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VARIATIONS IN INOCULA AND THEIR INFLUENCE ON THE PRODUCTIVITY OF ANTIBIOTIC FERMENTATIONS

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Summary

Inocula, used for the production of penicillin and griseofulvin, giving different yields, were investigated. Biochemical factors, such as the level of enzyme activity and efficiency, were at least as important as morphology in determining yield, being carried forward from the inoculum to the production stage.

INTRODUCTION

The importance of the inoculum in determining the productivity of industrial fermentations has long been recognised (Foster, 1949; Calam, 1976), but has been little investigated. Meyrath and collaborators have shown that inoculum size affected both the growth pattern and the efficiency of growth of cultures of Aspergillus oryzae (Meyrath and McIntosh, 1965). Inoculum morphology, i.e. mycelial pellet size, is known to be important in the manufacture of citric acid by A. niger (Chaturvedi et al., 1978). The work described here is concerned with the production of penicillin by Penicillium chrysogenum and griseofulvin by P. patulum. P. chrysogenum was studied in shake-flask culture, inoculum quality being varied by the use of different concentrations of spores for seeding the inoculum culture. In the case of P. patulum commercial production cultures were investigated. In addition to studying the behaviour of inocula, it was also hoped to find a rapid method for assessing the quality of inocula.

MATERIALS AND METHODS

Penicillium chrysogenum

The culture was a mutant (JV 101) obtained from I.C.I. Ltd., Pharmaceuticals Division, Trafford Park, Manchester. It was held in soil culture after preliminary plating and selection of mosaic free parental colonies. It gave 5000 u/ml of penicillin. Subcultures were made fortnightly, second generation slopes being used for laboratory work. Spore suspensions were made by rubbing off spores into 10 ml water so as to give a uniform green suspension. Spore concentrations were measured with a haemocytometer slide. Germination was 90%.

Media: For slopes: lactose 30 g, corn-steep solids (Manbre and Garton, London) 20 g, Solution C 20 ml, agar 15 g, pH adjusted to 6.2, water to 1 litre. Solution C: $MgSO_4.7H_2O$ 2.5 g, $FeSO_4.7H_2O$ 0.6 g, $MnSO_4.4H_2O$ 0.2 g, $CuSO_4.5H_2O$ 0.2 g, water 1 litre.

For inoculum: Corn-steep solids 20 g, glucose 25 g, KH_2PO_4 1.5 g, $NaNO_3$ 1.5 g, $ZnSO_4$ 0.04 g, $CaCO_3$ 2 g, $MgSO_4.7H_2O$ 0.25 g, water to 1 litre, pH adjusted to 5.3-5.5: distribute 50 ml per 250 ml Erlenmeyer flask. 0.5 ml Arachis oil (BP) was added per flask.

For production: Corn-steep solids 14.7 g, lactose 38.5 g, $CaCO_3$ 6.2 g, KH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 0.25 g, $Na_2S_2O_3.10H_2O$ 1 g, $NaNO_3$ 2.5 g, phenoxyacetic acid 2 g, water to 1 litre, pH adjusted to 6.2. Distributed 45 ml per 250 ml Erlenmeyer flask, with addition of 1 ml of Arachis oil (BP) and 0.5 ml White Oil (Shell, London).

Flasks were plugged and sterilised in an autoclave at 120° for 20

minutes. After adding the appropriate quantity of spore suspension, the flasks were incubated at 25° on an orbital shaker giving 5 cm circles at 220 rpm. Production flasks were inoculated with 5 ml of inoculum. The inoculum was grown for 40-42 hours, when a thick culture developed but some glucose remained, as shown by testing with Clinistix (Ames Co., Stoke Poges, Berks.). Normally triplicate flasks were used and experiments repeated 3-5 times.

Penicillium patulum

Samples were obtained from industrial fermenters in the course of production batches. The production method was similar to that described by Glaxo Laboratories (1959). Griseofulvin assays were carried out in the works laboratory.

Analytical methods

Dry mycelial weight: Samples of culture (15-60 g) were weighed, filtered, rinsed and dried to constant weight at 60° and weighed. Results were corrected by extraction with light petroleum (Soxhlet) to remove fat, and, after re-weighing, ashing to allow for insoluble matter,

Penicillin assay: An agar plate method was used with Bacillus subtilis (NCIB 8739) as test organism. The procedure was similar to the British Pharmacopoeia method (1973), using paper discs (6 mm diameter) to apply standard and test solutions, with crystalline benzyl-penicillin as standard. Results are given in International Units (u/ml). For each sample three plates were used, each with six circles (three for tests, three standards).

Enzyme extraction and assay: Within five minutes of removal, the culture sample was weighed, filtered and rinsed with Tris buffer (pH 7.2, 0.1M). The mycelial pad was then homogenised in a Griffiths tube with 9 ml of Tris buffer for five minutes. The homogenate was centrifuged (3500 rpm, 15 minutes) and the milky supernatant transferred to clean test tubes in an ice bath, for assay. Assays were carried out using test kits (Boehringer Corporation Ltd., London), No. 15993 (Glucose 6-phosphate dehydrogenase (G6PDH), No. 15933 (Iso-citrate dehydrogenase (ICDH) and No. 15974 (Aldolase), without deproteinisation. The results were calculated as described in the instruction sheet.

Glucose was estimated colourimetrically using kit No. 635 (Sigma Ltd., Poole, Dorset).

RESULTS

Penicillium chrysogenum

When flasks of inoculum medium were seeded with different concentrations of spores and incubated on the shaker, swelling of the spores occurred between 12-16 hours, and germination after 17 hours. Growth developed at different rates, depending on spore concentration (Table 1). Growth increases with spore concentration. The critical level for good growth and productivity was 5×10^3 spores/ml, although at higher levels growth was better.

Table 2 expands this information, indicating the morphology of the cultures and results in the production stage. In our work the morphology of the pellets was expressed simply as dense pellets (DP), having dense amorphous centres with short hyphae protruding from it, and open pellets (OP), having no more than a single growth point at the centre. Two intermediate forms were also recognised, semi-dense pellets (SDP) having an amorphous centre with longer out-growths, and semi-open pellets (SOP)

having a cluster of mycelium at the centre, consisting of distinguishable hyphae. Open pellets are similar to filamentous or mycelial forms, though they are considered by us to be a form of pellet.

Table 1. Growth of inocula with different spore concentrations

Spores/ml	Dry weights, g/l, at ages:- 25	30	42 hours
5×10^2	0.1	0.33	1.35
5×10^3	0.3	1.1	3.8
5×10^4	0.9	2.3	11.2
5×10^5	1.3	3.3	12.8

Table 2. Spore concentration, inoculum growth and penicillin production

Averaged results from five experiments.

Inoculum, Spores /ml	42 hours old.. Dry mycelium, g/l	Growth type*	Pellet diam. (mm)	Production, 120 hours: Mycelium g/l	Penicillin u/ml
5×10^2	1.35	DP	0.9	7.5	550
1×10^3	2.7	DP	0.7	13	1800
5×10^3	3.8	SOP	0.5	26	4800
1×10^4	10.0	SOP + OP	0.4	27.5	4950
1×10^5	12.9	OP	0.2	28	5000
1×10^6	14.5	OP	0.1	-	-

*For explanation, see text.

A variety of experiments on the effect of conditions on growth on inocula, are summarised in Table 3. (1) Inocula, grown from low spore concentrations, were either concentrated by centrifugation, or allowed to grow on longer, to give an equal weight of cells as found in inocula grown from high spore concentrations; the effect of extending production time was also investigated. (2) Pellets were mechanically disrupted by blending, so as to produce a mycelial form of growth in the production stage. (3) Synthetic media were tested. (4) Inocula were grown in a well-stirred 5-litre fermenter (660 rpm, 3 litres of air/min); to avoid formation of dense pellets, 1×10^4 spores/ml were used, giving semi-open pellets of satisfactory appearance. None of the conditions tested gave inocula with a productivity equal to the standard method with 5×10^3 spores/ml.

These results suggested that inocula can be divided into two groups, (1) dense pellets which give low yields, (2) other types which are not readily distinguishable from each other and which may give high or low yields. As an alternative method of assessment, enzyme assays were made on mycelium, of aldolase, G6PDH and ICDH, representing differing areas of metabolic activity. It is recognised that the significance of *in vitro* tests is unclear, but they do give some indication of potential biochemical activity.

Inocula, 42 hours old, gave the following results:-

Spores/ml	Aldolase	G6PDH	ICDH	(mU/mg)
10^3	2.8	10.0	11.9	
10^5	4.8	36.0	65.6	

With 10^5 spores/ml the results were not only higher, but the ratios between the enzyme concentrations were different.

Table 3. Experiments with inoculum production

Triplicate tests: controls with 5 x 10^3 spores/ml

Inoculum preparation:		Penicillin production, u/ml Test	Control
1. Quantity of inoculum (10^3 spores/ml)			
Increased by centrifugation		2763	4950
Extra inoculum growth time:	42 hrs	1813	
	46	2025	
	50	2391	
Extended fermentation (max at 7 days)		2660	
2. Mechanical disruption:	Blended		
(Pellets, grown from	0 secs	403 DP*	
4 x 10^2 spores/ml)	20	1390 DP	
	90	2433 OP	
	180	2778 OP	
3. Synthetic medium (Jarvis & Johnson, 1953)		750	4900
Synthetic medium (Jarvis & Johnson, 1953) + 0.5% corn-steep liquor		2100	
4. Inoculum grown in stirred culture:	Spores/ml		
	5 x 10^3	2520 DP*	5000
	1 x 10^4	3415 OP	

*For explanation, see text.

Table 4. Inoculum variations and productivity

			Age (hrs)	Spore concentration/ml 10^3	5 x 10^3	10^4	10^5
Inoculum	Dry weight,	g/l	42	2.7	3.7	10	12.9
	Y_G	g/g		0.16	0.27	0.5	0.5
	ICDH	mU/mg		10	14	22	26
	G6PDH	mU/mg		12	42	62	69
	Pellet shape			DP	SOP	OP	OP*
Production	Dry weight,	g/l	24	6.0	7.0	11.5	14
	ICDH	mU/mg		8	11	18	20
	G6PDH	mU/mg		12	21	36	38
	Penicillin	u/ml		30	60	120	150
	Q_{pen}	(0-24 hrs)		0.4	0.68	0.86	0.82
	Dry weight	g/l	72	11.5	17.9	21	22
	ICDH	mU/mg		11	16	18	18
	G6PDH	mU/mg		14	18	16	11
	Penicillin	u/ml		240	1500	2000	2500
	Q_{pen}	(48-72 hrs)		0.8	3.2	3.7	4.3
	Dry weight	g/l	120	17	28	28	28
	ICDH	mU/mg		12	35	27	9
	G6PDH	mU/mg		11	15	5	5
	Penicillin	u/ml		1900	4800	5000	5000
	Q_{pen}	(96-120 hrs)		2.9	2.9	2.3	1.5

*For explanation, see text.

Table 4 gives the results of an experiment which surveyed enzyme concentrations, growth, productivity and efficiency. It confirmed the results obtained before and showed how the properties of the inoculum were carried forward into the production stage.

Penicillium patulum

Inoculum and production cultures were tested for growth and enzyme activities. Batches were classed as giving high or low yields, the ratio being 1:0.55. The results are given in Table 5.

Table 5. Griseofulvin production by P. patulum

Averaged data from graphs.

Inoculum						Production						
	High	Four Low yield batches				Age (hrs)	Cells g/l		G6PDH mU/mg		ICDH mU/mg	
Age (nrs)	41	31	36	42	47		High	Low	High	Low	High	Low
G6PDH	9.4	45	10	15	11	0	-	-	9.7	11	7.7	4.5
ICDH	9.0	6	1.6	4.5	4.8	50	25	18	28	35	20	25
G6PDH / ICDH	1.1	7.7	6.3	3.3	2.3	100	35	23	30	25	18	27
						125	36	24	20	18	10	28
						150	36	25	8	11	6	20
						250	36	33	1	3	3	4

Variations in production appear to be associated with the balance of metabolism, the reverse of that described by Hostalek (1969). Low yielding batches show retarded growth and a high level of ICDH in the period 50-150 hours, while high-yielders show good early growth with a rapid decay of ICDH. This suggests, in the low yield batches, a lack of balance between TCA-cycle activity and growth. Inefficient use of sugar was observed in the low-yielding batches, as was observed previously by Calam and McCann (1972). High- and low-yielding inocula also show characteristic differences. In preparing inocula the fermenter is seeded and allowed to grow until cell density and sugar concentration reach the correct levels. By a coincidence, the four low-yielding inocula show an ascending time requirement for this, but the results seem to show a consistent pattern. G6PDH was always higher in the low-yielders, than the high-yielders, as was the ratio of G6PDH/ICDH. It is possible that storage material was being formed instead of growth. In the production stage this relationship continued at first, but later G6PDH/ICDH became much lower than in the high yielding batches.

Both P. chrysogenum and P. patulum show characteristically different enzyme patterns in high- and low-yielding inocula. This suggests that measurements of enzyme activity could be of practical value in testing inocula before use, as they are quick and easy to carry out. This could be particularly interesting in the case of P. patulum, as high and low yielding inocula are morphologically indistinguishable.

The main conclusion from the enzyme assays is that high and low yielding batches are clearly different from the start, in biochemical terms, and that this effect remains in existence for a long time.

DISCUSSION

Antibiotic production was strongly influenced by the quality of the inoculum. Variations in inocula were produced:-

1) In association with morphological variations, by seeding with different levels of spores.
2) By altering medium and conditions of growth, with or without variations in morphology.
3) With P. patulum, occasional chance variations arose in a standard process, unaccompanied by obvious morphological changes.

It is surprising that the problem has been so little investigated. The explanation is probably that the cause is obscure and difficult to resolve, and that, in practice, it can usually be overcome by a combination of trial and error and experience.

Although it may be of use in a limited range of conditions, morphological observation appears to be a poor method of assessing inoculum quality. Biochemical tests provide a better system, which also has the advantage of giving readily handleable numerical results. With both the fermentation systems studied, good results were associated with a correct balance of enzyme activities in the inoculum, presumably established by some control mechanism set up by the conditions used for growth. This control system remains operative in the production stage where, again, a particular system of enzyme production, balance and decay is required.

From a theoretical point of view, the main point of interest is the mechanism used to develop and retain the type of long-term control system we have observed, which remains operative over many growth generations. Since this is related to the number of spores used and the conditions of growth, it is concluded that inoculum quality must be determined soon after the moment of seeding or shortly after germination. It is also apparent that the conditions of inoculum preparation must be in balance with those used in the production fermenter. The biochemical forces involved in this situation are at present almost unknown. It is hoped to report further work in this area on a later occasion. It is of interest that an investigation of a practical problem should have led so far in this direction.

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