Some Observations on the Growth of *Aspergillus niger* from Spore Inoculum

P. S. KRISHNAN, VIOLOT BAJAJ, AND S. P. DAMLE

Division of Biochemistry, National Chemical Laboratory, Poona, India

Received for publication May 4, 1954

One of the uncertainties in mold culture is the nature of the spore inoculum. Spores are harvested from agar slants by one of two methods, namely, either by flooding the slants with water, or dry, without allowing water to come in contact with the slants. The two types of spore inocula may be expected to give rise to different growth performances in a synthetic medium, since the inoculum prepared by flooding the slants with water would contain material leached out from the mycelium and the agar slant.

It was also thought likely that simple washing of the spores with water might yield an inoculum giving reproducible growth performances. Experiments reported in a recent communication from this laboratory (Bajaj, Damle, and Krishnan, 1954) showed that when the spores of *Aspergillus niger* are collected from synthetic liquid cultures or from agar slants by flooding with water, considerable amounts of inorganic and esterified phosphates pass into solution. McCallan and Wilcoxon (1936), investigating the fungicidal action of Bordeaux mixture, found that malic acid and some amino acids are present in aqueous extracts of spores. The work of Shu and Johnson (1947) indicates that some trace elements can be removed from the conidia by the simple process of washing with water. A number of authors (Mandels and Norton, 1948) have used washed mold spores for biological studies and have reported a high percentage of germination. Perlman (1951) used washed spores of *Memnoniella* and *Aspergillus* for inoculating synthetic media and found that the washed spore suspensions could be stored in the cold and used over a period of weeks.

During the course of experiments designed to study the phosphate metabolism of molds in surface and submerged cultures (Krishnan and Bajaj, 1953a, b; Bajaj, Damle, and Krishnan, 1954), the authors have repeatedly found that the technique of collection of the spores, namely by flooding the slants with water or by removing them dry, affects the cultural characteristics of *A. niger* and also that the cultures inoculated with washed spores differ from those inoculated with the unwashed spores. Furthermore, the addition of an aqueous extract of potato-dextrose-agar medium has a pronounced influence on the germination of the spores. An account of these observations is recorded in the following pages.

MATERIALS AND METHODS

A. niger NRRL 67 was used in all the experiments. Unless otherwise stated potato-dextrose-agar (PDA) served as the sporulation medium and was always made up with distilled water. This medium was dispensed into either 25- x 150-mm test tubes, or litre Roux bottles. A stock soil culture was subcultured thrice and the spores from the last used for inoculating the fermentation medium. The spores were harvested by two different techniques. In one, the spores were removed by the suction method of McCallan and Wilcoxon (1936); in the other, the agar slants were flooded with water and the spores dislodged by gentle scraping. The spore count was adjusted to 4 to 7 million conidia per ml. A portion of the spore suspension was set aside for the inoculation of control cultures. The major bulk of the spore suspension was measured, filtered by gravity through Whatman filter paper No. 1 and washed twice on the funnel with distilled water. The entire operation of filtration and washing took 15 to 30 minutes. The residual spore material was resuspended in water and made up to the original volume. The two supplementary washings of the spores were rejected in some experiments; in others, they were added to the main filtrate and the final volume made up to twice the original volume. The fermentations were carried out under surface and submerged conditions, using 250-ml Erlenmeyer flasks for the former and 250- or 500-ml flasks for the latter. Submersion was effected by shaking either in a reciprocal shaker with an amplitude of 1 in at the rate of 127 strokes per minute, or in a rotary shaker describing a circle 3 in in diameter at a speed of 156 rpm. Two-ml aliquots of the spore suspension were used for inoculation of 50 ml portions of Currie's medium made up with 12 per cent dextrose and with the pH adjusted to 1.9.

The cultures were divided into the following groups:

1. Control cultures inoculated with the unwashed spores.
2. The rest of the flasks, after prior supplementation as indicated below, inoculated with the washed spores:
   a) Washed spore cultures, receiving only water.
   b) Supplemented cultures, receiving 2 ml of the spore filtrate when only the main solution was used, but 4 ml when the two supplementary washings also were combined.

TABLE 1. *Surface culture of* Aspergillus niger *inoculated with spores of three different ages, collected by flooding slants with water*

	Days of Incubation, No.	Acidity	Utilization of Glucose	Mycelium plus Spores, Dry Wt.*
		ml N/10 NaOH	*per cent*	*mg*
4 days agar slants				
Control	4	35	8.7	163
Washed		43	9.1	150
Supplemented		18	7.0	97
P.D.A.		70	10.1	258
Control	7	326	29.6	598
Washed		188	13.0	304
Supplemented		254	18.8	389
P.D.A.		304	26.2	575
Control		564	52.2	884
Washed		390	39.2	652
Supplemented	12	390	39.2	676
P.D.A.		688	60.0	1012
Control		603	55.6	847
Washed		778	68.2	1138
Supplemented	15	501	50.0	823
P.D.A.		885	69.7	1092
6 days agar slants				
Control	4	38	3.6	159
Washed		34	4.9	177
Supplemented		32	4.2	178
P.D.A.		51	5.9	212
Control	7	233	24.1	525
Washed		250	21.1	445
Supplemented		219	21.4	444
P.D.A.		261	25.2	518
Control	12	416	38.8	780
Supplemented		343	37.4	590
P.D.A.		564	51.4	834
Control	15	424	43.6	787
Washed		436	45.9	695
Supplemented		424	42.2	714
P.D.A.		681	57.6	945
9 days agar slants				
Control	4	26	7.9	145
Washed		18	4.6	117
Supplemented		29	10.5	147
P.D.A.		47	14.0	144
Control	7	251	18.2	420
Washed		202	15.2	331
Supplemented		273	21.0	482
P.D.A.		211	17.4	357

* Analysis of mycelium plus spores showed an average of 10.35 μg phosphorus and 45.4 μg nitrogen per mg dry weight.

c) PDA cultures, receiving 2 ml of an extract of the PDA medium. This extract was prepared by flooding the uninoculated test tube slants with 5 ml portions of water and draining after 15 to 20 seconds.

The volumes were equalized by adding the requisite amount of distilled water to the cultures. All incubations were carrried out in weak diffused light at room temperature (26–28 C), which, during the course of any one complete series of experiments was held constant within ±0.5 C. Further details of the culture of the organism, and of the techniques of assay of the mycelium and of the metabolism fluid, have been reported elsewhere (Krishnan and Bajaj, 1953a, b). No attempt was made to separate the spores from the mycelia; the whole mass was dried to constant weight, powdered and sampled. The total nitrogen contents of the mycelia were also determined, being a better indication of protoplasmic synthesis than dry weight yields. The dried material was digested with H_2SO_4 aided by H_2O_2 and the same digest used for phosphate and nitrogen estimations (Damle and Krishnan, 1954).

EXPERIMENTAL RESULTS

Surface Culture with Spores Collected by Flooding

Sporulation on the agar slants commenced by about the 2nd day after subculturing. At the end of 4, 6 and 9 days the spores were collected by flooding the slants with water and gentle scraping with a blunt inoculating needle. The two supplementary washings were rejected, the main filtrate only being used in the supplemented cultures. These filtrates had pH values of 3.81, 3.67 and 3.52 on the 4th, 6th and 9th days respectively. The analytical data for glucose utilization, acid production, dry weight yields of mat per 100 ml culture solution, and the total phosphate and nitrogen contents per mg of the dried mycelia are recorded in table 1.

It is apparent from the above table that the mycelial yield, glucose utilization and acid production were the highest in the PDA cultures. The initially formed mycelium in these cultures had a lower nitrogen content per mg dry weight than the mycelium from the others, but with older cultures such a difference was not apparent. The analytical data for total phosphate content per mg dry weight of the mycelia did not show any systematic variation between the different treatments.

There were significant differences in the morphology of the various cultures. In the control cultures a thin adherent mycelium was formed on about the 3rd day; sporulation commenced within 5 days and was profuse with further incubation. In the washed spore cultures, however, the mycelium was formed in patches and never joined up during the entire incubation period; also, sporulation was sparse. Supplementation with spore washing induced better, though discontinuous mycelium formation and heavier sporulation than were obtained in the washed spore cultures. In the PDA cultures using spores from 4- and 6-day-old agar

slants a well formed mycelium was obtained and sporulation was profuse.

Surface Cultures with Spores Collected by the Suction Technique

The design of this experiment was the same as that of the previous one, the only difference being that the two supplementary spore washings were combined with the main filtrate used in the supplemented cultures. The solutions had pH values in the range 5.9 to 6.1, which was higher than that of the spore filtrates from the flooded spores. The analytical data calculated on the basis of 100 ml culture solution are recorded in table 2.

The data in table 2 show marked differences in the dry weight yields of the mycelia, glucose utilization, and acid production in the various cultures on the 5th day of the incubation. The control and the supplemented cultures resulted in low yields of mycelia, the washed spore culture in higher yield, and the PDA culture in the highest yield. The glucose utilization and acid production, also, were the highest in the PDA cultures. With further incubation these differences tended to level out in the various cultures. The data for total phosphate and nitrogen contents per mg dry weight of the mycelia did not show any significant variation due to the different treatments.

The washed spore cultures grew in patches and resembled more or less the corresponding cultures obtained with spores collected by the flooding technique. However, the control cultures behaved quite differently; the germination of the spores was delayed and the mycelial formation was very poor even on the 5th day; sporulation, when it set in, was less intense. The supplemented cultures also showed inhibited growth. The PDA cultures, as before, yielded a well formed mycelium.

Surface Culture on Zn-Supplemented Medium with Spores Collected Dry

Spores from 6-day-old agar slants were used in the experiments. The fermentation medium contained in addition to Currie's salts, Zn in a final concentration of M per 100,000, added in the form of B.D.H. AnalaR $ZnSO4 \cdot 7H_2O$. Besides the four treatments described earlier a fifth set of cultures was simultaneously run, which was inoculated with the unwashed spores after adding PDA extract. These served as control plus PDA cultures. The analytical data for the Zn supplemented, as well as for the control basal medium, calculated on the basis of 100 ml culture solution are recorded in table 3.

The data for the 3-day-old culture show that the control cultures grew very poorly, that mycelium formation was increased on washing the spores, and that on supplementing the washed spores with the

TABLE 2. *Surface culture of* Aspergillus niger *inoculated with spores of three different ages, collected by suction*

	Days of Incubation	Acidity	Utilization of Glucose	Mycelium Plus Spores, Dry Wt.*
	no	*ml N/10 NaOH*	*per cent*	*mg*
4 days agar slants				
Control	5	50	10	145
Washed		94	9.4	216
Supplemented		51	7.5	150
P.D.A.		128	14	301
Control	8	236	22.6	357
Washed		296	20.4	380
Supplemented		251	18.5	338
P.D.A.		390	34	528
Control	12	389	36	480
Washed		518	42.9	608
Supplemented		467	39.8	542
P.D.A.		541	45.1	622
Control	15	639	53.1	706
Washed		638	54.9	661
Supplemented		659	56.9	691
P.D.A.		705	56.2	685
6 days agar slants				
Control	5	70	5.6	161
Washed		116	11.1	245
Supplemented		83	10.4	206
P.D.A.		156	14.8	335
Control	8	232	20.7	347
Washed		375	29.3	488
Supplemented		287	25.3	390
P.D.A.		371	29.4	486
Control	12	475	34.3	448
Washed		455	36.2	510
Supplemented		551	45.1	628
P.D.A.		506	39.1	586
Control	15	486	49.9	613
Washed		583	56.0	666
Supplemented		527	57.7	642
P.D.A.		484	43.5	608
9 days agar slants				
Control	5	17	4.7	81
Washed		46	8	157
Supplemented		31	10.2	129
P.D.A.		83	12.5	211
Control	9	206	25.3	329
Washed		292	24.6	386
Supplemented		267	28.5	405
P.D.A.		355	32.3	550
Control	12	226	27.5	378
Washed		376	35.4	473
Supplemented		337	35.7	452
P.D.A.		357	34.8	478

* Analysis of mycelium plus spores showed 11.71 μg phosphorus and 50.37 μg nitrogen per mg dry weight.

TABLE 3. *Surface culture of* Aspergillus niger *in Currie's medium, with and without Zn, using spores collected by suction from 6 day slants*

	Days of Incubation	With Zn, Dry Wt.	Without Zn Dry Wt.
	no	*mg*	*mg*
Control plus P.D.A.	3	446	242
Control		65	71
Washed		245	159
Supplemented		93	57
P.D.A.		392	266
Control plus P.D.A.	5	1023	468
Control		619	288
Washed		748	362
Supplemented		539	232
P.D.A.		920	500

spore washing, the growth was again retarded. The addition of PDA extract either to the washed or the unwashed spores considerably increased the dry weight yields of mycelium. Figure 1 illustrates the morphological appearances of the various cultures on the fourth day in Zn-supplemented medium. On the fifth day of growth, the above differences still persisted but were much less pronounced. The presence of Zn in the medium gave rise to increased mycelial formation and suppressed sporulation as compared to the control basal medium.

Submerged Culture with Spores Collected Dry and by Flooding

The agar slants used for the collection of spores in these experiments were 6 days old. The fermentation was allowed to proceed for only 3 to 5 days, since in the surface cultures the maximum difference between the various treatments was observed in the initial stages. The deposition of conidia on the sides of the flasks and the subsequent surface growth of mycelium, referred to by Camici, Sermonti, and Chain (1952) were considerably minimized by removing the flasks and shaking at frequent intervals and by rotating the flasks against their own axis while the shaker was in motion. As a rule, agitation of 500-ml Erlenmeyer flasks containing 75 ml medium in the reciprocatory shaker yielded the filamentous type of mycelial formation, whereas agitation in the rotary shaker gave rise to a predominantly pellet type mycelium. In the rotary shaker the PDA cultures consistently showed the smallest pellet formation, somewhat larger pellets were found in the washed spore cultures, and by far the largest in the supplemented and the control cultures. It may be recalled that Moyer (1953) recommended the addition of about 0.45 per cent agar to shake cultures to reduce clumping in the preparation of vegetative inoculum. Results obtained in typical experiments are recorded in table 4, the data being represented on the basis of 100 ml culture solution.

The data with the reciprocatory shaker are difficult to interpret, but with the rotary shaker the PDA cultures resulted in the highest dry weight yield of mycelial material on the third day. Also, as was observed in the surface cultures, washing of the spores collected dry led to better mycelial formation than that observed in the corresponding control cultures. Similarly, the various treatments of the spores gave rise to mycelial material of fairly constant composition with respect to total phosphorus and nitrogen. It may also be pointed out that in submerged cultures the acid production was much lower than in surface cultures using the same medium.

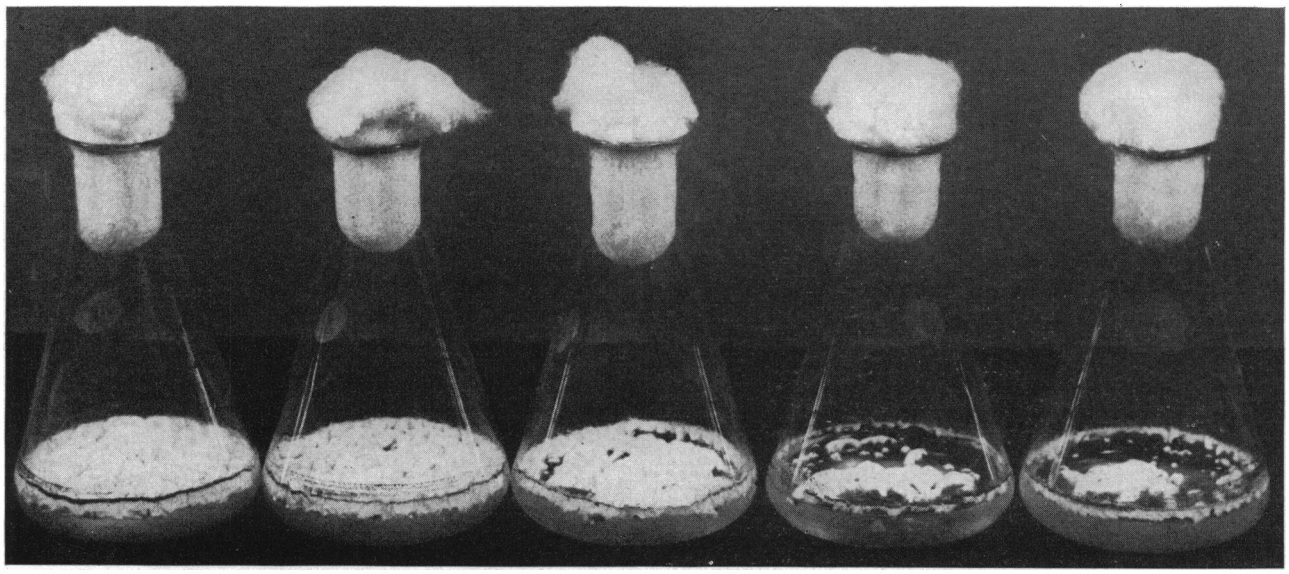

FIG. 1. Effect of washing the spores and of addition of an extract of PDA medium on the growth of *Aspergillus niger* NRRL 67 in Currie's medium. Growth 4 days. Left to right: Control plus PDA; PDA; Washed; Supplemented; Control.

TABLE 4. *Submerged cultures of* Aspergillus niger *inoculated with spores from 6 day old agar slants, collected by flooding and by suction*

	Days of Incubation, No.	Acidity	Utilization of Glucose	Mycelial Material, Dry Wt.*
		ml N/10 NaOH	*Per cent*	*mg*
Reciprocatory Shaker				
Spores by flooding				
Control	3	10	10.4	103
Washed		11	8.9	96
Supplemented		13	6.6	102
P.D.A.		12	11.1	106
Spores collected dry				
Control	3	10	9	88
Washed		12	8.7	103
Supplemented		10	8.7	87
P.D.A.		15	12.2	96
Rotary Shaker				
Spores by flooding				
Control	3	14	9.7	279
Washed		15	11.3	241
Supplemented		13	13	336
P.D.A.		14	14.9	436
Control	5	32	14.5	405
Washed		32	19.1	439
Supplemented		27	15.7	448
P.D.A.		28	15.2	446
Spores collected dry				
Control	3	15	10.9	217
Washed		17	8.3	268
Supplemented		15	5.6	214
P.D.A.		17	17.7	322
Control	5	33	16.1	416
Washed		29	14.3	433
Supplemented		32	18.6	435
P.D.A.		37	23.2	611

* Analysis of mycelium plus spores showed 6.91 μg phosphorus and 52.3 μg nitrogen per mg dry weight.

TABLE 5. *Mechanism of the agar effect in surface culture of* Aspergillus niger *with spores collected by suction*

	Dry Wt Yields of Mycelium	
	3 days	5 days
	mg/100 ml medium	
Control	410	1287
Ash	517	1254
Agar alone	605	1284
P.D.A.	839	1388

Nature of the Agar Effect

The following surface cultures were started using unwashed suction collected spores:

1. Control cultures, with water added to equalize volumes.
2. PDA cultures, which received the usual extract of the solidified sporulation medium.
3. Ash cultures, which received an aqueous solution of the ash obtained from the above extract.
4. Agar cultures, which received an extract from slants prepared with agar alone, without added potato.

The ash cultures as well as the control cultures grew in patches; the cultures with an extract of agar alone showed much better mycelial formation, but by far the best growth was observed in the cultures which received an extract of the whole PDA medium. The dry weight yields of mycelia calculated on the basis of 100 ml culture solution obtained in a typical experiment are recorded in table 5.

The data for the mycelial yields in table 5 indicate that ash by itself effected a certain stimulation, that an extract of agar alone increased mycelial formation significantly, and that the highest yields were obtained on supplementing with an extract of the whole medium. The agar effect is therefore a complicated factor, derived in part from the inorganic constituents and to a great extent from the organic constituents of agar and of potato. Whether the stimulation of the spore germination by the organic constituents of PDA is due to a surface effect or/and a growth factor can be decided only by further experimentation.

DISCUSSION

The experimental data recorded in the above pages indicate the following:

The spores of *Aspergillus niger* NRRL 67 contain some material inhibitory for surface and, to a less extent, also for submerged growth in Currie's synthetic medium. This inhibition is prominent in the cultures inoculated with spores collected by suction, but is completely suppressed in cultures with spores collected by flooding.

This inhibitory material can be washed out with water, so that the washed, suction collected spores grow better than the unwashed spores. On supplementation of the washed spore cultures with the spore washing the growth is again retarded. The inhibition in growth is most marked in the initial stages of mycelial formation and tends to disappear with progress in incubation of the cultures.

Quite independent of the inhibitory factor present in, or associated with the spores, is a stimulatory factor present in the PDA medium used for sporulation. On allowing water to remain in contact with the uninoculated slants for about 15 seconds, enough of this active principle is leached out to give a remarkable spurt in the initial mycelial formation in the shake (rotary) and much more so in the surface cultures. This is especially prominent in the case of the spores collected by suction, where the inhibition in growth normally observed with the unwashed spores is totally suppressed by the addi-

tion of an extract of the PDA medium. A spore inoculum prepared by flooding slants with water would contain a certain, though variable, amount of this active factor. With such an inoculum the effect of the inhibitory material normally associated with the spores is suppressed and the net effect is a fair growth of the mycelium. The growth stimulating effect of the extract of PDA is most prominent in the early stage of mycelial formation; with further incubation the differences between the PDA and the other cultures wear out.

The experiments of Robbins (1939) showed that the incorporation of agar into an otherwise complete synthetic liquid medium resulted in the stimulation of mycelial growth and gamete production in *Phycomyces blakesleanus*. Potato and some other naturally occurring material were likewise found to have a growth promoting effect on the organism. The accelerated growth response of *A. niger* on the addition of an extract of PDA medium observed in the present series of investigations is probably analogous to the observations of Robbins on the effect of agar on the culture of *Phycomyces*. It is obvious that this agar factor has to be reckoned with in the normal process of inoculation with spores collected by flooding. Varying amounts of the active material will be removed depending upon the type and the amount of agar and potato used in compounding the medium, the period for which water is allowed to remain in contact with the slant, the evenness with which the mycelium spreads over the agar surface, and also the manner in which the spores are dislodged, namely by gentle scraping with a blunt rod, by shaking with glass beads (Dirkx, 1952), or sand (Moyer, 1953), and also the extent to which the active factor has been depleted from the slants by the growing organism.

It has to be borne in mind that when water is added to the slants, some of the material elaborated by the growing organism, and secreted into the medium such as organic acids may get dissolved out. During the brief period that the water remains in contact with the slants it can also solubilize material from any autolyzed cells of the mycelium and any cells ruptured during the process of scraping. Such material may exert an influence on the growth of the culture. In the present series of experiments no attempt was made to study this factor separately.

A. niger NRRL 67 is known to convert sugar primarily into citric acid under the conditions of pH and sugar concentration used in the present experiments. No attempt was made to estimate the proportion of the total acidity which can be accounted for by citric acid. The data for total acid production given in tables 1 and 2 show that inocula prepared by the flooding technique give rise to higher acidity, the highest yield being from cultures containing PDA extract and inoculated with spores from 4-day-old agar slants.

ACKNOWLEDGMENT

Two of us (V. B. and S. P. D.) are indebted to the Council of Scientific and Industrial Research and to the Director, National Chemical Laboratory, for permission to work in this Laboratory.

SUMMARY

Spores of *Aspergillus niger* NRRL 67 contain a material inhibitory for surface or submerged growth of the culture in Currie's synthetic medium. The inhibitory material can be removed from the spores by washing with water. When the water washings are added back to washed spores, growth is inhibited.

The potato-dextrose-agar medium used for sporulation of *A. niger* contains a stimulatory factor for growth. Water extracts of the medium are active in stimulating growth in both surface and submerged cultures. Acid production by *A. niger* is greater in cultures containing water extract from PDA agar than in cultures containing no supplement.

REFERENCES

BAJAJ, V., DAMLE, S. P., AND KRISHNAN, P. S. 1954 Phosphate metabolism of mold spores. 1. Phosphate uptake by the spores of *Aspergillus niger*. Arch. Biochem. and Biophys., **50**, 451–460.

CAMICI, L., SERMONTI, G., AND CHAIN, E. B. 1952 Observations on *Penicillium chrysogenum* in submerged culture. 1. Mycelial growth and autolysis. Bull. World Health Organization, **6**, 265–276.

DAMLE, S. P., AND KRISHNAN, P. S. 1954 One step digestion procedure for the estimation of total phosphorus and nitrogen in mold tissue. Anal. Chim. Acta., **2**, 225–228.

DIRKX, J. 1952 L'acide ribonucleique des moisissures. 1. Une technique rapide de dosage. Biochim. et Biophys. Acta, **8**, 194–201.

KRISHNAN, P. S., AND BAJAJ, V. 1953a The polyphosphatases of *Aspergillus niger*. 1. The nonidentity of metaphosphatase with apyrase and pyrophosphatase. Arch. Biochem. and Biophys., **42**, 174–184.

KRISHNAN, P. S., AND BAJAJ, V. 1953b The polyphosphatases of *Aspergillus niger*. 2. Metabolic role of the polyphosphatases. Arch. Biochem. and Biophys., **47**, 174–184.

MANDELS, G. R., AND NORTON, A. B. 1948 Studies on the physiology of spores of the cellulolytic fungus *Myrothecium verrucaria*. Research report from the Quartermaster General Laboratories, Microbiology series No. 11, 1–50.

MCCALLAN, S. E. A., AND WILCOXON, F. 1936 The action of fungus spores on Bordeaux mixture. Contrib. Boyce Thompson Inst., **8**, 151–165.

MOYER, A. J. 1953 Effect of alcohols on the mycological production of citric acid in surface and submerged cultures. Appl. Microbiol., **1**, 1–7.

PERLMAN, D. 1951 The effects of biologically active agents on fungi at different stages of growth. Am. J. Botany, **38**, 652–8.

ROBBINS, W. J. 1939 Growth substances in agar. Am. J. Botany, **26**, 772–778.

SHU, P., AND JOHNSON, M. J. 1947 Effect of composition of sporulation medium on citric acid production by *Aspergillus niger* in submerged culture. J. Bacteriol., **54**, 161–167.