THE DETERMINATION OF OPTIMAL INOCULUM QUALITY FOR SUBMERSE FERMENTATION PROCESS

Petr ETTLER

Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4

Received November 22, 1990
Accepted June 28, 1991

The quantity and quality of inoculation material play a crucial role in bioprocess results. Instead of inexact decrease of pH value and subjective evaluation of the inoculum maturation, we proposed for the *Streptomyces noursei* cultivation to determine the rheological behaviour of the broth reflecting the growth of the culture and his physiological activity. For the rotational system cylinder–cylinder we found the optimal level of shear stress 80—120 mPa at shear rate 698 s^{-1} for the transfer of inoculum to production stage and achievement of maximal process productivity.

The history and physiological state of the microbial culture play an important role in achieving the maximal productivity of the bioprocess. The course of a fermentation is considerably affected not only by spore or vegetative type of inoculum, but by its quantity and also quality.

Brown and Zainudeen[1] indicated the effect of increased inoculum quantity on biomass production. The influence of four nominal volumetric levels (0·5; 1; 3 and 5%) of *Trichoderma viride* inoculum on growth kinetics and specific oxygen uptake rate was reported.

Gomez and Goma[2] mentioned that the increase of traditional ratio of inoculum size 10% of total volume causes higher concentration of toxic substances transfered to the production stage.

Calam[3] described the relation between inoculum quality and productivity during cultivation of various types of microorganisms. The formation of antibiotics connected mainly to the growth of filamentous fungi is related to several stages which the culture should pass through: after first stage of rapid growth, the oxygen is becoming a limiting factor and cells should adapt themselves to changing conditions. The biomass must reach an "adequate concentration" to ensure a fast rate of antibiotic production.

Martin and Mc Daniel[4] used the term "cell maturation time" instead of "adequate inoculum concentration" for the biosynthesis of polyene macrolide antibiotics. Hillinger and Nitzsche[5] compared various methods for the *Streptomycetes* growth patterns: photometry, the protein contend and dry weight determination.

The aim of this study was to demonstrate on an example of *Streptomyces noursei* cultivation and nystatin biosynthesis that if the growth of the producing microorganism is not quantified, the criteria for the transfer of inoculum from seed fermentor to production level are selected according to subjective evaluation.

EXPERIMENTAL

Microorganism and fermentation media. The industrial production strain *Streptomyces noursei* 153 received after systematic mutagenic treatment was used throughout. The number of vital spores in 1 ml of suspension used for inoculation was $2{\cdot}0 \cdot 10^9$. Inoculation media contained 2% of glucose and 0·5% of cornsteep liguor as main C- and N- sources.

Apparatus. The experiments were performed in a stainless-steel pilot plant fermentor with following geometry of the vessel and mixing:

$$D_t = 0{\cdot}65\ \text{m}\,, \quad d = \tfrac{1}{3}D_t\,, \quad H_L/D_t = 1{\cdot}2\,, \quad V_L = 0{\cdot}17\ \text{m}^3\,.$$

Flat blade turbine impeller according to ČSN 691021 was used as the mixing system. Process conditions:

$$Q = 0{\cdot}5\ \text{VVM}\,, \quad n = 300\ \text{min}^{-1}\,, \quad \text{ITS} = 3{\cdot}4\ \text{m s}^{-1}\,, \quad t = 28°\text{C}\,.$$

Laboratory experiments proceeded in 500 ml cultivation flasks with 40 ml working volume and 10% volumetric inoculum ratio.

Analytical methods. Simple sugars were determined manganometrically[6], ammonia nitrogen according to Hanuš[7].

Nystatin was assayed by microbiological titration using *Saccharomyces cerevisiae* as the test microorganism. The volume of packed biomass was expressed as sediment after 20 min of centrifugation in laboratory centrifuge Janetzki T30 at rotation speed $3\,000\ \text{min}^{-1}$. Another method called "Free sediment determination" leads to the value of biomass content after 1 h of sedimentation at laboratory temperature without centrifugation in calibrated column. The determination of rheological behaviour was performed on rotational viscometer Contraves 30 (Switzerland) with the slot 16 mm between two cylinders.

The pH value was detected by Ingold sterilisable senzor type 2293 (Switzerland).

RESULTS AND DISCUSSION

Our attention was focused to optimal design of the inoculation step and to finding of simple and exact method for the quick determination of "optimal quality" of seed inoculum.

It has been always recognised that the origin (spores or vegetative inoculum) and quality of inoculum have a considerable effect on the profile of the fermentation[1–4]. We agree with the literature statement, that once a fermentation is started, it can be made worse but not better[4].

The differences in the viability of spores, variations in spores inputs into seed fermentor, variation of quality of raw material used are the reasons for the fluctuations in process results.

In Fig. 1 is demonstrated the influence of two spores input on the velocity of germination and mycelia differenciation.

The survival of spores after preservation and their total input influences the velocity of mycelia differenciation and consequently the yield of antibiotic at the end of the production phase. The characterisation of the *Streptomyces noursei* growth on complex media was performed by sediment determination. The intensive growth of the culture between 36 and 42 h of cultivation seems to be the best period for the incolum transfer to production fermentor, but any exact criteria for it quantification has been missing.

The following step of optimization of the inoculum preparations was the determination of the substrate consumption and pH changes during the biomass growth (Fig. 2). In the first period of cultivation non-substantial change in biochemical activity occurs, due to the spores germination. The slow decrease of C- and N-sources in first 25 h refers to the adaptation of germinating spores to new environmental conditions. Experimental phase of the *Streptomyces noursei* growth corresponds with elevation of biomass content and free sediment volume. Ammonium ions regulate the formation of biomass. Concentration of C- and N- sources gradually decrease in association with the intense growth of the culture. The results showed that no substrate limitation is present during the cultivation in seed fermentor. The

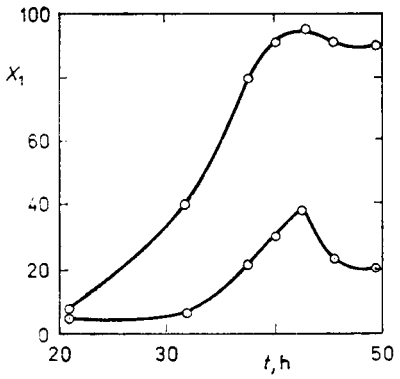

FIG. 1

The influence of spores input on the growth of *Streptomyces noursei* producing strain: ○ $4 . 10^9$ spores. ml^{-1}, ⊙ $2 . 10^9$ spores. ml^{-1}

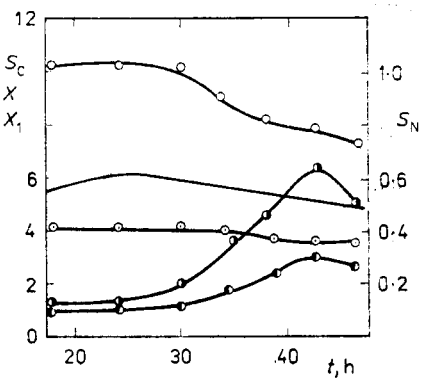

FIG. 2

The course of *Streptomyces noursei* cultivation in seed fermentor: ○ S_N-ammonia nitrogen concentration ($g\,l^{-1}$), ⊃ S_C-reducing sugars ($g\,l^{-1}$), ◑ "free sediment" X_1 (% of total volume), ◐ packed biomass volume X (% of total volume), — continuously measured pH value

decrease of pH value which was formely used as the only criterion for transfer of inoculum to production stage is continuously smooth and stepless. We tried to find out a method and criterion reflecting better the growth of *Streptomyces noursei* and physiological state of the culture than the pH value. The biomass dry weight determination is for the estimation of inoculum maturation unflexible and unsuitable for the case of suspension substates contained in complex nutrient media. The nature of the broth flow pattern and the magnitudes of interval stresses and applied forces depent primary on the geometry of the system, the rate of fluid motion and the intrinsic rheological properties dependent on physiological state of the culture. Broth rheology is a more reproducible method characterizing immediately the flow properties of broth, concentration and morphological state of the mycelia important for process design and operation[8]. A study of broth rheology was initiated using rotational viscometer Contraves 30 with various cylindrical spindles for the evoluation of apparent viscosity. After tesing of several systems we have choosen system A with the slot of 16 mm between the two rotationg cylinders to prevent inhomogeneity, channelling, settling and particle interaction[9].

Precautions were taken to ensure a fair reproducibility of the viscosity measurement: samples of broth we deaerated and homogenised.

Morphological conditions exert a very profound effect on the nature of broth rheology. *Streptomyces noursei* exhibits a style of growth characterized by linear elongation through accumulation of biomass at the growing tips of the hyphae filaments. This process is accompanied by lateral branching of the hyphae to produce

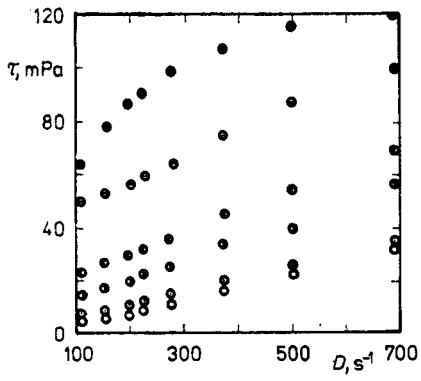

FIG. 3

The flow curves of *Streptomyces noursei* inoculation broth: ○ 0 h, ⊙ 24 h, ◑ 34 h, ◐ 38 h, ⊖ 44 h, ● 48 h

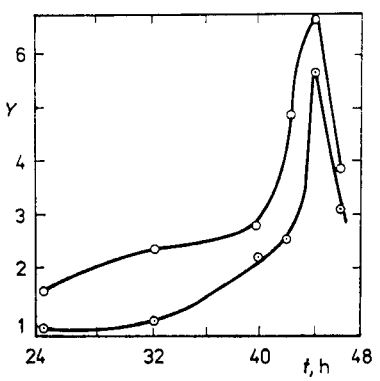

FIG. 4

The determination of optimal maturation time of inoculating material: ○ seed fermentor inoculum, ⊙ laboratory inoculum

new growing tips. The result of this pattern is the growth and formation of three--dimensional structure of the mycelia in seed fermentor. The rheological behaviour of *Streptomyces noursei* broth (Fig. 3) exhibits at the beginning of the cultivation Newtonian behaviour with the shift to non-Newtonian from the 40 h of cultivation. These data are in good agreement with literary statement[9-11]. The viscosity of the broth at the end of the cultivation is no more a constant, but dependent on the shear stress and velocity gradient.

No detailed analysis of fluid mechanics, flow models formulation or microscopic studies of hyphae branching were performed. A simple and quick rheological measurement serves as a marker for the maturity of inoculating material. For the determination of optimal physiological state of the culture and achievement of maximal yield of nystatin at the end of production stage, we transferred sterile samples of the broth at various periods of mycelia development from laboratory scale and pilot plant fermentor to cultivation flasks with production media (Fig. 4). We found a good agreement of results from both scales. The optimal time for inoculation of production fermentor was the 44 h of cultivation, which corresponds to the level of shear stress between 80 and 120 mPa at shear rate 698 s^{-1}. This values are strictly dependent on properties of selected strain and media composition.

Thanks are due to Mrs Šůsová for laboratory collaboration and assistance.

SYMBOLS

d	impeller diameter, m
D	shear rate, s^{-1}
D_t	fermentor diameter, m
H_L	height of liquid, m
ITS	impeller tip speed, m s^{-1}
Q	volumetric gas flow rate, VVM
S_N	ammonia nitrogen concentration, g l^{-1}
S_C	reducing sugars concentration, g l^{-1}
X	packed biomass volume (% of sediment)
X_1	biomass volume without centrifugation (%, "free sediment")
Y	yield (biologically defined potency in international units)
τ	shear stress, mPa

REFERENCES

1. Brown D. E., Zainudeen M. A.: Biotechnol. Bioeng. *20*, 1045 (1978).
2. Gomez J., Goma G.: Biotechnol. Lett. *8*, 833 (1986).
3. Calam C. T.: Proc. Biochem. *4*, 7 (1976).
4. Martin J. F., Mc Daniel L. E.: Biotechnol. Bioeng. *17*, 925 (1975).
5. Hillinger M., Nitzsche Ch.: Z. Allg. Microb. *14*, 193 (1974).
6. Grégr V., Rychtera M.: *Analytické metody ke cvičení z kvasné chemie a technologie*, Vol. III. SNTL, Praha 1966.

7. Janíček G., Šandera K., Hampl B.: *Rukověť potravinářské analytiky*. SNTL, Praha 1962.
8. Ettler P.: Collect. Czech. Chem. Commun. *55*, 1730 (1990).
9. Bongenaar J. J. T. M., Kossen N. W. F., Metz B., Meijboom F. W.: Biotechnol. Bioeng. *15*, 201 (1973).
10. Blanch H. W., Bhavarajn S. M.: Biotechnol. Bioeng. *18*, 745 (1976).
11. Leduy A., Marsan A. A., Coupal B.: Biotechnol. Bioeng. *16*, 61 (1974).

Translated by the author.