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Seed stage development for improved fermentation performance: increased milbemycin production by *Streptomyces hygroscopicus*

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Fermentation development for improved culture productivity can be achieved in a number of ways. Conventional approaches usually concentrate initially on optimisation of the final stage fermentation. However an understanding of the seed stage and its further development can lead to an improvement in final stage productivity. A significant increase in the production of milbemycin VM44866 by *Streptomyces hygroscopicus* was achieved by manipulation of several factors associated with the seed stage fermentation. Juvenile seeds and seed media containing reduced levels of carbohydrates overcame the detrimental effects of passaging and seed age associated with the standard (control) process. The effect of final stage inoculum level was seed medium-dependent and seed fermentation incubation temperature also affected subsequent milbemycin VM44866 production. These findings were extended to a second milbemycin-producing strain and these results have demonstrated the potential benefits of seed stage optimisation for improved final stage production.

Keywords: milbemycin; inoculum development; seed stage fermentation; *Streptomyces hygroscopicus*

Introduction

The overall aim of fermentation development is usually to increase the productivity of the fermentation process. This is most commonly achieved by optimisation of the final stage fermentation medium and incubation conditions which can result in many-fold increases in metabolite production. However, a number of steps upstream of the final fermentation stage can also have major effects on the metabolite yield of a fermentation, for example: culture selection, culture storage and inoculum development. Therefore a complementary approach to final stage optimisation is to investigate and develop the seed stage fermentation in pursuit of increased culture productivity.

Although there are many examples in the literature where modification of the final stage medium and/or incubation conditions have resulted in improved fermentation titres [5,6], there are fewer examples of work in which the effects of inoculum preparation have been systematically investigated. The importance of both the biochemistry and the morphology of the inoculum as factors determining the efficiency of penicillin production have been recognised [3] and other inoculum-related factors shown to affect the performance of specific bacterial and fungal fermentations include seed age [20], aeration [18] and seed medium [16].

In this work the effects of changes to the seed stage fermentation on the production of milbemycins by two strains of *Streptomyces hygroscopicus* have been investigated. Milbemycins are macrolides with anthelmintic activity and this work is directed to increasing the production of one of these, milbemycin VM44866 [9]. The final stage fermentation is unusually long, up to 17 days incubation, and therefore any increase in final stage titres or reduction in incubation time would be beneficial in terms of overall productivity. Titres of milbemycin VM44866 have been increased by optimisation of the final stage medium [22] and this work demonstrates further possible improvements by development of the seed stage fermentation. Thus, a number of seed-related factors: medium, age, temperature, inoculum level and passage number were systematically investigated for their effect on the subsequent production of the antibiotic.

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Materials and methods

Culture

Streptomyces hygroscopicus RB4569D and MB6 were maintained as 1-ml aliquots of vegetative mycelium at −70°C. Recovery of the stored culture was by inoculating 50 ml of the appropriate seed medium with 1 ml of the thawed stock culture.

Media

All seed media used were variations of a common base medium (F1) containing Arkasoy 50 (10 g L^{-1}) (British Arkady Co, Manchester, UK), casein (BDH Light white soluble) (2 g L^{-1}), $CaCO_3$ (5 g L^{-1}) and $MgSO_4 \cdot 7H_20$ (1 g L^{-1}). Carbohydrates were added to this medium such that, for example, F1GD(20/20) contained glucose (20 g L^{-1}) and collofilm dextrin (20 g L^{-1}) (Amylum NV, Burchstraat 10, B9300, Aalst, Belgium) and F1GD(10/0) contained glucose (10 g L^{-1}) and no dextrin. The final stage fermentation medium used throughout this work was F1FW80(20/50) containing F1 base medium plus fructose

(20 g L^{-1}) and Avedex W80 starch (50 g L^{-1}) (Tunnel Avebe Ltd, Gillingham, Kent, UK).

Incubation

Unless otherwise stated, seed stage evaluation experiments were carried out using a primary seed stage in F1GD(20/20) medium followed by inoculation to test seed media. All seed flasks were inoculated with 4% (v/v) inoculum and incubated at 28°C on a rotary shaker (240 rpm, 5 cm throw) for 48 h unless specified otherwise. Final stage shake flasks were incubated as above with triplicate shake flasks being prepared for each time point (usually 14 or 17 days post-inoculation).

Analyses

Growth of the culture was assessed by measuring the packed cell volume (PCV) in 10 ml whole broth centrifuged at 1200 × *g* for 15 min at 5°C. Carbohydrate concentrations were obtained using standard Boehringer or Roche Diagnostics test kits adapted for use on a COBAS Bio centrifugal analyser (Roche Products, Welwyn Garden City, Herts, UK) [22].

Milbemycins were extracted from whole broth into acetone (2 volumes of acetone:1 volume of whole broth) for 1 h followed by filtration through Whatman GF/A and GF/F glass fibre filter papers. Quantification was by HPLC using a Waters WISP 710B autosampler fitted with a C18 bondapak 5-μm column using a methanol:water (84:16) solvent system at a flow rate of 1.5 ml min^{-1}, column eluates were monitored at 244 nm.

Experimental data were evaluated by Analysis of Variance (ANOVA) using standard RS/Explore statistical software. All tests carried out have an underlying null hypothesis that the experimental treatment will have no effect on final stage milbemycin titres. The threshold *P* value chosen to minimise the chances of making a type 1 error (falsely rejecting the null hypothesis) was 0.005.

Results

Figure 1 shows a typical seed stage fermentation profile obtained from shake flasks sacrificed at the appropriate time to provide time-course data. While dextrin was not utilized during the fermentation, the rate of glucose utilization increased until approximately 30 h and then declined until glucose was depleted at about 65 h. Biomass (measured as PCV) also increased up to 30 h and remained constant after that time; during the initial growth phase there was a constant biomass yield of approximately 3.5 PCV % units g^{-1} glucose consumed. Carbon dioxide evolution profiles obtained from stirred tank fermentations indicate that exit gas carbon dioxide concentration increased up to approximately 14 h and then remained constant for the rest of the seed stage fermentation.

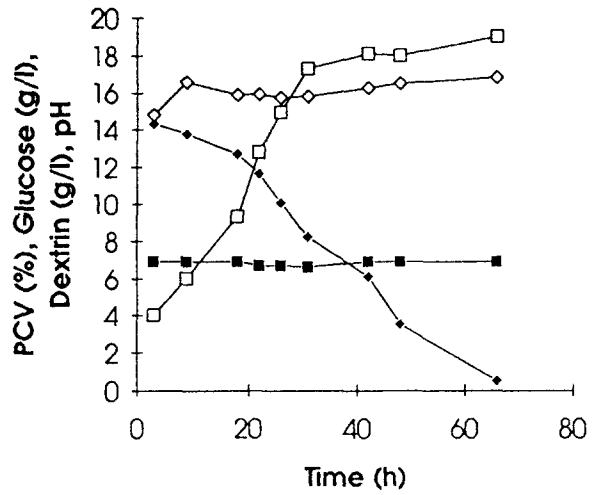

Figure 1 Seed stage fermentation profile (from smoothed data) of *S. hygroscopicus* RB4569D. (■) pH, (□) packed cell volume %, (◆) glucose (g L^{-1}), (◇) dextrin (g L^{-1}).

Effect of carbohydrate

Figure 2 shows titres of milbemycin VM44866 produced in final stage fermentations inoculated with seeds grown for 48 h in media containing different levels of carbohydrates. These results show that dextrin can be omitted from the seed medium and that the initial glucose concentration can be reduced to as little as 5 g L^{-1} without significantly affecting subsequent milbemycin production; final stage titres varied by less than 10% from the control.

Effect of inoculum age

Figure 3 shows the effect of seed age on subsequent milbemycin production. For each seed medium tested, final milbemycin titres were reduced as the time of seed incubation increased. Thus, milbemycin VM44866 titres in fermentations inoculated with 10-h seeds were on average 38% higher than those inoculated with 48-h-old seeds. Analysis of variance showed that for seeds grown in F1GD(20/20) up to 94% of these differences were due to differences in the seed age ($F = 28.1$, $P = 0$).

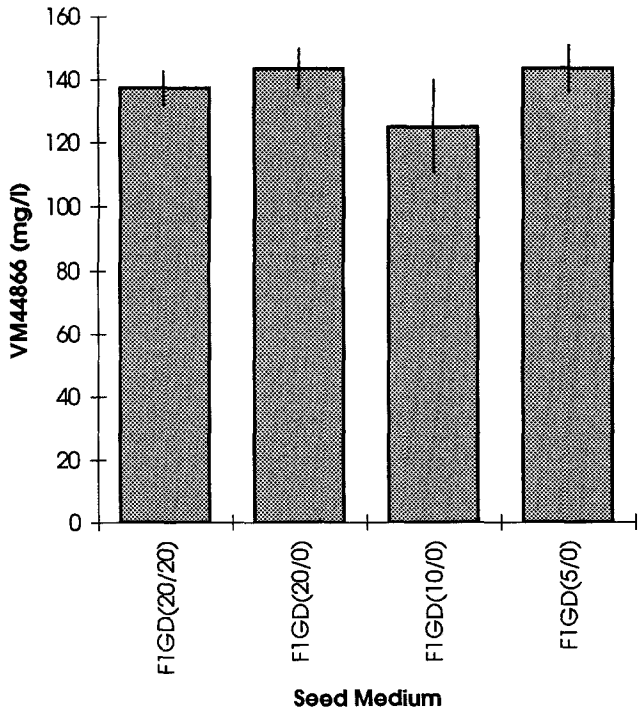

Figure 2 Mean (and standard deviation) final stage fermentation (14-day) titres of milbemycin VM44866 produced by *S. hygroscopicus* RB4569D after seed fermentation in different seed media.

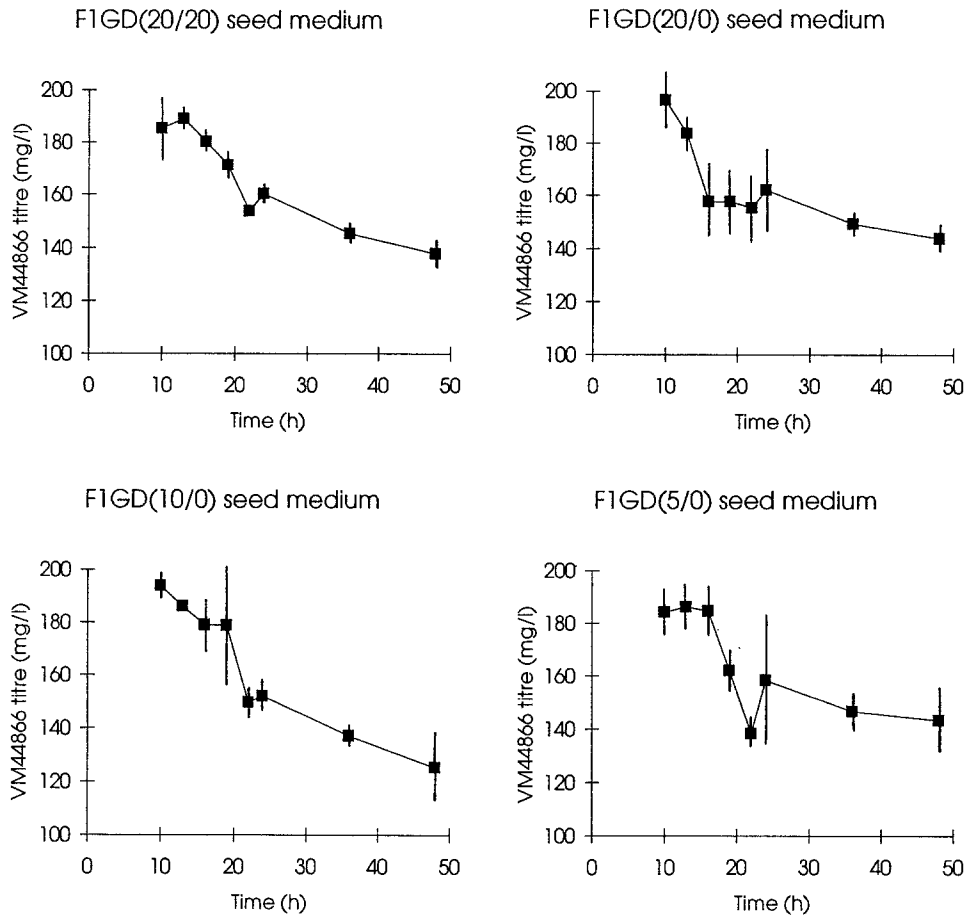

Figure 3 Mean (and standard deviation) final stage (14-day) titres of milbemycin VM44866 produced by fermentation of *S. hygroscopicus* RB4569D inoculated from different seed media after different seed incubation times.

Effect of inoculum level

At inoculum levels greater than 1% the titre of milbemycin VM44866 was relatively unaffected by the amount of inoculum or the seed medium used although inocula grown in F1GD(10/0) consistently produced slightly higher titres than those grown in F1GD(20/20) medium. At inoculum levels below 1% the final titre of milbemycin was dependent on the seed medium (Figure 4).

For inocula grown in F1GD(20/20) medium final milbemycin titres decreased by almost 50% with decreasing inoculum level down to 0.2%; the corresponding decrease for inocula grown in F1GD(10/0) medium was only 17%. In contrast variation in inoculum level into the seed fermentation followed by a constant (4%) transfer into the final stage had little effect on subsequent milbemycin production (data not shown).

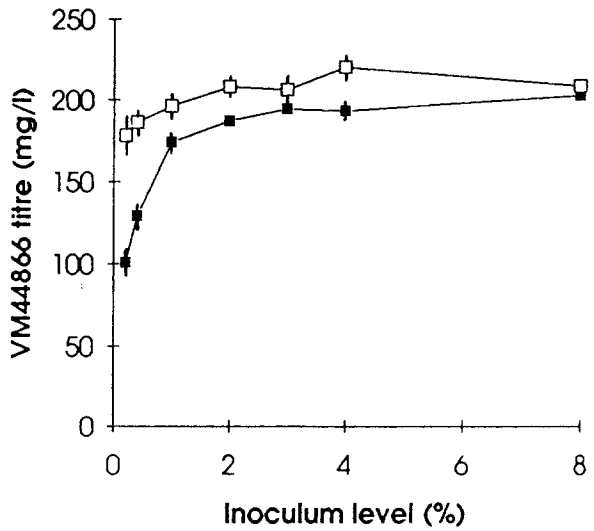

Figure 4 Mean (and standard deviation) titres of milbemycin VM44866 produced by *S. hygroscopicus* RB4569D in final stage (14-day) fermentations inoculated with different levels of culture grown in seed medium. (■) F1GD(20/20) or (□) F1GD(10/0).

Effect of temperature

Milbemycin production can be enhanced by increases in the final stage incubation temperature (unpublished data). Results in Table 1 indicate that the incubation temperature of the final seed stage can also have a significant effect on subsequent milbemycin production. These data show that in general, higher final titres were produced when seeds are incubated at 32°C compared to the standard 28°C. This effect was particularly pronounced after three passages. Similar results were obtained with other inoculum level/seed age regimes and statistical analysis confirmed that seed incubation temperature had a strong effect on the subsequent production of milbemycin VM44866.

Table 1 Final stage (14-day) titres of milbemycin VM44866 (mg L^{-1}) produced by *S. hygroscopicus* RB4569D after seed stage incubation in F1GD(10/0) medium at different temperatures

Passage number	Age of seed at transfer	28°C	30°C	32°C
2	24 h	176	163	173
	48 h	134	150	144
3	24 h	123	108	141
	48 h	108	140	144

Effect of passage number

In common with many other industrial fermentations, large scale fermentations of this organism have utilized multiple seed stages for adequate inoculum build-up. These have conventionally used F1GD(20/20) seed medium and each seed stage was routinely incubated for approximately 48 h. Results shown in Figure 5 indicate that the use of these seed conditions leads to a decline in final stage milbemycin titre with increasing number of seed stages. However this effect was negated when an alternative seed medium F1GD(10/0) was used and the seed incubation time was reduced to 24 h. Under these conditions there was no significant difference in final titres when up to four seed stages were used (Figure 5).

Improved strain MB6

During this work an alternative strain of *Streptomyces hygroscopicus*, strain MB6, became available. Results of an experiment carried out with this strain to assess the effects and interactions of seed age, seed and final stage inoculum level and passage number are shown in Figure 6. These data show that, as with strain RB4569D, seed age has a major effect on subsequent milbemycin production; juvenile seeds generally giving the highest titres. Increased passage number was also shown to have a detrimental effect on production of milbemycin VM44866, particularly

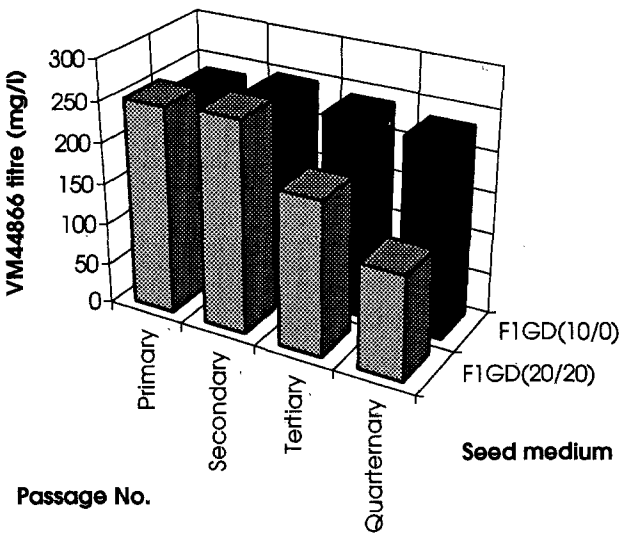

Figure 5 Mean final stage (14-day) fermentation titres of milbemycin VM44866 produced by *S. hygroscopicus* RB4569D after different numbers of seed stage passages in different seed media.

with longer seed incubation times. Inoculum level into the seed stage (Figure 6a) or into the final stage (Figure 6b) had very little effect as indicated by the similar overall shape of surface plots and by similar titres for any given seed age/passage number combination.

Discussion

Overall, these results demonstrate that the production of the natural product, milbemycin VM44866, by *Streptomyces hygroscopicus* in final stage fermentations is significantly influenced by optimisation of the preceding seed stage. Reduction in passage number, the use of juvenile seeds and higher incubation temperatures generally resulted in higher milbemycin production by both strains of *S. hygroscopicus* used here.

Although the importance of inoculum preparation has long been recognised [12,17] it has been the subject of surprisingly little published work. The 'quality' of inoculum is often stated as an important factor in determining the performance of a final stage fermentation although how this is best assessed is open to question.

Many industrial processes rely on carbon dioxide evolution rate (CER) as a key factor in determining the time of transfer of a fermenter-grown inoculum to the final stage production fermenter [2,19]. Transfer at or near to peak CER results in the use of an actively growing inoculum which is generally beneficial to final stage performance. Transfer of actively growing culture is also implicit in the beneficial effects of juvenile seeds reported here. Biomass within seed fermentations increased until approximately 25–30 h and highest milbemycin titres were achieved in fermentations inoculated with seed cultures transferred before this time (Figure 3). Juvenile seeds were also reported to improve the production of lysergic acid derivatives by *Claviceps paspali* [20] and to increase culture productivity in *Lactobacillus casei* fermentations for lactic acid production [11]. In contrast, the use of older inocula gave higher rates of phytic acid hydrolysis in the solid state fermentation of canola meal by *Aspergillus ficuum* [15].

Other measures of inoculum quality which have been used to enhance final stage performance include cell motility in *Clostridium acetobutylicum* fermentations [7] and levels of specific intracellular enzymes in *Saccharomyces cerevisiae* fermentations [13]. Similarly, measurement of aldolase, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase has been suggested as a rapid means of assessing inoculum quality in *Penicillium* fermentations [21].

Morphology of the inoculum is not generally used as an indicator of inoculum quality although some assessment of morphology is implicit in the use of 'expert' visual examination of the inoculum to determine transfer times in some fermentations [23]. Metabolite production by mycelial organisms can be markedly affected by differences in culture morphology, and inoculum size plays a key role in determining final stage morphology [1]. However, morphology of the inoculum is also known to influence critically the production of penicillin by *Penicillium* spp [21] and of citric acid by *Aspergillus niger* [4]. It would be interesting to generate numerical assessments of inoculum mor-

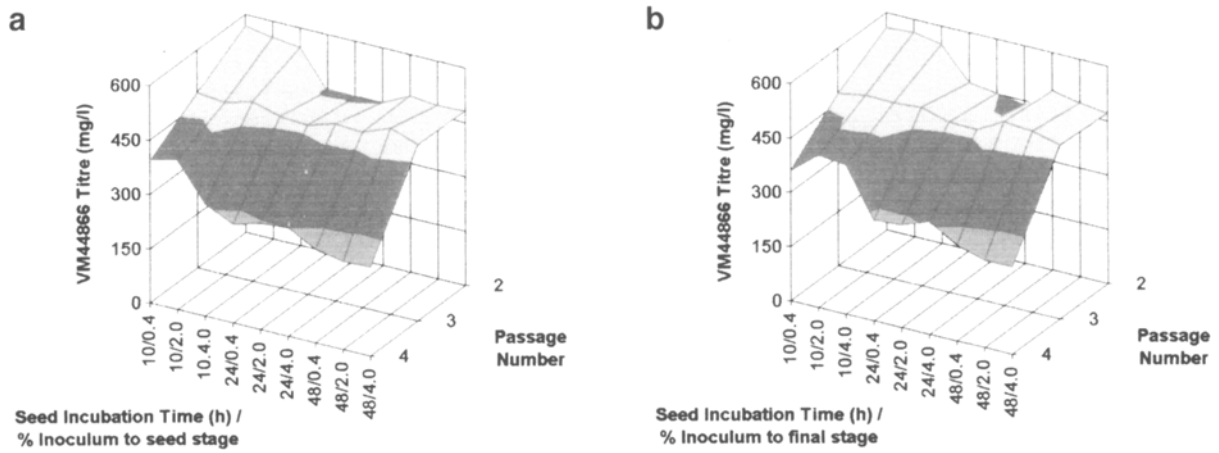

Figure 6 Mean final stage (14-day) fermentation titres of milbemycin VM44866 produced by *S. hygroscopicus* MB6 inoculated after different seed incubation times and numbers of seed stage passages. (a) Seed stage inoculated with different inoculum levels; and (b) final stage inoculated with different inoculum levels.

phology using improved image analysis techniques and to investigate how these may correlate with differences in final stage metabolite production.

The beneficial effects of increased seed incubation temperature reported here may also be explained in terms of biomass; elevated temperatures leading to increased growth rates will result in increased levels of biomass for a given seed incubation time (at least until a specific nutrient or some other factor becomes limiting). Alternatively, activities of specific biosynthetic enzymes may be enhanced at higher temperatures leading to reduced lag times in final stage fermentations.

The effect of passage number is particularly important in large scale industrial fermentations where multiple seed stages may be required to build up inoculum volume. Increased passage number is detrimental to penicillin production by *P. chrysogenum*, novobiocin production by *Streptomyces niveus* [19] and to tobramycin production by *Streptomyces cremeus* [14]. This work has demonstrated that the detrimental effect of serial passages can be negated by manipulation of the seed medium and incubation conditions (Figure 5).

Seed medium composition affects the final stage performance of a number of fermentations. It has been stated that antibiotic-producing activity of actinomycetes tends to be decreased by the use of metal deficient media, prolonged seed incubation time and higher temperatures [8] and it is generally accepted that seed media will be optimised for rapid growth enabling the generation of high levels of biomass with high levels of biosynthetic activity.

The seed medium composition was an important factor in a titre development program for avermectin production by *Streptomyces avermitilis* [16]. When combined with improved cultures, alternative seed media resulted in a 10-fold enhancement of avermectin production. However not all improved *S. avermitilis* mutants responded in the same way to alternative seed media and this resulted in the development of a generic seed medium for avermectin production based on the nutritional requirements of the organism. Similarly, enhanced cephalosporin production has been achieved by manipulation of the seed medium in fermentations of *Cephalosporium acremonium* [10].

Variability in yields of final stage fermentations is an important problem in the large scale fermentation industry. Undoubtedly there are many contributing factors to variation in the final stage performance of a given process and it is likely that inoculum-related factors are at least partly responsible. This work has shown that final stage fermentation performance can be improved by optimisation of the preceding seed stage and variation in the initial inoculation process has been identified as causing significant differences in subsequent performance of *S. cerevisiae* fermentations [23]. Even before this stage, appropriate methods of culture maintenance and preservation must be used to minimise culture heterogeneity and future losses in viability and productivity.

This work has shown that the production of the natural product, milbemycin VM44866, by *Streptomyces hygroscopicus* is enhanced by optimisation of the final seed stage and it is likely that further improvements will be achieved by development of the complete inoculum build-up process. These results have demonstrated that seed stage development is a valid complementary approach to final stage optimisation for titre improvement and may lead not only to increased titres but also to reduced variation in final stage performance.

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