THE FRAGMENTATION OF THE MYCELIUM OF PENICILLIUM NOTATUM AND PENICILLIUM CHRYSOGENUM BY A HIGH-SPEED BLENDER AND THE EVALUATION OF BLENDED SEED

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Penicillin is currently being produced almost entirely by a submerged fermentation for reasons already stated by Moyer and Coghill (1946). For this fermentation, a reduction in the volume of seed and a reduction in the number of steps in production would have the advantage of simplifying the preparation of seed and minimizing the risk of contamination. Reducing the number of steps would also hold to a minimum any loss of penicillin productivity from seed deterioration (Clutterbuck *et al.*, 1932; Foster *et al.*, 1943). Any concomitant sacrifice in yield or delay in the final fermentation cycle would, of course, be undesirable. This is a report of experiments, which have been conducted on a small scale, testing a method of preparing seed which may find application in larger scale production.

Most fungi, including the penicillin-producing penicillia, produce both mycelium and spores. Fermentations brought about by these fungi may vary in their course and extent with the quantity and type of seed unit employed, a seed unit being any viable spore, spore cluster, hypha, or hyphal cluster. Certain members of the *Penicillium notatum-chrysogenum* group produce such large mycelial clusters that they are visible as discrete pellets, 1 to 5 mm in diameter. Since hand shaking of a flask of these pellets causes no fragmentation, we tried various mechanical methods in an effort to induce separation of the mycelium at its cross walls. Of the methods tried, blending in a high-speed propeller type blender (Waring "blendor") proved to be most satisfactory, and throughout this report we shall refer to vegetative seed so fragmented as "blended" seed.

We have not been interested solely in measuring the increase in seed units brought about by blending, for mere growth of fragments would be no guarantee of efficiency in penicillin production. Rather, we have been interested in determining how small an amount of blended seed will substitute adequately for 10 per cent seeding with unblended vegetative pellets, which serve as a control.[1]

Two strains of penicillia, *Penicillium notatum*, NRRL 832, and *Penicillium chrysogenum*, NRRL 1951, both obtained from the Northern Regional Research Laboratories at Peoria, Illinois, have been submitted to parallel treatment. To eliminate the factor of strain variation during the study, we used spores from two large reserves of aqueous spore suspensions, stored at 4 C, for the preparation of vegetative seed as needed. The suspension of NRRL 832 spores contained 0.5×10^9 spores per ml; that of NRRL 1951 spores contained $2.0 \times$

[1] Ten per cent vegetative seed was considered essential at the time these studies were started.

10^9 scores per ml. These suspensions were kindly made and counted for us by Dr. A. J. Whiffen of these laboratories.

EXPERIMENTAL PROCEDURE

Erlenmeyer flasks of 500-ml capacity were used routinely as "shaker flask" fermenters. Each flask contained 100 ml of sterile corn steep medium, prepared according to the following formula:[2]

Lactose	20.0 g
Corn steep liquor (50 per cent solids)	40.0 ml
$MgSO_4$	0.125 g
KH_2PO_4	0.250 g
$NaNO_3$	1.00 g
Tap water to	1,000 ml
pH, after sterilization, 4.5	

Seeding was accomplished with measured volumes of spore or mycelial suspensions. Triplicate flasks were seeded at each level, and the fermentation was conducted at 24 C in a shaking machine of the oscillating type. The length of stroke was 4 inches; there were 95 such complete oscillations per minute.

Samples were taken daily, starting usually on the third day of the cycle. A minimal amount, about 3.5 ml, was removed from each of the three replicate flasks to give a pooled sample for that level. This gave a sufficiently large sample for assay and determination of pH, without materially diminishing the beer volume in each flask.

All pooled samples were allowed to stand for 10 to 15 minutes. One-ml volumes of the clear, supernatant beer were then transferred to sterile vials and submitted for assay of penicillin content by a hollow-cup agar-plate method. This method, as described in Circular 198 of the Department of Agriculture (1931), was adapted to the assay of penicillin by Dr. J. F. Norton of these laboratories. Each assay value was determined by averaging four zones of unknown from a single plate and translating this average diameter into Oxford units per ml from the standard curve prepared for that day.

TITRATION OF OPTIMAL SPORE SEED LEVEL

Spore seeding was performed at various levels in order to establish the optimal rate of seeding. In figures 1 and 2 are shown the influence of rate of spore seeding upon the fermentation cycle. The curve for each level of spore inoculum is "set over" five days from the curve for the preceding level, to avoid the confusion of too many overlapping curves.

From figures 1 and 2 it may be seen that too small or too large an inoculum is not productive of maximal potency levels. Seeding rates between 1,000 and

[2] This formula was communicated to us by Dr. A. J. Moyer of the Northern Regional Research Laboratories in a copy of one of the restricted reports to Dr. A. N. Richards, chairman, Committee on Medical Research, Office of Scientific Research and Development. Its composition is given here since it is not included in the recent publication of Moyer and Coghill (1946).

1,000,000 spores per ml caused the fermentation to reach maximal potencies in almost identical lengths of time. Strain NRRL 832 was capable of producing penicillin levels of approximately 80 u (Oxford units) per ml and strain NRRL 1951 was able to produce about 100 u per ml at optimal spore seed levels.

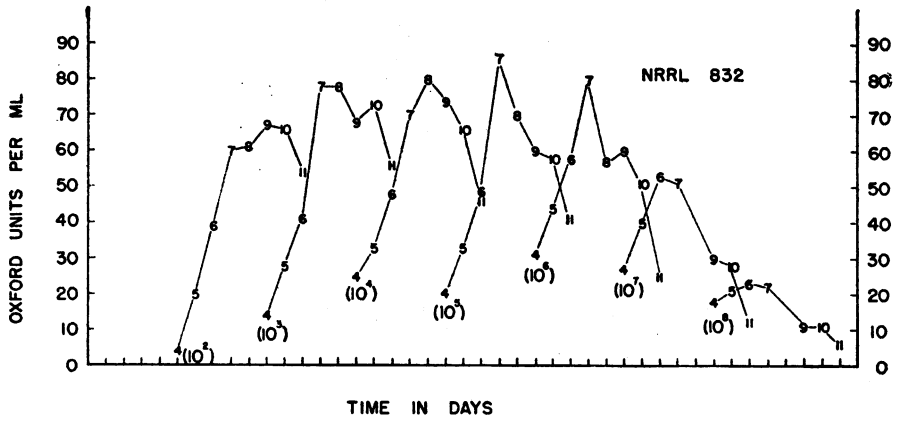

FIG. 1. PENICILLIN FERMENTATION CURVES SHOWING THE INFLUENCE OF RATE OF SPORE SEEDING FOR P. NOTATUM, NRRL 832

Numbers in parentheses refer to the number of spores per ml of substrate to give the corresponding curve. Numbers are used as data points to indicate the day of the fermentation cycle.

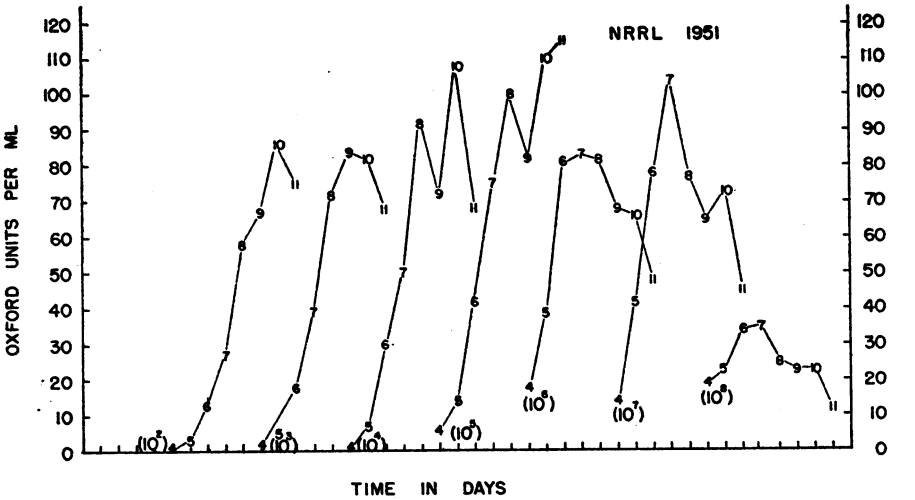

FIG. 2. SAME AS FIGURE 1, BUT FOR P. CHRYSOGENUM, NRRL 1951

STUDY OF BLENDING TIME

Mycelial growth of these two fungi in a shaker flask reaches a maximum in from 3 to 6 days. At this time most of the liquid volume is occupied by pellets of mycelium. One flaskful of this seed, approximately 100 ml, is a convenient volume to blend at one time in the usual 1-quart-size blender jar.

This operation is performed aseptically in a jar equipped with a special type of metal cover and splash plate, as shown in figure 3.

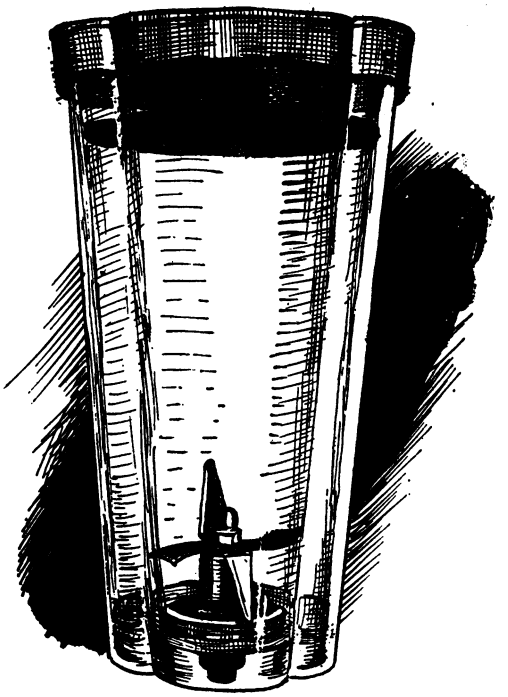

FIG. 3. THE TYPE OF COVER WITH BAFFLE MADE OF SHEET COPPER USED WITH WARING "BLENDOR" JAR TO CARRY OUT STERILE BLENDING OPERATION

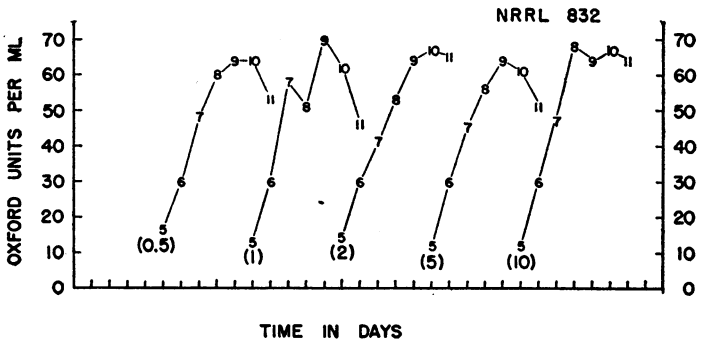

FIG. 4. INFLUENCE OF BLENDING TIME UPON PENICILLIN FERMENTATION CURVE
Seeding rate is constant, at 1:100,000. Culture is *P. notatum*, NRRL 832. Numbers in parentheses refer to minutes of blending time.

During the first half minute of blending, a visible change in the consistency of the slurry occurs. A "titration" of the blended seed for increase in seed units was conducted at the time intervals of 0.5, 1, 2, 5, and 10 minutes. The fermentation curves obtained from seed blended for these various time intervals are shown in figures 4 and 5.

A continuous blending for more than two minutes is detrimental to these fungi, since, during the blending operation, heat develops from the operation of the high-speed (10,000 rpm) blending blade. The seed prepared for the 5- and 10-minute blending-time studies shown in figures 4 and 5 was protected from this heat effect by cooling the blender jar and contents between each blending period of not more than 2 minutes.

From data of figures 4 and 5, we adopted 2 minutes as a desirable length of time for routine blending. In 2 minutes, most of the hyphal fragments contain between 1 and 10 cells, with an average of about 4 cells per fragment. About 20 per cent of the fragments are created by actual fracture of the cell wall. The terminal cells of the rest of the fragments are undamaged, and these frag-

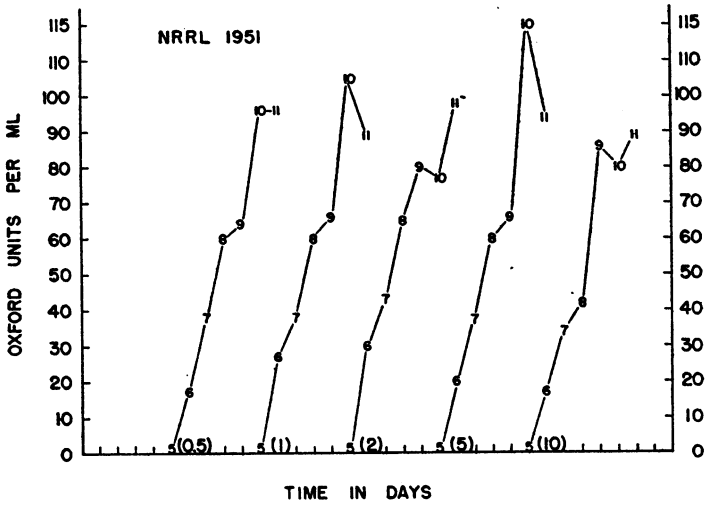

FIG. 5. SAME AS FIGURE 4, BUT FOR P. CHRYSOGENUM, NRRL 1951

ments appear to have been formed by fragmentation at the septa, under the physical stress of blending.

TITRATION OF BLENDED SEED

Six-day-old vegetative seed was blended for 2 minutes and diluted in sterile medium so that final dilutions of 1:2,500; 1:10,000; 1:40,000; 1:100,000; 1:250,000; and 1:500,000 could be fermented parallel to 1:10 dilutions of the unblended seed, which served as a control. Data so obtained are shown as solid lines in figures 6 and 7.

Similar results were obtained with 4-day-old seed blended on the second as well as the fourth day, and with 6-day-old seed, blended on the third as well as the sixth day. Twice-blended seed, since it was able to mend between its first and second blending periods, is referred to as "B.M.B." seed. Comparisons of singly blended seed (B.) with doubly blended seed (B.M.B.) are also shown in figures 6 and 7.

Blended seed seems to reach slightly higher peaks about 2 days later than the

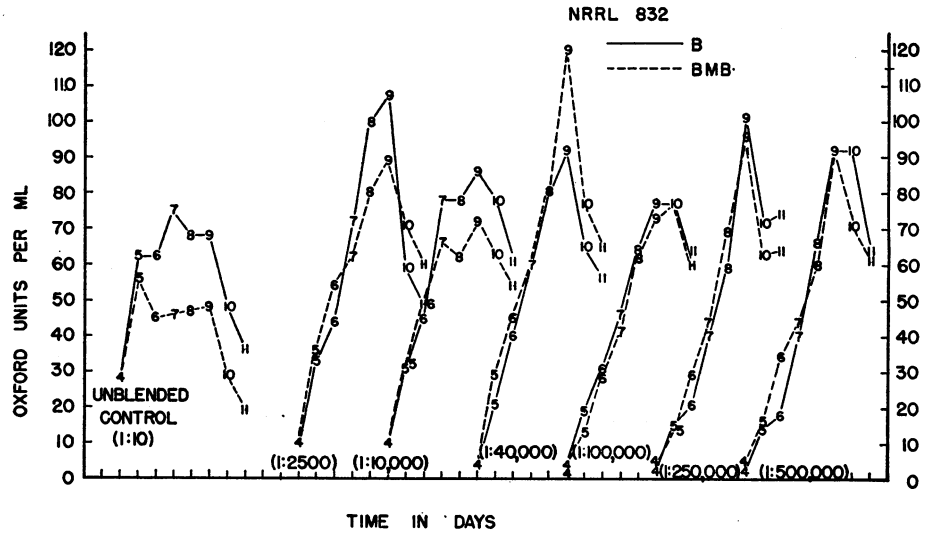

FIG. 6. TITRATION OF TWO-MINUTE BLENDED SEED SHOWING THE INFLUENCE OF DILUTION UPON THE FERMENTATION CURVE

Unblended seed was run as a control at a seeding rate of 1:10. Numbers in parentheses refer to the dilutions of blended seed giving the corresponding fermentation curves for *P. notatum*, NRRL 832.

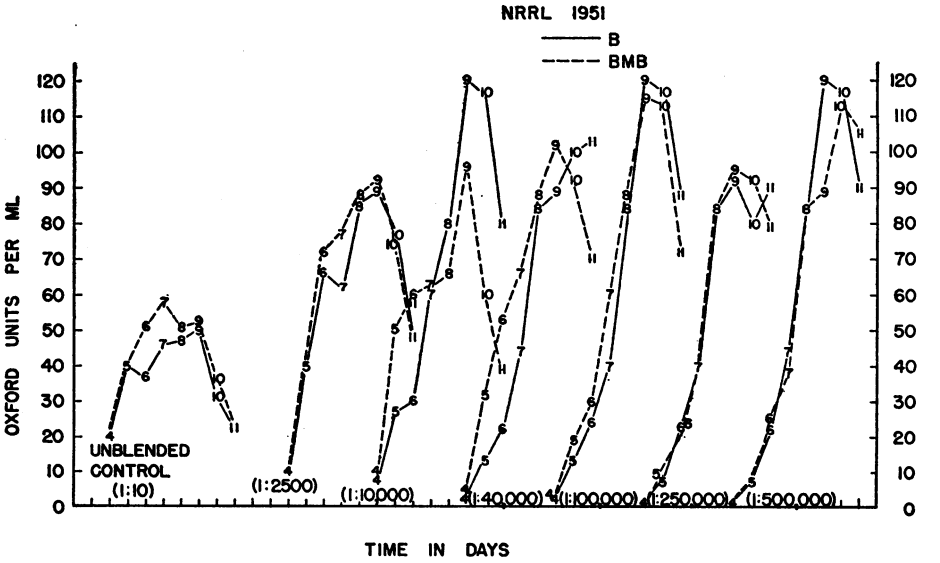

FIG. 7. SAME AS FIGURE 6, BUT FOR P. CHRYSOGENUM, NRRL 1951

controls. At the peak for the controls (seventh day), blended seed, diluted as much as 1:40,000, gives as good production as the controls, and the productions from the 1:100,000, 1:250,000, and 1:500,000 series are almost as good.

DISCUSSION

Some fungi, e.g., the oidia among transitional yeastlike fungi (Henrici, 1930) and *Nocardia* among the actinomycetes (Waksman and Henrici, 1943), tend to fragment automatically or with very little mechanical stress applied. Apparently fragmentation can be mechanically induced in certain fungi whose mycelium resists fragmentation under the small stresses of hand shaking and ordinary routine handling. At any rate, two penicillia have been shown to endure fragmentation by blending, with no apparent disturbance of growth and fermentative properties. This property of withstanding damage under blending operation may allow blended seed to be used as a substitute for much larger volumes of unblended seed.

During the delay in publication of this work, there have been isolated and tested new members of the *P. notatum-chrysogenum* group which give higher yields and which grow as much smaller pellets or clusters of mycelium. It may well be that this type of vegetative seed can be used wisely at such low seeding rates as 1:10,000 or 1:40,000 without blending. The principle of using very small seed volumes, whether the seed is blended or unblended, will have to be evaluated in terms of current practice with large fermenters.

SUMMARY

Two fungi, *Penicillium notatum*, NRRL 832, and *Penicillium chrysogenum*, NRRL 1951, have been shown to endure blending of their vegetative mycelium by a Waring "blendor" with little if any injury to their growth and fermentative capacities. Fragmentation seems to occur usually at the septa.

Such blended seed, when diluted as much as 1:40,000 times, adequately substitutes for unblended seed at a 1:10 seeding rate, in shaker flasks.

Blended, mended, and blended seed is not superior to singly blended seed.

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