tation (Gottschalk and Grupe, 1992). The inoculum development programme described by McNeil and Kristiansen (1986) is given in Table 6.2. The stock culture is heat shocked to stimulate spore germination and to eliminate the weaker spores. The production stage is inoculated with a very low volume and this corresponds with Lurie's (1975) description of the South African acetone–butanol fermentation in which a 100,000 dm^3 fermenter was inoculated with only 10 dm^3 of seed. The use of such small inocula necessitates the achievement of as near perfect conditions as possible to prevent contamination and to avoid an abnormally long lag phase.

THE DEVELOPMENT OF INOCULA FOR MYCELIAL PROCESSES

The preparation of inocula for fermentations employing mycelial (filamentous) organisms is more involved than that for unicellular bacterial and yeast processes. The majority of industrially important fungi and streptomycetes are capable of asexual sporulation, so it is common practice to use a spore suspension as seed during an inoculum development programme. A major advantage of a spore inoculum is that is contains far more 'propagules' than a vegetative culture. Three basic techniques have been developed to produce a high concentration of spores for use as an inoculum.

Sporulation on solidified media

Most fungi and streptomycetes will sporulate on suitable agar media but a large surface area must be employed to produce sufficient spores. Parker (1950) described the 'roll-bottle' technique for the production of spores of *Penicillium chrysogenum*. Quantities of medium (300 cm^3) containing 3% agar were sterilized in 1 dm^3 cylindrical bottles, which were then cooled to 45° and rotated on a roller mill so that the agar set as a cylindrical shell inside the bottle. The bottles were inoculated with a spore suspension from a sub-master slope and incubated at 24° for 6 to 7 days. Parker claimed that although the use of the 'roll-bottle' involved some sacrifice in ease of visual examination, it provided a large surface area for cultivation of spores in a vessel of a convenient size for handling in the laboratory.

Hockenhull (1980) described the production of 10^{10} spores of *Penicillium chrysogenum* on a 300-cm^2 agar layer in a Roux bottle and El Sayed (1992) quoted the use of spore suspensions derived from agar media containing between 10^7 and 10^8 cm^{-3}. Butterworth (1984) described the use of a Roux bottle for the production of a spore inoculum of *Streptomyces clavuligerus* for the production of clavulanic acid. The spores produced from one bottle containing 200-cm^2 agar surface could be used to inoculate a 75-dm^3 seed fermenter which, in turn, was used to inoculate a 1500-dm^3 fermenter. The clavulanic acid inoculum development programme is illustrated in Fig. 6.4. Some representative solidified media for the production of streptomycete and fungal spores are given in Tables 6.3 and 6.4 respectively.

Sporulation on solid media

Many filamentous organisms will sporulate profusely on the surface of cereal grains from which the spores

TABLE 6.2. *The inoculum development programme for the clostridial acetone–butanol fermentation* (Spivey, 1978)

Stage	Cultural conditions	Incubation time (hours)
1.	Heat-shocked spore suspension inoculated into 150 cm^3 of potato glucose medium	12
2.	Stage 1 culture used as inoculum for 500 cm^3 molasses medium	6
3.	Stage 2 culture used as inoculum for 9 dm^3 molasses medium	9
4.	Stage 3 culture used as inoculum for 90,000 dm^3 molasses medium	

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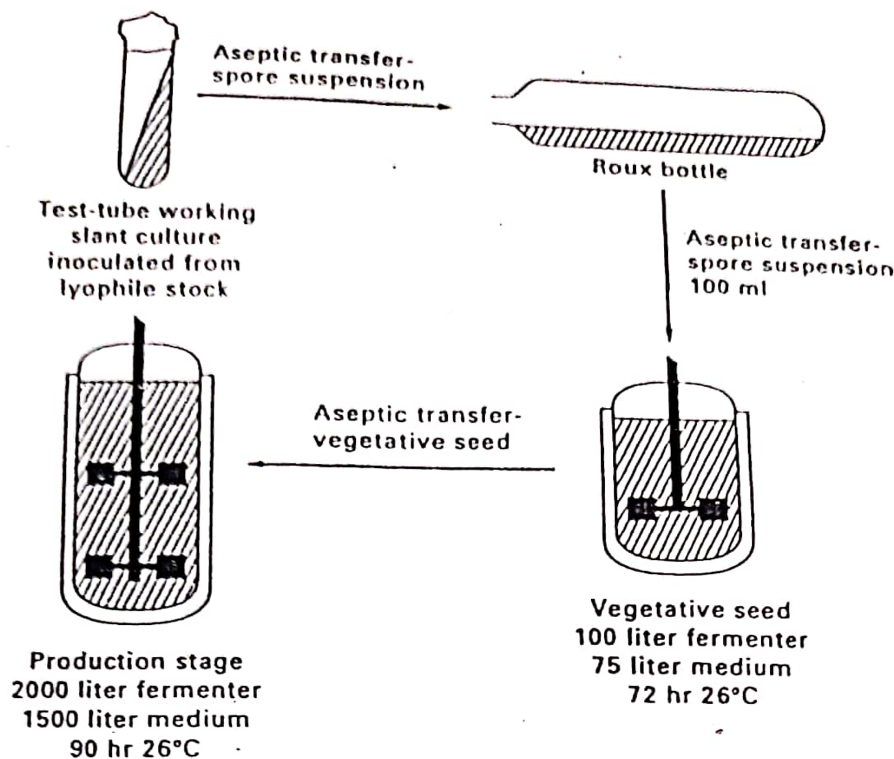

FIG. 6.4. The inoculum development programme for the production of clavulanic acid from *Streptomyces clavuligerus* (Butterworth, 1984).

may be harvested. Substrates such as barley, hard wheat bran, ground maize and rice are all suitable for the sporulation of a wide range of fungi. The sporulation of a given fungus is particularly affected by the amount of water added to the cereal before sterilization and the relative humidity of the atmosphere, which should be as high as possible during sporulation (Vezina and Singh, 1975). Singh *et al.* (1968) have described a system for the sporulation of *Aspergillus ochraces* in which a 2.8-dm^3 Fernbach flask containing 200 grams of 'pot' barley or 100 grams of moistened wheat bran produced 5×10^{11} conidia after six days at 28° and 98% relative humidity. This was 5 times the number obtainable from a Roux bottle batched with Sabouraud agar and 50 times the number obtainable from such a vessel batched with Difco Nutrient Agar, incubated for the same time period. Vezina *et al.* (1968) have published a list of fungi which are capable of sporulating heavily on cereal grains. El-Sayed (1992) quoted the use of cooked rice for the production of spores of *Penicillium* and *Cephalosporium* in penicillin and cephalosporin inoculum development. Sansing and Cieglem (1973) described the mass production of spores of several *Aspergillus* and *Penicillium* species on whole loaves of white bread and Podojil *et al.* (1984) quoted the use of millet for the sporulation of *Streptomyces aureofaciens* in the development of inoculum for the chlortetracycline fermentation (see Fig. 6.5).

Sporulation in submerged culture

Many fungi will sporulate in submerged culture provided a suitable medium is employed (Vezina *et al.*, 1965). This technique is more convenient than the use of solid or solidified media because it is easier to operate aseptically and it may be applied on a large scale. The technique was first adopted by Foster *et al.* (1945) who induced submerged sporulation in *Penicillium notatum* by including 2.5% calcium chloride in a defined nitrate–sucrose medium. An example of the use of this technique for the production of inoculum for an industrial fermentation is provided by the griseofulvin process. Rhodes *et al.* (1957) described the conditions necessary for the submerged sporulation of the griseofulvin-producing fungus, *Penicillium patulum*, and

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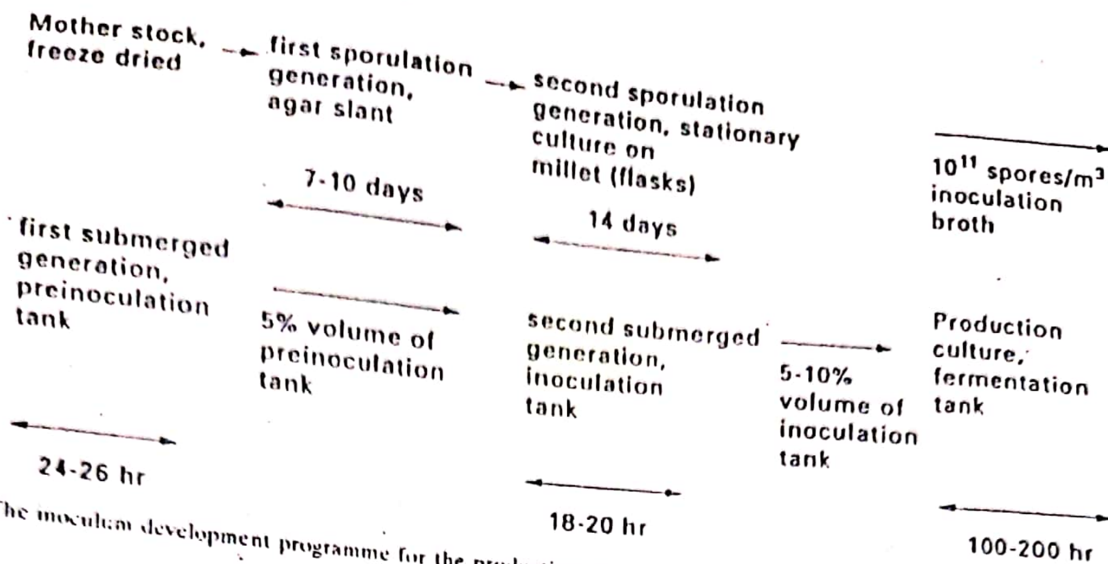

Fig. 6.5. The inoculum development programme for the production of chlortetracycline by *Streptomyces aureofaciens* (Podojil, 1984)

the medium utilized is given in Table 6.5. These authors found that for prolific sporulation the nitrogen level had to be limited to between 0.05 and 0.1% w/v and that good aeration had to be maintained. Also, an interaction was demonstrated between the nitrogen level and aeration in that the lower the degree of aeration the lower the concentration of nitrogen needed to induce sporulation. Submerged sporulation was induced by inoculating 600 cm^3 of the above medium, in a 2-dm^3 shake flask, with spores from a well-sporulated Czapek-Dox agar culture and incubating at 25° for 7 days. The resulting suspension of spores was then used as a 10% inoculum for a vegetative seed stage in a stirred fermenter, the seed culture subsequently providing a 10% inoculum for the production fermentation. The vegetative seed and production media are given in Table 6.1.

Most actinomycetes do not sporulate in submerged culture (Whitaker, 1992) and, thus, solid or solidified media tend to be used for the production of spore inocula.

The use of the spore inoculum

The stage in an inoculum development programme

TABLE 6.5. *Media for the submerged sporulation of selected fungi*

Rhodes *et al.* (1957): *Penicillium patulum*		
Whey powder, to give	Lactose 3.5%	
	Nitrogen 0.05%	
KH_2PO_4		0.4%
KCl		0.05%
Corn-steep liquor solids to give approx. 0.04% N		0.38%
Foster *et al.* (1945): *Penicillium notatum*		
Sucrose		2.0%
$NaNO_3$		0.6%
KH_2PO_4		0.15%
$MgSO_4 \cdot 7H_2O$		0.05%
$CaCl_2$		2.5%
Vezina *et al.* (1965): *Aspergillus ochraceus*		
Glucose		2.5%
NaCl		2.5%
Corn-steep liquor		0.5%
Molasses		5.0%

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at which a large-scale spore inoculum is used varies according to the process: it appears to be common practice that the penultimate stage is so inoculated, but this will depend on the scale of the production fermentation. In the inoculum development programme for the early penicillin fermentation described by Parker (1950) the penultimate stage was inoculated with a spore suspension (from a 'roll-bottle') and this stage may have produced either a vegetative or a submerged spore inoculum for the final fermentation. For the griseofulvin process, Rhodes *et al.* (1957) stated that the spore suspension obtained from the submerged sporulation stage could either be used for direct inoculation of the production fermentation or it could be germinated in an inoculum development medium to yield a vegetative inoculum for the final fermentation. The latter course was preferred and an inoculum volume of 7–10% was used. From Figs 6.4 to 6.6 it can

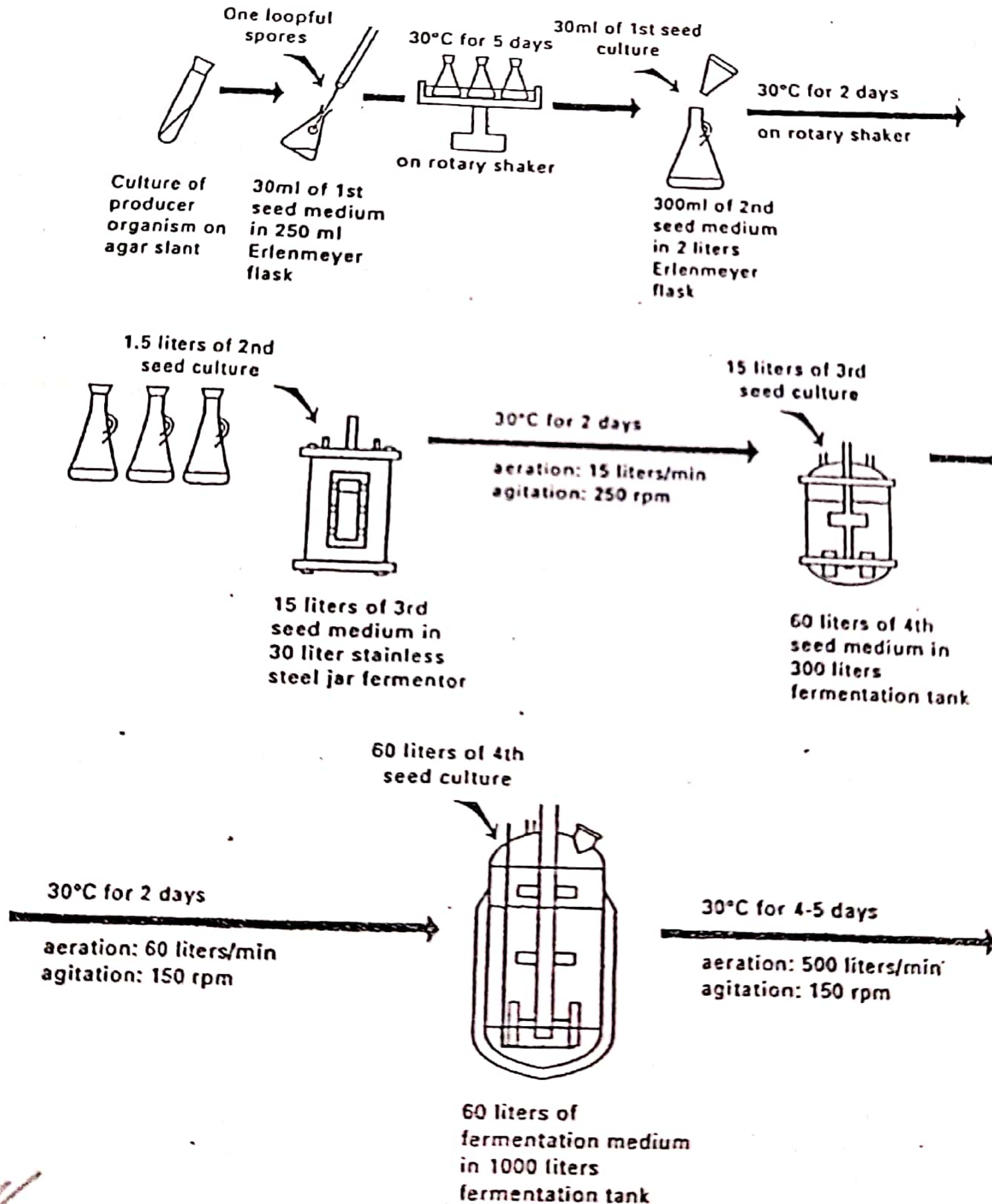

Fig. 6.6 The inoculum development programme for the production of sagamicin by *Micromonospora sagamiensis* (Podojil, 1984).

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be seen that in the clavulanic acid process the spore inoculum is used to inoculate the final seed stage, in the chlortetracycline process a vegetative stage is interspersed between the spore inoculated batch and the production fermentation, and in the sagamicin process the spore inoculum is used at a very early stage followed by vegetative growth.

When considering the production of gluconic acid by *Aspergillus niger*, Lockwood (1975) discussed the merits of inoculating the final fermentation directly with a spore suspension as compared with germinating the spores in a seed tank to give a vegetative inoculum. Direct spore inoculation would avoid the cost of installation and operation of the seed tanks whereas the use of germinated spores would reduce the fermentation time of the final stage, thus allowing a greater number of fermentations to be carried out per year. However, labour costs for the production of the vegetative inoculum could be almost as high as for the final fermentation although some of these costs may be recovered, in that gluconic acid produced in the penultimate stage would be recoverable from the final fermentation broth and would contribute to the buffering capacity throughout the fermentation. Thus, Lockwood claimed that the choice of inoculum for the production stage depends on the length of the cycle of the fermentation process, plant size and the availability and cost of labour.

Inoculum development for vegetative fungi

Some fungi will not produce asexual spores and, therefore, an inoculum of vegetative mycelium must be used. *Gibberella fujikuroi* is such a fungus and is used for the commercial production of gibberellin (Borrow *et al.*, 1961). Hansen (1967) described an inoculum development programme for the gibberellin fermentation. Cultures were grown on long slants (25 × 10 mm test tubes) of potato dextrose agar for 1 week at 24°. Growth from three slants was scraped off and transferred to a 9-dm^3 carboy containing 4 dm^3 of a liquid medium composed of 2% glucose, 0.3% $MgSO_4 \cdot 7H_2O$, 0.3% NH_4Cl and 0.3% KH_2PO_4. The medium was aerated for 75 hours at 28° before transfer to a 100-dm^3 seed fermenter containing the same medium.

The major problem in using vegetative mycelium as initial seed is the difficulty of obtaining a uniform, standard inoculum. The procedure may be improved by fragmenting the mycelium in an homogenizer, such as a Waring blender, prior to use as inoculum. This method provides a large number of mycelial particles and therefore a large number of growing points. Worgan (1968) has given a detailed account of the use of this technique in the preparation of inocula for the submerged culture of the higher fungi.

The effect of the inoculum on the morphology of filamentous organisms in submerged culture

When filamentous fungi are grown in submerged culture the type of growth varies from the 'pellet' form, consisting of compact discrete masses of hyphae, to the filamentous form in which the hyphae form a homogeneous suspension dispersed through the medium (Whitaker and Long, 1973). The filamentous type of habit gives rise to an extremely viscous broth which may be very difficult to aerate adequately, whereas the pellet type of habit gives rise to a far less viscous, but also less homogeneous, broth (see Chapter 9). In a pelleted culture there is a danger that the mycelium at the centre of the pellet may be starved of nutrients and oxygen due to diffusion limitations. Also, there is considerable evidence that the morphological form of the organism influences the productivity of the culture, but whether this is due to the phenomena already mentioned or to some form of metabolic control is far from clear. Thus, some fermentations are carried out with the fungus in a filamentous habit, whereas others are carried out with the organism growing as pellets. For example, filamentous growth has been claimed to be optimum for penicillin production by *P. chrysogenum* (Smith and Calam, 1980), whereas pelleted growth has been claimed to be optimum for citric-acid production from *Aspergillus niger* (Al Obaidi and Berry, 1980) and lovastatin from *Aspergillus terreus* (Gbewonyo *et al.*, 1992; see also Chapter 9). The necessity for filamentous growth is taken to the extreme in the ICI–Rank Hovis McDougal mycoprotein process where *Fusarium graminearium* is produced for human consumption. A highly filamentous morphology is required to produce the desired texture in the product which resembles the strength and eating texture of white and soft, red meats (Trinci, 1992). Thus, in this process a median hyphal length of 400 μm is required.

The relevance of this consideration of mycelial morphology to inoculum development is that the morphology may be influenced considerably by both the concentration of spores in a spore inoculum and the inoculum development medium. Usually, a high spore inoculum will tend to produce a dispersed form of growth whilst a low one will favour pellet formation (Foster, 1949). The effect of the concentration of a

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spore inoculum on the morphology of *P. chrysogenum* is given in Table 6.6. Thus, in the commercial production of fungal products it is critical to grow the organism in the desired morphological form which necessitates the use of an inoculum which achieves this end. If the production fermentation is to be inoculated with a spore suspension then the spore concentration must be such as to produce the production culture in the desired morphological form; if a vegetative inoculum is to be used for the production fermentation then, again, the concentration of its spore inoculum must be such as to produce the vegetative inoculum in the desired morphological form. Although the effects of media on morphological form can be extremely varied dispersed growth is more likely in rich, complex media and pelleted growth tends to occur in chemically defined media (Whitaker and Long, 1973). Thus, the medium used in the spore germination stage must be optimized in terms of the morphology of the inoculum.

An interesting series of experiments on the effects of inoculum conditions on the morphology of *Penicillium citrinum* were reported by Hosobuchi *et al.* (1993). This *Penicillium* species synthesizes compound ML-236B, a precursor of pravastatin which is a cholesterol-lowering drug. Optimum productivity was achieved when the organism grew as compact pellets in the production fermentation. The vegetative inoculum for the production fermentation had to contain an optimum number of short, filamentous propagules in order to initiate pellet formation in the final culture. This was achieved by using a four-stage inoculum development programme (initiated by a spore-inoculated shake flask) with very rich media in the third and fourth cultures. Thus, this system required a dispersed vegetative inoculum to generate a pelleted production fermentation.

The information available on the morphology of actinomycetes in submerged culture is very limited compared with that on fungi. However, Whitaker (1992) has reviewed the area and it is obvious that actinomycetes are capable of producing a wide range of morphological types. Also, it appears to be accepted that a dispersed mycelial morphology is desirable for most industrial actinomycete fermentations. Mycelial forms have been shown to be desirable for the production of streptomycin by *Streptomyces griseus* and turimycin by *S. hygroscopicus*, whereas the pelleted form of *S. nigrificans* was better for glucose isomerase production (Whitaker, 1992). As already discussed for the fungi, the concentration of spores in the inoculum has also been shown to influence the morphology of certain streptomycetes (Lawton *et al.*, 1989). These workers also demonstrated that medium composition and the shear forces operating during culture also affect morphological form. Thus, the principles applied to the optimization of fungal inoculum development regimes are also relevant to actinomycete processes. Hunt and Stieber (1986) described the optimization of the inoculum regime of a small-scale streptomycete cephamycin C fermentation. Pellet formation was observed to be detrimental to product formation and

TABLE 6.6. *The effect of spore concentration and medium on the morphology and penicillin productivity of* Penicillium chrysogenum

Medium	Spore concentration in the medium	Morphology
Camici *et al.* (1952):		
Corn-steep dextrin	More than 10×10^5 dm^{-3}	Filamentous
	Less than 10×10^5 dm^{-3}	Pellets
Czapek-dox	More than 3.0×10^5 dm^{-3}	Filamentous
	Less than 3.0×10^5 dm^{-3}	Pellets
Glucose, lactose and ammonium lactate	More than 2.0×10^5 dm^{-3}	Filamentous
	Less than 2.0×10^5 dm^{-3}	Pellets
Spore concentration in the inoculum (cm^{-3})	**Penicillin yield (units cm^{-3})**	**Morphology**
Calam (1976):		
10^2	500	Dense pellets
10^3	1800	Dense pellets
2×10^3	4000	Open pellets
10^4	5000	Filamentous

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the key factor in establishing the correct form appeared to be the concentration of iron in the seed medium, a higher iron concentration giving the optimum inoculum.

THE ASEPTIC INOCULATION OF PLANT FERMENTERS

The inoculation of plant-scale fermenters may involve the transfer of culture from a laboratory fermenter, or spore suspension vessel, to a plant fermenter, or the transfer from one plant fermenter to another. Obviously, it is extremely important that the fermentation is not contaminated during inoculation but if the process is a contained one then it is equally important that the process organism does not escape. Thus, the nature of the inoculation system will be dictated by the containment category of the process (see Chapter 7).

At Containment levels 1 and B2, the addition of inoculum must be carried out in such a way that release of micro-organisms is restricted. This should be done by aseptic piercing of membranes or connections with steam locks. At Containment level 2 and B3/4, no micro-organisms must be released during inoculation or other additions. In order to meet these stringent requirements, all connections must be screwed or clamped and all pipelines must be steam sterilizable (Werner, 1992).

Inoculation from a laboratory fermenter or a spore suspension vessel

Several systems have been described in the literature which are suitable for inoculating fermentations requiring only Good Industrial Large Scale Practice (GILSP, see Chapter 7). To prevent contamination during the transfer process it is essential that both vessels be maintained under a positive pressure and the inoculation port be equipped with a steam supply. Meyrath and Suchanek (1972) described a system for the inoculation of a plant fermenter from a laboratory vessel. The apparatus is shown in Fig. 6.7. The connecting point A is normally covered with the blank plug a and prior to inoculation this plug is slightly loosened, valve E closed and valve F opened to allow steam to exit at A. Valve F is then closed and E opened so that when a is removed sterile air will be released from the vessel. After removal, plug a is placed in strong disinfectant. Blank plug b is then removed and a coupling made between B and A. Valve E is closed and an air line attached to point C, establishing a pressure inside the inoculum fermenter greater than in the plant vessel. Valve E is then opened and the inoculum will be forced into the plant fermenter. After closing valve E the inoculum fermenter may be removed, plug a replaced, and the line steamed out by opening valve F. This system may be modified by using the quick connection devices which are now available (see Chapter 7).

Jackson (1958) has described a very similar system for the introduction of a spore suspension into a plant fermenter. The apparatus is shown in Fig. 6.8 and its operation is identical to that described by Meyrath and Suchanek.

Parker (1958) described a more complex system for inoculation of a plant fermenter from a spore-suspension vessel. The apparatus is shown in Fig. 6.9. The sterile spore-suspension vessel is batched with the spore suspension in the sterile room. The plant vessel, containing the medium and with blank plugs screwed on at A and B, is sterilized by steam injection. The plugs A and B are slightly loosened to allow steam to emit for 20 minutes when the whole system is under steam pressure. The blanks are then tightened, the valves E and G shut and sterile air is allowed into the plant fermenter by opening valves D, F and C. The spore suspension vessel is loosely connected at A and B and valves D, H, I and C closed and E, F and G opened. After 20 minutes steaming A and B are tightened and G and E are closed. D is then opened to establish a positive pressure in the pipework. When the pipework has cooled, the pressure in the plant vessel is reduced to approximately 5 psi, valve F is closed and H, I and C are opened. This procedure allows the spore suspension to be forced into the fermenter. Valves D, H, I

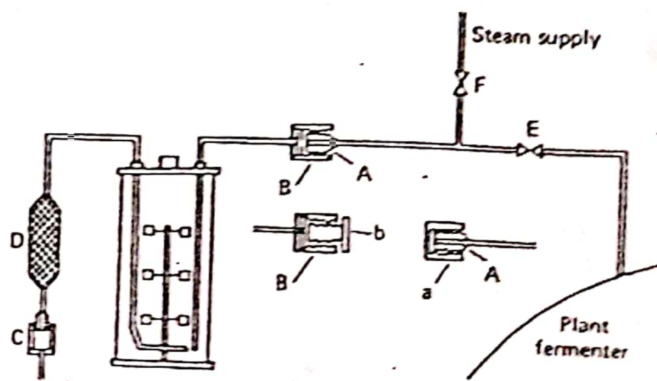

Fig. 6.7. Inoculation of a plant fermenter from a laboratory fermenter (Meyrath and Suchanek, 1972).

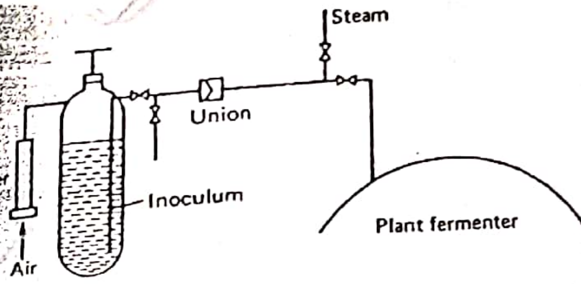

FIG. 6.8. Inoculation of a plant fermenter from a spore suspension vessel (Jackson, 1958).

and C are closed and the suspension vessel replaced by blank plugs at A and B and the pipework steamed by opening valves E and G.

GILSP and category 1 fermentations may also be inoculated by aseptic piercing of a membrane. In this system the inoculum vessel is connected to an inoculating needle assembly (as shown in Fig. 6.10) and the sterile needle pierces a rubber septum set into a fermenter port. However, the use of a needle presents safety problems and many companies prohibit such systems. Also, aerosols may be created on the removal of a needle.

The inoculation of level 2 or B3/4 contained fermentations requires the use of modified systems. It must be possible to steam sterilize all the pipework

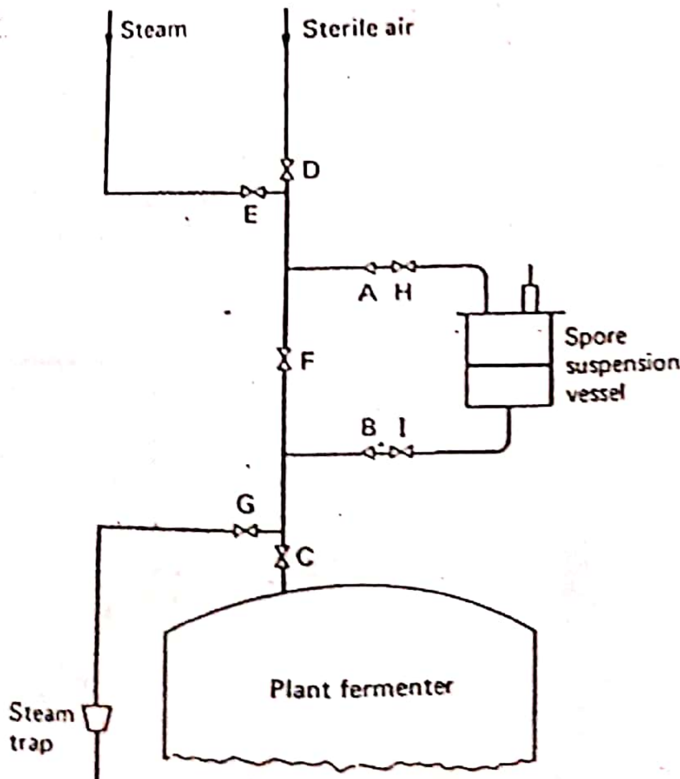

FIG. 6.9. The inoculation of a plant fermenter from a spore suspension vessel (Parker, 1958).

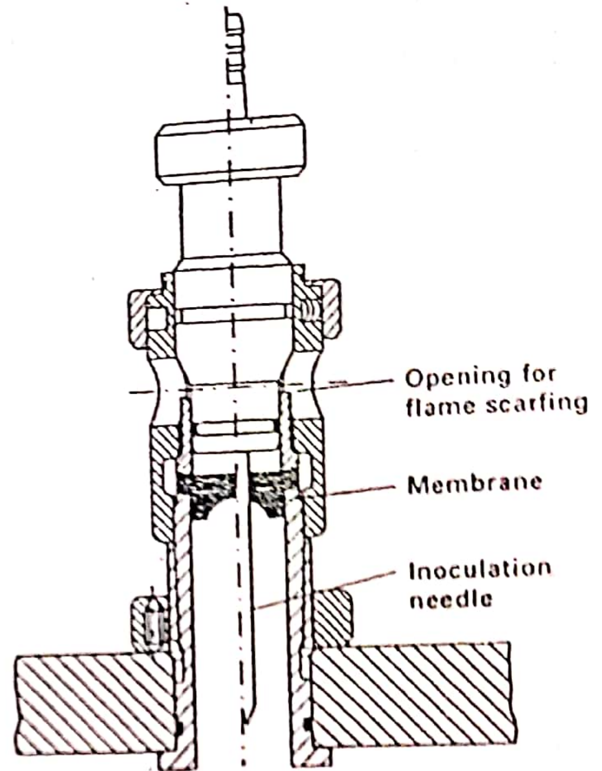

FIG. 6.10. Needle inoculation device (Werner, 1992).

after the inoculation and the condensate from the sterilization must be collected in a kill tank (see Chapters 5 and 7). The inoculation flask is then removed after inoculation and sterilized in an autoclave. One such system is shown in Fig. 6.11 (Vranch, 1990).

Inoculation from a plant fermenter

Figure 6.12 illustrates the system described by Parker (1958). The two vessels are connected by a flexible pipeline A–B. The batched fermenter is sterilized by steam injection via valves G and J, valves D, I, A, B, H, E and F being open and valve C closed. Valves H and I lead to steam traps for the removal of condensate. After 20 minutes at the desired pressure the steam supply is switched off at J and G and the steam-trap valves I and H are closed. F, E and D are left open so that the connecting pipeline fills with sterile medium. The medium in the fermenter is sparged with sterile air and when it has cooled to the desired temperature the pressure in the seed tank is increased to at least 10 psi whilst the pressure in the fermenter is reduced to about 2 psi. Valve C is opened and the inoculum is forced into the production vessel. After inoculation is

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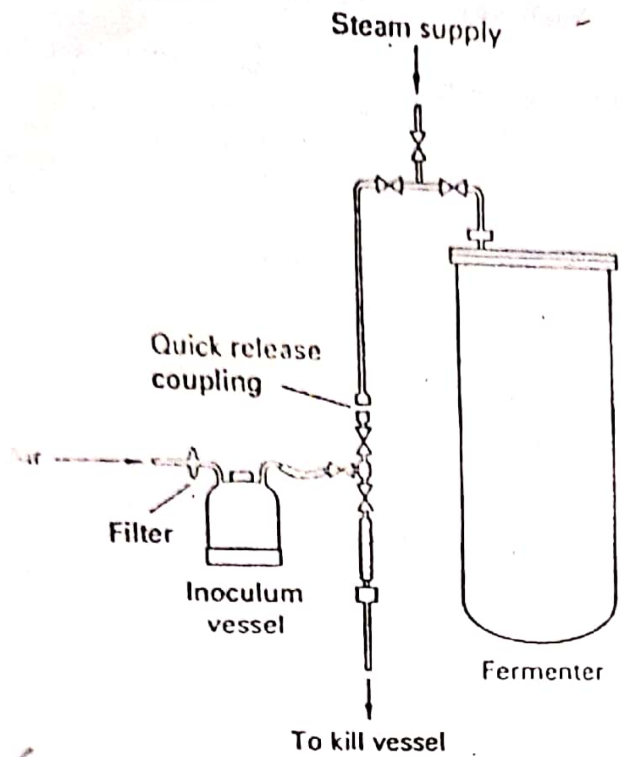

FIG. 6.11. Inoculum system suitable for contained fermentations (Vranch, 1990).

complete the connecting pipeline is resterilized before it is removed. For a contained fermentation the condensate from the two steam traps attached to valves I and H would be directed to a kill tank.

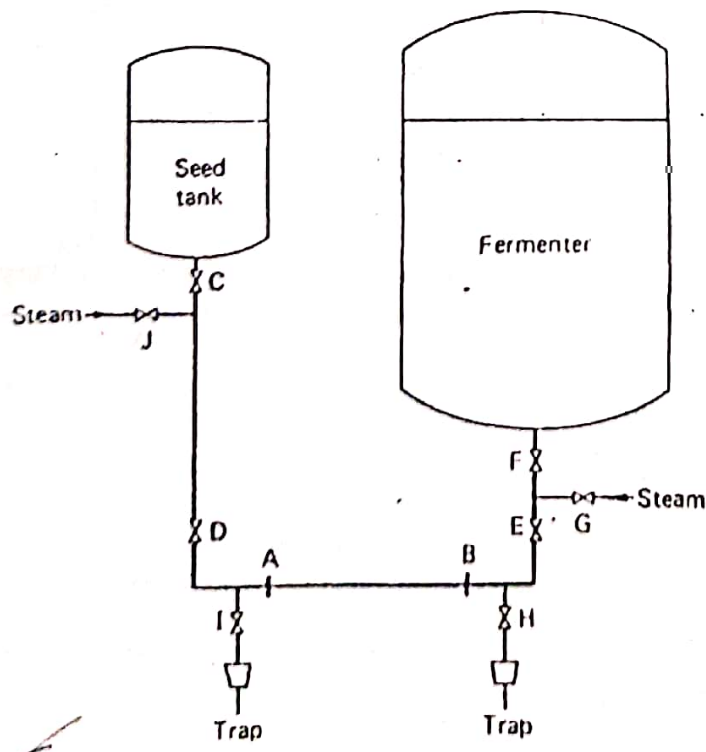

FIG. 6.12. Inoculation of a plant fermenter from another plant fermenter (Parker, 1958).

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