SCALE-UP AND SCALE-DOWN TECHNIQUES FOR FERMENTATIONS OF POLYENE ANTIBIOTICS

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Our philosophy of successful biotechnology transfer to industrial scale covers the comparison of complex sets of microbiological, analytical and bioengineering data from cultivations in various scales and different geometries of mixing with laboratory findings. In particular, the availability of nutrients to producing microorganism should be understood, therefore for quick scaling-up procedure of polyene antibiotics produced by *Streptomyces noursei* we recommend to use physiological marker as total dehydrogenase activity determination. The utility of scale-down tests for identification of process fluctuation, validation of new substrate batches and simultaneous control of inoculum quality was proved.

Conditions at laboratory scale differ widely from conditions at production scale. Therefore, the results of small – scale experiments are often of little use for scale-up. But each level of the bioprocess development has its unchangeable importance which cannot be either underestimated or overestimated. Starting from the geometric similarity of reactors, kinematic similarity (in each point of both vessels all components of vectors are equal), the dynamic similarity are commonly used[1].

A number of scale-up methods exist[2]:
1. Fundamental method (solving the microbalances for momentum-, mass-, and heat transfer. 2. Semi-fundamental method (solving simplified balances). 3. Dimensional analysis (including regime analysis). 4. Rules of thumb. 5. Trial and error.

Another scheme of scaling-up procedures is done according to different kinds of phenomena[3]: thermodynamic phenomena; microkinetic phenomena; transport phenomena with phenomena directly related to flow and diffusion as shear, mixing, mass transfer, heat transfer, etc.

Einsele[4] mentions scale-up rules for different biosynthetic processes of European fermentation industries. These criteria are all closely related (constant P/V, constant K_La coefficient, constant ITS, constant D.O.C.) and refer mainly to oxygen transfer.

According to Hubbard et al.[5] maintaining D.O.C. at a specific value is taken to be the limiting factor in designing any aerobic fermentation system. Our access, which we have used during the process development of antibiotics biosynthesis are[6-8]: utilisation of theoretical relations of data from testing of equipment; "exten-

sion" of data from two scales; extrapolation of laboratory results with the knowledge of process mechanism.

Selection of appropirate method depends on program priority, on time for disposal, equipment instrumentation, etc. Some scientists consider in most cases for scale-up procedure microbiological aspects only and this frequently results in misinterpretation or in conclusion of highly limited applicability. During the biosynthesis of polyene antibiotics for optimal process design a good understanding of the interaction between shear and mycelial morphology is needed. Therefore, the finding and maintenance of suitable (and similar) environmental factors in all scales for growing and producing microorganism for maximal product recovery is the objective of this paper.

EXPERIMENTAL

Results of the batch and fed batch cultivations of *Streptomyces noursei* at various nominal volume levels of fermenters are reported. The producing strain is an obligatory aerobic actinomycete forming substrate and aerial mycelium and producing polyene antibiotic with elemental composition $C_{46-47}H_{73-75}O_{18}N$. The product of the biosynthetic process is known under commercial trade marks Fungicidin, Nystatin, Nurseothricin, Moronal, Mycostatin, Stamicin.

TABLE I
Fermentors dimensions[a]

Parameter	Dimensions					
Volume brutto m^3	0·002	0·014	0·3	0·3	15	26
Fermentor diameter, m	0·105	0·23	0·5	0·65	2·1	2·51
Fermentor height, m	0·22	0·47	1·5	1·16	4·81	5·63
Impeller diameter, m	0·066	0·078	0·17 0·25	0·21 0·32	0·78	0·9
Number of impellers	1	2	2	2	2	2
Number of blades	6	6	6	6	6	6
Type of sparger	jet	ring	ring	ring	ring	ring
Number of baffles	4	4	4	4	cooling coils	0
Impeller speed, min^{-1}	770	410	170—500	370	80	90
Air flow Q/V	0·2	0·2	0·2	0·2	0·2	0·2
Power input installed, kW	—	—	5·5	3	28	40

[a] Flat blade turbine impeller according to ČSSR norm No. 691021.

The final antifungal properties of the product are dependent on the ratio of components in the mixture, as by most of polyene antibiotics. The complete structure of the product was described by Borowski et al.[9]. The cultivation media contain glucose and soya bean meal as mains C and N sources. The cultivation period was 120 h. The temperature of the cultivation was maintained during the whole biosynthesis at 29°C. The foam level was controlled by additions of emulsions of soya bean oil with silicone oil, type SAG 471 (Union Carbide), when necessary. Glucose feed was added during the production phase. Analytical methods correspond to our previous reports[6-8]. Total dehydrogenase activity was determined according to Novikova and Makarevitch[10] as the rate of methylene blue decolorization expressed as absorbance at 660 nm after 10 minutes of exposition. The ratio of A_{660} and packed cell volume of biomass expressed in % of sediment in suspension media designated as q_{OX} represents specific dehydrogenase activity quotient for 10 minutes reaction.

Statistical tests for comparing the mean of a new product or a variable with a standart according to Himmelblau[11] were proved successful in microbiological practice as LSD test. Deviation for the determination of active substance content was less than 4%. Variability of other biochemical methods fluctuate in range of 5—10% and is effected mainly by unhomogeneity of samples.

The geometry of experimental equipment and the geometry of mixing is summarized in Table I.

Streptomyces noursei, producing strain of antifungal antibiotic has several specific properties which form a suitable engineering model organism from it: sensitivity to oxygen supply, to pH value, substrate concentrations and to mechanical shear. Simultaneously with scale-up procedure several optimization steps for maximal product recovery were performed.

RESULTS AND DISCUSSION

Laboratory Level

After mutagenic treatment and selection of producing strain *Streptomyces noursei* in genetic laboratories a large quantity of mutants should be tested on ability to grow, to form spores and components of active substance.

Flask cultivations can be performed in large series without continuous supervision, without any risk of contamination. The OTR in the flask is determined by the mechanical characteristics of the shaker, by filling volume (Fig. 1), by flask closure and shape of flask. The determination of this parameter was the first information about the sensitivity of *Streptomyces noursei* to oxygen supply.

The ability of the producing strain to metabolize various C and N substrates in various concentrations has allowed us to perform the optimal media design with respect to maximal product concentration. The additions of antifoaming agents cannot be examined at this level, oils and silicones are washed out on wall of shaking flasks. For solving of many fermentations problems, for development of process technology, this level is unsufficient. Installation of oxygen electrodes in shaking flasks changes hydrodynamical behaviour of broth, cause foaming and shearing of growing mycelia.

In laboratory fermentors, the decision about "the maturation" of inoculating material can be done according to pH change, dry weight determination or other biochemical parameter. Our proposal is to use viscosity measurement (Fig. 2) for quick and accurate information. It reflects in a complex way the morphological and physiological state of the culture in relation to substrate concentration. The achievement of maximal productivity of the process corresponds to inoculation period with maximal apparent viscosity. The volume of samples from these fermentors does not change substantially the hydrodynamical behaviour of the broth. The screening of substrates depends on the accessibility of raw material on domestic market and on enzymatic equipment of the producing strain. Missing the amylase and glucoamylase (E.C.3.2.1.3.) activity, the use of starch as main C- source was excluded. The high power input per unit volume and small mixing times resulting in perfect homogeneity of the broth in laboratory fermentors are not attainable in larger vessels. The losses of installed power input form approx. 50%.

Pilot Plant Level

After receiving the basic information about the growth and productivity of selected producing strain *Streptomyces noursei*, solution of the process design is transferred to pilot plant scale. Mass transfer, power input, biochemical and microbiological

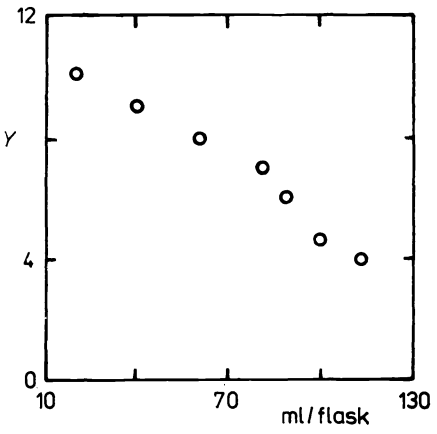

FIG. 1

The relation between flask filling volume representing OTR and yield of product (in arbitrary units)

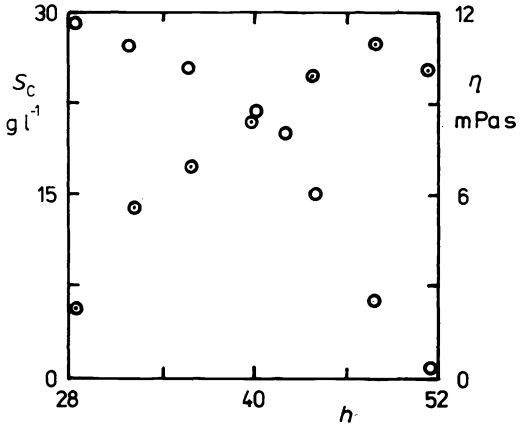

FIG. 2

The determination of optimal inoculum quality during Nystatin biosynthesis. S_C concentration of C source (○), η apparent viscosity (⊙)

parameters, foaming can be better controlled at this level. The experimental results of the determination of hydrodynamical behaviour and OTR in simulated media showed that at apparent viscosities 1 – 10 mPas in 300 l fermenter, the suitable range of impeller speed is about 300 min^{-1} at aeration rate 0·6 – 0·8 VVM from the point of power input 2·6 – 2·8 kW m^{-3} and coefficient $K_L a$ 160 – 200 h^{-1} determined with static method.

The air flow rate has relatively smaller effect on $K_L a$ value in comparison with impeller speed under given experimental configuration. The cultivation experiments with complex cultivation media involve intensive foaming at higher aeration rates. Therefore the volume of incoming air was diminished to 0·2 VVM assuring that no oxygen limitation occurs. The working volume can be after that elevated for maximal productivity of the vessel.

The process of secondary metabolite production can be improved in this scale by substrate additions to prolong the phase of intensive product biosynthesis. Methods for the OTR measurement in the absence and with presence of microorganisms with their basic assumptions and corresponding procedures were reviewed by Sobotka et al.[12]. The $K_L a$ value may play a key role in some processes, but its application where no oxygen limitation in process scale occurs (Fig. 3) is of secondary importance. The D.O.C. can be used as physiological marker for substrate feeding to the culture in several processes. Whenever the substrate in the medium is about to be completely consumed and therefore becomes a limiting factor, the D.O.C. increases rapidly. During the biosynthesis of nystatin on complex media with metabolizable oil additions, this parameter of control is not directly effective in scale- up procedure.

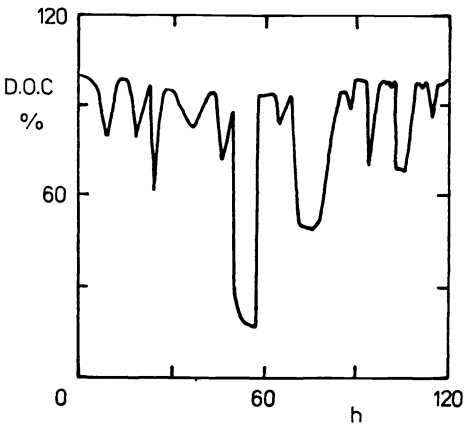

FIG. 3
The course of D.O.C. during the Nystatin biosynthesis in 26 m^3 fermentors

Various sterilization regimes were examinated from the point of view of media component preservation, especially in the case of C source and biofactors contained in complex organic substrates. Economical aspects and the aim to reduce the lag phase of the biosynthetic process have encouraged the use of various amounts of inoculum additions (3–10%), Experimental information from 300 l vessel indicates that maximal product formation was achieved at 10% of inoculum volume.

Industrial Scale

The objective in the process design should be to maintain "optimal environmental conditions" for preservation of the growth and productivity of the culture. The assurance of several functions of bioreactor in industrial scale during cultivation of mycelia suspension should be solved with compromises: assuring high OTR without mechanical damage of mycelia, forming of large interfacial area without problems of foaming, recovery of maximal working volume of fermenter without danger of contamination. Unfortunately, the instrumentation of industrial equipment is rather underestimated. Einsele[4] reports, that the measurement of oxygen transfer data are rarely made in this scale. Therefore D.O.C. and K_La determined with balance method cannot be accepted as scale-up criteria for reactor and process characterization.

The formal application of dimensional analysis published by several authors[2,3,5] leads to technically unrealistic situations or to conclusions of highly limited applicability. Parameters like P/V, t_{mix}, etc. (Table II) can be taken as arbitrary and can

TABLE II
Correlation among various scale-up criteria during nystatin process design

Criterion	Value at fermenters (in m^3 brutto)					
V	0·002	0·014	0·3	0·3	15	26
H/D	2·09	2·04	3	1·79	2·29	2·24
d/D	0·62	0·33	0·33 0·5	0·32 0·49	0·37	0·35
H_L/D	1·04	1·13	2·4	1·2	1·67	1·45
ITS, $m\,s^{-1}$	2·66	1·67	1·51–4·45 2·22–6·54	3·29 5·02	3·26	4·24
P/V kW inst. m^{-3} netto	—	—	32	17·6	2·54	2·35
t_{mix}, s^{-1}	—	<6	147–16	—	—	—

serve as a useful tool for scaling up, but high power input per unit volume and short mixing times can be achieved in laboratory scale only, are not attainable in large vessels. The transfer of the power is in industrial scale complicated with losses in the sealing. The comparison of several plant evaluations in Table II demonstrates that even achieving of exact geometric similarity is not realistic recommendation for facilitation of the process transfer to already existing fermentation line.

Recommendations About Scale-up Procedure

An engineering scientist is basically concerned with the solution of continuity and field equations which represents balances of momentum, mass and energy. A microbiologist prefers to focus his attention to utilization of various substrates by producing strain after genetical treatment at laboratory level. This comparison is quite schematic, but reflects the real situation in the bioprocess development.

Our access to the design of biotechnological process from laboratory scale to industrial level is based on good understanding of tasks and possibilities of each level: The identification of basic concept of the bioprocess at laboratory level, with the determination of growth properties and potency of selected strain.

The development of optimal sterilization regime, impeller configuration, detection of the sensitivity of the culture to D.O.C., antifoam concentration, possible mechanical damage at pilot plant level. The transfer of technology to industrial level is performed by "extension" of determined microbiological, biochemical and engineering data from two scales or from several vessels with different geometry of mixing. All known scale-up criteria should be combined with engineering optimization techniques, modelling, simulation etc. As key problem during nystatin biosynthesis in all scales we estimate the achieving of physiological homogeneity of mixed biosuspension, as mentioned above. We performed the selection of key physiological functions connected with mass transfer and correlating with product formation. As marker of physiological state of the culture we have used a quick analytical method[10] which determines the total dehydrogenase activity. Reflecting oxidation-reduction activity of the population under various external conditions, under various level of transport phenomena which are governing[2] for scale-up procedure and are influenced by pH, D.O.C., respiration, etc.

Typical profile of this metabolic parameter which is related with process results[10] is shown on Fig. 4. The maximum of the enzymatic activity was observed after accumulation of the most active part of biomass in the first stage of the biosynthetic process. The slope of the decrease of dehydrogenase activity reflects environmental conditions of each batch. This parameter can be applied for process control and for process improvements in various scales, see Fig. 5. Ryu and Hospodka[13] reported, that there are definite correlations between the productivity of the process

and physiologically important variables as specific uptake rates of carbon, nitrogen, oxygen, phosphate, sulphate and precursor. Our results confirmed that a certain level of specific growth rate must be maintained in order to exploit the full biosynthetic capacity of the selected strain.

Scale-down Tests

Scale-down experiments during the cultivation of *Streptomyces noursei* were performed by transferring the corresponding amounts of fermentation broth from industrial fermentors to laboratory scale, where the process was continued at different cultivation conditions. One of the goals of such studies is the control of inoculum quality and quantity of raw material and influence of process variables on growth of producing strain, ramification of mycelia and product formation for transferring associated with process improvements from laboratory scale to the plant scale (Table III). Many problems associated with process improvements can be solved by scale-down experiments: determination of optimal sterilization regime, the determination of optimal cultivation period for inoculation of production fermentor, elimination of hydrostatic pressure on growing cells, design of fed-batch process, etc.

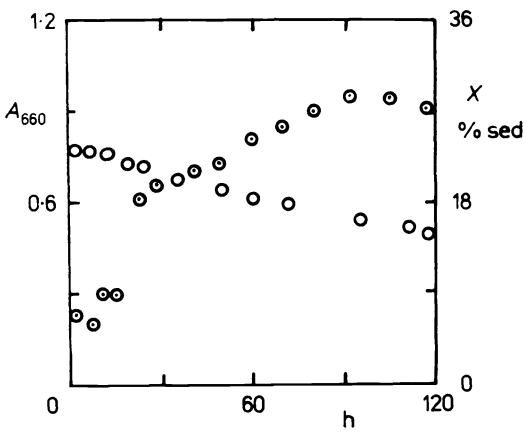

FIG. 4

Correlation between packed cell volume (○) and total dehydrogenase activity (⊙) during Nystatin biosynthesis

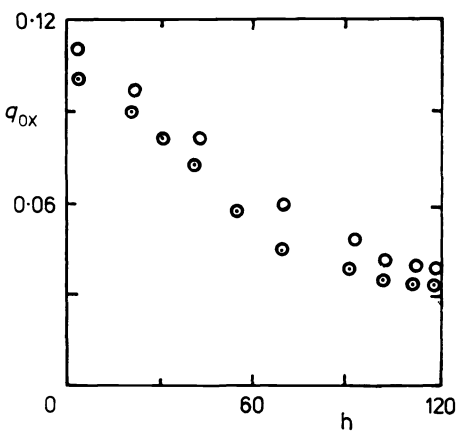

FIG. 5

Profiles of specific dehydrogenase activity quotient in two scales: 0·002 m^3 FT (○), 26 m^3 FT (⊙)

TABLE III

Eliminating scale-down tests

Experiment No.	Origin of medium	Sterilization in	Inoculum in	Comments
1	laboratory	autoclave	laboratory	laboratory control in shaking flasks
2	plant (0·2; 15; 26 m^3)	autoclave	laboratory	fermentor presterlization sample
3	plant (0·3; 15; 26 m^3)	plant (0·3; 15; 26 m^3)	laboratory	fermentor poststerilization sample
4	plant (0·3; 15; 26 m^3)	plant (0·3; 15; 26 m^3)	plant (0·3; 15; 26 m^3)	fermentor postinoculum sample for comparison with exp. No. 1
5	laboratory	laboratory	plant	determination of optimal inoculation
6	stock	autoclave	laboratory	raw material quality control
7	lab. + plant	autoclave	various batches of inoculum	inoculum quality control
8	plant	plant	without inoculation	control of sterilization conditions

Sometimes, morphological changes caused by D.O.C. and CO_2 fluctuations between operation in shaking flask and stirred vessel make achieved data difficult to interpret. This scale-down technique is a big aid in troubleshooting, in control of process regime too.

SYMBOLS

FT	fermentor
ITS	impeller tip speed, m s^{-1}
H	height of fermentor, m
t_{mix}	mixing time, s^{-1}
$K_L a$	volumetric mass transfer coefficient, s^{-1}
P/V	power input per unit volume, kW m^{-3}
D.O.C.	dissolved oxygen concentration in the broth, (% of saturation)
X	packed biomass volume, (% of sediment)
VVM	aeration rate Q/V
Q	volumetric gas flow rate, m^3
V	fermentor volume, m^3
D	fermentor diameter, m
d	impeller diameter, m
H_L	height of liquid, m
n_B	number of baffles
S_C	carbon substrate concentration, g l^{-1}
η	apparent viscosity, mPa s
Y	yield (biologically defined potency in international units)
q_{OX}	specific dehydrogenase activity quotient (absorbance at 660 nm in 10 minutes. packed cell volume^{-1})

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