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Rheological Behavior of Fermentation Broths in Antibiotic Industry

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ABSTRACT

An experimental study of rheological behavior of three morphological types of fermentation broths in relation to process conditions was carried out in industrial and pilot-plant bioreactors. Three industrially manufactured antibiotic broths were studied: Cephalosporin C (fungal), tylosin (actinomycetic), and apramycin (proactinomycetic), in which the rheology may cause real problems.

A viscometer with coaxial cylinders and six-blade impeller was used for rheological measurements. The shear rates range in which both the instruments give similar results was determined.

During the experiments rheological and morphological behavior, pH, activity, biomass concentration, fats, glucose, and nitrogen concentrations were studied.

All the broths studied exhibited nonNewtonian behavior, which could be described with Ostvald-de-Waele power-law model.

It was found that the rheological behavior of the broths during the process is closely related to biomass concentration and morphological changes of mycelia, so that viscosity could be used as a parameter for process monitoring and regulation. A set of experiments on oxygen-transfer improvement in the case of the most viscous tylosin broths by water addition was also done.

Index Entries: Fermentation broth rheology; antibiotic industrial fermentation; tylosin; cephalosporin; apramycin.

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Abbreviations: A—activity, %; B—biomass concentration, %; K —consistency index, Pa.s^{-n}; n—flow index, –; t—time, h; $\dot{\gamma}$—shear rate, s^{-1}; $\mu_{app.}$—apparent viscosity, Pa.s; μ_p—plastic viscosity, Pa.s; τ—shear stress, Pa; τ_o—yield stress, Pa.

INTRODUCTION

Many important industrial bioprocesses (e.g. antibiotic, citric acid, and penicillin production) involve rheologically complex fermentation broths. It is well established that filamentous microorganisms in these broths even in very low concentrations cause nonNewtonian behavior of such suspensions *(1–19)*. An important feature of fermentation processes is the change of rheological properties of the broths with fermentation time because of changes in mycelial concentration and morphological char-acteristics of media. There is a lack of information on this question in literature *(1,6,13,15,17)*. A serious problem in biological process investigations is the reproducibility of the results of different fermentation cycles.

There is no unique opinion on the type of viscometers most effective in the rheological measurements of fermentation broths *(1,6)*. Some difficulties emerge when the conventional types of absolute measurement techniques (such as coaxial cylinder or tube viscometers) are used. The complications are owing to *(4)* the dimensions of large particles (especially in the case of pellets), near to that of the annulus between the cylinders, which may cause a destruction of mycelia during the measurement; possible "slip effect;" settling of the suspension.

To avoid these complications, other types of viscometers have been proposed—relative measurement systems with other revolving elements, such as a turbine impeller with 6 or 8 blades or helical-ribbon impeller *(4,9–11,15)*.

The main difference between the relative and the absolute measurement techniques is that the fluid flow is so complex that the constitutive equations for rheological variables cannot be readily solved *(6)*. As a result, a relative measurement system must rely on empirical "calibration" with fluids of known rheological properties by calibration method of Metzner and Otto *(20)*. In such measurement systems, the laminar flow exists in quite a narrow range of shear rates, which is more important for low-viscosity liquids.

Comparative studies *(1,2,6,10,14)* on the methods for fermentation broths viscosity measurement had been performed and several comprehensive reviews *(3,5,6,14,21)* of the main results had been published. In many cases, serious discrepancies between the results obtained by different technics were found *(1,6)*.

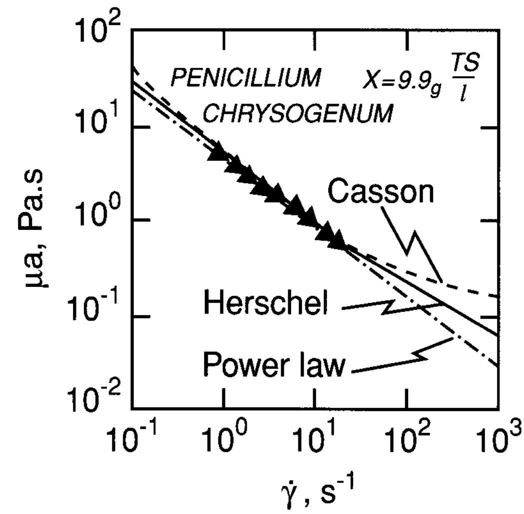

Fig. 1. A comparison of Casson, Herschel, and Bulkley and power-law plot for apparent viscosities of a suspension of Penicillium Chrysogenum, obtained from turbine impeller measurements (adapted from ref. *14*).

This asks the question to study the conditions under which the measurements of different rheometers coincide.

The most often used models for description of rheological behavior of fermentation broths are:

model of Casson *(1,6,15)*:

$$\sqrt{\tau} = \sqrt{\tau_0} + K_c\sqrt{\dot{\gamma}} \tag{1}$$

model of Bingham *(4,6,7,16)*:

$$\tau = \tau_0 + \mu_p \dot{\gamma} \tag{2}$$

power-law model of Ostvald-de-Waele *(1,2,6,8,12,14,17,19)*:

$$\tau = k \dot{\gamma}^n \tag{3}$$

model of Herchell-Bulkley *(1,6,14)*:

$$\tau = \tau_0 + k \dot{\gamma}^n \tag{4}$$

Even for a single type of microorganisms, several different rheological models have been proposed *(1,4,6–8,14,15,19)*. This fact can be attributed mainly to the range of shear rates studied because the rheological models proposed are empirical relationships, but not physical laws. Some of the authors *(1,6,14,15)* emphasized that in a sufficiently narrow range of shear rates, all the rheological models could fit the experimental data fairly well (*see* Fig. 1) *(14)*. In that case, the choice of the constitutive equation should be based on the model simplicity.

Morphological conditions should exert a profound effect on the nature of broth rheology. It could be expected that disperse filamentous growth should tend to "structure" the entire suspension and cause yield stress, τ_o, (Casson or Bingham models); pseudoplasticity (Ostvald-de-Waele model) or both *(5)*. In some of the investigations, the relation between the morphology of biological systems and their rheological parameters had been studied *(7,15)*. Roels et al. *(15)* attempted to correlate these factors theoretically and obtained satisfactory results. The work of Mitard and Riba *(13)* is devoted to morphology and growth rate of mycelial structures at constant shear rates in Couette flow conditions. Van Suijdam *(18)* had done a complex study on shear-stress influence on the morphology and growth of filamentous fermentation broths.

There are some studies concerning the dependence between the fermentation time and the rheological parameters. Richards *(22)* noticed sinusoidal changes of yield stress and plastic viscosity with time. Other authors *(2,9,14,19)* had shown that there is a maximum in the consistency index K and a minimum in flow index n. This fact can be attributed to morphological changes of fermentation broths. As Mitard and Riba showed *(13)*, during the fermentation, the microorganisms form two structures, the pellet form and the filamentous form. The average shear stress at $\dot{\gamma}$ = const. increases with fermentation time. That is why the growth-rate evolution curve shows two maxima (*see* Figs. 5–7 in ref. *13*) characterizing the pass of mycelia from one structure to another, whereas the minimum between them shows the destruction of the pellet form. It is one of the probable explanations of the sinusoidal shape of the rheological parameter's curves with fermentation time. Another one could be the relation between the biomass and the apparent viscosity growth (viscosity decreases as a result of lysis process beginning).

It is evident that there is no unique approach to explain the rheological behavior during the fermentation cycle. Moreover, the disagreements arose from the different experimental conditions used by investigators, so it is difficult to establish general conclusions. Because of that, a reason for further experimental rheological studies of different fermentation broths under industrial conditions exists.

AIM

This chapter concerns the rheology of different fermentation broths of industrially manufactured antibiotics in large scale (pilot plant and industrial equipment). Its relation to the biochemical and the morphological parameters in respect to process control by viscosity was investigated.

MATERIALS AND METHODS

Microbial Cultures

The strains used for rheological experiments were: *Cephalosporium acremonium* (fungal culture); *Streptomyces fradiae* (actinomycetic); and *Streptomyces cremeus v. tobramycini* (proactinomycetic), giving antibiotical products Cephalosporin C, tylosin, and apramycin. These processes were chosen because of their industrial and economical importance, and the rheology may cause real problems.

The compositions of the media used for various fermentations and the experimental conditions are summarized in Table 1. The investigations were done at least for two parallel fermentation cycles for each culture. Previous experiments in the same industry showed a satisfactory reproducibility of the results *(23)*. The experiments on rheology, and biochemical and microbiological parameters were done, studying for each culture pH, activity, fat concentration, glucose, nitrogen, biomass concentration, morphology, and rheological behavior during the fermentation.

Rheological Measurements

For rheological studies, a coaxial cylinder viscometer Rheotest RV2 (Germany) was used. The data obtained were compared with those derived by use of a six-blade turbine impeller, attached to Rheotest RV2 measuring head (*see* Fig. 2) in cases of more viscous "structured" broths. It was calibrated by the method of Kemblowski and Kristiansen *(9)*, using polyacrilamyde solutions with known rheological parameters. The range of shear rates where the two instruments used give similar values of shear stress was found. It was up to approx 80 s^{-1}. This range is sufficient for antibiotical fermentation broths investigations, because the stirrer speed in bioreactors for the strains studied does not exceed 170 min^{-1} [which corresponds to mean $\dot{\gamma}$ values of about 32.58 s^{-1} *(24)*]. Above these values of $\dot{\gamma}$, the impeller instrument gives higher values for shear stress τ, which can be attributed to nonlaminar flow in this range (Fig. 3). This fact may also explain the higher values for τ obtained by other authors using the impellers for viscometrie *(2,6,14)*.

RESULTS

The rheological behavior for the three types of cultural broths during the full industrial fermentation cycles was investigated. All three types of fermentation broths were found to show definite nonlinear flow curves without intercept, which could be described with the power-law rheological model. Figure 4 presents the flow curves for the three types of fermentation broths on the 72nd h of fermentation. It can be seen that the tylosin

Table 1
Composition of Fermentation Media

Antibiotic	Strain	Component	Working conditions
Cephalosporin C	*Cephalosporin acremonium* (fungal	corn steep extract, Proflo, dextryn, soy meal, $MgSO_4$, urea, KH_2PO_4, $MnSO_4$, $FESO_4$, $(NH_4)_2SO$ $ZnSO_4$, $CuSO_4$, $CaCO_3$ (*pH* stabilizer), soy oil, silicon SAG-antifoam and C-source	working volume $V = 20\ m^3$ stirrer speed $n = 180\ min^{-1}$ overpressure 0.5 atm
Tylosin	*Streptomyces fradiae* (actinomycetic)	fish meal, corn meal $NaCI$, $(NH_4)_3PO_4$, $CaCO_3$, soy-oil, Proflo, betain Silicon, beetroot molasses, KCI, $MiSO_4$	Working volume $V = 44\ m^3$ pH = 7, 3–7, 6 t = 30°C overpressure 0,5 atm gradual aeration $n = 170\ min^{-1}$
Apramycin	*Streptomyces cremeus var tobramycini* (proactinomycetic)	soy groats, NH_4CI, $Ca\ CO_3$, NH_4NO_3, sodium glutamat - antifoam, glucose	Working volume V = 50 l t = 37°C $n = 300\ min^{-1}$

broths are the most viscous, followed by cephalosporin C and apramycin (which show the lowest shear-stress values at given $\dot{\gamma}$ values).

Cephalosporium acremonium Fermentations

The first fermentation studied was that of *Cephalosporium acremonium*, producer of the antibiotic Cephalosporin C. The results of its two industrial fermentation runs are presented here. The main difference between them was the time of the beginning of starch hydrolizate solution adjusting (18th h for the first run; first hour for the second one) in order to maintain the glucose content in the medium in 0.9–1.4% intervals.

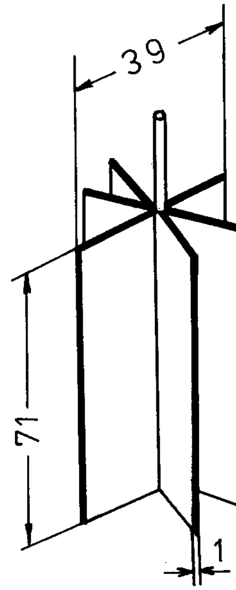

Fig. 2. Measuring element, used in tylosin fermentation broths rheological measurements (dimensions given in mm).

The duration of one fermentation for Cephalosporin C biosynthesis was about 130 h. The pH value of the media before inoculation was about 6.23. It decreased with fermentation time; its values were maintained about 5.8–6.0 by NH_4OH adjusting until the end of the process. The biomass quantity was measured after the 10th h of the process. Every 8 h, the main parameters, e.g., pH values, nitrogen, glucose and fats concentration, bio-

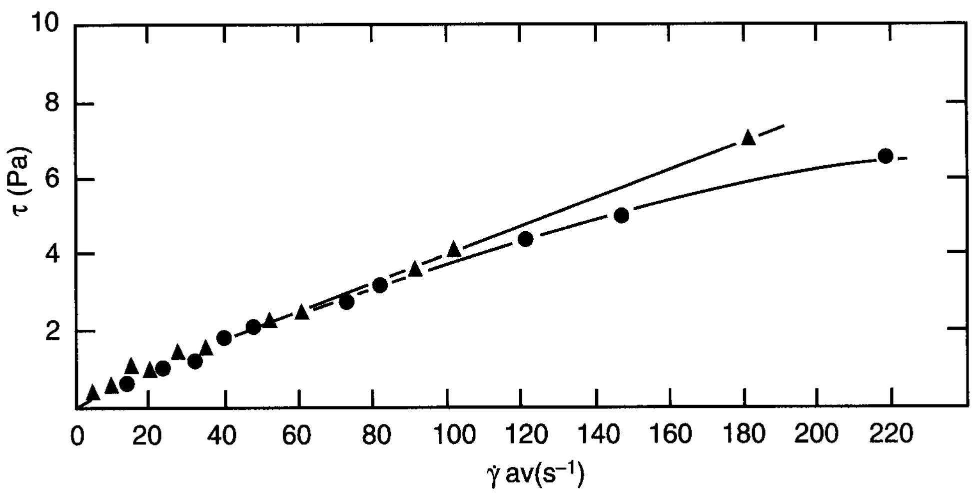

Fig. 3. To the comparison of data, obtained with different measuring elements (▲) six-blade impeller; (●) Sl cylinder.

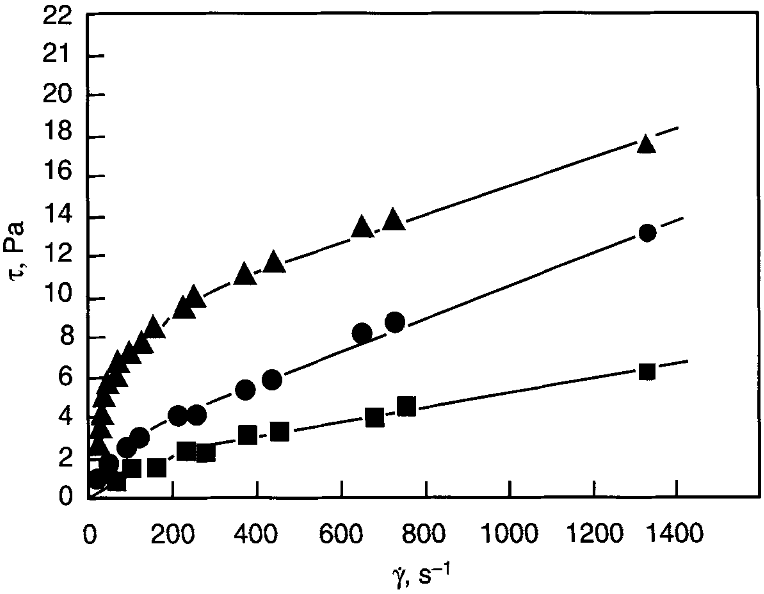

Fig. 4. Flow curves for cephalosporin (●), tylosin (▲), and apramycin (■) fermentation broths on 72 h of fermentation.

mass, and activity were monitored. At the same time rheological measurements and morphological observations were performed.

In Fig. 5, the flow curves of cephalosporin C during the full fermentation cycle are given. It can be seen that the flow curves taken for the later fermentation time lie below those from earlier h. This suggests that the shear stress (at $\dot{\gamma}$ = const.) passes through maximum.

From the flow curves, using the least squares method, the rheological parameters of power-law model K and n were found. In Fig. 6, (A,B) the changes in rheological parameters for Cephalosporin C two fermentation cycles are given. It is clear that the rheological indexes K and n also show clear extrema during the fermentation (K passes through maximum when n passes through minimum). In the same figure, the curve of biomass concentration vs. fermentation time is given. It can be seen that the biomass-concentration curve resembles that of consistency index K vs fermentation time. So that the extrema in rheological indexes K and n could be attributed to changes in biomass concentration. The correlation between the biomass-concentration dynamics and the apparent viscosity values observed could be used for process regulation by starch hydrolyzate solution dosage in dependence of biomass increase.

Figure 6C presents the time evolution of the apparent viscosity for two fermentation runs of Cephalosporin C biosynthesis. It can be seen that μ_{app} increased almost linearly until the 40th h of the fermentation, when the culture reached the maximum of the growth phase. The highest values of

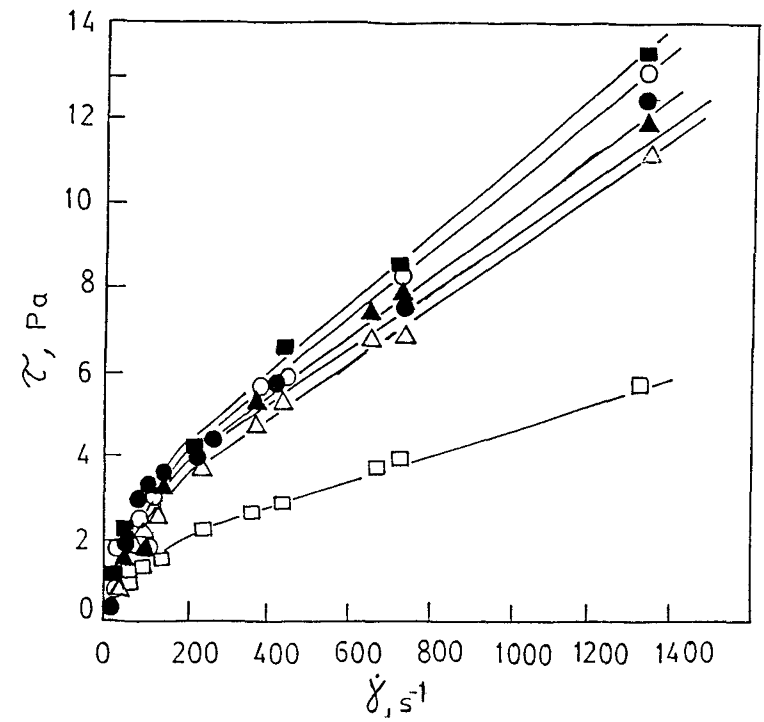

Fig. 5. Flow curves of cephalosporin C fermentation broths during the full fermentation cycle (□) 0 h (▲) 28 h (■) 52 h; (○) 76 h (●) 100 h; (Δ) 128 h.

the apparent viscosity were observed about the 90th h of the process. This was followed by a slight decrease of μ_{app} which could be explained with the beginning of the lysis process. But this μ_{app} decrease after the 120th h of the process results in high-strain productivity in spite of the lysis beginning. In the second fermentation run, the biomass concentration was lower than in the first. In the same figure, it can be seen that the maximal apparent viscosity values during this cycle were observed earlier than during the first one—about 50th–60th h, and they were lower than those observed during the first fermentation. The biomass concentration was maintained constant by adjusting the hydrolized starch solution.

These differences in process conditions resulted in serious differences in the antibiotic activity measured: during the second run the activity values increased more rapidly and reached higher values (73% for the first fermentation and 109% for the second one).

Except the rheological studies the morphological observations had also been done. During the fermentation sensible changes in mycelia morphology were observed. The first phase (until the 40th h) was characterized by forming of fine unique hyphae with spreaded septa (*see* photographs—Fig. 7A). After that, the hyphal diameters increased (Fig. 7B) and the thick-

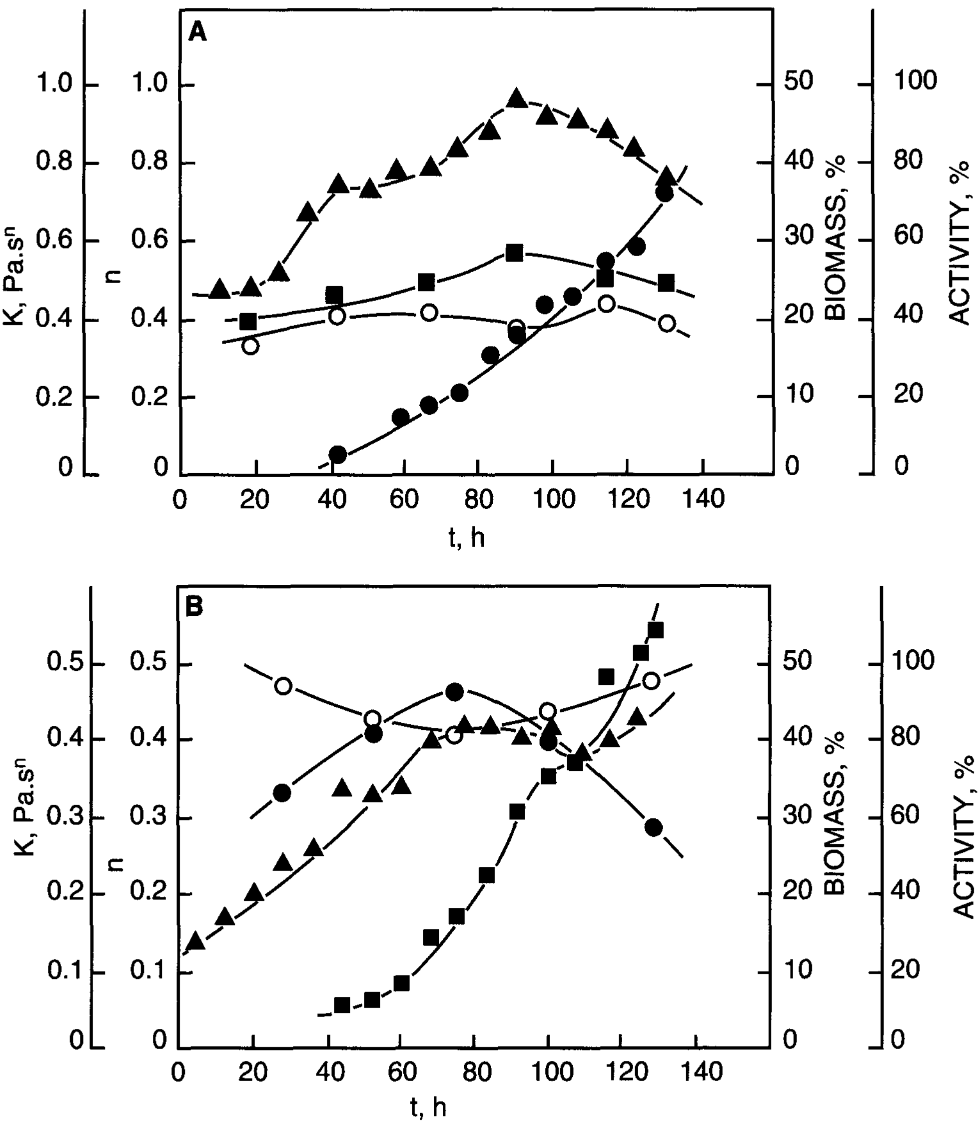

Fig. 6(A). Changes in rheological parameters *K* (■) *n* (○); biomass concentration (▲) and activity (●) during the second industrial fermentation of cephalosporin C. (**B**) Changes in rheological parameters *K* (●) *n* (○), biomass concentration (▲) and activity (■) during the first industrial fermentation of cephalosporin C.

est of them (15–20 µm) formed arthrospores (Fig. 7C, D). This morphological picture was observed in 60th–120th h range during the phase of maximal productivity. Among the hyphae, spaces larger than their diameters were formed. They developed during the process by lengthening and septation in the ends without forming netlike structures. This could be the reason of relatively slight changes in viscosity of the fermentation broths

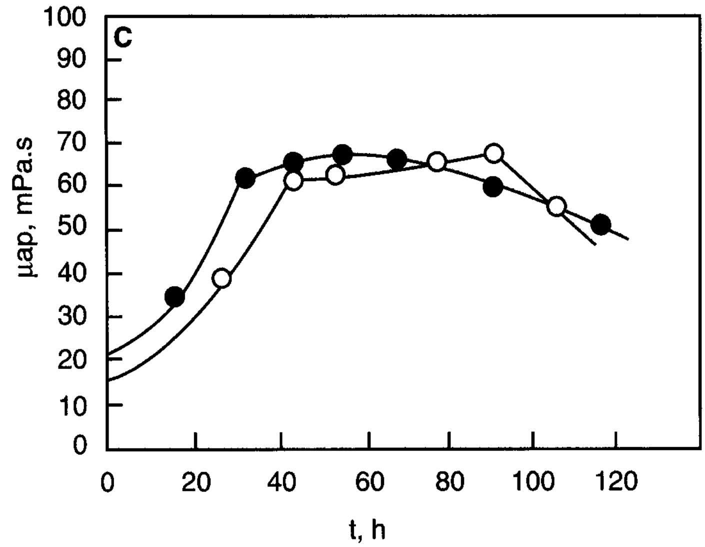

Fig. 6.(*continued*) **(C)** Dependence of apparent viscosity μ_{app} on fermentation time for two fermentation cycles of Cepfalosporin C at $\dot{\gamma} = 27\ s^{-1}$: (○) first run; (●) second run.

throughout the different phases of the process. At the end of the process (130–140 h) conidia were formed.

This morphological picture is not in contradiction with the results of other authors *(18)*, who have observed forming of "rather short and very fine hyphae" and have reported that *Cephalosporium Acremonium* is generally insensitive to shearing forces.

Streptomyces fradiae Fermentations

The second culture studied was *Streptomyces fradiae*, producer of the antibiotic tylosin, used exclusively in veterinary medicine. Similar to *Cephalosporium Acremonium*, rheological behavior was observed during the tylosin fermentation. But as has already been mentioned the tylosin fermentation broths show quite higher viscosities than the other ones studied. That is why for these broths we used not only coaxial cylinders for rheological measurements, but also a six-blade turbine impeller. More details on these experiments are given elsewhere *(23)*.

It is well known that viscosity is one of the limiting oxygen-transfer factors in aerobic fermentations *(3,5,18,25)*. The high viscosity of suspensions of filamentous mycelia causes serious problems with mass transport from the gas to the liquid phase, which decreases as a result of the strongly enlarged coalescence of bubbles. Tylosin fermentation is highly aerobic. In batch fermentation, the total oxygen consumption increases dramatically with the accumulation of cell mass during the rapid-growth phase and thus, the concentration of dissolved oxygen may gradually fall *(25)*. This is why we attempted to improve the process by adding several times (two or

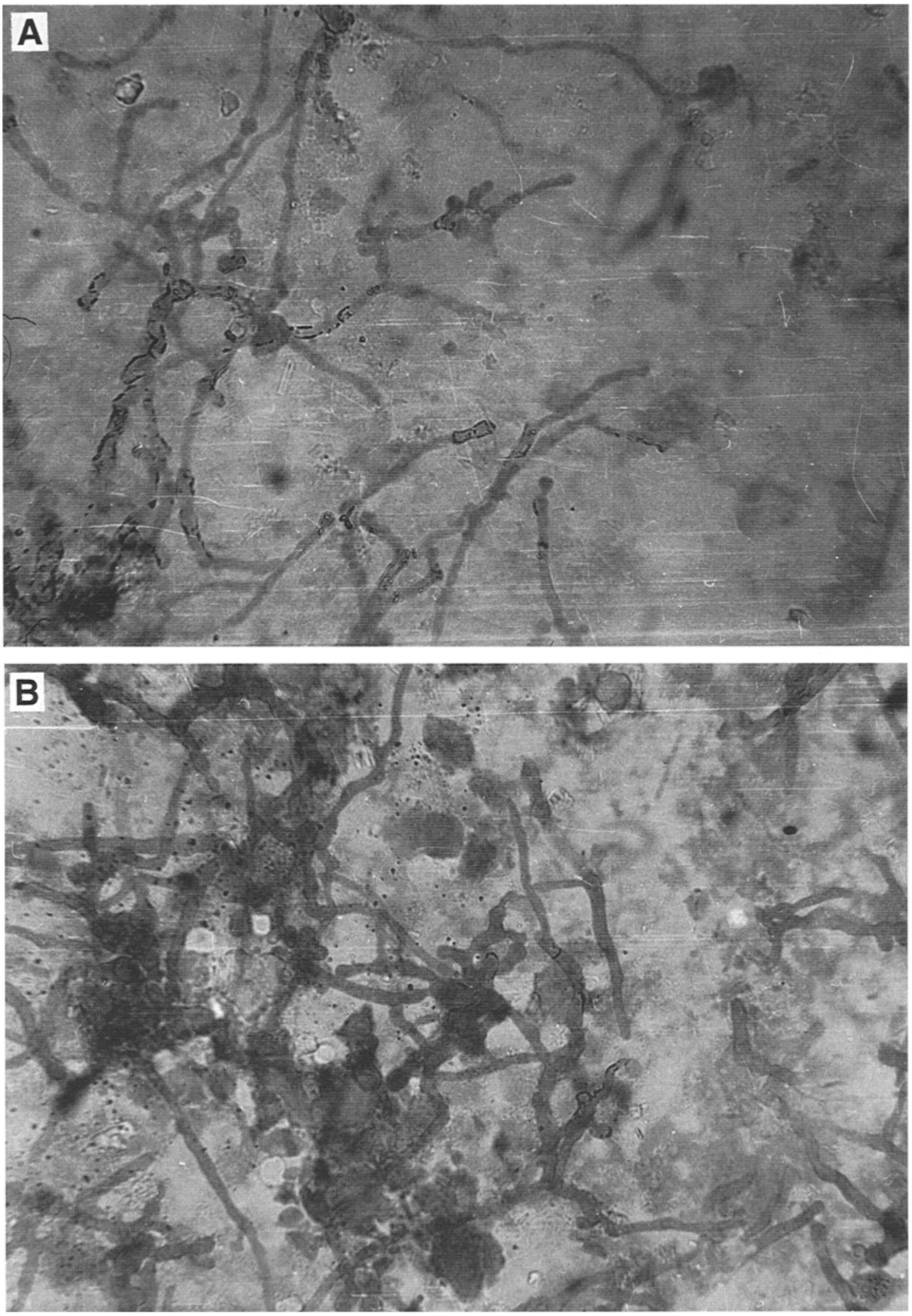

Fig. 7. Morphological characteristics of *Cephalosporium Acremonium* during its growth: (**A**) 12–20 h unique hyphae with spreaded septa, (**B**) hyphae growth and budding 20–35 h, (**C**) 35–60 h, (**D**) 110–120 h forming of arthrospores and conidia (1000 × final magnification).

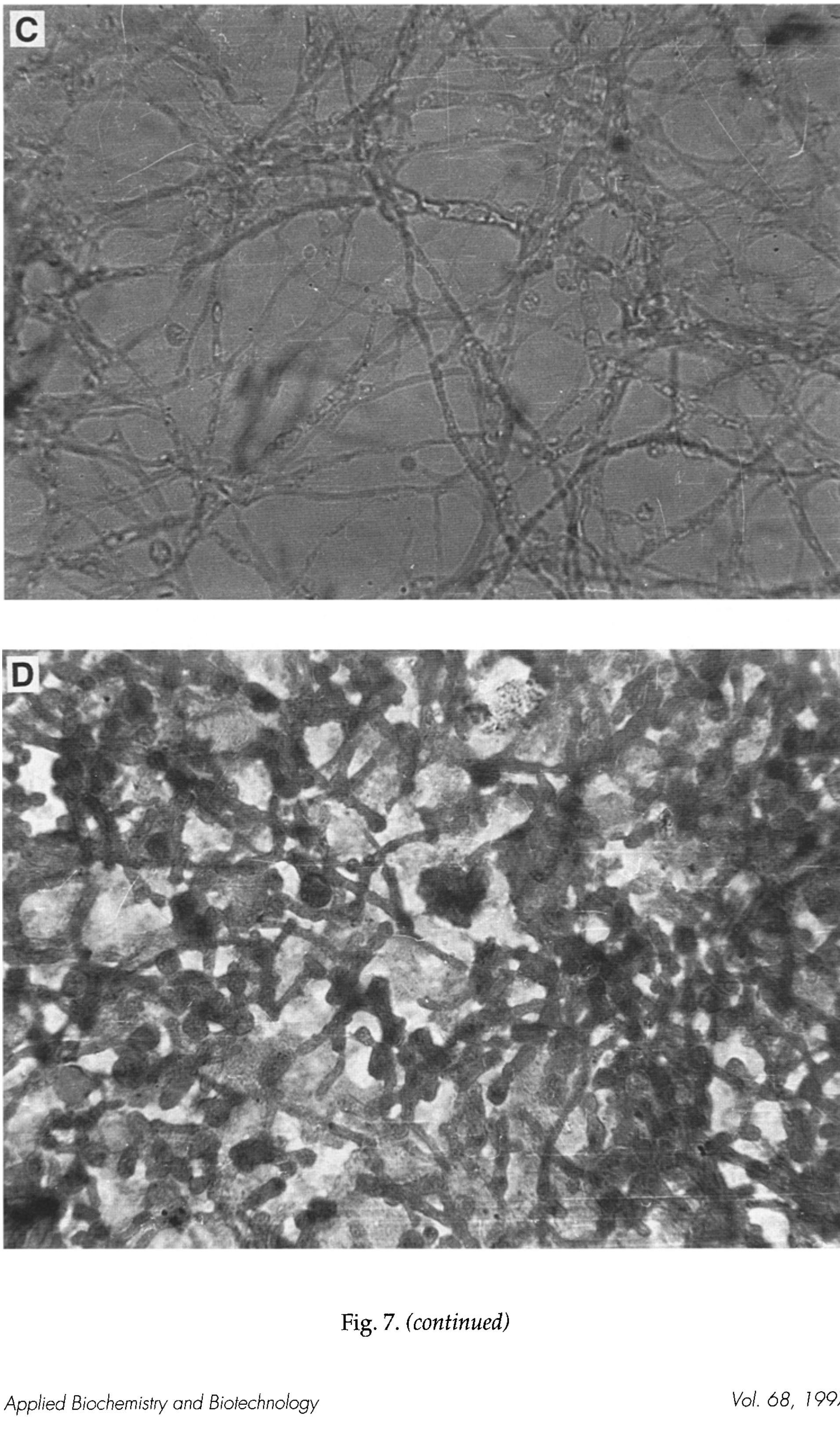

Fig. 7. *(continued)*

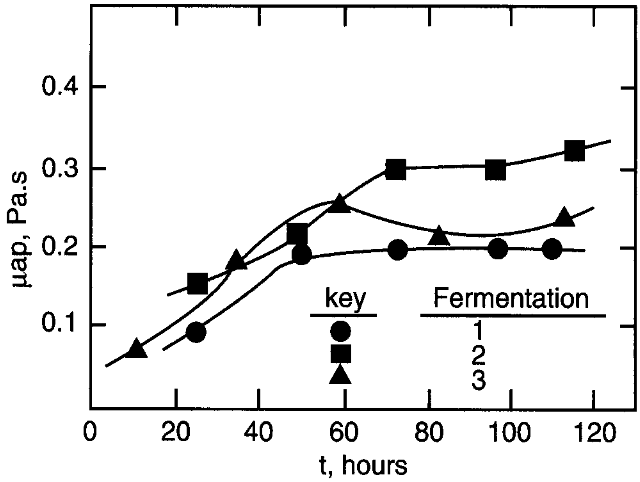

Fig. 8. Dependence of apparent viscosity μ_{app} on fermentation time for three fermentation cycles of tylosin fermentation at $\dot{\gamma}$ = 27 s^{-1}.

three) a definite quantity of sterilized water in order to maintain the apparent viscosity of the broths in given range (Fig. 8). There are some data in literature concerning the marked reduction of viscosity, obtained by diluting the broth with water *(3,21)*. During the first fermentation cycle, water was added three times in the quantity of 4% of working volume. The water quantity was chosen after previous studies so that it could provide an apparent viscosity decrease without sensitive decrease in biomass concentration. The time of water addition during the first fermentation was 40th, 65th, and 90th h of the process. During the second fermentation, water was added twice, without addition on 90th h of the process. In the Fig. 9A, the rheological index curves during the second fermentation are given. In the same figure the curves of biomass concentration (with double-fold dosage of sterilized water) and activity are shown. It is clear that in spite of the fact that biomass concentration during the second run is not higher than in the first one, the activity curve suggests a clear delay. It could be attributed to high apparent viscosity values measured on the 96th and 110th h. If the activity for the first fermentation is considered as 100%, during the second one we would have 95% activity due to the negative influence of the apparent viscosity on oxygen consumption. In Fig. 8 is shown the μ_{app} dynamics for the three fermentation cycles at shear rate $\dot{\gamma}$ = 27 s^{-1}, corresponding to the stirrer speed during the tylosin fermentation (170 min^{-1}) in industrial conditions. In Fig. 9B rheological indexes curves together with shear stress vs fermentation time curves at $\dot{\gamma}$ = const. for tylosin fermentation are given. As for the case of cephalosporin C, the consistency index *K* shows a clear maximum when the rheological index *n* shows a minimum. At the same time the shear-stress curves passed

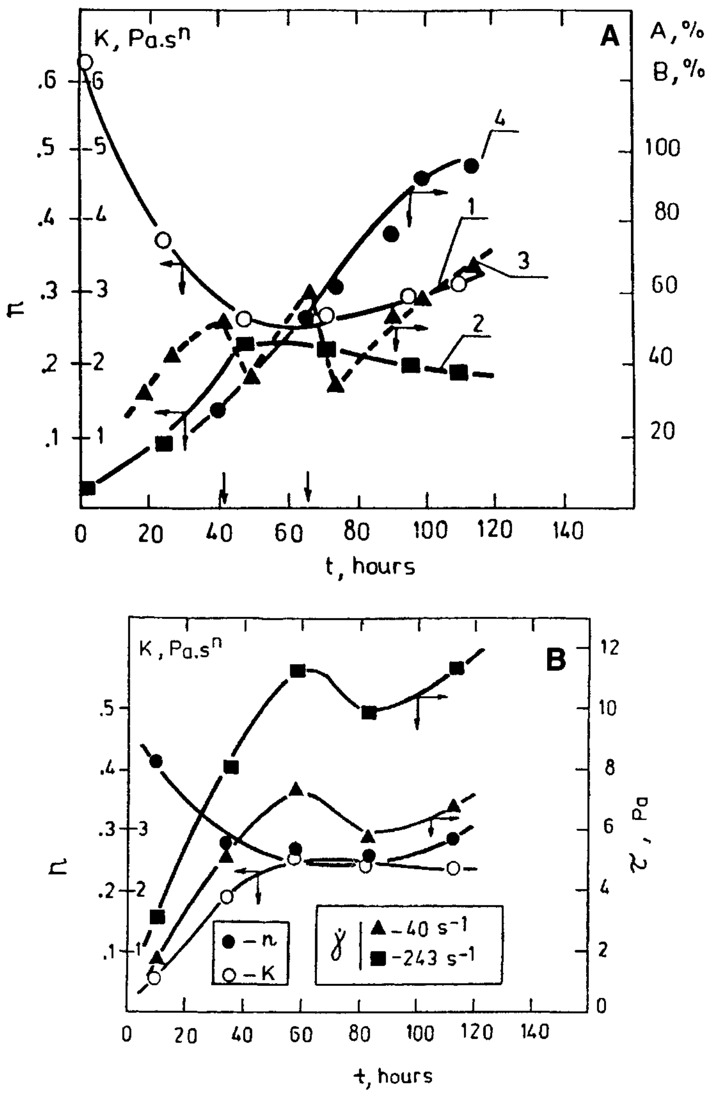

Fig. 9.(A) Dependence of rheological indexes *K* (■), *n* (○), activity (**A**, ●) and biomass concentration (**B**, ▲) on fermentation time for tylosin fermentation, second run. (**B**) Dependence of rheological indexes *K* (○) and *n* (●) on fermentation time. Dependence of shear stress τ at $\dot{\gamma} = 40\ s^{-1}$ (▲) and $\dot{\gamma} = 243\ s^{-1}$ (■) on fermentation time for tylosin fermentation, first run.

through maximum. (We must say that the rheological index curves characterize the whole range of shear rates for the full fermentation cycle, whereas the shear-stress curves are given at definite $\dot{\gamma}$ values.)

Experiments on oxygen consumption improvement in pilot-plant scale by stirrer-speed regulation were also carried out. More details on them are given elsewhere *(27)*.

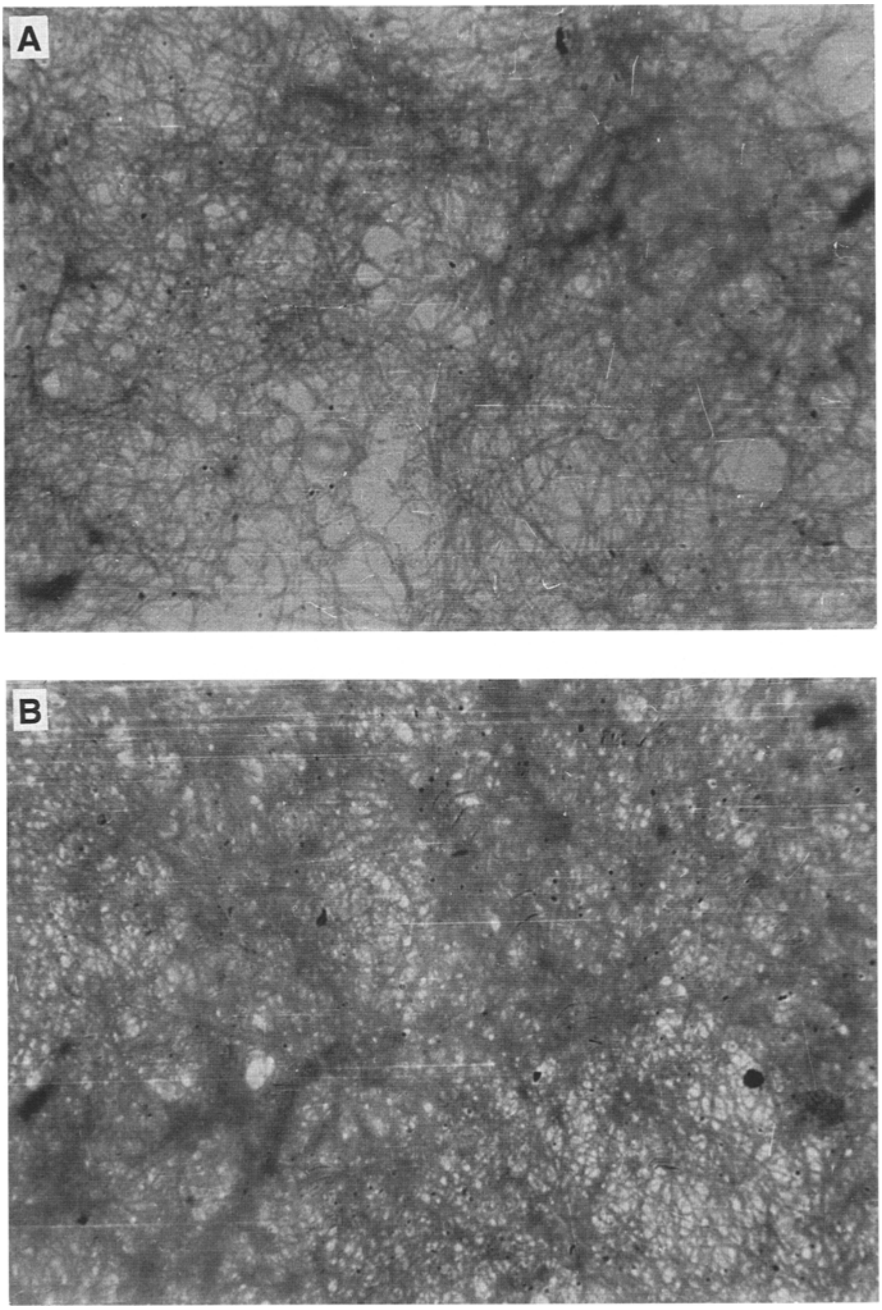

Fig. 10. Morphological characteristics of *Streptomyces fradiae* during 24–48 h (**A**) and 48–96 h (**B**) of its growth, (1000 × final magnification).

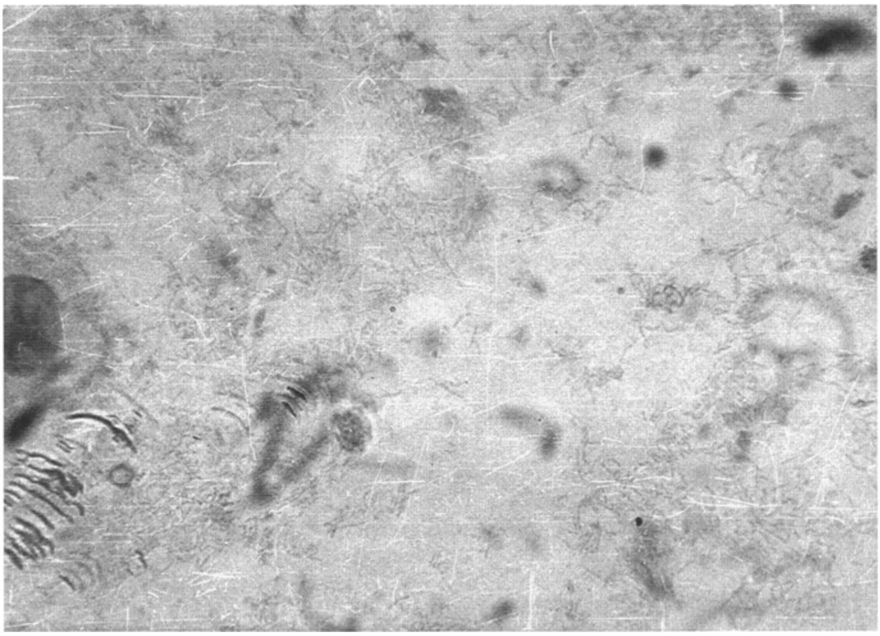

Fig. 11. Morphological picture of *Streptomyces cremeus v. tobramycini* fermentation with 1000 × final magnification.

The morhological observations during the fermentations were done. *Streptomyces fradiae* is a typical actinomycetic culture. During the fermentation, it formed long netlike structures with large length to diameter ratio (*see* photographs—Fig. 10A, B). The pellet formation was noticed almost from the beginning of the growth phase. It continued during the productive phase. Stable structures of the hyphae during the whole fermentation were observed. For actinomycetic cultures, a clearly determined nucleus is typically missing. Their reproduction is similar to that of mycelial cultures without forming transverse walls. The structuring of the fermentation broths during the process reflected in serious apparent viscosity increase.

Streptomyces cremeus v. tobramycini Fermentations

The third culture studied was the proactinomycetic *Streptomyces cremeus v. tobramycini*, from which the veterinary antibiotic Apramycin was produced. During its fermentation, the rheological picture was quite similar to that of two other fermentations studied. The fermentation broths exibited pseudoplastic behavior with the lowest apparent viscosity values of the medium before inoculation and an increase with process development.

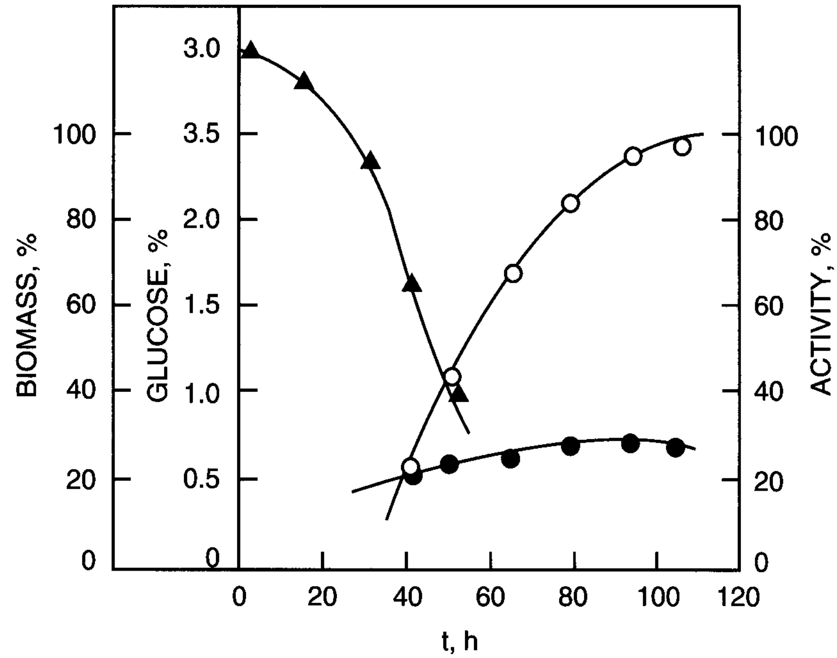

Fig. 12. Process parameters' dynamics for apramycin biosynthesis. (○) activity; (●) biomass concentration; (▲) glucose.

However, because of low values of apparent viscosities, there was no need to add water to improve the process.

The morphological observations showed that during the first h of fermentation, the morphological picture was quite similar to that of actinomycetic cultures—relatively long unfragmented hyphae were formed (Fig. 11). After the 24th h of the process, the fragmentation began and the morphological picture resembled that of bacterial cultures—fine hyphae with length-to-diameter ratio near unity during the growth phase. At the end of the growth phase most of them fragmented and only a small part maintained the secondary culture growth. The picture after septing was quite similar to that of bacterial cultures, which makes its behavior closer to them than to typical actinomycetic cultures like *Streptomyces fradiae*.

Glucose, as the main carbon source, was consumed intensively, and by the 54th h of the process had been consumed entirely. After this time the biomass concentration remained constant (*see* Fig. 12).

The small dimensions of cells without agglomeration and relatively low biomass concentration (24–27%) are the reason for fairly low apparent viscosity values, quite lower than in the cases of two other fermentations studied. These relatively low apparent viscosity levels do not cause serious problems in apramycin production.

DISCUSSION

In this article, we have described the rheology and the morphology of the fermentation broths of three cultures: fungal, actinomycetic, and proactinomycetic, in respect to the study of the dependence between the rheology and other process parameters. In other works, different rheological models have been proposed. For example, Deindoerfer and West *(8)* used the Bingham model to describe the rheological behavior of *Streptomyces griseus* broths and mentioned that it became Newtonian towards the end of the process as a result of hyphal fragmentation and lysis.

For the three organisms investigated, even the nutrient media were nonNewtonian (Fig. 5). The fermentation broths also demonstrated definite non-Newtonian behavior. Their flow curves are nonlinear without intercept, so they could be described by the Ostvald-de-Waele power-law rheological model (Eq. 3). This is why the rheology of the three cultures was described in terms of the consistency index K and the power-law index n. In all fermentations, the rheological constants passed through extremums (K—through maximum; n—through minimum). This fact is consistent with the results of other authors for other fermentation broths *(2,9,14)*.

Cephalosporin C broths were observed to have more or less similar profiles for biomass concentration and K index, a fact noted by Warren et al. *(26)* for actinomycets in submerged culture. In tylosin broths, not only the biomass concentration, but also the morphology strongly influenced K. The different morphology of the strains studied influenced their rheological parameters; because all were pseudoplastic, they possessed extremely different consistency as a result of their morphology. The tylosin fermentation broths are most viscous (Fig. 4) as a result of the strong structuring (Fig. 10). This compelled us to try different ways to improve the process of tylosin fermentation (by water addition and by changing the stirrer speed), which influenced the oxygen consumption.

The time at which K declined depended on the organism and the fermentation conditions. In *Streptomyces cremeus v tobramyciny* fermentation, the apparent viscosities reached were quite low and the changes in the rheological parameters were very slight owing to the morphological picture of the culture, which, as already mentioned, after 24th h of fermentation more closely resembled that of bacterial cultures and not that of filamentous microorganisms.

CONCLUSIONS

An experimental study of the rheological behavior of three morphological types of fermentation broths in relation to other process parameters was carried out in industrial and pilot-plant bioreactors. It showed that the rheological behavior of the broths during the process is closely related to biomass concentration and morphological changes of mycelia, so that viscosity could be used as a parameter for process monitoring and regulation. A set of experiments on oxygen-transfer improvement in the case of the most viscous tylosin broths by water addition were also carried out.

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