9. W. M. DION, A. CARILLI, G. SERMONTI and E. B. CHAIN. — **The effect of Mechanical Agitation on the Morphology of Penicillium chrysogenum Thom in Stirred Fermenters.**

Summary. — The morphology of *P. chrysogenum* strain 47.1564 was studied in submerged culture with varying intensities of mechanical agitation, in both the baffled and vortex systems of aeration. When the agitation was mild the hyphae were long, attenuate and little branched, whereas with vigorous agitation the hyphae were short, thickened and very branched. The degree of shortening and branching, up to a certain limit, was dependent upon the intensity of the agitation. The introduction of oxygen gas into fermenters with mild agitation also resulted in a shortening and thickening of the hyphae.

Mycelial autolysis occurred regularly with mild agitation, due to insufficient aeration, and with very high intensities of agitation, due to excessive mechanical damage. The penicillin yields were found to be best with a short mycelium when the aeration was adequate and the agitation not sufficiently intense to cause excessive mechanical damage.

Changes in aeration rates and intensities of agitation found in scaling up from pilot plant to industrial size fermenters are discussed.

Riassunto. — Si è studiata la morfologia di *P. chrysogenum* ceppo 47.1564 in coltura sommersa con intensità variabili dell'agitazione meccanica, in entrambi i sistemi di aerazione, con tramezzi e a vortice. Quando l'agitazione era mite, le ife erano lunghe, attenuate e poco ramificate, mentre con una agitazione vigorosa le ife erano corte, ispessite e molto ramificate. Il grado di accorciamento e di ramificazione, fino a un certo limite, era dipendente dalla intensità dell'agitazione. L'introduzione di gas ossigeno nei recipienti di fermentazione con mite agitazione pure ebbe per risultato un accorciamento e un ispessimento delle ife.

Un'autolisi del micelio ebbe luogo regolarmente con una agitazione mite, a causa di insufficiente aerazione, e per contro, con intensità molto forti dell'agitazione, a causa di un eccessivo danno meccanico. I rendimenti migliori di penicillina si constatarono con un micelio corto quando l'aerazione era adeguata e l'agitazione non era abbastanza intensa per causare un eccessivo danno meccanico.

Si discutono i mutamenti della entità dell'aerazione e delle intensità

dell'agitazione riscontrate nel passare di scala dall'impianto pilota ai recipienti di fermentazione di grandezza industriale.

Résumé. — On a étudié la morphologie de *P. chrysogenum*, souche 47.1564, en culture submergée avec des intensités variables de l'agitation mécanique, en se servant du système d'aération à chicane et a vortex. Lorsque l'agitation est moderée, les hyphes sont longues, atténuées et peu ramifiées, tandis que dans le cas d'agitation vigoureuse, les hyphes sont courtes, épaisses et très ramifiées. Jusq'à un certain point, le degré de ramification et les courtes dimensions dépendent de l'intensité de l'agitation. L'introduction d'oxygène dans les cuves de fermentation avec une agitation moyenne a aussi comme résultat de raccourcir les hyphes et de les rendre plus épaisses.

L'autolyse du mycelium a lieu régulièrement avec une agitation moyenne en raison de l'aeration insuffisante; il en est de mème avec une très forte agitation, l'effort mécanique étant excessif. Les meilleurs rendements en pénicilline ont été obtenus avec un mycelium court quand l'aération est suffisante et l'agitation pas assez intense pour causer des dommages mécaniques excessifs.

On expose les changements des taux d'aération et des intensités d'agitation observés, en passant de l'echelle de l'installation pilote à celle des cuves de fermentation de dimensions industrielles.

Zusammenfassung. — Der mechanische Effekt der Rührungsintensität in verschiedenen Belüftungssystemen (Vortexsystem und Belüftung durch Luftverteiler in Gegenwart von Prallblechen) auf die morphologische Formbildung von *P. chrysogenum* Stamm 47-1564 in Tiefenkultur wurde untersucht. In Gegenwart langsamer Rührung waren die Hyphen lang, dünn und wenig verzweigt, in Gegenwart starker Rührung hingegen waren die Hyphen kurz, verdickt und stark verzweigt. Der Grad der beobachten Verkürzung und Verzweigung der Hyphen war bis zu einem bestimmten Grenzwert abhängig von der Rührungsintensität. Belüftung mit reinem Sauerstoff in Gegenwart langsamer Rührung hatte den gleichen Effekt wie intensive mechanische Rührung, nämlich die Bildung von kurzen und verzweigten Hyphen.

Autolyse des Mycels erfolgte regelmässig in Gegenwart langsamer Rührung als eine Folge des Sauerstoffmangels; sie trat ebenfalls auf in Gegenwart intensiver Rührung als eine Folge der zu starken mechanischen Schädigung. Die höchsten Penicillinausbeuten wurden mit der kurzen, verzweigten Hyphenform erhalten, in Gegenwart ausreichender Belüftung

und einer Rührungsintensität von nicht so hohem Grade dass mechanische Schädigung des Mycels eintrat.

Aenderungen der Sauerstoffdiffusionsgeschwindigkeit und der Rührungsintensität, die beim Uebergang von Fermentergefässen von Laboratoriums und Versuchsanlagedimensionen zu Fermentern industrieller Ausmasse auftreten, werden diskutiert.

Introduction

In a previous communication (Camici, Sermonti and Chain 1952) observations on the organization of the mycelium of *P. chrysogenum* Thom in submerged culture in shake flasks were recorded. In particular, the conditions pertinent to obtaining mycelial growth either in pellet form or the filamentous form were established.

During the course of studies being pursued in this department on factors influencing the biological production of penicillin in mechanically stirred fermenters, it was noted that the mycelial growth occured in various forms of differing morphological appearance, depending, among other factors, on the degree of intensity of the mechanical agitation (*). In view of the considerable differences in the morphological sutructure it was felt that the different hyphal forms may also exhibit a different metabolic behaviour. Furthermore, as the aeration efficiency in stirred fermenters is proportional to the degree of air-water turbulence created by the propeller blades, i.e. to the agitation intensity, and as the agitation intensity affects the morphology of the mycelium, it is clearly impossible to evaluate correctly the biochemical effects of aeration without taking into account the effect of mechanical agitation on the type of mycelium produced. Reproducible biochemical results in stirred aerated fermenters, and the correct interpretation of these, could only be hoped for if reproducible conditions for the formation of the different types of mycelium could be established.

For this purpose, and in order to correlate biochemical and mycological observations it was considered essential to undertake a systematic study of the different mycelial forms growing in aerated stirred

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(*) The first observations of the influence of intensive mechanical agitation on the morphological appearance of the mycelium of *P. chrysogenum* were made in collaboration with S. Paladino during the first experiments on penicillin production with the vortex system of aeration.

fermenters. Duckworth and Harris (1949) have dealt with the development of the mycelium of *P. chrysogenum* in submerged culture in a stirred fermenter; the observations reported in their paper refer, however, only to one set of conditions of agitation.

Methods

Strain. — The strain Q.176 471564 (*) of *P. chrysogenum* Thom. was used. The stock cultures were maintained in sterile soil and every month transfers were made from these to agar slants. Conidial suspensions from the agar cultures were used to inoculate small flasks containing 5-10 gms of corn meal moistened with 1-2ml of a solution containing 0.1% asparagine and 3% glycerol in tap water. (Whiffen and Savage 1947).

When sporulation was well advanced after about two weeks, 50 ml of sterile distilled water was added to the flasks and the resulting conidial suspension used for inoculation of the seed tanks.

Conditions of growth

1. - *Fermenters.* — The mycelium was grown in stainless steel agitated fermenters. (Chain, Paladino, Ugolini, Callow and Van der Sluis, 1954). Most of the morphological observations were made on mycelium grown in ten litre fermenters, containing five litres of culture medium, but in addition some experiments were run in a fermenter of 3000 litres capacity containing 2200 litres of culture medium, and in fermenters of 12.000 litres capacity with 10.000 litres of culture medium.

2. - *Aeration.* — Aeration in stirred fermenters is achieved by dispersing the air into fine bubbles by means of a rotating propeller. The air may enter the culture fluid through a sparger or may be sucked into it by a vortex formed by a fast rotating propeller. When a sparger is used for the introduction of air, baffle plates may be inserted into the culture fluid to facilitate air dispersion.

Factors influencing aeration efficiency and extensive measurements of aeration rates under different conditions of agitation have been re-

(*) Obtained from University of Wisconsin, Madison, Wis.

ported in other communications (Chain, Paladino, Callow, Ugolini and Van der Sluis, 1952, Chain and Gualandi, 1954).

a) *Sparger system.* — In the ten litre fermenters for aeration with the sparger system in the presence of baffle plates an eight bladed propeller of the disk turbine type (see Chain et al. 1952, 1954) of 75 mm diameter was used at three different speeds of agitation. The aeration rates at these speeds, in the presence and absence of mycelium, with an air flow through the sparger of 2.5 litres of air per minute and an overpressure of 0.5 atm. are given in Table 1. They were determined by the amperometric method by means of the rotating platinum electrode (Chain and Gualandi, 1954).

Table 1.

Aeration rates in 10 L fermenters containing 3 L of culture medium with a disk-turbine propeller blade of 75 mm diameter, and an air flow of 2.5 l/min. at an overpressure of 0.5 atm.

Agitation speed r. p. m.	Aeration rates (ml O_2/100 ml soln hour)	
	in absence of mycelium	in presence of mycelium (1.5% dry weight)
360	60	45
600	185	90
830	360	190

In the 3000 litre fermenter in the absence of baffles a propeller of 350 mm diameter was used with an agitation speed of 350 r.p.m. and an air flow through the sparger of one volume of air per volume of medium per minute. These conditions gave an aeration rate of 326 ml O_2/100 ml of culture fluid per hour at one atmosphere over pressure.

In the 12.000 litre fermenter an eight bladed disk-turbine propeller of 600 mm diameter was used at an agitation speed of 160 r.p.m., with an air flow through a one hole sparger of 10 m^3 per min., at an overpressure of 1 atm. This gave an aeration rate of 400 ml O_2/100 ml of culture fluid/hour.

Vortex system. — For the ten litre fermenters two eight bladed disk-turbine propellers, of 65 mm and 90 mm diameter respectively, were used. The agitation speeds employed during the experiments and the corresponding aeration rates are listed in Table 2.

TABLE 2.

Aeration rates in 10 L fermenters containing 5 L of culture medium obtained with the vortex system with an air flow over the surface of the culture fluid 2.5 l/min.

Diameter of propeller blade (mm)	Agitation speed r. p. m.	Air overpressure atm.	Aeration rates (ml O_2/100 ml soln hour)	
			in absence of mycelium	in presence of 1.5% dry weight of mycelium
65	900	0.5	105	65
	1200	0.5	175	105
	1450	0.5	230	45
	1850	0.5	315	210
90	560	1.5	150	100
	750	1.5	275	225

The agitation speed and aeration rates for the 3000 litre fermenter are given in Table 3.

TABLE 3.

Aeration rates in 3000 litre fermenter containing 2200 litres of culture medium, obtained with the vortex system with an air flow over the surface of the culture fluid of 300 litres/min. and an overpressure of 1 atm.

Diam. of propeller blade (mm)	Agitation speed r. p. m.	Aeration rate (ml O_2/100 ml soln hour) (in absence of mycelium)
350	720	140
200	1460	160

3. - *Culture medium.* — A corn steep medium, containing 3% corn steep solids, 3% lactose 0.7% calcium carbonate and 0.1% sodium sulphate was used for the majority of the experiments. A few fermentations were run with the synthetic medium of JARVIS and JOHNSON (1947).

Foam was controlled by the addition every six hours of 0.1% grape seed oil containing 3% alkaterge C. In later experiments the content of alkaterge was increased to 30%, but this had no effect on the appearance of the mycelium.

4. - *Growth of Inoculum.* — The seed for the small ten litre fermenter was always grown in a 10 l fermenter with the vortex system

of aeration, and an agitation speed of 900 r.p.m. using a propeller of 65 mm diameter. An air flow across the surface of the medium of 2.5 litres per minute was used with an overpressure of 1.5 atm. After 35-40 hours growth under these conditions, at which time the dry weight of the mycelium was about 0.8%, an amount of the seed culture corresponding to 5 per cent was transferred to the experimental fermenters.

The seed for the 3000 litre fermenter was grown in a baffled fermenter of 300 litres capacity, with an agitation speed of 190 r.p.m. using a propeller of 250 mm diameter and an air flow of 1 volume per volume of medium per minute, at 1 atm. pressure.

For the 12,000 litre fermenters the seed was grown in an unbaffled fermenter of 500 litres capacity with an agitation speed of 190 r.p.m. using a propeller of 360 mm diameter and air flow of 1 volume/volume of culture medium/min. at 1 atm. pressure.

Results

The two main types of mycelium of P. chrysogenum *formed in stirred fermenters*

While it is difficult to describe in precise terms an organism as variable as a fungal mycelium it has been possible to distinguish clearly two main types of growth, which appeared in the stirred fermenters according to the intensity of the mechanical agitation.

1. *A long filamentous mycelium* (fig. 1). — This is what is considered to be the normal growth form for *P. chrysogenum* in submerged culture in the absence of vigorous mechanical agitation.

The units, consisting of a hypha and its laterals, are always longer than 250 µ. The diameter of the hyphae is about 2-3 µ and the septa are 30 µ-40 µ apart. There is little branching. The lateral hyphae are also long and taper gradually towards the tips. While this type of mycelium is much longer than the « short » mycelium described below the hyphae rarely reach the length attained by those grown on solid media or in shake flasks.

2. *A short fragmented mycelium.* (figs. 2, 3 and 4). — This type of mycelium is characteristic of growth under conditions of vigorous mechanical agitation. The hyphae are short, branched, and thickened, forming small rigid units. The degree of shortening and thickening is

largely dependent on the degree of agitation. The hyphae vary from about 40 μ-250 μ in length and 2.5 μ-4 μ in diemeter. The lateral hyphae are numerous, with a pronounced narrowing towards the tips, especally in a young mycelium.

Hyphal development in stirred fermenters with sparger aeration.

1. *10 litre laboratory fermenters, provided with baffle plates.* — Three different agitation speeds were used (Table 1). With the lowest speed, 350 r.p.m., the mycelium was always of the long filamentous type. In the early stages of growth after inoculation the hyphae continued to elongate and branch forming a tangled network of long, attenuate hyphae. The lateral branches were widely spaced, and of about the same diameter as the parent hyphae. A few of the « wide forms » described by Duckworth and Harris, which were slightly thickened and sometimes distorted hyphae, were present, but they were rare and usually disappeared after the first day (*).

A small number of the cells was always in a state of autolysis in these fermentations, but complete collapse and regrowth was never observed,. About 70 or 80 hours after transfer the long hyphae were sometimes observed to break up into short unbranched fragments composed of living cells often attached to empty hyphal walls. These short pieces of hyphae, although falling within the length measurements of a short mycelium, differ in the mode of formation, diameter and length of cells from a true short mycelium and are not characterized as such.

When the rate of stirring was increased to 600 r.p.m. the course of the mycelial development was conspicuously altered. For 20 hours after transfer the hyphae remained long, but became slightly thickened with a diameter of 2.7 μ to 4.0 μ and with septa much closer together. They produced a large number of lateral branches in the first period of growth. These branches were commonly formed in bunches, one cell of the parent hypha producing two or three laterals which were often branched themselves. These bunches were composed of short, rigid

(*) These thickened hyphae appeared irregularly in these experiments and were more numerous at the higher rates of agitation. They were sometimes formed from enlarged cells which survived in the long fine hyphae after they broke up, and sometimes from thickened hyphae which were present in the inoculum at the time of transfer. Since these thickened hyphae never amounted to more than 1% of the total mycelium little work was done with them, but on the few occasions when these forms were transferred to an agar medium they always gave rise to long, attenuate hyphae indistinguishable from the normal type.

twig-like hyphae (fig. 5) often of finer diameter than the parent, and all tapered very sharply towards the tips. Between 30 and 40 hours after transfer the longer parent hyphae broke up and emptied, releasing the bunches of short lateral hyphae. These now constituted a short mycelium (fig. 2). Among these, « witches broom » formations were conspicuous. The empty walls of the original long hyphae were a characteristic feature of vigorously agitated cultures. They sometimes persisted for 70 hours, as fragments attached to one or more of the living lateral hyphae.

The short hyphae, at the above rate of agitation, very rarely showed any signs of mechanical damage. No evidence has been obtained to show that they are formed by the breakage of long hyphae into short pieces by the mechanical action of the propeller. They are, in fact, the lateral branches produced profusely under the influence of mechanical agitation in the early stage of growth.

After the development of the short mycelium at about 36 hours there was usually little change in the hyphae. They became rounded at the tips, losing the characteristic pointed shape of the early growth period, but remained short throughout the course of fermentation. Apparently they were prevented from elongating by the intense mechanical agitation. There was usually an increase in the number of empty cells as the culture aged. The hyphae would become vacuolated, and often the cell contents would round off. There was usually no autolysis in the young cultures in this type of fermentation in contrast to a small amount of continuous autolysis at 350 r.p.m.

When the agitation was very intense with a stirring speed of 800 r.p.m., the mycelium was short and branched after the first day. However, in these cultures there was always a considerable amount of autolysis. This was apparent as early as 24 hours after transfer and increased until at 74 hours about half the hyphae were empty and the living hyphae were broken up into short, unbranched, fine fragments. There was a little new growth from these fragments but the cultures generally remained in a very poor condition.

The effect of vigorous mechanical agitation was not only to shorten the hyphal unit, but also the length of the individual cells within one unit. This was shown by micrometric measurements of the total length of the hyphal unit and the distances between two septa within the hyphal unit. The results of such measurements are reported in table 4.

TABLE 4.

Effect of agitation on hyphal dimensions (10 l fermenters, fully baffled system, air sparger.)

Diameter of propeller blade	r. p. m.	Mycelial Characteristics					
		24 hrs after inoculation			72 hrs after inoculation		
		Mycelial type	Length of units	Length of cells	Mycelial type	Length of units	Length of cells
75 mm	360	long	> 250 μ	35.28 μ	long	> 250 μ	32.76 μ
75 mm	600	long	250 μ	27.38 μ	short	170 μ	17.01 μ

With mild agitation in presence of baffle plates the cell length, about 35 μ, remained approximately constant for 72 hours; with increased agitation intensity the cell length was shorter after 24 hours and became still further reduced after 72 hours.

The frequency of branching is another mycelial characteristic which is greatly affected by the intensity of the agitation. This factor is difficult to estimate quantitatively. In a long mycelium it is almost impossible to observe the number of branches on a long tangled hypha, but in conditions of mild agitation it was normal for the shorter hyphal units of 250 μ-300 μ long in a 70 hours culture to be unbranched. With more vigorous agitation hyphal units 170 μ long after 72 hours growth had an average of 1.5 branches for each unit, whereas hyphal units 80 μ long of the same age, but produced under the vortex system of agitation, contained an average of 2.4 branches for each unit. Thus, the frequency of branching increases with increasing intensity of agitation. It is obvious that since there is no significant difference between the growth rates of cultures grown with mild or vigorous agitation, the change in growth habit brought about by mechanical agitation is a change from mere elongation with few branches formed, to the production of many short hyphae with profuse branching.

Effect of oxygen on hyphal development in stirred fermenters.

In order to study the mycelial development in the presence of an excess of oxygen supply but under conditions of mild agitation, experiments were set up in ten litre baffled fermenters in which oxygen gas instead of air was passed through the culture fluid while the pro-

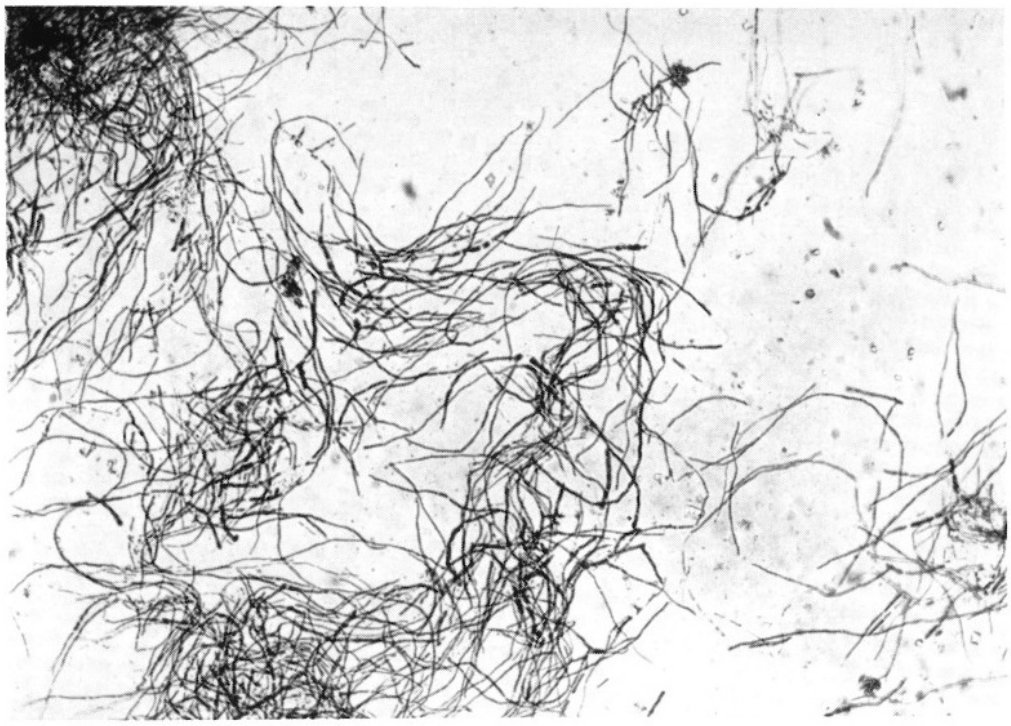

Fig. 1. - Long, filamentous mycelium after 58 hours growth. (Baffled system, 360 r.p.m., 75 mm φ) × 150.

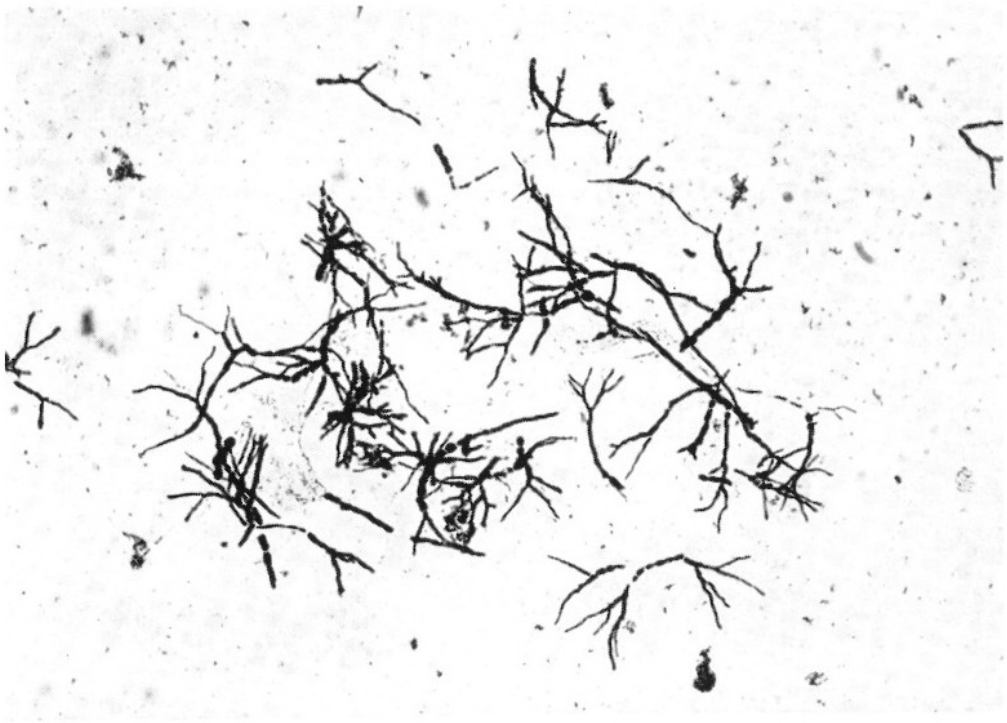

Fig. 2. - Short, branched mycelium after 36 hours growth. (Baffled system, 600 r.p.m., 75 mm φ) × 150.

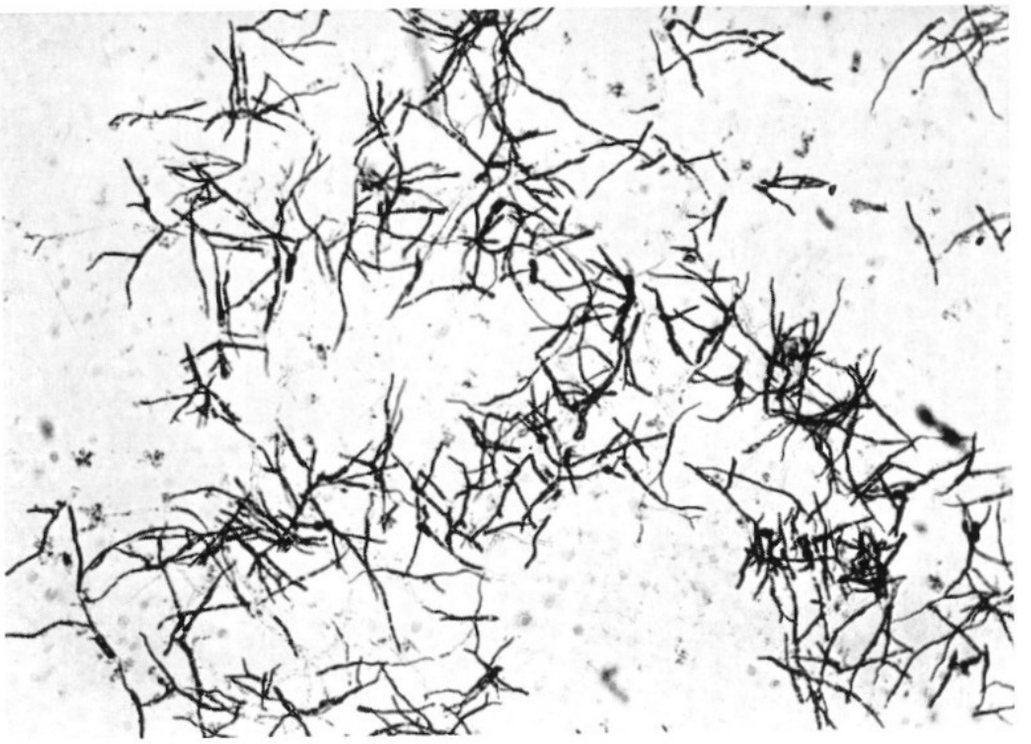

Fig. 3. - Short, branched mycelium after 48 hours growth.
(Vortex system, 750 r.p.m., 90 mm φ) × 150.

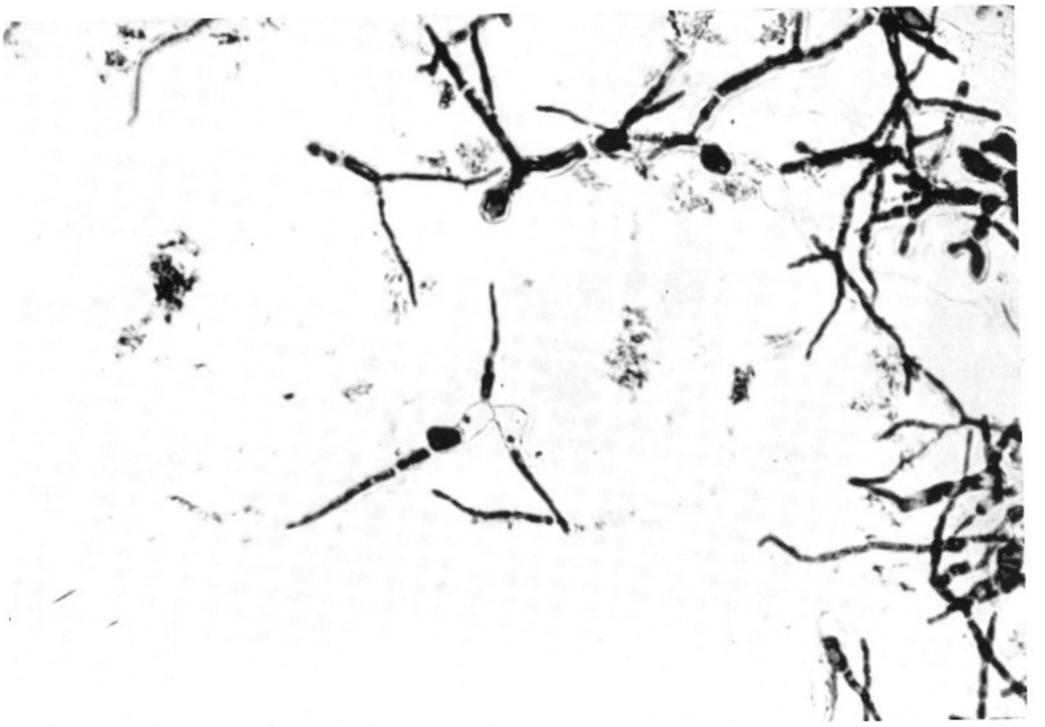

Fig. 4. - Short mycelium after 72 hours growth showing empty cells in the center of the hyphal units.
(Vortex system, 750 r.p.m., 90 mm φ) × 450.

Fig. 5. - Lateral production in a vigorously agitated culture 24 hours after transfer. (Baffled system, 620 r.p.m., 75 mm φ) × 150.

Fig. 6. - Mycelium produced under conditions of mild agitation when the air stream is replaced by oxygen gas.
(Baffled system, 350 r.p.m., 75 mm φ) × 150.

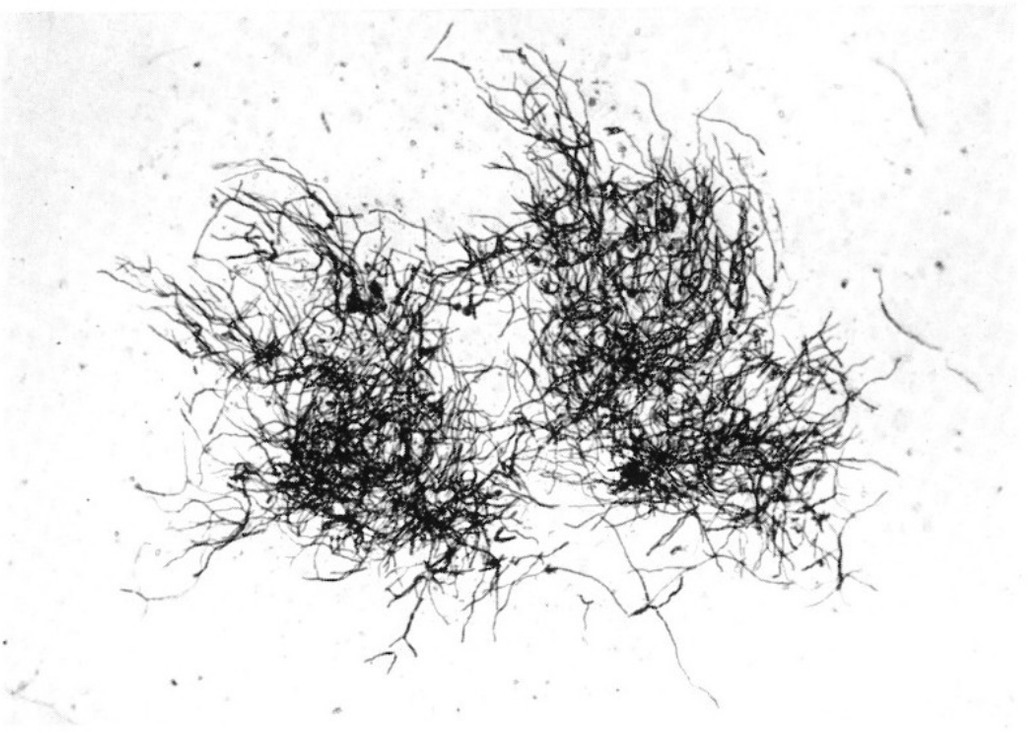

Fig. 7. - Long, filamentous mycelium after 72 hours growth in 3000 litre fermenter. (Air sparger system, 350 r.p.m., 350 mm φ) × 150.

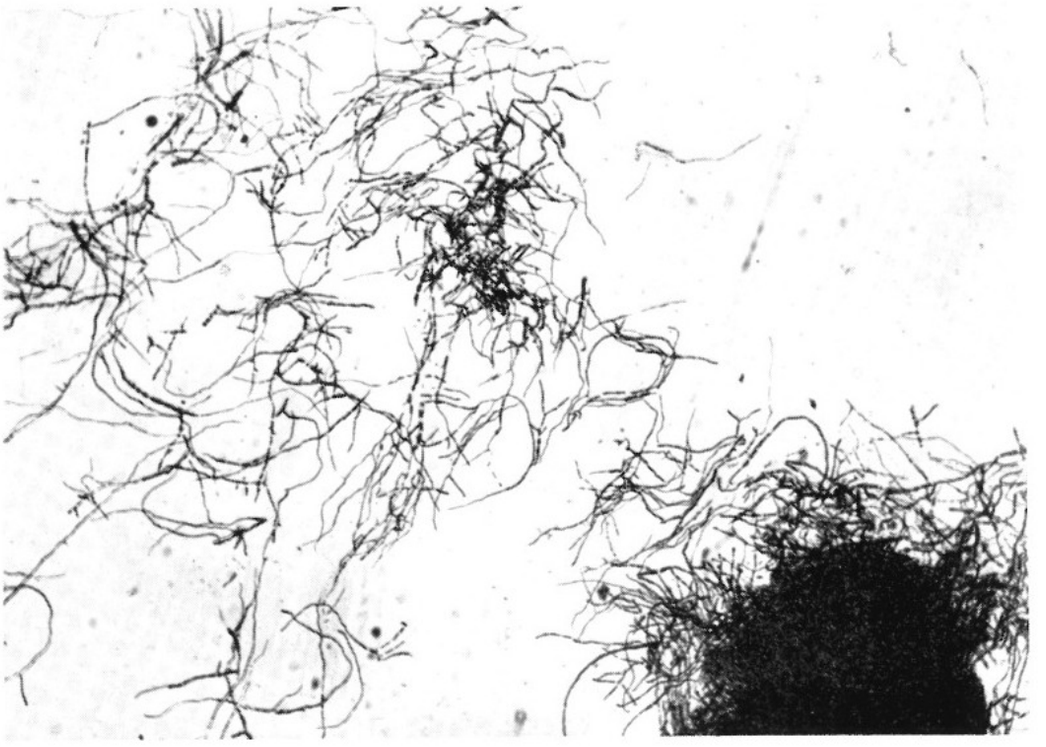

Fig. 8. - Long, filamentous mycelium and pellet after 47 hours growth in 12000 litre fermenter.
(Baffled system, 160 r.p.m., 600 mm φ) × 150.

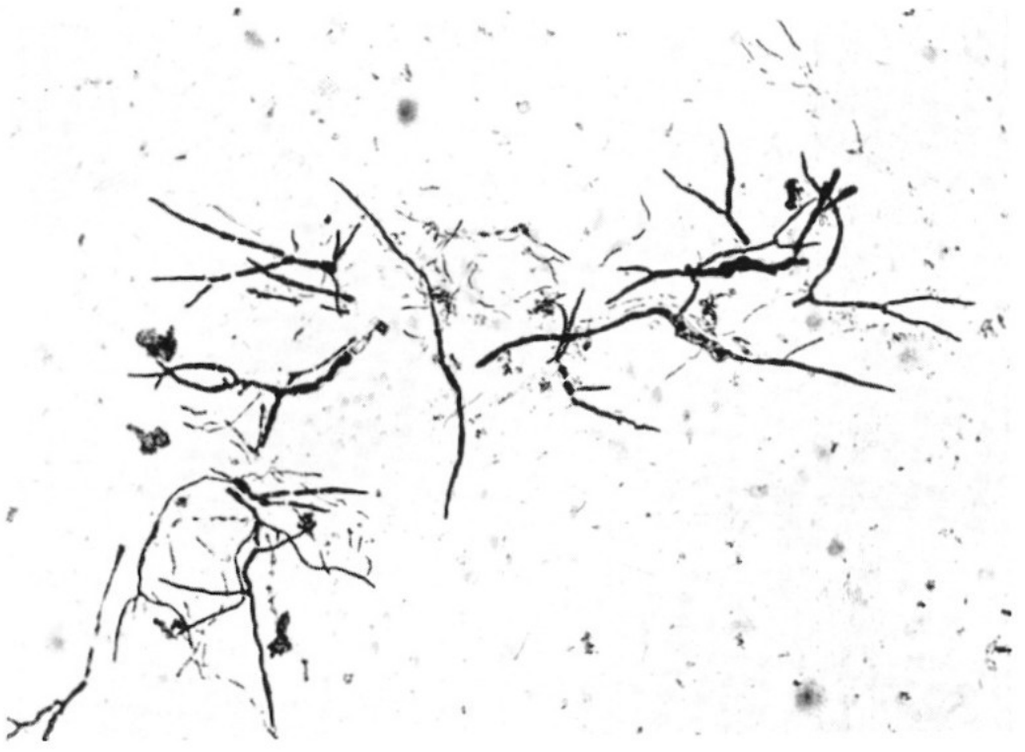

Fig. 9. - Showing the effect of excessive mechanical agitation after 46 hours growth. (Vortex system, 1850 r.p.m., 65 mm φ) × 300.

Fig. 10. - New growth of thickened, branched hyphae after sudden and complete autolysis of the culture.
(Vortex system, 750 r.p.m., 90 mm φ) × 300.

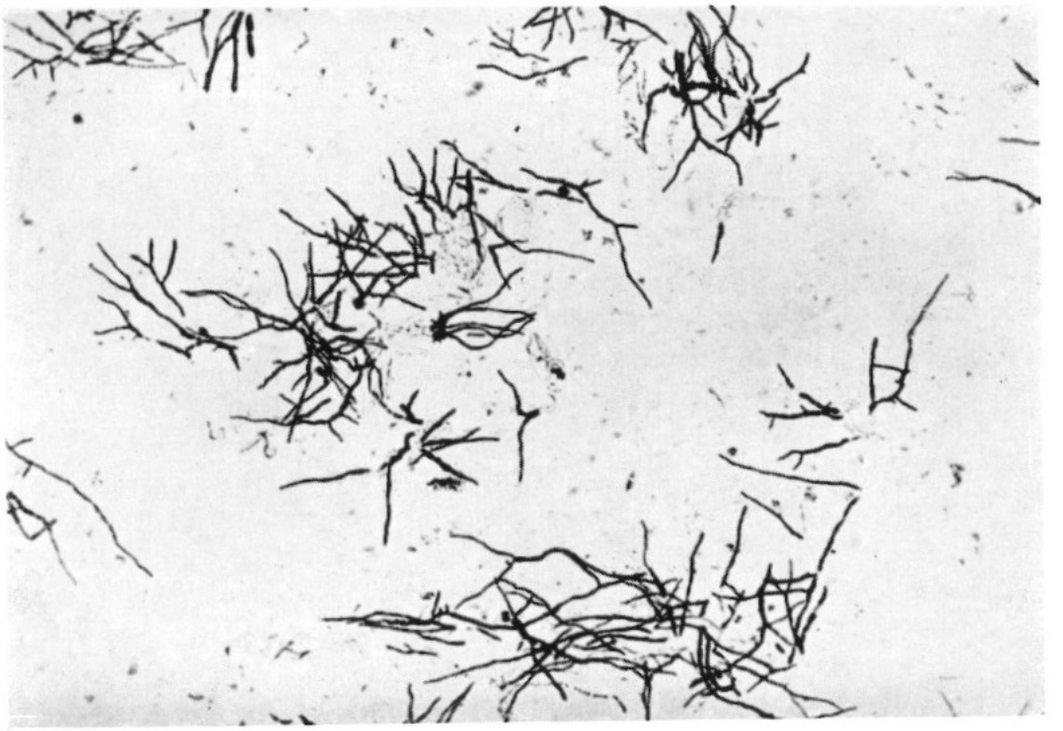

Fig. 11. - Short branched mycelium after 72 hours growth in 3000 litre fermenter with vigorous agitation.
(Vortex system, 760 r.p.m., 350 mm φ) × 225.

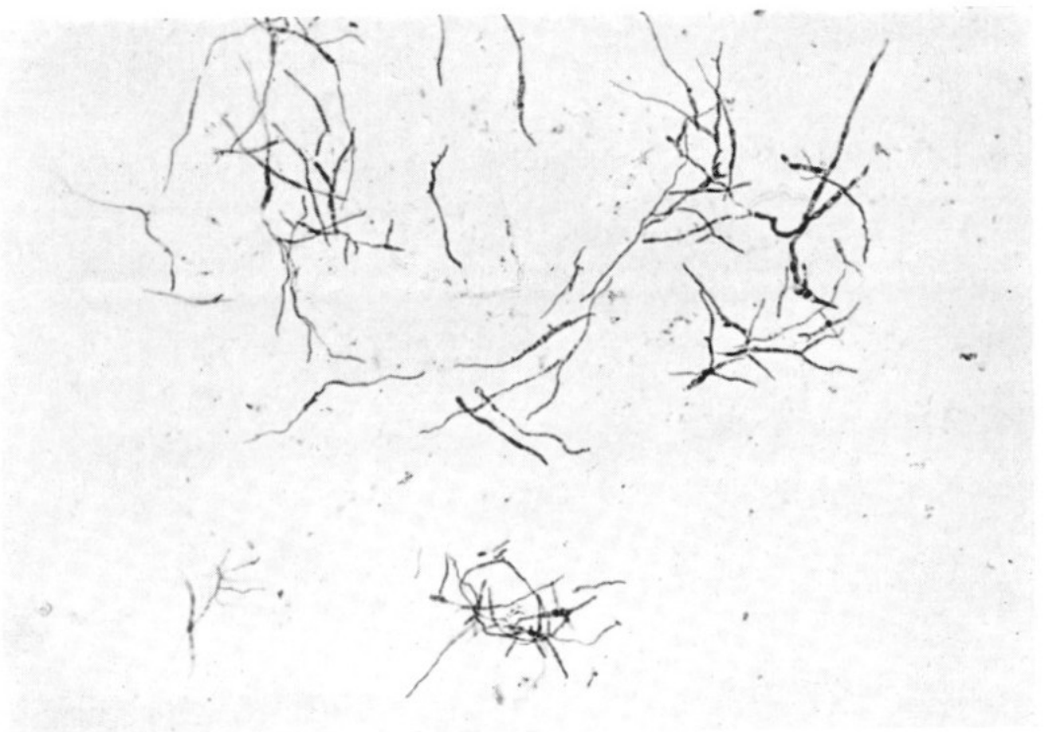

Fig. 12. - Short, fine little-branched mycelium after 72 hours growth in 3000 litre fermenter. This mycelium was grown under the same rate of aeration as that shown in Fig. 11 but with different conditions of agitation.
(Vortex system, 1460 r.p.m., 200 mm φ) × 225.

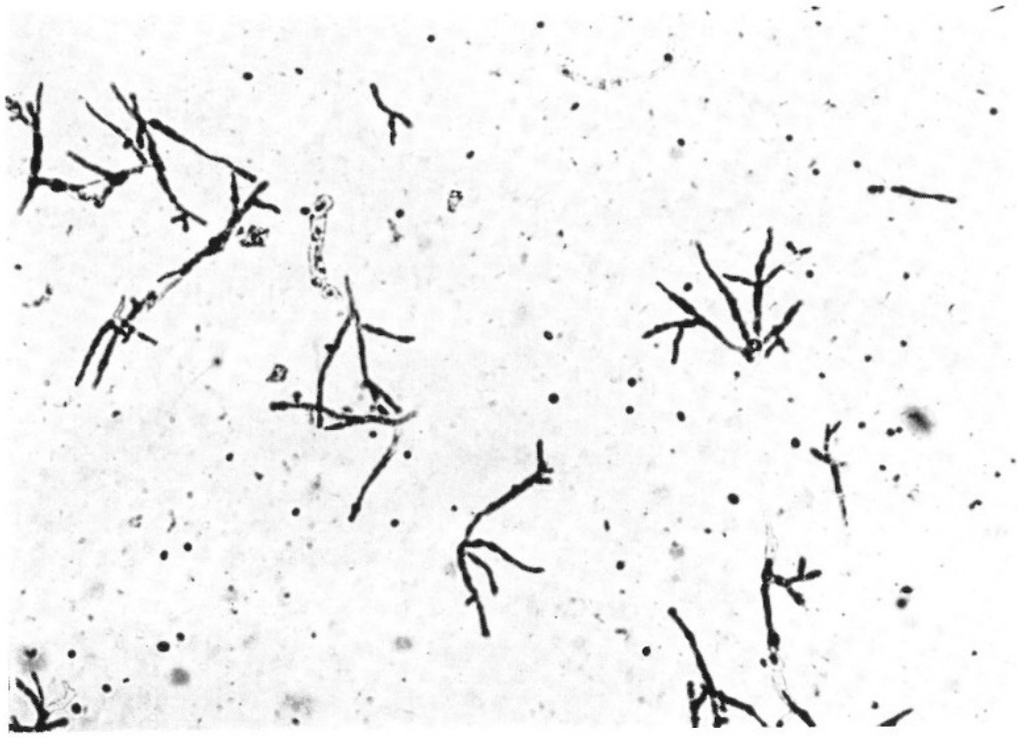

Fig. 13. - Conidial production under conditions of vigorous agitation. The phialides are born directly on the fragmented mycelium.
(Vortex system, 750 r.p.m., 90 mm φ) × 250.

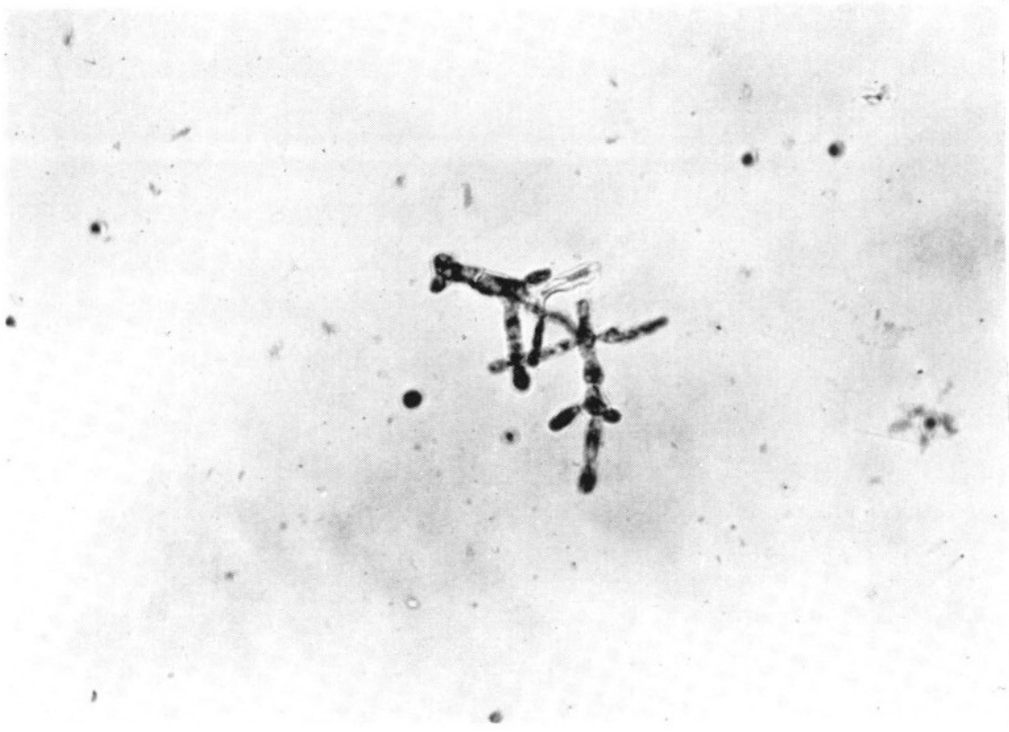

Fig. 14. - Short hyphal unit producting conidia.
(Vortex system, 750 r.p.m., 90 mm φ) × 450.

peller (75 mm diameter) speed was reduced to the low value of 350 r.p.m. It was hoped to obtain in this way, under conditions of adequate aeration, a mycelium of the long filamentous type which, as was shown above, develops at this propeller speed at which the aeration rate is below the minimal requirement for optimal penicillin production.

There was a marked difference in the appearance of the mycelium in comparison to the normal conditions when aeration was effected by an air stream. The hyphae became thickened and deeply staining and were shorter and more pointed, resembling the twig-like laterals of the vigorously agitated cultures. After 48 hours growth the mycelium broke up into shorter irregular fragments which were thicker and more branched than those produced under normal conditions (fig. 6). Oxygen thus appears to have a similar effect to vigorous mechanical agitation.

The primary aim for which the experiment was planned, i.e. to obtain a long mycelium under conditions of adequate aeration was thus not attained in the small fermenters. In fact, it does not seem possible to achieve these conditions except in fermenters of larger size.

The effect of oxygen on the morphological appearance of the mycelium of *P. chrysogenum* is particularly marked in the synthetic medium of JARVIS and JOHNSON, (CHAIN, PIRT and MILLER, 1954).

These experiments show that substitution of air by oxygen in a fermentation may have consequences which are not necessarily limited to an augmentation of the aeration rate, and that the greatly increased saturation concentration of oxygen in the solution, due to the increased partial pressure, may cause specific changes of a more or less profound nature in the metabolism of the organism under investigation. It is therefore not always possible to increase the aeration rate by the simple expedient of increasing the oxygen tension without causing unpredictable metabolic disturbances; it has to be ascertained in each individual case whether these occur or not.

3000 litre fermenter. — In general, in larger volumes of culture medium the hyphal units were less uniform in length than in the small fermenters. In the 3000 litre fermenter the mycelium was predominantly of the long filamentous type (fig. 7). However, the mycelium contained also many short, fine unbranched fragments. As the culture became older the hyphae tended to become shorter, and autolysis began to appear and became marked after 72 hours. Occasionally a tendency towards pellet formation was observed.

12000 litre fermenters. — The aeration rate was high in these fermenters so that the culture medium always contained an excess of

oxygen in solution during the whole course of the fermentation. The hyphae formed were always of the long filamentous type, irrespective of whether baffle plates were present or absent (fig. 8). After forty eight hours some autolysis became evident which increased in intensity with the increasing age of the culture.

A characteristic feature of the cultures grown in these large fermenters was the formation of pellets, a small proportion of which have been shown to be of a sclerotic nature (GOPALKRISHNAN & THIRUMALACHAR unpublished observations) while the larger part is similar in nature and appearance to the pellets formed in shake flasks. (CAMICI, SERMONTI & CHAIN, (1952). The number and size of the pellets varied greatly in the different fermentations, and the factors responsible for their formation are not yet fully understood. It has been shown (THIRUMALACHAR & GOPALKRISHNAN, 1953) that in the small fermenters sclerotic pellets appear under special, well defined conditions. These require a mild mechanical agitation that does not interfere with colony formation, and at the same time an adequate aeration rate must be maintained in the culture fluid. Such conditions are satisfied in the ten litre fermenters if the agitation speed (using the 75 mm diam. propeller) is reduced to 350 r.p.m. and at the same time good aeration efficiency is achieved by dispersing the air (equal volume of air per volume of liquid per minute) through a large porous stainless steel sparger. Under these conditions large numbers of mycelial pellets appear in the culture fluid and they have been recognised as resting vegetative forms of sclerotial nature.

The aeration efficiency E_r is about the same for the small and large fermenters, i.e. the total power consumption per unit volume for aeration in the 10 litre baffled fermenter under conditions of agitation and aeration under which the short hyphal form appears, is about the same as the total power consumption per unit volume for aeration in the 12000 litre fermenter, in which the mycelium grows in the filamentous and pellet form (CHAIN and GUALANDI, 1954). The total power required to produce a given aeration rate is composed of two variables, the energy required for the production of air and that required for the dispersion of air in the culture fluid by means of mechanical agitation. Though the total power consumption per unit volume of culture fluid does not change much with increasing volumes, the ratio of power consumed for mechanical agitation to power consumed for air production decreases with increasing fermenter size This signifies that with increasing volumes of a culture fluid, the rate of air flow remaining constant, a larger proportion of the energy spent for me-

chanical agitation is utilized for the dispersion of air and less energy is absorbed by the culture medium. Thus, the agitation intensity per unit volume is reduced with increasing fermenter volume, and with it the intensity of the shearing forces in the culture medium to which the mycelium is exposed, and which are responsible for the shortening of the hyphae, fragmentation, and other morphological effects described above as the results of intensive agitation.

This is the explanation of the appearance of long hyphae and sclerotial pellets in the large fermenters, and one of the most important phenomena encountered in scaling up from laboratory to industrial size.

Hyphal development in stirred fermenters with vortex aeration.

1. *10 litre laboratory fermenters.* — Two propellers with different agitation speed were used as indicated in table 2.

With the propeller of the smaller size diameter (65 mm) and an agitation speed of 900 r.p.m. a short mycelium developed in much the same way as in the baffled fermenters at an agitation speed of 600 r.p.m. For the first 24 hours after inoculation the hyphae were long. During this period they produced many side branches; the long hyphae then broke up, releasing the short lateral branches. The mycelium was then converted into the « short » form.

At 48 hours the average length of the hyphal units was a 127.9 μ and the hyphae were very fine with a diameter of 2 to 3 μ. There was a little continuous autolysis in these cultures The individual cells were already very short after 27 hours growth and decreased still further in length after 72 hours.

When the rate of agitation was increased to 1200 r.p.m. and higher values, the course of the hyphal development in the early fermentation stages was similar to that which was observed at 900 r.p.m., but the establishment of a characteristic morphological type in the later stages of the fermentation was prevented by the occurrence of mycelial autolysis. The degree of autolysis became the more pronounced the higher the agitation speed and was severe at 1450 and 1850 r.p.m. As many as half of the hyphae autolysed continuously at 1850 r.p.m. The mycelium had a broken and fragmented aspect at these high speeds (fig. 9). The hyphal fragments ranged from 10 μ to 200 μ in length and often showed jagged ends where they had been broken. The mycelium was composed of a mixture of fine and thicken-

ned hyphae. The latter represented new growth from the autolysed debris, and many of these thickened hyphae had clavate tips and large spherical intercalary cells.

With a propeller blade of 90 mm diameter and an agitation speed of 500 r.p.m. the long hyphae after transfer from the seed tank produced many short pointed twig-like laterals. These formed the short mycelium after the original hyphae broke up. The mycelial units were 110 μ long at 48 hours and the hyphae were about 2 μ to 3 μ in diameter. There was slight increase in length later, but the mycelium always remained short. As the culture aged there was increase in the number of empty cells, but general autolysis was not observed until about 90 hours after transfer.

When the number of revolutions was increased to 750 r.p.m. the lateral production in the early stages of growth was very much increased and « witches broom » formations were often seen. When the short mycelium developed the average length of the fragments was 80 μ, at 72 hours, or less than than half the length of the units formed when the stirring speed was 360 r.p.m. (fig. 2). These hyphal units contained an average of 2.4 branches for each unit, and the hyphae were slightly thickened being 3 μ to 4 μ wide and sometimes continued to grow, forming very fine extensions 1 μ wide, from the tips of the thickened fragments. There was an increase in the number of empty cells particularly in the centre of the hyphal units but general autolysis was not observed in these cultures.

Shortening of the individual cells within a hyphal unit was again a characteristic feature of the mycelium grown under these conditions, as shown in table 5.

TABLE 5.

Effect of agitation on hyphal dimensions (Vortex system of aeration).

Diameter of propeller blade	r. p. m.	Mycelial characteristics					
		24 hours after inoculation			72 hours after inoculation		
		Mycelial type	Length of units	Length of cells	Mycelial type	Length of units	Length of cells
90 mm	750	long	250 μ	15.33 μ	short	80 μ	9.13 μ

During the course of experiments in 10 litre fermenters with the synthetic medium of Jarvis and Johnson, it was found that the effects of mechanical agitation in the vortex system were similar to those

obtained in a cornsteep medium: that is, the hyphae became shorter and more branched with increasing agitation intensity. The hyphal units of strain Q-176 when grown with a 65 mm propeller and a stirring speed of 620 r.p.m. averaged 213 μ in length with 2.07 branches per unit after 72 hours growth, whereas with a stirring speed of 1100 r.p.m. they averaged 93 μ in length with 2.91 branches per unit. It was noticed that strain Q-176 tended to become more thickened and branched at high agitation intensities in synthetic medium than other strains.

3000 litre fermenter. — With an agitation speed of 700 r.p.m. and a propeller of 350 mm diameter, the mycelium was short and thickened, developing in much the same way as in the small fermenters, though the hyphal units were less uniform in length. Although lateral production was profuse, the witches broom formations were not observed. The mycelium was a typical short mycelium with thickened, pointed hyphae with an average length of 98 μ at 72 hours (fig. 11). These cultures showed an increasing number of empty cells with age, a characteristic of the short mycelium.

When the size of the propeller was reduced to a diameter of 200 mm and the agitation speed increased to 1460 r.p.m., the aeration rate was identical with the previous experiment (table 3) but the mycelium formed was longer, with an average hyphal unit length of 113 μ (fig. 12). In these cultures the hyphae were very fine, less branched and did not have the characteristic pointed shape of the short mycelium. The penicillin yields were low, of the order of 400 units/ml, in contrast to yields of about 1000 units/ml produced by the shorter, more branched mycelium.

Autolysis and New Growth. — In all cultures there was a gradual increase in the number of empty cells as the mycelium aged, due to the migration of the cytoplasm into the tips and branches of the hyphae. This was particularly noticeable in the « short » mycelium.

In contradistinction to this process, in some cultures the entire hyphal contents became disorganised and disappeared, leaving behind the collapsed walls and fragments of cell contents. This phenomenon was called autolysis.

In experiments in the ten litre fermenters where the rate of agitation was adjusted to give sufficient aeration without causing excessive mechanical damage (e.g. with the 90 mm propeller rotating at 750 r.p.m.) there was very little autolysis. In other experiments with higher

agitation speeds a certain proportion of the hyphae was continuously undergoing autolysis although the mycelial dry weight was steadily increasing. On some rare occasions the whole mycelium autolysed suddenly in the course of a few hours; the authors have no plausible explanation to offer for this phenomenon. New growth occurred in some cases of complete autolysis, but not always. This regrowth when it did occur, consisted only of thick, clavate, hyphae which grew very slowly and often aggregated in pellets (fig. 10).

Duckworth and Harris (1949) have reported alternative phases of hyphal growth and autolysis as a characteristic feature of submerged fermentation in stirred fermenters in corn steep medium. The authors have observed this phenomenon only in exceptional cases in media containing corn steep liquor though it did occur in synthetic media (Chain, Pirt and Miller, 1954).

Spore Production. — The production of spores was relatively rare in these experiments and was only encountered when the mycelium was very short. No spores were ever produced by a long mycelium in the ten litre fermenters within the duration of these experiments, which was generally not longer than 140 hrs, although a few conidia were produced in the 12,000 litre fermenter in the presence of baffles. Spore production was commonest in the vortex system in the ten litre fermenter with a 90 mm propeller and an agitation speed of 750 r.p.m., but also occurred in the baffled fermenters with a 75 mm propeller at 600 r.p.m. Under these conditions both conidiophores and typical penicilli were absent and the mycelium produced flask-shaped phialides about 5 μ-10 μ in length and 2.5 μ-3.5 μ in diameter (figs 13 and 14). These phialides were produced singly, or occasionally in a whorl of two or three, directly on the vegetative hyphae. The contents of the mycelium would be gradually used up in the production of spores, and in an old fermentation it was possible to see two or three living phialides attached to a completely empty fragment of mycelium. The conidia were normal sized and on one occasion were produced in sufficient numbers to turn the medium green.

Correlation between agitation intensity and penicillin production.

Table 6 shows the correlation between agitation intensity and penicillin production. As can be seen the penicillin yields are low at high rotational speeds both in the vortex and fully baffled systems of aeration. This is probably due to damage to the mycelium. In choos-

TABLE 6.

Correlation between agitation intensity and production of penicillin in ten litre fermenters.

r. p. m.	Diameter of Propeller	Maximum Yield of Penicillin Units ml
	Baffled System	
360	75 mm	460
530	75 mm	785
600	75 mm	1330
830	75 mm	1020
	Vortex System	
900	65 mm	625
1200	65 mm	510
1450	65 mm	400
1850	65 mm	390
560	90 mm	565
750	90 mm	1435

ing the most suitable propeller speed and dimensions it is evidently necessary to strike a balance between too low an agitation intensity causing no damage to the cells but giving insufficient aeration rates, and too high an agitation intensity giving high aeration rates but causing damage to the cells. In the vortex system of aeration the best yields of penicillin were obtained in the 10 litre fermenters with the propeller of 90 mm diameter and an agitation speed of 750 r.p.m. Under these conditions the aeration rate was about the same as that obtained with the propeller of 65 mm diameter and an agitation speed of 1450 r.p.m., but the faster rotating propeller caused much autolysis and the penicillin yields were low.

DISCUSSION

The studies reported above show that the shearing action induced by intensive mechanical agitation has a marked effect on the morphological appearance of the mycelium of *P. chrysogenum* strain 47-1564 when grown in stirred fermenters.

The most apparent effects of mechanical agitation are the shortening of the hyphal unit, shortening and thickening of the individual

cells within the hyphal unit, and the stimulation of branching. The changes are the more pronounced the more intensive the agitation, but when the agitation intensity exceeds a certain limit extensive autolysis sets in.

Although the overall growth, as measured by the dry weight, is the same for cultures grown with different intensities of agitation, the proportion of young hyphae per unit dry weight is greater in the short branched mycelium developed under the influence of vigorous agitation than it is in the long filamentous mycelium which appears when the agitation is mild. It can therefore be expected that the metabolic activity of mycelium grown under conditions of intensive mechanical agitation will be quantitatively and qualitatively different from mycelium grown under conditions of mild mechanical agitation, or in its complete absence.

This is an important factor which must be taken into consideration in the interpretation of the differences observed in the course of fungal fermentations carried out in fermenters of different sizes. It may be responsible, for instance, for the frequently observed fact that certain metabolic products which have been obtained in high yields in small laboratory fermenters appear in much lower yields in fermenters of industrial scale though all controllable variables such as the composition of the culture medium and the aeration rates, are maintained strictly identical.

On the other hand, as the aeration rates are dependent on the degree of mechanical agitation it may not always be possible to achieve the desired degree of aeration in small fermenters without excessive damage to the fungal mycelium and concomitant profound changes in its metabolism. In such cases satisfactory results may be obtained only by the use of fermenters of larger capacity where high aeration rates may be obtained with a lower intensity of mechanical agitation per unit volume than is possible in small fermenters.

Although these experiments have demonstrated the general morphological changes which occur during growth in submerged culture with various intensities of mechanical agitation, the authours are aware that the actual size of the hyphal units and the degree of branching are influenced also by other factors such as the composition of the medium, and the amount and type of oil used as an antifoam-agent. It has also been noticed that the various strains of *P. chrysogenum* differ considerably in their response to mechanical agitation.

Roma — Istituto Superiore di Sanità - International Research Centre for Chemical Microbiology.

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