microbiological laboratory is in the actual design of the existing PDC buildings. Specific points of concern are listed:

- all existing laboratories have doors which open directly to the open air, and this poses general contamination problems

- the high ceilings and the presence of ceiling fans in the existing laboratories will cause an excessive air movement which again will increase microbial contamination problems

- the laboratory in question is next door to a toilet which is in a dirty and generally unhygienic condition and, further more, has a door which is normally left open to the common courtyard

- an open drainage ditch lies alongside one of the laboratory walls, and the area both around the walls and adjacent to them seems to be used to dump rubbish indiscriminately

All of these factors, in addition to the high ambient temperatures in Delhi in the summer months, could contribute to the ingress of unwanted microorganisms into the microbiological area. The major concern is obviously with contamination by microorganisms pathogenic to humans, especially as these would be likely to thrive on at least some of the microbiological growth media in routine use. For example, media containing dried blood and brain-heart infusions have been recommended for the large scale growth of Bacillus thuringiensis.

Such contamination possibilities are recognised even in areas where high standards of hygiene are practiced, and it is becoming necessary to monitor all larger scale culture batches for the presence of certain common pathogens. This is now a legal requirement for commercial batches of Bacillus thuringiensis in certain countries.

In an earlier document on Bacillus thuringiensis production ("Guidelines for the production of B.thuringiensis H-4" UNDP/World Bank/WHO, 1982) it was suggested that all laboratories involved in Bacillus thuringiensis production should consult with local WHO laboratories on the subject of pathogen monitoring.

I hope these comments underline the need to ensure that all steps are taken to minimise contamination in the microbiological laboratory and to provide adequate cleaning services both in the laboratory and in the surrounding environment.

Detailed microbiology laboratory designs

A basic design for the laboratory itself, and for specific benching is given in Appendix 8. The laboratory has been designed to minimise contamination problems where possible.

I propose that the existing chemical laboratory is completely stripped out and revamped to create three areas as shown:

- A large working laboratory is effectively split into two by a central bench, and thus provides separate working space for general microbiological operations and for fermentation and cell culture

- A small room with direct access from the main laboratory is provided to house all incubation equipment. Location of such equipment in a common area will facilitate any air conditioning or temperature control that may be necessary. It will also remove noisier and continuously running equipment

from the day to day working environment

- A third room for the preparation of media, storage of media chemicals, and for the location of sterilising facilities and washing up services. Access from the central laboratory to this room is via a sliding door to maximise the use of available space

- A small entrance foyer is created by fitting double doors at the entrance. This is largely to minimise contamination from the outside courtyard. Contamination in the laboratory and incubator rooms is further minimised by their location as they can only be entered via the media preparation room. Contamination will be further minimised by fitting an air curtain generator on the inside of the outside door. This will provide a curtain of filtered air and effectively provide a barrier between the outside environment and the laboratory.

Building and services specifications

During the laboratory revamp certain standards should be taken into consideration, all of which are designed to provide a cleaner working environment and thus to reduce the problems of contamination:

- all floors need to be of a smooth, washable material (eg.tiles, polyvinyl sheeting) to facilitate cleaning and to minimise accumulation of dust etc.

- walls should have a smooth finish (gloss or silk finish paint for example). Rough plasterwork or cement walls provide crevices for accumulation of dirt and dust and make effective cleaning difficult

- air supply fans should be removed and relocated to avoid generation of areas of turbulence. They should also be fitted with filters to clean up the incoming air. Ceiling fans should be removed, and air conditioning units, fitted with filters, located as indicated in the diagrams

- the ceiling should be lowered both to facilitate effective air conditioning, to minimise the areas for dust accumulation, and to allow the flush fitting of lights. The enclosed ceiling space generated can effectively be used for running hidden services across the laboratory and hence avoiding major building works in digging up concrete floors to relay services (e.g. piping and electrical supplies)

- services such as compressed air, vacuum, chilled water, and steam can all enter the laboratory from the outside as indicated into an existing alcove which can serve to locate control valves, pressure gauges, and associated equipment

- all sinks should be fitted with elbow operable taps to allow workers to switch them on when hands are contaminated

Basic benching design plans are attached (Appendix 8). They are largely conventional but it is essential that surfaces are washable and well sealed. The fermentation equipment will need to be located on a split level bench to allow easy operator access to the reactor vessel

Some provision must be made for back up electrical services in case of

power failures at times when the laboratory is not manned The failure of incubators at these times, or of fermenters when running continuously, would result in loss of experiments. As microorganisms tend to want to grow during antisocial hours then such provisions are essential.

At present power cuts regularly occur during the working day and usually at about the same time (late morning and lunch times). It should be possible to liaise with the local electricity company to receive some prior warning when cuts can be predicted. I suspect that the supply is diverted at peak times. The generator could then be cut in prior to a problem occurring rather than afterwards. The greatest problems are encountered with computer controlled equipment

This is an area requiring urgent attention.

Recommendations

One local company, Kartos International , are recommended to instal a laboratory incoprporating all of the features discussed. The designs were discussed with them in detail and a final quotation for carrying out all works was left with the General Manager at PDC. Kartos Int. were prepared to employ subcontractors to carry out the various stages of the work and to oversea the entire project. Given their work in other laboratories, and their role as a supplier of air purification and microbiological equipment, I was confident that they could build the laboratory to the specifications desired. When I left PDC all arrangements had been made for them to initiate the work on provision of final authorisation for the allocation of funds.

APPENDICES

APPENDIX 1

MICROBIAL INHIBITION TESTING

There are a number of relatively simple techniques for assessing the inhibitory effects of a chemical or a process on the growth and viability of a microorganism. These are briefly discussed below.

1. Use of solid medium in plates

Preparation of plates

An appropriate liquid growth medium is prepared and 2.5% agar added. The mixture is then sterilised in screw capped bottles in a pressure cooker operated at 15psi (this gives a temperature of 121C) for 20 minutes. The media bottles should be only loosely capped during sterilisation to avoid problems of liquid expansion. After sterilisation the media is allowed to cool to about 45C and 20ml aliquots dispensed aseptically into previously sterilised Petri dishes. The dispensing operations should ideally be carried out in a microbiological cabinet, but may be performed on the open bench if they are carried out speedily and plates are left open for a minimum period of time. All liquid transfers should be carried out with sterile pipettes. As the medium cools it will solidify and the resulting plates can be used for the culture of microorganisms.

Inhibition testing

Solid media as described above can be used for inhibition testing in a variety of ways:

a) Incorporation of the test chemical into the medium

Here a known amount of the chemical under test is added to the medium during preparation. The final solid medium then contains a known concentration of chemical and a series of concentrations can be prepared. Care should be taken to ensure that the test chemical is not effected by the sterilisation process - if it is heat labile then it can be sterilised by filtration at ambient temperatures and added to the medium as it is cooling.

The appropriate microorganism is then streaked onto the plate using a platinum wire, and the plates incubated at the appropriate temperature for 24 - 48 hours, or until the control plate containing no test chemical shows growth. By observation of microbial growth on the different plates the

concentration of test chemical which is toxic can be determined.

b) Use of wells in the plate

In this technique the solid medium is inoculated by spreading approximately lml of a liquid microbial culture onto the surface of the medium. The liquid will be quickly taken up and the microbes will have been evenly inoculated over the surface of the plate. A series of small wells are then drilled into the solid plate using a sterile cork borer, and varying concentrations of the test chemical carefully pipetted into these wells. The plates are then incubated for an appropriate period. Microbial growth well occur evenly over the plate unless the test chemical is toxic. One will then observe an inhibition zone around the well containing the toxic concentration of chemical. Toxicity can be roughly quantified by comparing the diameters of inhibition zones (Figure 1).

2. Growth and inhibition testing in liquid media

There are a number of ways in which such tests can be performed. In all cases media are prepared as described previously but without incorporation of agar. Essentially a series of liquid media are prepared incorporating varying concentrations of the test chemical. The medium is then inoculated with a microorganism and the cultures incubated for an appropriate time. Such experiments can be carried out in flasks of varying sizes, or in micotitre plates.

Microtitre plates are used to facilitate rapid testing of large numbers of samples. They are normally plates of about 5 X 12 cm containing a number of wells. The wells are filled with sterile medium, inoculated with microorganisms, test chemical added, and the plates incubated. Growth can then be assessed visually or using a specially designed plate reader. Such miniaturised tests require little space or materials and are used in laboratories where a large number of tests need to be performed on a routine basis.

Assessment of growth and inhibition

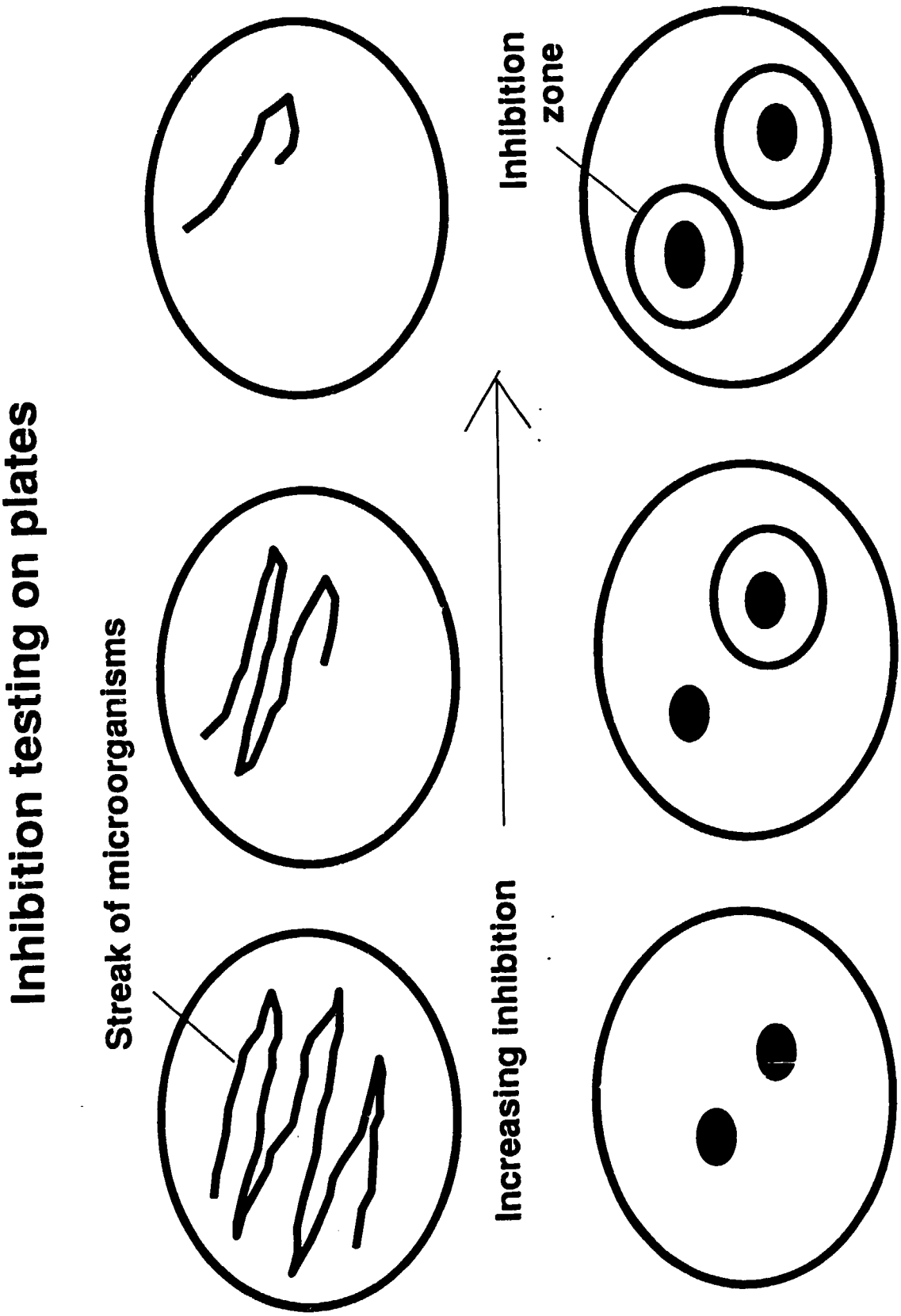
Growth can then be assessed visually (from the cloudiness of the medium), or, more accurately, using a spectrophotometer to assess optical density at around 500 to 600 nanmeters. By plotting optical density against time an actual growth curve can be plotted, and any inhibitory effects quantified by comparison with a control experiment (Figure 2).

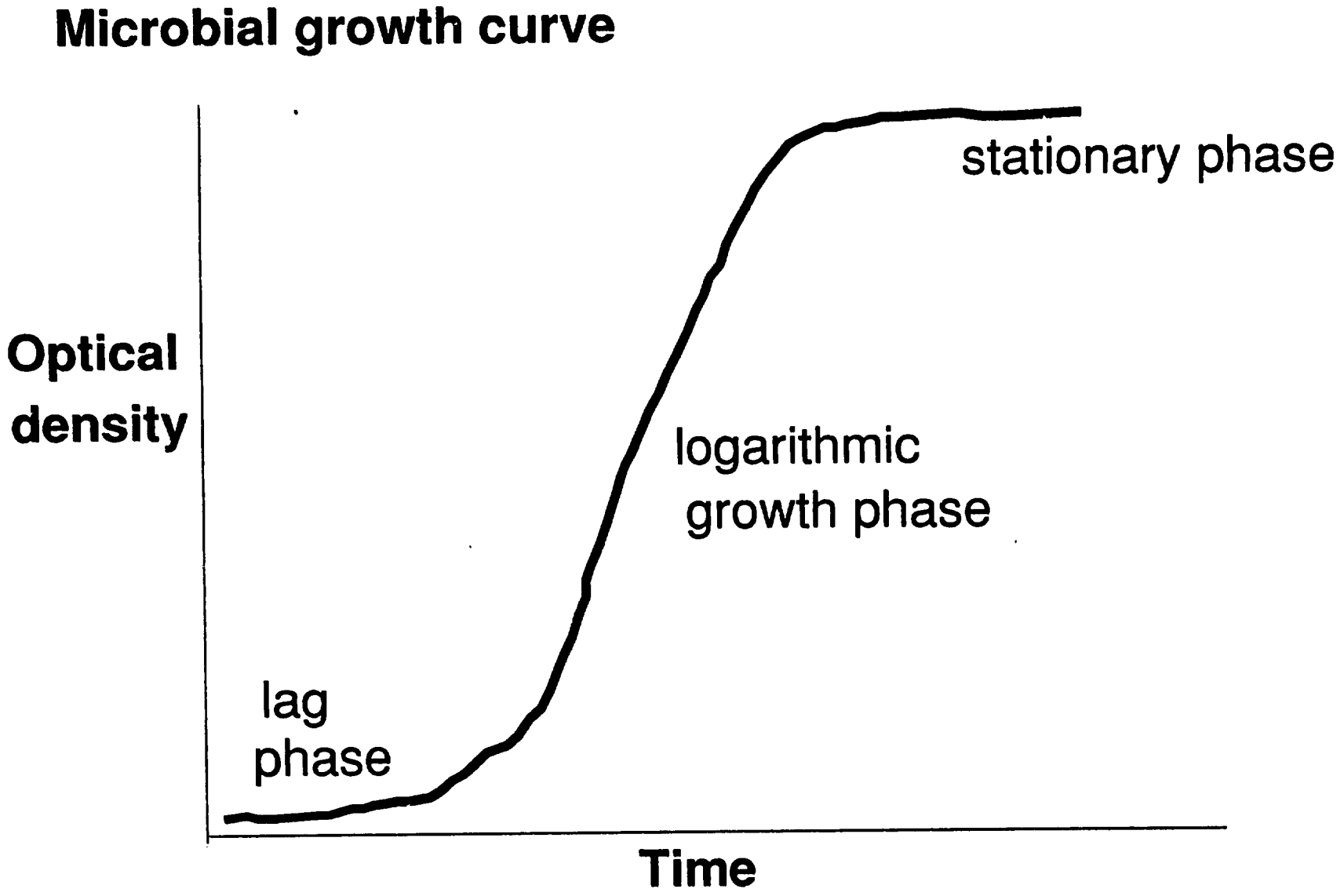
Optical density measurements : For the assessment of optical density, samples can either be removed from the growth flask aseptically, and pipetted into optical cuvettes, or a side arm flask can be used. In this latter case no samples need to be taken. The culture flask is simply tipped so that the culture runs into a side arm which fits directly into the photometer in some models. Appropriate attachments to normal spectrophotomers can be used which allow such a side arm to be inserted.

Enumeration of microorganisms.

While the tests described above give a general qualitative estimation of toxicity they do not allow enumeration of microorganisms in the tests. Where the effects of processes on microbial viability are being studied it is

Figure 1


Inhibition testing on plates
Streak of microorganisms
Inhibition zone
Increasing inhibition


Microbial growth curve
Optical density
stationary phase
logarithmic growth phase
lag phase
Time

often necessary to be able to determine the actual loses in microbial numbers. The most commonly used technique for estimating viable numbers is the plate count.

A known weight or volume of either a pure microbial culture, or a soil or water sample, is serially diluted into sterile saline solution. An aliquot of each dilution (0.1ml) is then spread onto a plate of solid medium using a glass spreader, and the plates incubated. As each individual bacterium grows it will divide and eventually form a bacterial colony visible to the naked eye. Each such colony will theoretically have been derived from a single bacterium present in the original solution. Hence back extrapolation allows us to calculate the numbers of microorganisms in the original sample.

Example. A 1g sample of solid formulation is added to 9mls of sterile saline contained in a sterile, capped test tube. The contents are mixed well and 1ml of this solution is transferred to another tube containing 9.0ml of saline. As the process is repeated a series of 10 fold dilutions are prepared. An aliquot of each solution is then counted and the numbers in the original deduced eg. if there are 125 bacteria/ml in a 1/1000 dilution, then then original solution contained 125 X 1000 bacteria.

APPENDIX 2

VISITS AND DISCUSSIONS

Indian Institute of Immunology, 15.2.90

The purpose of the visit was to look at the microbiological and fermentation laboratories. The overall facilities were excellent and the staff were most helpful in providing details on suppliers of microbiological chemicals, equipment and services. Their laboratory furniture was designed by a company from Lucknow (Newton Scientific), and their grade 1 microbiological laboratory, designed for work with the AIDS virus, by Kartos International (also local).

The Alfa - Laval fermentation system (similar to the one PDC wish to purchase) was situated in a normal microbiological laboratory, while an adjacent room housed all the necessary services (compressed air, chilled water, and steam). The buildings in which the institute was housed were relatively new and because of limited access to the outside environment the problems of running clean microbiological laboratories were minimised.

I was provided with a list of local suppliers addresses and put in touch with the laboratory design companies.

In view of the experience of this institute with microbiology in general, and with Alfa - Laval fermenters in particular, I would recommend that the new PDC microbiologist establishes contact with the local staff on a regular basis.

Indian Agricultural Research Institute (IARI)

(i) Dr K.V.B.R.Tilak, Head, Division of Microbiology, and Dr S.T.Shide, Senior Applied Scientist

This department was visited largely to ascertain their interests in the use of microorganisms in agriculture. The major interests in the department were in biological nitrogen fixation, waste recycling, biofertilisers, and phosphate mobilisation.

Nitrogen fixation

They are actively involved in growing up strains of Rhizobia, Azotobacter and Azospirillum and providing these to farmers as biofertilisers. All cultures were either grown in small fermenters, or in shake flasks, and then

harvested and mixed with a carrier. Peat is a good carrier but is not widely available in India. Considerable research had been carried out into alternatives such as charcoal, lignite, clays, and farmyard manure. and current preferences were for the use of charcoal and lignite. Hence bacterial culture broths were mixed with lignite to give a mixture with a final water content of about 40%. This material was then sealed in plastic bags and provided to the farmer. They claimed a shelf life of about 2-3 months for this material.

Different inocula were recommended for different crops:

Rhizobium : clovers, lucerne, pulses
Azotobacter : mustard, cotton, maize, sugar cane, potato, wheat Azospirillum : sorghum, millet, maize, barley, oats

Once delivered the farmers mixed the material with 10% sugar solution and used this to coat both seeds and seedling roots. The application rate is about 1kg/ha and the cost of inoculum for this is only about 40Rs. Given this very low cost they expressed little interest in looking at other formulations.

While I didn't see any detailed results they claimed that they could see improvements in yield in field trials and stated that about one third of the required nitrogen for a crop could be provided by the inoculum (60 - 100kg/ha for sorghum and millet). Inocula proved most useful in areas where there was a minimal or no application of synthetic nitrogen.

Phosphate mobilisation

They were also using strains of Pseudomonas striata, Aspergillus awamori, and Bacillus polymyxa for phosphate solubilisation. I didn't see any data to demonstrate# that phosphate was actually solubilised solubilisation was inferred from yield data from field trials. They were also looking at the effectiveness of VA mycorrhizae in this area in cooperation with workers at Oregon State University, USA.

The phosphate mobilising preparations were used with rock phosphate and they stated that they achieved their best results in acidic laterite soils where chemical conditions obviously also favoured phosphate dissolution.

The activities described above constituted the major current interests although there was also an activity on the use of cellulolytic fungi as composting enhancers.

A leaflet detailing the production of microbial inocula for use in the areas described is available from the institute.

Formulation activities

The staff we talked to showed little interest in improving the formulation of their microbial materials. Their current techniques were extremely cheap and they felt that farmers would not pay for anything more sophisticated. They also felt that treatment of seed before it was sent to the farmers was not feasible because of problems with seed storage - I must admit to not fully understanding their objections to prior seed treatment.

In discussion staff felt that the shelf life of their inocula could be improved, and they were also looking at granular formulations or use with crops such as sugar cane. I suspect that there could be a role for PDC in cooperating with IARI in this area but this would be largely for experimental interest - I doubt that any industry would be interested in supplying such products because they are unlikely to be commercially viable, and because it is extremely difficult to demonstrate that such products actually work in practice..

(ii) Dr Ramakrishnan, IARI

Dr Ramakrishnan has extensive experience in insect pathology and worked on Bt and Bs for many years before moving on to bacullo -viruses. He attributed the lack of registration of any Bt products in agriculture in India to concern over their toxicity to silk worms. While silk is now produced in a number of areas, 4 main states produce the bulk of the 9600 ton/annum currently produced. It is hoped that this production figure can be increased by another 5000 ton.

Research on Bt and Bs in India

A number of institutes mentioned Dr Ramakrishnan are listed below:

ICMR (Indian Council of Medical Research) with headquarters in Delhi, have an interest in these microorganisms for mosquito control. The Vector Control Research Institute at Mysore is a part of ICMR and has an active interest in the field. The institute director is Dr P.K.Rajagopolan, and a Dr Balaraman works on Bs and Bt. There is a current interest in slow release formulations, and the institute has some fermenter capacity.

Dr K. Jayaraman at the University of Madras is also active in this field of research and some of PDC,s samples of Bt and Bs originated from her laboratory.

Other targets for biological control

Dr Ramakrishnan identified Heliothis (American boll worm), Spodoptera (tobacco caterpillar) and Amsacta (red hairy caterpillar) as potential biocontrol targets because of development of resistance problems. Plutella (diamond back moth) is also of interest. In India 60% of current insecticide use is on cotton and paddy.

Location of workers

Heliothis / Amsacta TNAU (Tamil Nadu Agricultural University)

Heliothis, Spodoptera, Amsacta, Dibrotica (Indo-US collaboration project)

Bt and Bs VCRS (Bt & Bs 27396, 27397)

An all India coordinated research project on biological control of pests of insects and weeds is in operation (ICAR funded). This is centred at Bangalore and Dr S.P.Singh is coordinator. Projects were reviewed in 1989 at

the Lucknow sugar cane institute. The documents from the meetings of this group are a valuable source of information on biocontrol projects in India and list locations, workers, and basic project details. The relevant documents are referenced below:

Proceedings of the first biocontrol workers group meeting, Organised by the Biological Control Centre, Bangalore, July 29-30, 1988. Technical document No. 22

All India Coordinated Research Project on the Biological Control of Crop Pests and Weeds. Annual Report, 1988. Technical document No.23

EFC Memo of the All India Coordinated Research Project on the Biocontrol of Crop Pests and Weeds. The 7th plan, 1985-90

These documents have been obtained by PDC staff.

(iii) Department of Biotechnology - Dr Ghosh

Dr Ghosh was in charge of coordinating a number of government funded research programmes on Bt and Bs, as listed :

a) Use of Bt and Bs in mosquito control

b) Use of MPV viruses for lepidopteran control on sugar cane, cotton, and tobacco

c) Transfer of Bt toxin genes into natural leaf colonising bacteria

Mosquito control

Groups in both Lucknow and Madras are working in this area, as is a private company in Bombay but Dr Ghosh would not name the company. It was difficult to determine exactly what had been done but apparently the Lucknow group was looking at formulation, and had decided that particle size was very important. They were also having problems in growing these organisms because of the timing of toxin expression. I found this surprising as none of the western companies which I've talked to seem to have problems in this area. At present , while no detailed production costings had been done, Dr Ghosh felt that they could produce the microorganisms for a lower cost than chemicals such as malathion, but more expensively than DDT.

Again it was evident that there are considerable regulatory hurdles to overcome before a Bt product will be registered. This is largely due to fears of toxicity to silk worms. Indeed the only rationale I can find for people working on Bs is in it's supposedly lower silk worm toxicity. However none of the scientists I spoke to have either surveyed the literature in the area, or carried out the toxicity assessments themselves. This strikes me as a major folly - one does not base an entire programme on an organisms simply on the assumption that it is more environmentally acceptable, especially when it is obvious that Bs performs very poorly when compared to Bt. The fact that a number of Japanese companies are now showing an interest in Bt, in spite of their concern over silk worms, would indicate that maybe some of the technical and toxicity problems have been overcome.

Transfer of Bt genes

Dr Ghosh would not reveal the location where this work was being carried out- I am baffled as to why as the results will eventually be offered to others for development. Apparently they had carried out some work on Bt survival in the field on leaf surfaces and obtained poor results - by 10 days no live cells were detectable. I wasn't surprised at this as the cells were just added in medium or in an isotonic solution and not, according to Dr Ghosh, formulated in any way. With no protection from dessication, nutrient source etc I find it hard to see why organisms should be expected to survive. Be that as it may, they felt that they would be better off transferring the Bt toxin gene into a natural leaf coloniser, and in principle at least, this is a sensible approach. They claimed that they were getting better results but I could't substantiate this without looking at some hard data. They felt that Bt would be more easily registered in this area as it would be used in the cotton belt where there was no silk industry.

Commercialisation

Apparently any product would be offered to either public or private sector for development. In this case I find it difficult to understand why he would not reveal the location of the work.

Project coordination

The various teams involved meet 3 times a year to review progress and I wonder if it would be possible for someone from PDC to sit in. In my view it would probably be better to establish contact with individual scientists involved especially as PDC staff members already have contacts in both Madras and Lucknow.

Dr Ghosh admitted that production and formulation were comparatively weak areas and would welcome an expert group with such capabilities. This contact should be used in any future programme devepopment.

APPENDIX 3

MICROBIOLOGICAL SAFETY

Basic laboratory safety procedures

I have recommended a number of basic texts on the subject for inclusion in the PDC library. The notes below outline basic procedures that should be adopted while carrying out any work with microorganisms.

General practices

- No eating, drinking or smoking should be allowed in the laboratory, neither should any food or drink be stored in the laboratory area. In essence any activities involving hand to mouth contact should be avoided if possible, and stringent conditions of hygiene should be adopted.

- Laboratory coats should be worn at all times within the laboratory but should not be taken out of the area. Coats should be removed and left in the laboratory when staff go to lunch, tea etc. Coats should be laundered weekly with prior autoclaving if necessary.

- the laboratory areas should be cleaned daily and care taken not to generate dust. It is thus preferable to use a vacuum cleaner or wet mop to using a broom.

Laboratory operations

Before carrying out any microbial transfer work microbial hoods and benches in the appropriate areas should be wiped down with an appropriate disinfectant solution or with alcohol.

Pipettes to be used for microorganisms should be plugged (in the neck) with cotton wool and sterilised before use. Pipette bulbs should be used for pipetting operations - mouth pipetting should be avoided.

Used pipettes should be disposed of in a container filled with disinfectant prior to washing. Containers should be charged with fresh disinfectant on a daily basis.

All other contaminated glassware should be stored in metal boxes which can be autoclaved with their contents. Contaminated material should be autoclaved on a daily basis and not left lying round in the laboratory for extended periods.

All solutions containing microorganisms must be sterilised before they are discarded. Fermenter effluents generated on a continuous basis should be collected in a sterile vessel equipped with an air outlet filter, and sterilised before it is disposed of.

Used syringes, needles, Pasteur pipettes and other sharp materials should be placed in a beaker of disinfectant and allowed to soak for at least 24 hours before disposal.

APPENDIX 4

PURCHASE OF FERMENTATION EQUIPMENT

A number of quotes had been received from different companies before I arrived. However based on my own past experience and on expert advice from European colleagues I decided that Alfa - Laval not only provided excellent equipment, but were also in the best position to offer a continuing service to PDC. Subsequently I arranged a discussion with the Alfa Laval representatives and the details of this are summarised below.

Present: Mr Kumar, PDC
Dr.K.A.Cook, UNIDO
Mr P.Dhotey, Alfa - Laval (India) Ltd., Pune Mr A.S.Bhat Alfa - Laval (India) Ltd., New Delhi

Alfa - Laval (India) Ltd. is a 60% Indian and 40% Swedish company and has offices and service facilities in 14 Indian cities. Chemap, the actual company providing fermentation equipment, is 100% owned by Alfa - Laval.

Since the last quotation was sent to PDC in 1989, a number of changes in equipment specifications had occurred and prices have changed accordingly. The purpose of the discussions held at PDC were to identify the most suitable equipment for PDC,s use. My discussion document on the overall possibilities for biological programmes at PDC discusses the question of fermentation facilities, and concludes that a small scale fermentation unit is most suitable for PDC,s short and medium term needs. Hence our discussion was restricted to fermentation vessels with a maximum working volume of 30 litres.

Vessel size : The Chemap fermenter range is a modular system which can accommodate vessels of 7, 14, 20, and 351 total volume. For general experiments on the effects of various parameters on growth yield a working volume of about 10l is useful, while a larger vessel will give the additional capacity to grow up bulk material for field trials. Hence I would recommend purchasing both 14l and 35l vessels to give a level of flexibility

Agitator systems : Seven distinct systems are available but the basic paddle system used in conjunction with a ring-type air sparger is most suitable for general microbial growth studies.

Air filtration : Ceramic filters are available and these are adequate - they are interchangeable with more expensive filters should these be required.

Back pressure control : An automatic control system is available and this sounds useful in view of the very short period of the day when the laboratory is manned by scientific staff. However I am told by colleagues with experience with such systems that they are technically very difficult to set up and are not really necessary for laboratory scale fermentations. With larger scale fermenters they are useful on scale up to increase oxygen transfer rate and compensate for blockage of outlet filters. Therefore I would not recommend the purchase of such a control at this stage.

Air supply : The 60l/min rotameter available is recommended with a standard pressure gauge.

Harvesting valve: Disadvantages of standard membrane valves include a requirement for a higher positive pressure inside the vessel for the membrane to function, and they are prone to blockages during long fermentation runs.

Piston valves are generally preferred for reliability, ease of sterilisation, and elimination of dead space. However they are more expensive and also require more servicing - the rings need replacing at regular intervals. On balance piston valves should be used wherever possible.

Pumps : Two peristaltic pumps are required to service the fermenter but the use of double - headed pumps will allow for a reasonable capacity with little extra cost.

pH control : Normal acid / base control is considered to be sufficient. Use of gases is only recommended where the rapid adjustment of pH is essential (gases mix faster than liquid additions), or where there is a need to minimise the increase in volume of the fermenter. It can also be useful for shear - sensitive cells. For current purposes, therefore, the use of gases should be avoided.

Gas analysis : Oxygen and carbon dioxide are estimated via specific electrode systems.

Fermenter control : The new model fermenters are controlled by microprocessors which receive software instructions via a built in floppy disk. Control parameters are output as hard copy text or graphs to an attached printer.

Spares and servicing : Spares kits are provided for all components and are available for 2 or 5 years operation. I would recommend the purchase of a 2 year kit in combination with a service contract. The Delhi branch of Alfa Laval has a service engineer on call.

I cannot overemphasise the importance of good service facilities. There is no engineering effort at PDC capable of maintaining such a fermenter, although I would hope that the microbiologist who will be hired will become familiar with the equipment. Similarly there is no on site support for computing equipment. These facts make it essential to have a fast local call out engineer who can quickly rectify any technical faults. In addition to this the price of Alfa Laval equipment is inclusive of a 1 week training course in Switzerland for the operator.

The overall reputation of Chemap fermentation equipment, the availability of local engineering backup , and the inclusion of an expert training course at the companies laboratories in Switzerland are, in my opinion, sufficient justification to purchase the Alfa - Laval equipment and I would not recommend negotiations with other companies unless there is an overriding financial constraint.

A comprehensive quote from Alfa - Laval was left with PDC management but there are a few minor changes to be made in the light of additional information which I have obtained. Hence the following additions should be made to the quote:

- an additional 14 litre fermentation vessel

- piston type harvesting valves

This will not make an enormous difference to the final quote which will be in the order of Swiss francs 100.000.

AUXILIARY EQUIPMENT

While PDC technically have on site facilities for the provision of compressed air, steam, and chilled water, certain considerations should be taken into account.

Air compressor : This should be oil-free to avoid any contamination of the culture vessel contents

Steam generation : Steam is required for in situ sterilisation of the fermenter vessel and thus the times at which it is required should be predictable. However one should consider the cost of running the large steam generator at intervals solely to serve this small fermenter and the cost of running steam pipes into the new microbiology laboratory , as against the purchase of a small local steam generation plant. On consideration of these factors, and bearing in mind the space constraints in the new laboratory, I feel that the existing generator should be used.

APPENDIX 5

FORMULATION PROCESSES AND CHEMICALS

The pilot plant equipment was examined and the conditions of operation listed to allow an assessment to be made of the likely effects of the operations on preparations containing microorganisms.

The combinations of equipment and processes that would be likely to be used for different generic types of materials are summarised below:

1) Slurries / suspension concentrates :

Silverson mixer : pump : Dynomill

2) Solid/solid mixtures :

Ribbon blender : Hammer mill : Fluid energy mill

3) Solid/liquid mixtures :

Lodige mixer : Pin mill : Fluid energy mill
Hammer mill

4) Extrusion granules :

Sigma mixer : Condux cutter

5) Liquid / liquid mixtures :

Papenmeier mixer

PILOT PLANT EQUIPMENT

Lodige mixer (powder mixer)

This is used to mix basic formulations containing active ingredient (ai) surfactants and fillers). It contains large blades which rotate at about 250 rpm. Operating temperature is the same as ambient. The mixing process takes about 30mins.
If a liquid active ingredient is being used then this will be pumped into the apparatus under pressures of up to 100 psi (it is effectively added as a spray).

Double helical ribbon blender

This contains helical blades and is also used at ambient temperatures. Mixing time is up to 60mins.

Sigma mixer

This is effectively a machine with a kneading action and is used for the

preparation of extruded materials. It is operated at ambient temperatures for periods of up to 60 mins.

Silverson mixer

This is used for mixing slurries and is effectively a blender with recirculation of material, run at ambient temperatures for up to 60 mins.

Papenmeier mixer

A mixer used for liquid/liquid mixing, and for emulsifiable concentrates. This runs somewhat above ambient (40C) but has a facility for cooling. It mixes at about 5000 rpm and is used for up to 120 mins.

Fluid energy mill

Here the solid product is forced through a nozzle in a stream of compressed air at 100 psi. Smaller particles are generated and these are precipitated in a cyclone apparatus. Escaping particles are collected by a fine particle filter. It runs at about 3C over ambient at a continuous rate of 50 kg/h.

Hammer mill

Hammers within this mill rotate at .2880 rpm and grind any solids present. This apparatus gives a fairly coarse grinding by hammering. Material is thus under some pressure. the apparatus runs at about 15C above ambient and operates at 5 kg/h.

Ultra fine grinding mill

This is a commercial size piece of equipment and material is delivered by a screw feed and transported in an upward air flow. Particles are broken between the vessel wall and a set of hammers. The final product is blown over into a cyclone device, and escaping particles are extracted in a further filter. The machine operated at 1C above ambient and can process 100 kg /h of material.

Dyno mill (agitated ball mill)

This is used for slurries and material is usually fed from a Silverson mixer via a pump operated at 45 psi. The machine mixes with the aid of glass balls. It is cooled but still reaches temperatures up to 50C. It can process 50 kg/h of material.

Alpine mill

This is a rough grinding machine of the hammer mill type.

Pin Mill

This is a small piece of apparatus used to break up agglomerations. It runs at 10C above ambient at about 1kg/h.

Tri-Homo colloidal mill

This is similar to a Dyno mill but uses a cone system rather than glass beads. It operates at a high temperature (80C) and 25 psi, in a continuous mode.

Condus cutter

This is simply designed to cut extruded materials.

LABORATORY EQUIPMENT

The formulation laboratory largely contained smaller versions of the equipment in the pilot plant (eg Hammer mill, Dyno mill, Silverson mixer). with a few exceptions which are discussed below.

Spray drier

Here a solution is introduced into the dryer via a peristaltic pump, mixed with air, and sprayed through a noozle (c. 0.5mm diameter) into a chamber where the drying occurs. This can be operated at variable temperatures but a temperature of at least 80C is required to dry aqueous solutions.

Fluidised bed

This involves spraying a solution into a chamber in an air stream and conditions are similar to those within a spray dryer.

FORMULATION CHEMICALS

Chemicals commonly used in formulation are listed for reference.

Wettable powders

Kaolin clay, precipitated silica, alkyl arly sulphonates, sulphated fatty aids, lignin sulphonates

Aqueous suspension concentrates

Glycols, calcium dodecyl sulphonate, polyoxyethylene nonyl phenol, ethers, non-ionic emulsifiers, polysaccharides, formalin

Oil flowables

Mineral and vegetable oils, non-ionic emulsifiers, block copolymers, anionic surfactants, hydrophobic silica

Granules

Silica sand, marble chips, polyethylene glycol

Dusts

Soapstone, hydrophobic silica

APPENDIX 6

MICROBIOLOGICAL BOOKS FOR THE LIBRARY

I would recommend the purchase of the books listed below to form the nucleus of a microbiological section in the library. With the close proximity of other scientifi# libraries I would only recommend the purchase of a few general scientific journals, and summaries of scientific publications.

LIBRARY BOOKS

1. Bergey,s Manual of Systematic Bacteriology. Published by Williams and Wilkins in 4 volumes, each with different editors and publication dates.

2. The Procaryotes. M.P.Starr, H.Stalp, H.G.Truper, A.Balows & H.G.Schlegel. Springer Verlag, 1981 (2 volumes)

3. The filamentous fungi. Edited by Smith, J.E. & Berry, D.R. Published by Edward Arnold, 1976 (4 volumes)

4. The bacteria, a treatise on structure and function. Series editors I.C.Gunsalus, J.R.Sokatch & I.N.Ornston. In 9 volumes, Academic Press, 1960 - 1986

5. Maintenance of microorganisms. B.E.Kirsop & J.J.S.Snell. Academic Press, 1984

6. Methods in Microbiology. Edited by J.R.Norris & D.W.Ribbons. Published in 19 volumes by Academic Press, 1969 - 1989

7. Manual of Methods for General Bacteriology. Ed. in chief P.Gerhardt. American Society for Microbiology, 1981

8. Manual of Industrial Microbiology & Biotechnology. A.I.Demain & H.A.Solomon. American Society for Microbiology, 1986

9. Handbook of Microbiology. A.I.Laskin & H.A. Lechevalier. Published in 10 volumes by CRC Press, 1987

10. Data for Biochemical Research. R.M.C.Dawson, D.C.Elliot, W.H.Elliot & K.M.Jones. Clarendon Press, 3rd Edition, 1981

11. The biochemistry of bacterial growth. J.Mandelstam, K.McQuillan & I.Dawes. Blackwell Scientific 1982

12. The Enzymes. M.Dixon & E.C.Webb. Longman, 1979

13. Microbial Technology. H.J.Peppler & D.Perlman. Published by Academic Press in 2 volumes, 1979

14. Comprehensive Biotechnology. Series Editor M.Moo-Young. Published in 4 volumes by Pergammon Press, 1985

15. Biotechnology, a comprehensive treatise. Series editors H.J.Rehm & G.Reed. Published by Verlag Chemie in 8 volumes, 1983

16. Biochemical Engineering and Biotechnology Handbook. B.Atkinson & F.Mavituna. Macmillan Publishers, 1983

17. Principles of fermentation Technology. P.F.Stanbury & A.Whitaker. Pergammon Press, 1984

18. Principles of microbe and Cell cultivation. S.J.Pirt. Blackwell Scientific, 1975

19. Biochemical Engineering Fundam#ntals. J.E.Bailey & O.F.Ollis. McGraw - Hill International, 1986

20. Biochemical Engineering. S.Aiba, A.E.Humphrey & N>F.Mills. Academic Press, 1973

21. Dictionary of Antibiotics and related substances. Edited by B.W.Bycroft,Chapman Hall, 1988

22. The Nature and Practice of Biological Control of Plant Pathogens. R.J.Cook & K.F.Baker. American Phytopathological Society, 1983

JOURNALS

New Scientist
Nature
Science
Scientific American
Current Contents (this comes in sections covering various areas) Chemical abstracts summaries are already held

BOOKS ALREADY IN THE LIBRARY

There are only 3 relevant books on the shelves at present and these should be included in the new microbiology section.

Pesticide microbiology. Ed. I.R.Hill & S.J.L.Wright. Academic Press, 1978

Biotechnology for Crop Protection. Ed. P.A.Hedin, J.J.Menn & R.M.Hollingworth. ACS symposium series, 1987. American Chemical Society, Washington D.C, 1988

Biologically active natural products. Potential use in agriculture. Ed.H.G.Cutler. American Chemical Society, Washington D.C., 1988

APPENDIX 7

EQUIPMENT PURCHASE - COSTINGS AND RECOMMENDATIONS

Equipment has been discussed with a number of companies and estimates obtained. Only equipment specific to microbiological operations has been discussed - the additional equipment required for the laboratory is common to the chemical laboratories at PDC and is simply listed. The addresses of the relevant companies are given below, followed by recommendations, with reasons, and a list of prices. Detailed quotes from the companies in question are appended.

The specialised fermentation equipment required is discussed separately.

Suppliers contacted

Yorko Sales PVT Ltd,
11 Netaji Subhash Marg
New Delhi 110 002
Tele. 3264042 / 3278381 / 3278306

KRATOS INTL.
A - 52, Sector 57
Noida 201301 (UP)
Tele. 8922112

KRISHNAPLAST
GG-1/5-C, Vikas Puri
New Delhi 110 018

METREX - Agent for Mercantile Engineers
4/5 Swami Ram Tirath Nagar
Jhandewalan Extension
New Delhi 110 055
Tele. 520981 / 529472

HERAEUS SEPATECH
Heraeus India PVT Ltd.,
Post Box 4573
P.O. Hauz Khas
New Delhi 110 016
Tele. 666194

RECOMMENDATIONS

Microbiological hoods and air curtain generators

Kartos International have consider#ble experience in this area and have provided such equipment to other laboratories in the area. As they are the company that I will recommend for the overall refurbishing of the microbiological laboratory then I would also recommend that this type of microbiological equipment is purchased from them. Their prices are also competitive so that there is no financial penalty attached to this decision.

Autoclave

The 2 companies that I talked to that produce suitable autoclaves are effectively offering a similar model of autoclave at a similar price. I therefore have little preference here. Yorko are offering a 10% discount on orders above Rs 50000 but a similar discount may be negotiable with the other company. Delivery dates quoted are also similar.

Sterilising oven

The model offered by Yorko is most economical and looks similar to the Krishnaplast model. Again on balance I have no real preferences.

Incubators

A number of local companies manufacture incubators which look quite adequate - I only had discussions with Yorko. Heraeus produce a model from Europe which is reasonably priced by European standards but about 20% more expensive than locally available models. However as I intend to recommend that we purchase other equipment from Heraeus then it may be easier to obtain the incubators from them at the same time. I feel this decision should be made locally on the basis of financial considerations.

Continuous centrifuge

I have discussed this with Heraeus and with Alfa - Laval and feel that the Heraeus model recommended is the most versatile for the needs of PDC. It can be used as a normal bench centrifuge, but can also be operated continuously - this continuous operation is needed for harvesting microorganisms from the fermenter. I therefore recommend the purchase of the Heraeus model.

PRICES AND EQUIPMENT DETAILS

INCUBATORS

Yorko

5 - 50C, stainless steel linings, adjustable trays 6 cuft Rs 28000
10 Rs 29000
12 Rs 33000
15 Rs 42000

Metrex

830 X 505 X 415 mm (6.1 cu.ft) Rs 26500

Heraeus

550 X 554 X 644 mm Rs 34668 DM 3852
559 X 554 X 1366mm Rs 48150 DM 5350

AUTOCLAVES

Yorko

Horizontal, stainless steel lining (316), integral steam boiler optional, trolley and carriage

2 X 3 X 5 ft	2.2 lakh	Trolley RS 18000 Carriage Rs 6000
2 X 2 X 4 ft	1.4 lakh	Trolley Rs 15000 Carriage RS 5000

Krishnaplast

Horizontal, stainless steel liner, without boiler Rs 145,850 2 X 2 X 4 ft with boiler Rs 162000
with carriage Rs 15000

Vertical, 18" X 24" diameter Rs 45000

LAMINAR FLOW HOODS

Yorko

YSI-189	4 X 2 X 2 ft	fully equipped (manometer, light, door, gas point)	Rs 20000
YSI-190	4 X 2 X 2 ft	Model YBS 44	Rs 38500

Kartos Intl.

4 X 2 X 2	Rs 42750
Gas inlet	Rs 600
Manometer	Rs 1240
	Rs 44590

OVER DOOR AIR CURTAIN

Kartos Intl.

4 ft	Rs 12000
5 ft	Rs 15000

OVEN

Yorko

Stainless steel lining, 250C for sterilising, temperature controllers, digital display

Model VSI-431 605 X 605 X 605 mm Rs 22825

Krishnaplast

Sterilising, stainless steel lined, 2 X 2 X 2 ft Rs 32000

CONTINUOUS CENTRIFUGE

Heraeus

300MD	Rs 83367	DM 9263
Rotor Al	Rs 75600	DM 8400
Rotor 12 X 13 ml	Rs 13500	DM 1500
6 X 96 ml	Rs 27000	DM 3000
4 X 100 ml	Rs 6300	DM 700

APPENDIX 8

LABORATORY AND BENCHING PLANS AND DESIGNS

Fig.1 Scale drawing of laboratory

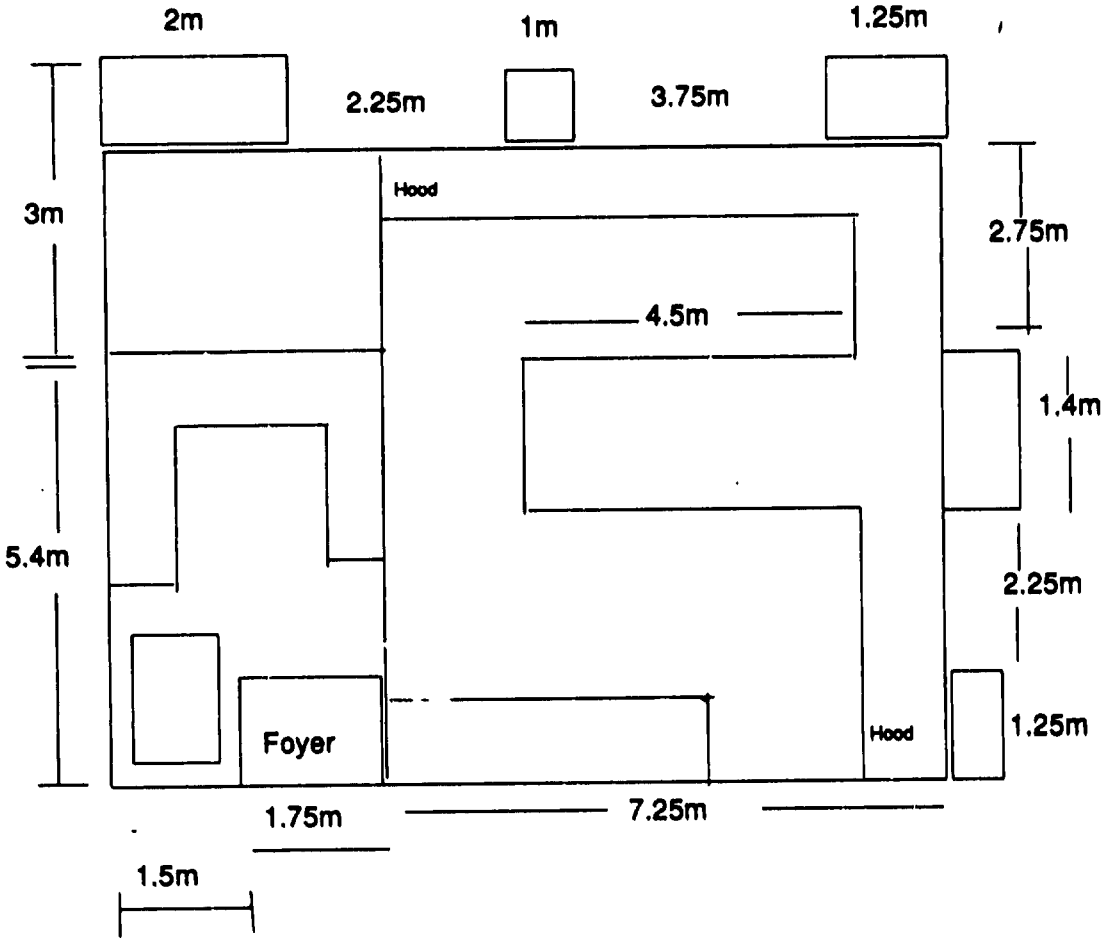
Fig.2 Laboratory plan

Fig.3 Design of fermentation banch

Fig.4 Design of normal laboratory bench

Microbiology laboratory

Scale drawing



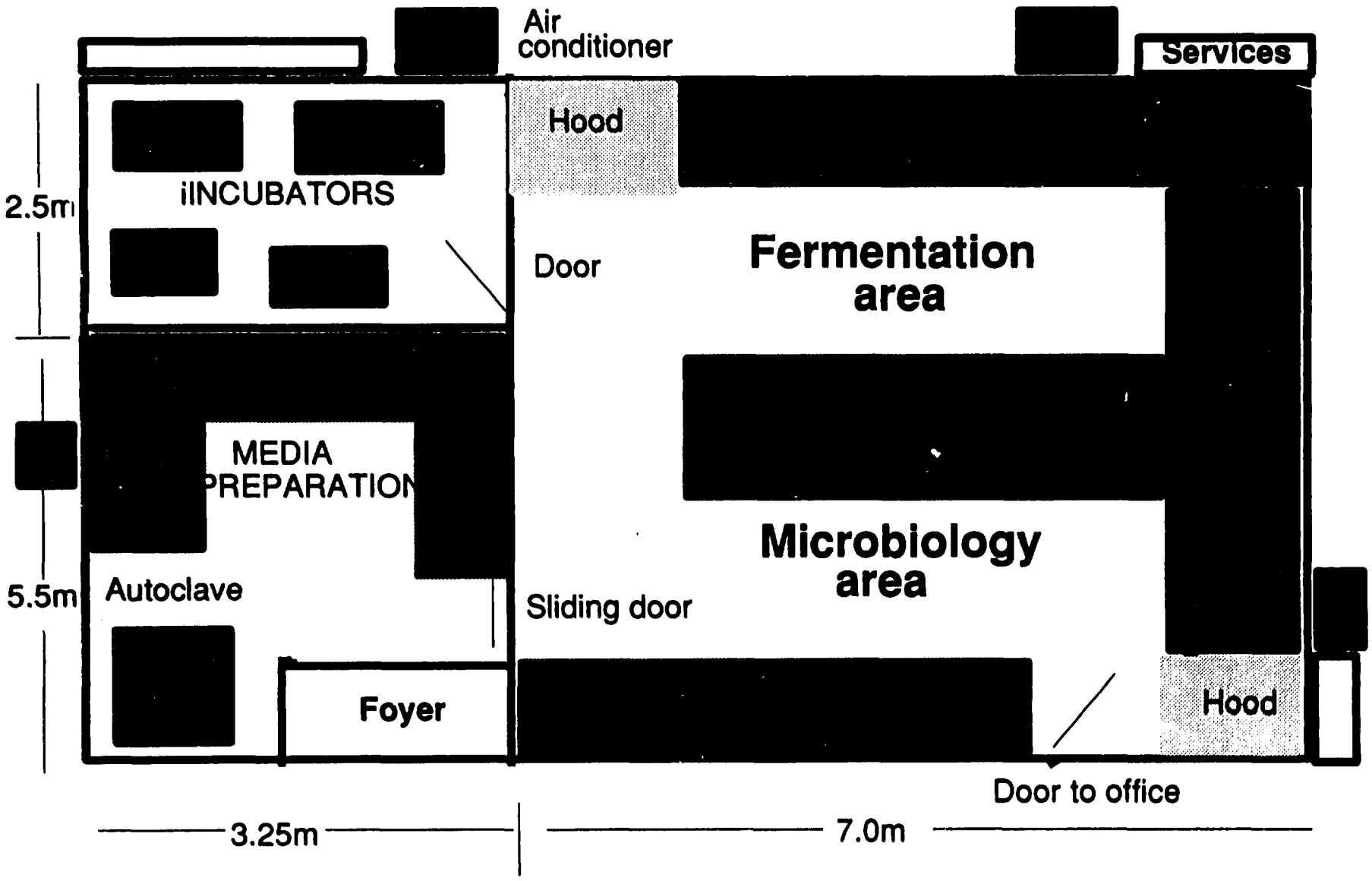
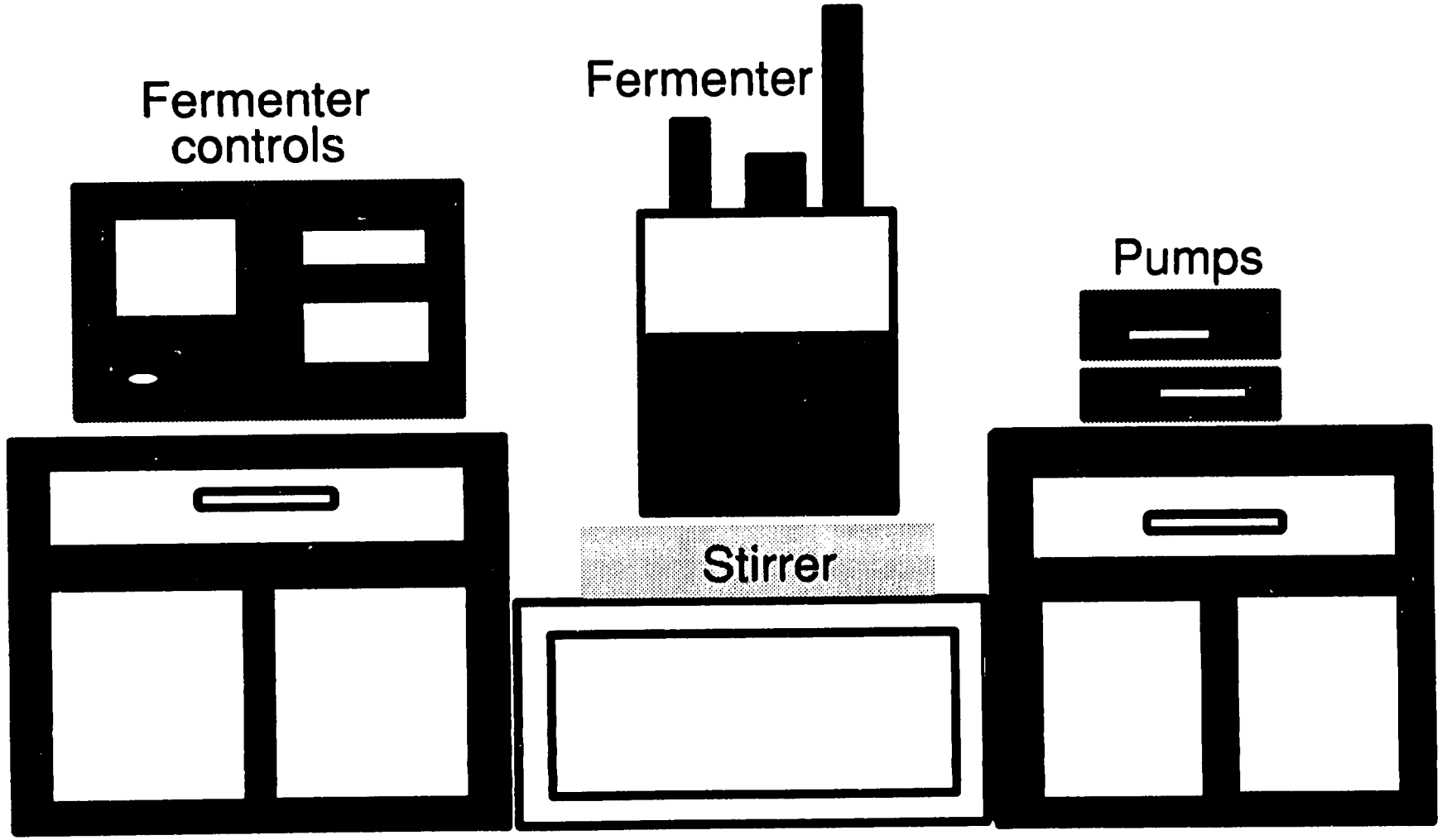

Air conditioner
Services
Hood
INCUBATORS
2.5m
Door
Fermentation area
MEDIA PREPARATION
Microbiology area
5.5m
Autoclave
Sliding door
Foyer
Hood
Door to office
3.25m
7.0m

Figure 2

Figure 3

FERMENTATION BENCH

Lowered bench required to allow access to fermenter



DESIGN OF STANDARD LABORATORY BENCH

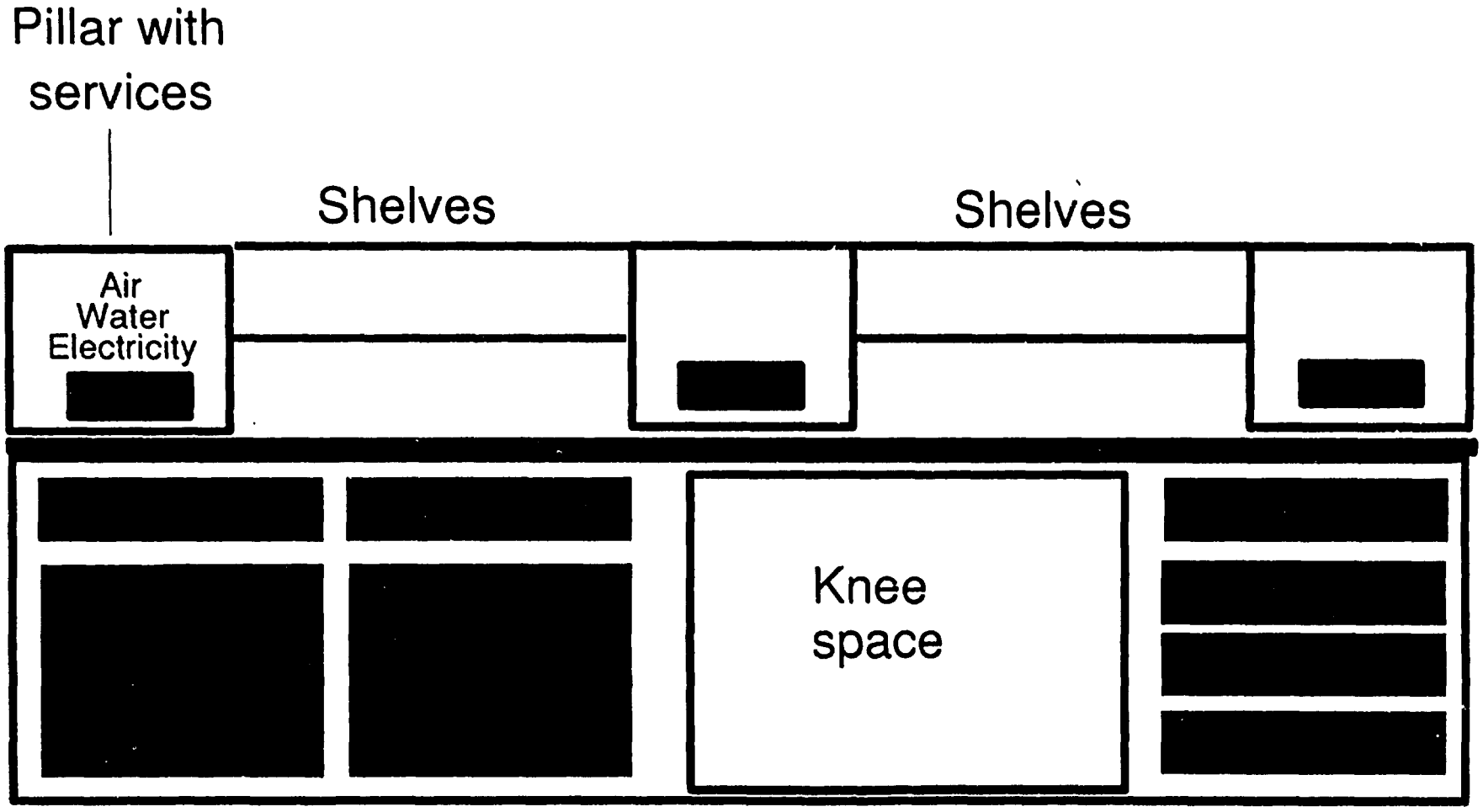


Figure 4