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The Development of Inocula for Industrial Fermentations

INTRODUCTION

IT IS ESSENTIAL that the culture used to inoculate a fermentation satisfies the following criteria:

1. It must be in a healthy, active state thus minimizing the length of the lag phase in the subsequent fermentation.
2. It must be available in sufficiently large volumes to provide an inoculum of optimum size.
3. It must be in a suitable morphological form.
4. It must be free of contamination.
5. It must retain its product-forming capabilities.

(The process adopted to produce an inoculum meeting these criteria is called inoculum development) Hockenhull is credited with the quotation "once a fermentation has been started it can be made worse but not better" (Calam, 1976). Whereas this is an over-statement it does illustrate the importance of inoculum development. Much of the variation observed in small-scale laboratory fermentations is due to poor inocula being used and, thus, it is essential to appreciate that the establishment of an effective inoculum development programme is equally important regardless of the scale of the fermentation. Such a programme not only aids consistency on a small scale but is invaluable in scaling up the fermentation and forms an essential part in progressing a new process.

A critical factor in obtaining a suitable inoculum is the choice of the culture medium. It must be stressed that the suitability of an inoculum medium is determined by the subsequent performance of the inoculum in the production stage. As discussed elsewhere (Chapter 4), the design of a production medium is determined not only by the nutritional requirements of the organism, but also by the requirements for maximum product formation. The formation of product in the seed culture is not an objective during inoculum development so that the seed medium may be of a different composition from the production medium. However, Lincoln (1960) stated that the lag time in a fermentation is minimized by growing the culture in the 'final-type' medium. Lincoln's argument is an important one, so the inoculum development medium should be sufficiently similar to the production medium to minimize any period of adaptation of the culture to the production medium, thus reducing the lag phase and the fermentation time. Furthermore, Hockenhull (1980) pointed out the dangers of using very different media in consecutive stages. Major differences in pH, osmotic pressure and anion composition may result in very sudden changes in uptake rates which, in turn, may affect viability. Hockenhull also emphasized that for antibiotic fermentations the inoculum medium should contain sufficient carbon and nitrogen to support maximum growth until transfer, so that secondary metabolism remains repressed during growth of the inoculum. If secondary metabolism is derepressed in the seed fermentation, then selection may enrich the culture with non-producing variants having a growth advantage over high-producing types. Hockenhull drew attention to Righelato's (1976) work in which it was shown that chemostat culture of *Penicillium chrysogenum* under carbohydrate-limited conditions led to a loss of penicillin synthesizing ability and an increase in the proportion of non-conidiated variants whereas this

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did not occur in ammonia-, phosphate- or sulphate-limited conditions. The relevance of this phenomenon is supported by Hockenhull's observation that *P. chrysogenum* inocula produced under non-limiting conditions are remarkably free from variants whereas variants arise relatively frequently during the carbon-limited production phase. Examples of inoculum and production media are given in Table 6.1, from which it may be seen that inoculum media are, generally, less nutritious than production media and contain a lower level of carbon.

The quantity of inoculum normally used is between 3 and 10% of the medium volume (Lincoln, 1960; Meyrath and Suchanek, 1972; Hunt and Stieber, 1986). A relatively large inoculum volume is used to minimize the length of the lag phase and to generate the maximum biomass in the production fermenter in as short a time as possible, thus increasing vessel productivity. Thus, starting from a stock culture, the inoculum must be built up in a number of stages to produce sufficient biomass to inoculate the production-stage fermenter. This may involve two or three stages in shake flasks and one to three stages in fermenters, depending on the size of the ultimate vessel. Throughout this procedure there is a risk of contamination and strain degeneration necessitating stringent quality-control procedures. The greater the number of stages between the master culture and the production fermenter the greater the risk of contamination and strain degeneration. Therefore, a compromise may be reached regarding the size of the inoculum to be used and the risk of contamination and strain degeneration. Another factor to be considered in the determination of the inoculum volume is the economics of the process. A seed fermenter 10% of the size of the production fermenter represents a considerable financial investment and must be justified in terms of productivity. A large-scale continuous fermentation for the production of biomass

TABLE 6.1. *Inoculum development and production media for a range of processes*

Process	Inoculum development medium		Production medium		References
Griseofulvin	Whey powder, Lactose } to give:		Lactose	7%	Rhodes *et al.* (1957)
	Lactose	3.5%	Corn-steep liquor solids to give:		
	Nitrogen	0.05%	Nitrogen	0.2%	
	Corn-steep liquor solids (to give approx. 0.04% N)	0.38%	Limestone	0.8%	
	KH_2PO_4	0.4%	KH_2PO_4	0.4%	
	KCl	0.05%	KCl	0.1%	
Clavulanic acid	Soybean flour	1.0%	Soybean flour	1.5%	Butterworth (1984)
	Dextrin	2.0%	Oil	1.0%	
	Pluronic L81 (Antifoam)	0.03%	KH_2PO_4	0.1%	
		($g\ dm^{-3}$)		($g\ dm^{-3}$)	
Vitamin B_{12}	Sugar beet Molasses	70		105	Spalla *et al.* (1989)
	Sucrose	—		15	
	Betaine	—		3	
	$NH_4H_2PO_4$	0.8		—	
	$(NH_4)_2SO_4$	2		2.5	
	$MgSO_4$	0.2		0.2	
	$ZnSO_4$	0.02		0.08	
	5-6 Dimethyl-benzimidazole	0.005		0.025	
Lysine	Cane molasses	5%		20%	Nakayama (1972)
	Corn-steep liquor	1%		—	
	$CaCO_3$	1%		-	
	Soybean meal hydrolysate	—		1.8%	

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would be expected to operate at steady state in excess of 100 days. Thus, the number of times that the fermenter is inoculated should be very few compared with batch or fed-batch systems. In such circumstances it may be more economic to compromise on the size of the inoculum and to tolerate a relatively lengthy period of growth up to maximum biomass than to invest a large seed vessel which would be used on very few occasions. This is particularly relevant for biomass continuous processes where one very large fermenter may be used and, thus, any seed vessel would only be servicing the one production vessel.

A typical inoculum-development programme will now be described in detail. The master culture (see Chapter 3 for an account of master-culture maintenance) is reconstituted and plated on to solid medium; approximately ten colonies of typical morphology of high producers are selected and inoculated on to slopes as the sub-master cultures, each sub-master culture being used for a new production run. At this stage, shake flasks may be inoculated to check the productivity of these cultures, the results of such tests being known before the developing inoculum eventually reaches the production plant. A sub-master culture is used to inoculate a shake flask (250 or 500 cm^3 containing 50 or 100 cm^3 medium) which, in turn, is used as inoculum for a larger flask, or a laboratory fermenter, which may then be used to inoculate a pilot-scale fermenter. Culture purity checks are carried out at each stage to detect contamination as early as possible. Although the results of these tests may not be available before the culture has reached the production plant, at least it is known at which stage in the procedure contamination occurred. For a sporulating organism the process may be modified to facilitate the use of a spore suspension as inoculum and this will be discussed in more detail later in this chapter.

Lincoln (1960) suggested a more elaborate procedure for the development of inoculum for bacterial fermentations which, with minor modifications, is applicable to any type of culture. The procedure involved the use of one sub-master culture to develop a bulk inoculum which was subdivided, stored in a frozen state and used as inocula for several months. A single colony, derived from a sub-master culture, was inoculated into liquid medium and grown to maximum log phase. This culture was then transferred into nineteen times its volume of medium and incubated again to the maximum log phase, at which point it was dispensed in 20-cm^3 volumes, plug frozen and stored at below −20°. At least 3% of the samples were tested for purity and productivity in subsequent fermentation and, provided these were suitable, the remaining samples could be used as initial inocula for subsequent fermentations. To use one of the stored samples as inoculum it was thawed and used as a 5% inoculum for a seed culture which, in turn, was used as a 5% inoculum for the next stage in the programme. This procedure ensured that a proven inoculum was used for the penultimate stage in inoculum development.

CRITERIA FOR THE TRANSFER OF INOCULUM

The physiological condition of the inoculum when it is transferred to the next culture stage can have a major effect on the performance of the fermentation. The optimum time of transfer must be determined experimentally and then procedures established so that inoculation with an ideal culture may be achieved routinely. These procedures include the standardization of cultural conditions and monitoring the state of an inoculum culture so that it is transferred at the optimum time, i.e. in the correct physiological state. The most widely used criterion for the transfer of vegetative inocula is biomass and such parameters as packed cell volume, dry weight, wet weight, turbidity, respiration, residual nutrient concentration and morphological form have been used (Hockenhull, 1980; Hunt and Stieber, 1986). Ettler (1992) demonstrated that the rheological behaviour of *Streptomyces noursei* could be used as the transfer criterion in the nystatin fermentation. The rheology of the seed fermentation changed from Newtonian to non-Newtonian behaviour and the optimum inoculum transfer time corresponded with this transformation.

Criteria which may be monitored on-line are the most convenient parameters to use as indicators of inoculum quality and these would include dissolved oxygen, pH (although pH would normally be controlled in seed fermentations) and oxygen or carbon dioxide in the effluent gas. Parton and Willis (1990) advocated the use of the carbon dioxide production rate (CPR) as a transfer criterion which requires analysis of the fermenter effluent air (see Chapter 8). This approach is suitable only when transfer is being made from a fermenter, but Parton and Willis stressed the importance of adopting this strategy even for the inoculation of laboratory-scale fermentations despite the fact that an adequate inoculum volume could be produced in shake flask. These workers provide an excellent example of the effect of inoculum transfer time on the productivity of a streptomycete secondary metabolite, as shown in Fig. 6.1a, b and c. The CPR of the

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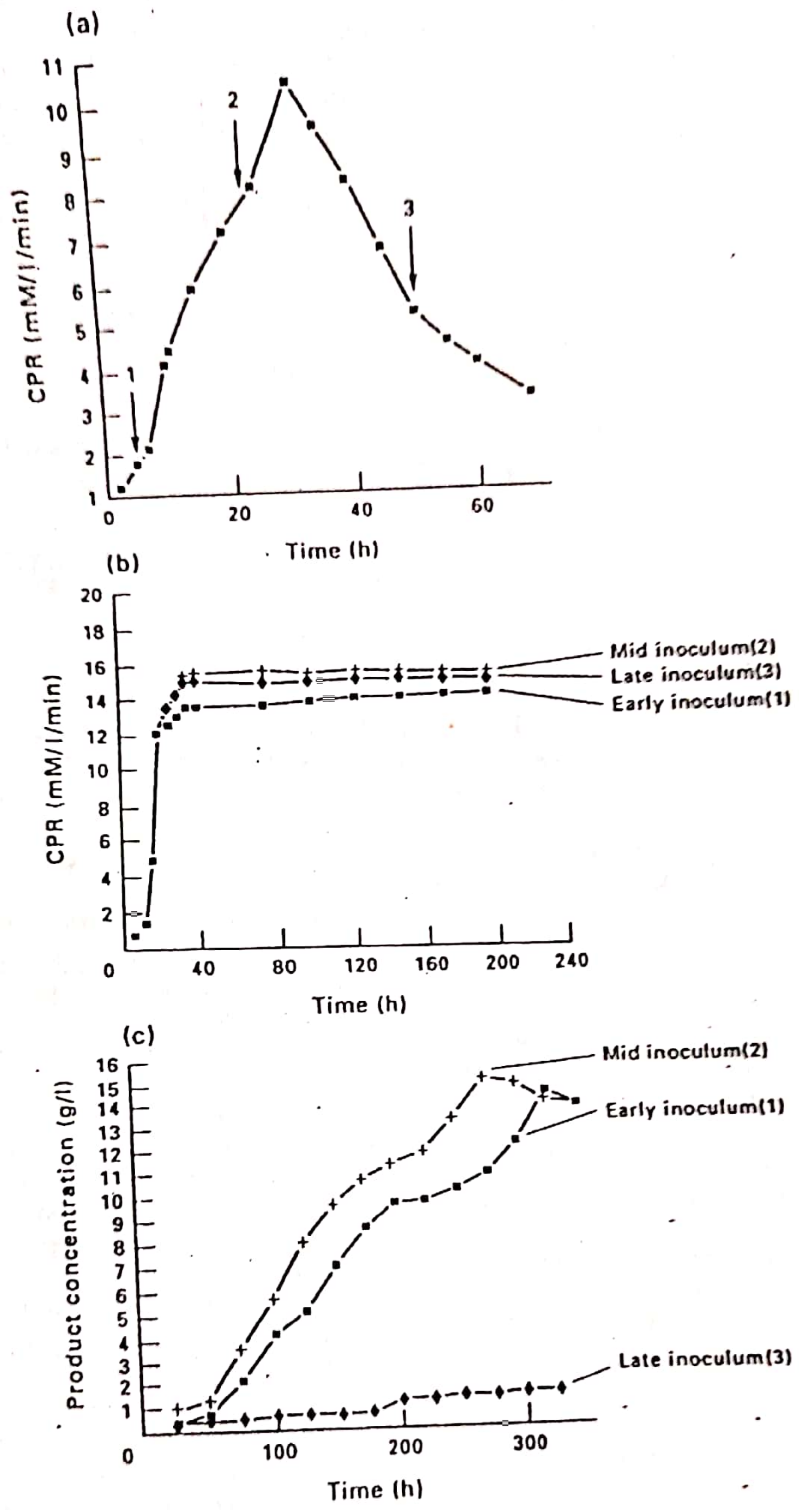

FIG. 6.1. The effect of inoculum age on growth and productivity in a streptomycete fermentation. (a) The carbon dioxide production rate (CPR) profile of the inoculum culture showing the points (1, 2 and 3) at which inocula were removed. (b) The effect of inoculum age on the CPR of the production fermentation. (c) The effect of inoculum age on productivity in the production fermentation (Parton and Willis, 1990).

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inoculum fermentation and the points at which inoculum was transferred are shown in Fig. 6.1a. The CPR of the subsequent production fermentations are shown in Fig. 6.1b, from which it may be seen that the three fermentations performed similarly. However, Fig. 6.1c illustrates the very different secondary metabolite production of the three fermentations. Thus, although the time of transfer had only a marginal influence on biomass in the production fermentation the effect on product formation was critical. It should be emphasized that the amount of biomass transferred was standardized for the three fermentations and, thus, the differences in performance were due to the physiological states of the inocula.

In recent years, probes have been developed for on-line assessment of biomass (see Chapter 8) and these could be invaluable in estimating the time of inoculum transfer. Boulton *et al.* (1989) reported the use of a biomass sensor (the Bugmeter) to control the yeast pitching rate (inoculum level) in brewing. The probe measures the dielectric permittivity of viable yeast cells and is unaffected by the presence of dead cells, air bubbles or detritus, making it ideal for the routine monitoring of yeast inoculum. Using the probe, these workers developed an automatic inoculum dispenser allowing a preset viable yeast mass to be transferred from a yeast storage vessel to the brewery fermentation.

Alford *et al.* (1992) reported the use of a real-time expert computer system to predict the time of inoculum transfer for industrial-scale fermentations. The system involves the comparison of on-line fermentation data with detailed historical data of the process. A problem with the interpretation of carbon dioxide production rate figures is that the data are not available continuously because the analyser is not dedicated to any one fermenter, but is analysing process streams from a large number of vessels via a multiplexer system (see Chapter 8). Thus, a fermentation may have passed a critical stage between monitoring times. Also, occasional false readings may be generated. The expert system enabled the verification of data points as well as prediction of the outcome of the fermentation from early information. Data from seed fermentations were analysed by the expert system and the transfer time predicted. As a result of this approach operators were able to plan their work more effectively, the need for manual sampling was reduced and early warning of contamination was provided if the seed-culture profile predicted from early readings was abnormal.

Smith and Calam (1980) compared the quality and enzymic profile of differently prepared inocula *Penicillium patulum* (producing griseofulvin) and demonstrated that a low level of glucose 6-phosphate dehydrogenase was indicative of a good quality inoculum. The enzyme profile of good quality inoculum was established early in the growth of the seed culture. Thus, this approach could be used to assess the cultural conditions giving rise to satisfactory inoculum, but would be of less value in determining the time of transfer.

Yeast, unicellular bacterial, fungal and streptomycete fermentations have different requirements for inoculum development and these are dealt with separately.

THE DEVELOPMENT OF INOCULA FOR YEAST PROCESSES

Whilst the largest industrial fermentations utilizing yeasts are the brewing of beer and the production of biomass, recent processes have also been established for the production of recombinant products.

Brewing

It is common practice in the British brewing industry to use the yeast from the previous fermentation to inoculate a fresh batch of wort. The brewing terms used to describe this process are 'crop', referring to the harvested yeast from the previous fermentation, and 'pitch', meaning to inoculate. One of the major factors contributing to the continuation of this practice is the wort-based excise laws in the United Kingdom where duty is charged on the sugar consumed rather than the alcohol produced. Thus, dedicated yeast propagation systems are expensive to operate because duty is charged on the sugar consumed by the yeast during growth. It can then be appreciated that the reduced cost of using yeast from a previous fermentation is an attractive proposition (Boulton, 1991). The dangers inherent in this practice are the introduction of contaminants and the degeneration of the strain, the most common degenerations being a change in the degree of flocculence and attenuating abilities of the yeast. In breweries employing top fermentations in open fermenters these dangers are minimized by collecting yeast to be used for future pitching from 'middle skimmings'. During the fermentation the yeast cells flocculate and float to the surface, the first cells to do this being the most flocculent and the last cells the least flocculent. As the head of yeast develops, the

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surface layer (the most flocculent and highly contaminated yeasts) is removed and discarded and the underlying cells (the 'middle skimmings') are harvested and used for subsequent pitching. Therefore, the 'middle skimmings' contain cells which have the desired flocculence and which have been protected from contamination by the surface layer of the yeast head. The pitching yeast may be treated to reduce the level of contaminating bacteria and remove protein and dead yeast cells by such treatments as reducing the pH of the slurry to 2.5 to 3, washing with water, washing with ammonium persulphate and treatment with antibiotics such as polymixin, penicillin and neomycin (Mandl *et al.*, 1964; Strandskov, 1964; Roessler, 1968; Reed and Nagodawithana, 1991a).

However, traditional open vessels are becoming increasingly rare and the bulk of beer is brewed using cylindro-conical fermenters (see Chapter 7). In these systems the yeast flocculates and collects in the cone at the bottom of the fermenter where it is subject to the stresses of nutrient starvation, high ethanol concentration, low water activity, high carbon dioxide concentration and high pressure (Boulton, 1991). Thus, the viability and physiological state of the yeast crop would not be ideal for an inoculum. The viability of the crop may be assessed using a biomass probe of the type described earlier, thus ensuring that at least the correct amount of viable biomass is used to start the next fermentation. However, the physiological state of the biomass will not have been influenced by such monitoring procedures. The situation is further complicated by the fact that the harvested yeast is stored before it is used as inoculum. Metabolic activity is minimized during this time by chilling rapidly to about 1°, suspending in beer and storing in the absence of oxygen. If oxygen is present during the storage period then the yeast cells consume their stored glycogen which renders them very much less active at the start of the fermentation (Pickerell *et al.*, 1991).

One of the key physiological features of yeast inoculum is the level of sterol in the cells. Sterols are required for membrane synthesis but they are only produced in the presence of oxygen. Thus, we have the anomaly of oxygen being required for sterol synthesis, yet anaerobic conditions are required for ethanol production. This anomaly is resolved traditionally by aerating the wort before inoculation. This oxygen allows sufficient sterol synthesis early in the fermentation to support growth of the cells throughout the process, that is after the oxygen is exhausted and the process is anaerobic. Boulton *et al.* (1991) developed an alternative approach where the pitching yeast was vigorously aerated prior to inoculation. The yeast was then sterol rich and had no requirement for oxygen during the alcohol fermentation.

The difficulties outlined above and the likelihood of strain degeneration and contamination mean that yeasts are rarely used for more than five to ten consecutive fermentations (Thorne, 1970; Reed and Nagodawithana, 1991a) which necessitates the periodical production of a pure inoculum. This would involve developing sufficient biomass from a single colony to pitch a fermentation at a level of approximately 2 grams of pressed yeast per litre. Hansen (1896) pioneered the use of pure inocula and devised a yeast propagation scheme utilizing a 10% inoculum volume at each stage in the programme and employing conditions similar to those used during brewing. However, modern propagation schemes use inoculum volumes of 1% or even lower and may use conditions different from those used during brewing. Therefore, continuous aeration may be used during the propagation stage which seems to have little effect on the beer produced in the subsequent fermentation (Curtis and Clark, 1957). Yeast inoculum produced in this way would also be sterol rich, obviating the need for aerated wort.

A number of yeast propagators (which are basically closed, aerated vessels) have been described in the literature. The simplest type of progagator is a single-stage system resembling an unstirred, aerated fermenter which is inoculated with a shake-flask culture developed from a single colony (Gilliland, 1971). Curtis and Clark (1957) and Thorne (1970) described two-stage systems which could be operated semi-continuously. Thorne's propagator consisted of two linked vessels, 1.5 and 150 dm^3 respectively. The smaller vessel was filled with wort, sterilized, cooled, aerated and inoculated with a flask-grown culture. After growth for 3 to 4 days the culture was forced by air pressure into the second vessel which had been filled with sterilized, cooled wort and aerated. An aliquot of 1.5 dm^3 was forced back into the first vessel after mixing. In a further 3 to 4 days the larger vessel contained sufficient biomass to pitch a 1000 dm^3 fermenter and the first vessel contained sufficient inoculum for another second stage. However, although this procedure should produce a pure inoculum there is a danger of strain degeneration occurring in such a semi-continuous system.

Boulton (1991) speculated that when the UK moves to an alcohol-based tax then the economics of propagating inoculum for each brew would be considerably more attractive. He suggested that the bakers' yeast aerobic fed-batch inoculum programme could be adopted to produce sterol-rich catabolite-derepressed

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cells. Such an inoculum would remove the necessity for aerating the wort prior to inoculation because sterol synthesis would not be necessary due to the high sterol content of the cells.

Bakers' yeast

The commercial production of bakers' yeast involves the development of an inoculum through a large number of aerobic stages. Although the production stages of the process may not be operated under strictly aseptic conditions a pure culture is used for the initial inoculum, thereby keeping contamination to a minimum in the early stages of growth. Reed and Nagodawithana (1991b) discussed the development of inoculum for the production of bakers' yeast and quoted a process involving eight stages, the first three being aseptic while the remaining stages were carried out in open vessels. The yeast may be pumped from one stage to the next or the seed cultures may be centrifuged and washed before transfer, which reduces the level of contamination. The yields obtained in the first five stages are relatively low because they are not fed-batch systems, whereas the last three stages are fed-batch (the fed-batch bakers' yeast fermentation is considered in more detail in Chapter 2). A summary of a typical inoculum development programme for the production of bakers' yeast is given in Fig. 6.2.

THE DEVELOPMENT OF INOCULA FOR BACTERIAL PROCESSES

The main objective of inoculum development for traditional bacterial fermentations is to produce an active inoculum which will give as short a lag phase as possible in subsequent culture. A long lag phase is disadvantageous in that not only is time wasted but also medium is consumed in maintaining a viable culture prior to growth. The length of the lag phase is affected by the size of the inoculum and its physiological condition (Meyrath and Suchanek, 1972). As already stated, the inoculum size normally ranges between 3 and 10% of the culture volume. Lincoln (1960) stressed that bacterial inocula should be transferred in the logarithmic phase of growth, when the cells are still metabolically active. The age of the inoculum is particularly important in the growth of sporulating bacteria, for sporulation is induced at the end of the logarithmic phase and the use of an inoculum containing a high percentage of spores would result in a long lag phase in a subsequent fermentation.

Keay *et al.* (1972) quote the use of a 5% inoculum of a logarithmically growing culture of a thermophilic *Bacillus* for the production of proteases. Aunstrup (1974) described a two-stage inoculum development programme for the production of proteases by *Bacillus subtilis*. Inoculum for a seed fermenter was grown for 1

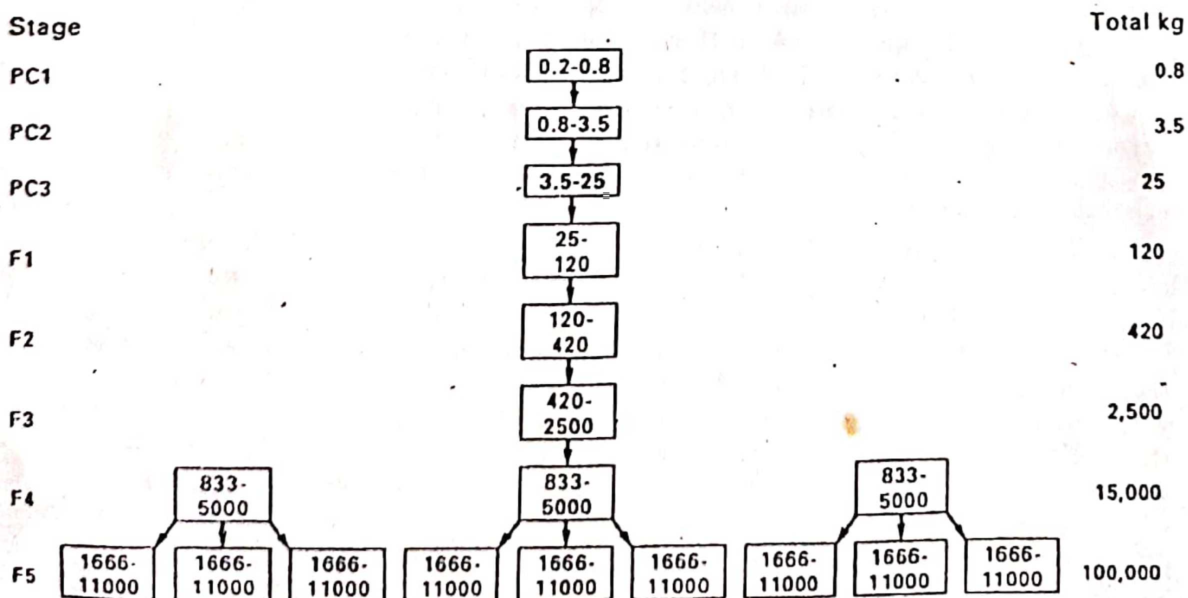

FIG. 6.2. The development of inoculum for the commercial production of bakers' yeast. PC 1, 2 and 3 are pure culture batch fermentations. F1 and 2 are non-aseptic batch fermentations. F3 and 4 are fed-batch fermentations and F5 is the final fed-batch fermentation (Reed and Nagodawithana, 1991a).

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to 2 days on a solid or liquid medium and then transferred to a seed vessel where the organism was allowed to grow for a further ten generations before transfer to the production stage. Priest and Sharp (1989) cited the use of a 5% inoculum, still in the exponential phase, for the commercial production of *Bacillus* α-amylase. Underkofler (1976) emphasized that, in the production of bacterial enzymes, the lag phase in plant fermenters could be almost completely eliminated by using inoculum medium of the same composition as used in the production fermenter and employing large inocula of actively growing seed cultures. The inoculum development programme for a pilot-plant scale process for the production of vitamin B_{12} from *Pseudomonas denitrificans* is shown in Fig. 6.3 (Spalla *et al.*, 1989).

The necessity to use an inoculum in an active physiological state is taken to its extreme in the production of vinegar. The acetic-acid bacteria used in the vinegar process are extremely sensitive to oxygen starvation. Therefore, to avoid disturbing the system, the cells at the end of a fermentation are used as inoculum for the next batch by removing approximately 60% of the culture and restoring the original level with fresh medium (Conner and Allgeier, 1976). The advantage of a highly active inoculum apparently outweighs the disadvantages of possible strain degeneration and contaminant accumulation. However, strain stability is a major concern in inoculum development for fermentations employing recombinant bacteria. Sabatic *et al.* (1991) demonstrated that plasmid stability and productivity in an *E. coli* biotin fermentation was greatly improved if stationary, rather then exponential, phase cells were used as inoculum. They postulated that the plasmid copy number may be higher in stationary cells than in exponential ones, resulting in a lower plasmid loss in the subsequent fermentation when a stationary culture is used as inoculum. A stationary phase inoculum would result in a lag phase, but this disadvantage was more than compensated for by the considerable improvement in plasmid retention and biotin production compared with that obtained using an exponential inoculum.

In the lactic-acid fermentation the producing organism may be inhibited by lactic acid. Thus, production of lactic acid in the seed fermentation may result in the generation of poor quality inoculum. Yamamoto *et al.* (1993) generated high quality inoculum of *Lactococcus lactis* IO-1 on a laboratory scale using electrodialysis seed culture which reduced the lactate in the inoculum and reduced the length of the lag phase in the production fermentation.

An example of the development of inoculum for an anaerobic bacterial process is provided by the clostridial acetone–butanol fermentation. The process was outcompeted by the petrochemical industry but there is still considerable interest in re-establishing the fermen-

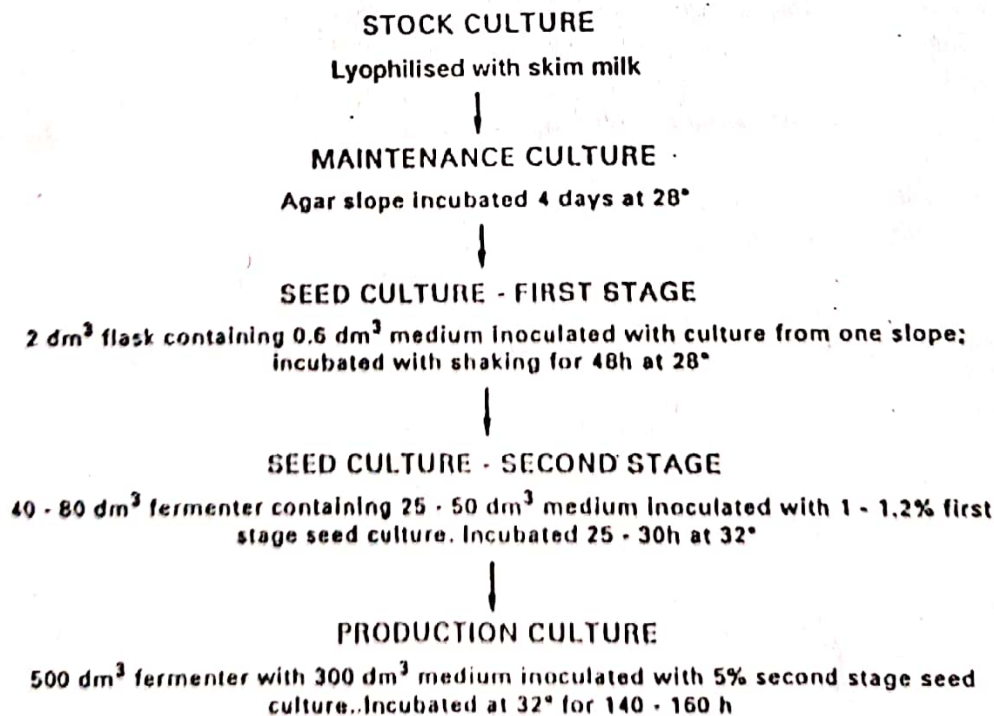

FIG. 6.3. The inoculum development programme for a vitamin B_{12} pilot scale fermentation using *Pseudomonas denitrificans* (Spalla *et al.*, 1989).

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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.

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